PRIMARY METABOLISM IN FRUITS

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PRIMARY METABOLISM IN FRUITS

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REVIEW
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The Starch Is (Not) Just Another Brick in the Wall: The Primary Metabolism of Sugars During Banana Ripening

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The monocot banana fruit is one of the most important crops worldwide. As a typical climacteric fruit, the harvest of commercial bananas usually occurs when the fruit is physiologically mature but unripe. The universal treatment of green bananas with ethylene or ethylene-releasing compounds in order to accelerate and standardize the ripening of a bunch of bananas mimics natural maturation after increasing the exogenous production of ethylene. The trigger of autocatalytic ethylene production regulated by a dual positive feedback loop circuit derived from a NAC gene and three MADS genes results in metabolic processes that induce changes in the primary metabolism of bananas. These changes include pulp softening and sweetening which are sensorial attributes that determine banana postharvest quality. During fruit development, bananas accumulate large amounts of starch (between 15 and 35% w/w of their fresh weight, depending on the cultivar). Pulp softening and sweetening during banana ripening are attributed not only to changes in the activities of cell wall hydrolases but also to starch-to-sugar metabolism. Therefore, starch granule erosion and disassembling are key events that lead bananas to reach their optimal postharvest quality. The knowledge of the mechanisms that regulate sugar primary metabolism during banana ripening is fundamental to reduce postharvest losses and improve final product quality, though. Recent studies have shown that ethylene-mediated regulation of starch-degrading enzymes at transcriptional and translational levels is crucial for sugar metabolism in banana ripening. Furthermore, the crosstalk between ethylene and other hormones including indole-3-acetic acid and abscisic acid also influences primary sugar metabolism. In this review, we will describe the state-of-the-art sugar primary metabolism in bananas and discuss the recent findings that shed light on the understanding of the molecular mechanisms involved in the regulation of this metabolism during fruit ripening.

Keywords: starch-degrading enzymes, starch degradation, starch ultrastructure, banana, ethylene, ripening, sucrose

BANANA STARCH STRUCTURE

During development, banana (*Musa acuminata*) fruit accumulates a large reserve of carbon in the form of starch. At the 3/4 diameter stage, which is considered to be the optimum point for commercial harvesting of bananas, the fruits have 12–35% starch, while the starch content at late ripening usually ranges from 15% to less than 1% (Soares et al., 2011). The mobilization of the starch reserve is followed by a concomitant increase in soluble sugars that may reach up to 20% of the fresh weight of the pulp in the ripe fruit, with sucrose accounting for approximately 80% of the soluble sugars in ripe bananas, whereas glucose and fructose make up almost all the remaining 20% of the soluble sugars in equal proportions (Henderson et al., 1959; Marriott et al., 1981; Hill and ap Rees, 1993; Cordenunsi and Lajolo, 1995; Mota et al., 1997; Shiga et al., 2011). However, maltose and other oligosaccharides, such as a trisaccharide correlate to invertase (INV) activity (Henderson et al., 1959), and fructooligosaccharides (FOS) (Der Agopian et al., 2008) are also detected during banana ripening. In this regard, the presence of 1-kestose, the first member of the FOS series, occurs when the levels of sucrose in the pulp reach 200 mg/g of dry matter, and an INV appears to synthesize the FOS by transfructosylation (Der Agopian et al., 2008).

The amount of starch in the fully developed fruit may vary significantly among banana varieties belonging to different species. Compared to Cavendish bananas (*M. acuminata*), the cooking varieties classified as plantains (*M.* × *paradisiaca*) accumulate more starch (up to 35%) and have larger amounts of undegraded starch when ripe, which means they are not as sweet as Cavendish bananas. As an example, the fully ripe plantain Brazilian cultivars (cv.), Terra (genotype group AAB) and Figo (genotype group ABB), have 8–16% and 6–9% residual amounts of starch and sucrose, respectively (Soares et al., 2011), which are equivalent to those values observed for plantains grown in Ghana and some dessert bananas purchased in the UK (Marriott et al., 1981). In contrast, the dessert bananas cv. Pacovan and Mysore (genotype group AAB) have approximately 1% of residual starch (Soares et al., 2011). Therefore, there is an indication that the pattern of starch accumulation and degradation is highly correlated with the banana specie.

Starch consists of linear amylose and highly branched amylopectin in the proportion 20:80. Amylose is formed by linear α-D-(1,4)-glucose units, whereas amylopectin consists of several short chains of α-D-(1,4)-glucose units interconnected by α-D-(1-6)-glucose units making up to 6% of the bonds in the molecule (Buléon et al., 1998; Hoover, 2001; BeMiller, 2019). These two macromolecules are arranged in the form of granules with a well-organized internal structure, alternating between semi-crystalline and amorphous layers, which are also known as growth rings.

The morphology of the starch granules differs according to the botanical origin, the cultivar of fruits, and the ripening stage (Peroni-Okita et al., 2010, 2013, 2015; Soares et al., 2011). Starch granules from unripe bananas were found to be oval and rounded for cv. Nanicão and small and leaf-like shaped for cv. Pacovan and Mysore. In plantains, the starch granules from unripe fruits were mostly round and elongated for cv. Terra and Figo, but some oval and rounded starch granules were present in the unripe fruit. In contrast, the partially degraded starch granules from both overripe bananas and plantains were narrow and elongated (Soares et al., 2011). Thus, as the small and round granules were degraded in bananas during ripening and almost disappeared in ripe plantains, it appears that granules with this shape and size have a greater susceptibility to enzymatic degradation (Peroni-Okita et al., 2010; Soares et al., 2011). Supporting this hypothesis, Gao et al. (2016) also reported rounded granules for unripe Cavendish varieties and smaller and ellipsoid granules in ripe plantains, which changed during ripening to an irregular shape in the first group of bananas and remained unchanged as ellipsoidal granules in the last group. As expected, starch granules appear decreasing in size during ripening. The size distribution analysis revealed that the granules from unripe bananas of cv. Nanicão averaged 28.9 μm, with 90% of the population being smaller than 49.6 μm and 10% being less than 10.3 μm. The granules from ripe fruits were smaller (25.4 μm), and the size distribution showed that 90% of the population was 45.4 μm and 10% was 7.6 μm (Peroni-Okita et al., 2010).

The investigation of starch granule surface during banana ripening, using microscopic and physical techniques, revealed that the inner part of the granule consists of a material with different viscoelastic properties (Peroni-Okita et al., 2010, 2013, 2015; Soares et al., 2011). In ripe bananas, the inner part of the granule is composed predominantly from large blocklets (80–200 nm), which consist of amylopectin lamellae with spherical structures that absorb less water than other parts. In contrast, green bananas have smaller blocklets (15–50 nm) in the inner part of the granule (Peroni-Okita et al., 2015).

The organization of the starch granule depends on the packing of amylopectin double helices; those from cereals, mango, and tapioca usually have the A-type pattern, which is associated with a monoclinic lattice with densely packed crystallites. In contrast, the granules from tubers and high amylose content starches have the B-type pattern composed of hexagonal crystalline unit cells that contain much more water (Gallant et al., 1997; Buléon et al., 1998; Thys et al., 2008). Banana starch granules show a typical C-type profile, resulting from the coexistence of A- and B-type allomorphs in the same granule (Millan-Testa et al., 2005; Peroni-Okita et al., 2010; Soares et al., 2011).

During banana ripening, the crystallinity index of starch granules decreases, although the total amylose content remained almost constant. Since the short chains of amylopectin are reduced, and the degree of crystallinity is dependent on the proportion of amylopectin, the amount of short A-type chains plays a role in the polymorphic forms of starch crystals. In fact, the amylopectin from cv. Nanicão has a large amount of short A- and B1-type chains and a reduced amount of long B-type chains. The ratios of the fraction fa/fb1 + fb2 + fb3 decreased during ripening. These fractions of amylopectin correspond to A-chains (fa, external short chains, DP 6–12),

B1 (fb1, DP 13–24), B2 (fb2, DP 25–36), and long B3 chains (fb3, DP > 37), and the ratio indicates the length and the degree of ramification of amylopectin chains (Hanashiro et al., 1996). Large proportions of short chains suggest a more crystalline starch granule. This clearly demonstrates that the degree of crystallinity is dependent on the branching patterns of amylopectin and may play an important role in determining the type of unit packing, the wide-angle X-ray diffraction (WAXD) pattern, and the susceptibility to enzymatic hydrolysis (Jane et al., 1997; Sanderson et al., 2006).

The amylose content in starch granule also plays an important role in accessibility to degrading enzymes, as the double helices formed by amylose acquire resistance to amylase hydrolysis. In general, the amylose content is greater in plantain than in Cavendish or dessert varieties and significantly decreases during ripening (Zhang et al., 2005; Peroni-Okita et al., 2010; Shiga et al., 2011; Chavez-Salazar et al., 2017). In this regard, the decrease in amylose content in starch granules from bananas cv. Figo during ripening appears to occur through the exo-corrosion of the amylose-rich layers of a granule population that is more susceptible to degradation. According to Soares et al. (2011), the small and round granules almost disappear at the same time that the A/B-type allomorph ratio reduces (2.03–1.27) during ripening, suggesting that the more susceptible round starch granules are predominantly A-type. Therefore, the application of atomic force microscopy (AFM), scanning electron microcopy (SEM), and WAXD to the analysis of starch granules from cv. Terra and cv. Nanicão demonstrated that the subtle changes observed at the surface were related to the lamellar organization of starch, suggesting that A-type crystallites located at the periphery of starch granules are preferentially degraded during ripening (Soares et al., 2011; Peroni-Okita et al., 2015). The granules of banana starch are highly resistant to enzymatic hydrolysis, and they appear to undergo the natural process of degradation by enzymatic corrosion of the surface, in a layer-by-layer process. Furthermore, the abovementioned studies show no evidence of porous structures at the granule surface, which would facilitate the access of hydrolases during ripening.

Data from AFM analysis also support the idea that the first layer covering the granule surface is composed of a hard or well-organized material. The removal of this first layer exposes new layers with alternate hard and soft regions repeated at regular intervals until a hard and well-organized semi-crystalline growth ring is attained. Results obtained by Peroni-Okita et al. (2010) and Soares et al. (2011) have suggested that this first layer is more resistant to the enzymes that degrade the starch granule in starches isolated from plantains than in Cavendish. **Figure 1** shows an interesting result obtained when granules isolated from cv. Terra (plantain) and Thap Maeo (Cavendish) were treated with amylase porcine for several hours. Some granules were very corroded in the interior, with the remaining shell being almost integrated in the case of cv. Terra (**Figures 1A**,**B**), which was not seen in cv. Thap Maeo (**Figures 1C**,**D**). According to Peroni-Okita et al. (2015), no migration of enzymes from the surface or signs of degradation was observed in the inner part of starch granules from unripe bananas, suggesting that the layers are more resistant to enzymatic corrosion. Since the core of the granules was weakly stained with iodine, the amount and distribution of amylose chains in the center of the particle were likely to be an important contributor to the resistance of banana starch to hydrolysis, alongside several other factors (Gallant et al., 1992, 1997; Faisant et al., 1995; Jiang et al., 2015).

STARCH-TO-SUCROSE METABOLISM DURING BANANA RIPENING

During banana development, a large amount of starch accumulates in the amyloplasts of cells from fruit pulp (Beck and Ziegler, 1989; Nascimento et al., 2000; Mota et al., 2002). However, during banana ripening, a complex regulatory mechanism shifts metabolism from starch synthesis to starch breakdown leading to the accumulation of soluble sugars, mainly sucrose, that will have a significant impact in fruit taste and flavor. This starch-to-sucrose conversion appears to be responsible not only for pulp sweetening but also for providing energy to metabolic processes that result in the development of other quality attributes of ripe bananas, such as color change, synthesis of volatile compounds, and even pulp softening, thereby strongly affecting final fruit quality. In this sense, the disappearance of the large stock of starch in favor of the accumulation of soluble sugars strongly contributes to pulp softening (Shiga et al., 2011).

Starch-to-sucrose metabolism has been extensively studied in model systems in the context of energy sources for plant growth and development, including *Arabidopsis* leaves (transitory starch) and the endosperm of germinating cereal seeds (storage starch). Both metabolism and energy supply in photosynthetic tissues clearly differ from the equivalent processes in heterotrophic tissues.

The starch breakdown in fleshy fruits such as bananas is less understood.

Taxonomically, the banana is a commelinoid monocot (Musaceae) and, therefore, is more closely related to cereal grasses than *Arabidopsis* (Brassicaceae). However, although highly heterogeneous, the starch breakdown during banana ripening appears to be more akin to the process in *Arabidopsis* leaves (**Figure 2A**) than that of endosperm from germinating cereal seeds (**Figure 2B**). In photosynthetic tissues, the assimilation of energy through the Calvin cycle results both in carbon transference for sucrose synthesis in the cytosol and the production of transitory starch within the chloroplasts, which is a short-term energy supply when the tissue is not able to perform photosynthesis (Santelia and Lunn, 2017). In the dark, this transitory starch is converted mainly into glucose and maltose in the chloroplast, which are shipped to the cytosol for the synthesis of sucrose that can be further transported through the phloem to sink tissues (Pfister and Zeeman, 2016).

The starch stored in banana pulp cells is compartmentalized within plastids, similar to the transitory starch stored in the chloroplasts of *Arabidopsis* leaves. Studies have shown the

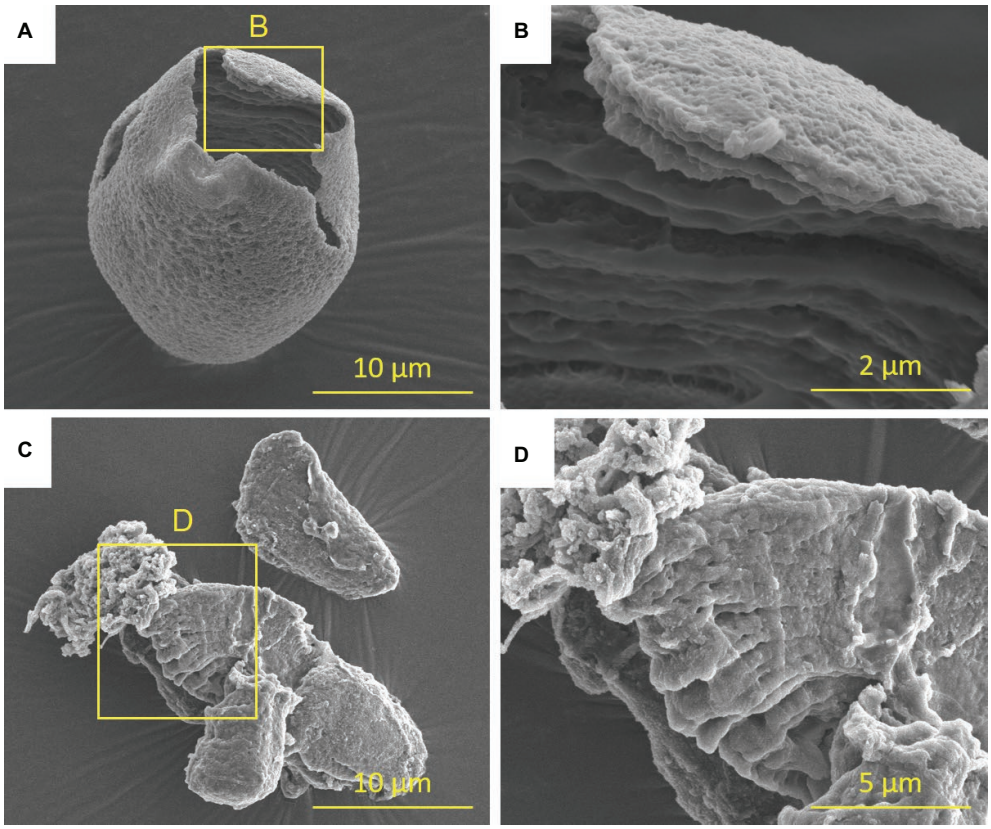

FIGURE 1 | Scanning electron microscopy (SEM) images of starch from unripe bananas. Starch granules isolated from unripe **(A)** Terra (plantain), **(B)** magnification of image, **(C)** Thap Maeo (banana), and **(D)** magnification of image, treated with porcine α-amylase (24 h; 37°C; 3 U/mg of starch). Images produced by SEM were acquired by the authors following the protocol described by Peroni-Okita et al. (2015).

presence and activity of several common starch-degrading enzymes (**Figure 2C**). In bananas, both AMY (e.g., MAmy) and β-amylases (BMY, EC 3.2.1.2) (e.g., bAmy) were associated with the starch granules in the amyloplasts of banana pulp (Peroni-Okita et al., 2013). A plastidic AMY identified in the banana (Junior et al., 2006) appears to act before BMY at the onset of starch breakdown, but the latter is essential for the complete breakdown and its upregulation is clearly correlated to a decrease in starch during fruit ripening (Purgatto et al., 2001). Alpha-amylase (AMY, EC 3.2.1.1) isoforms appear to play a crucial role in hydrolysis of starch in germinating cereal seeds (Radchuk et al., 2009) and is also present in the chloroplast of *Arabidopsis* leaves. However, AMY from *Arabidopsis* leaves, which have a strong preference for β-limit dextrin over amylopectin (Seung et al., 2013), is not necessary for the breakdown of transitory starch (Yu et al., 2005).

In *Arabidopsis* leaves, degradation of transitory starch at the end of the light cycle induces a transition from highly ordered to less ordered and hydrated granules by a complex process that involves the action of several starch-degrading enzymes. First, a group of starch-phosphorylating enzymes, termed glucan, water dikinase (GWD, EC 2.7.9.4), phosphorylates the C6 position of the glucosyl residues in starch (Ritte et al., 2006), and the steric hindrance of these (few) phosphorylated groups alters the intermolecular organization of the granule. This loss of structure favors the action of another group of starch-phosphorylating enzymes, termed phosphoglucan, water dikinase (PWD, EC 2.7.9.5) (Edner et al., 2007; Fettke et al., 2009), which acts downstream to GWD and phosphorylates the C3 position of the glucosyl residues. The role of phosphorylases including GWD and PWD in starch breakdown during banana ripening is less understood, but phosphorylation at the C3 and C6 position of the glucosyl residues in the starch of freshly harvested unripe bananas has already been found, as well as the presence of PWD and GWD associated with the granule through a proteomic analysis (Helle et al., 2018). Therefore, it is likely that GWD- and PWD-induced phosphorylation of banana starch favors granule hydration and phase transition from the crystalline state to the soluble state.

In *Arabidopsis* leaves, the neutral and phosphorylated glucans released from the granule surface undergo a complex net of enzymatic reactions. Starch-dephosphorylating enzymes prevent phosphate groups from obstructing the action of other enzymes, while starch-debranching enzymes from both the isoamylase-like protein 3 sub-family (DBE\ISA3, EC 3.2.1.68) and the limit dextrinase family (DBE\PUL, EC 3.2.1.142) hydrolyze side chains at the C6 position (Streb et al., 2012).

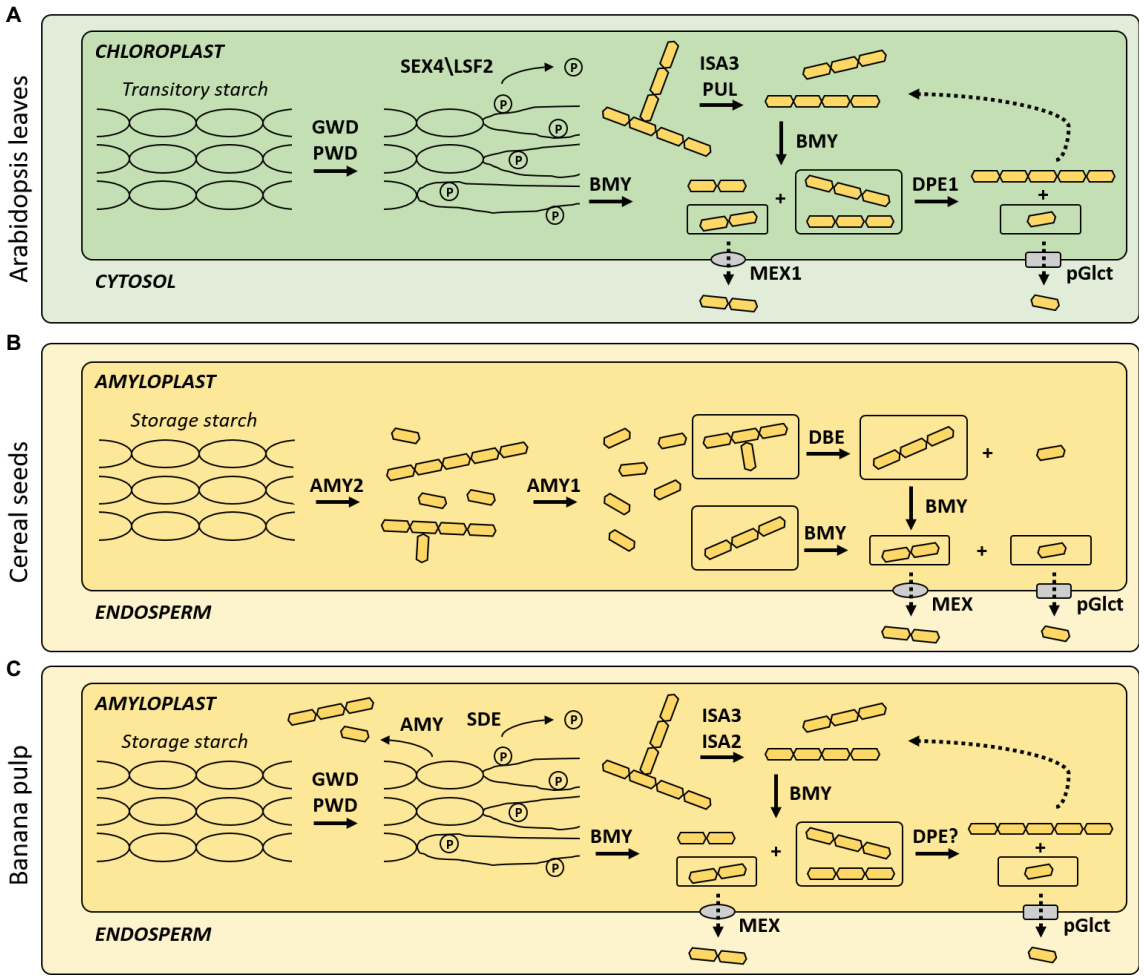

FIGURE 2 | Starch-to-sucrose metabolism in model systems and banana pulp. Main enzymes responsible for starch degradation in **(A)** Arabidopsis leaves, **(B)** germinating cereal seeds, and **(C)** banana pulp. GWD, glucan, water dikinase; PWD, phosphoglucan, water dikinase; ISA, isoamylase-like protein sub-family; PUL, limit dextrinase family; BMY, β-amylase; DPE1, starch-disproportionating enzyme 1; MEX1, maltose excess transporter 1; pGlcT, plastidic glucose translocator; AMY, α-amylase.

The breakdown of starch granules in amyloplasts during banana ripening is assumed to occur in a two-step process. First, AMY acts mainly in amylose-rich regions of the starch granule (amorphous lamella), thereby exposing amylopectin-rich regions (crystalline lamella). Then, the phosphorylation of residues at C3 and mainly C6 by PWD and GWD, respectively, favors the action of BMY. However, even if the correlation between BMY expression and starch breakdown is well established (Nascimento et al., 2006), other hydrolytic enzymes, such as a DBE\ISA3 (Maisa) with preference for β-limit dextrin (Bierhals et al., 2004), two DBE\ISA2 (Jourda et al., 2016), as well as starch phosphorylases (Mota et al., 2002) appear to contribute to starch degradation during ripening.

In photosynthetic tissues, the residual maltotriose produced by BMY (Li et al., 2017) can be used as substrates for starch disproportionating enzyme 1 (DPE1; EC 2.4.1.25), which transforms two molecules of maltotriose into glucose and maltopentaose (Critchley et al., 2001), and the latter could be a further target for BMY hydrolysis within the chloroplast (Moller and Svensson, 2016). Although the role of DPE is poorly understood in ripening bananas, genes encoding this enzyme were also found in the banana genome sequence[1] suggesting they may be acting at the mobilization of starch during ripening. Finally, maltose and lesser amounts of glucose are shipped from the chloroplasts of the photosynthetic tissues to the cytosol mainly through the maltose excess transporter 1 (MEX1) and the plastidic glucose translocator (pGlcT), respectively (Cho et al., 2011). In the cytosol, sucrose phosphate synthase (SPS, EC 2.4.1.14) (Bahaji et al., 2015) and mainly sucrose phosphate phosphatase (SPP, EC 3.1.3.24) appear to form complexes that are crucial for sucrose synthesis (Albi et al., 2016). In bananas, it is very likely that the resulting glucose and maltose are shipped from the amyloplast to the cytoplasm through MEX and pGlcT in a mechanism similar to that of *Arabidopsis* leaves. Then, sucrose is synthesized mainly by SPS, which increases in activity during ripening,

[1]http://banana-genome.cirad.fr/

mainly by transcriptional activation (Nascimento et al., 1997a,b; Rossetto et al., 2003).

HORMONAL AND GENETIC REGULATION OF STARCH-TO-SUCROSE METABOLISM IN BANANAS

Ethylene is the most strongly hormone correlated to fruit ripening, particularly in climacteric fruits such as bananas. Climacteric ethylene synthesis in bananas induces the conversion of starch to soluble sugars (Saraiva et al., 2018), particularly sucrose, whereas the treatment with ethylene inhibitor, 1-methylcyclopropene (1-MCP), delays such conversion over several days in a dose- and cultivar-dependent manner (Mainardi et al., 2006; Nascimento et al., 2006). In addition, there are several indications that other hormones are associated and form a network of signals that coordinate the phases during fruit development (Seymour et al., 2013).

In recent years, the increasing availability of transcript profiling tools and *in silico* genomic analysis has allowed for the identification of several transcripts of enzymes related to starch mobilization in bananas that were affected by ethylene during ripening. Jourda et al. (2016) have identified four *DBE*, 13 *AMY* and 13 *BAM* by *in silico* analysis of the *Musa* genome. They have found that the transcripts of three *DBE*, five *AMY*, and three *BAM* are expressed in several stages of fruit ripening after treatment with exogenous ethylene, while two *BAM* (*MaBAM6* and *MaBAM7*) were induced after 24 h of acetylene treatment. Miao et al. (2016) have identified 16 members of the *MaBAM* family after *in silico* analysis of *Musa* genome, and 10 *MaBAM* transcripts were observed at various stages of development and ripening in bananas of cv. BaXi Jiao and cv. Fen Jiao. The genes encoding isoforms *MaBAM9a*, *MaBAM9b*, and *MaBAM3c* showed high levels of relative expression after climacteric in both cultivars.

Recently, an extensive study (Xiao et al., 2018) has found 38 genes associated with starch metabolism in bananas including three GWD, three phosphoglucan phosphatases, eight BMY, seven AMY, two DBE, two α-glucan phosphorylases, two DPE, two MEX (*MaMEX1* and *MaMEX2*), and five pGlct. Among these, 17 presented high transcript accumulation in ethylene-treated fruits (*MaGWD1, MaSEX4, MaLSF2, MaBAM2-MaBAM4, MaBAM6-MaBAM8, MaAMY3, MaAMY3B, MaAMY3C, MaISA3, MaMEX1, MapGlcT2-1, MapGlcT4-1*, *and MapGlcT4-2*). The same pattern was also observed at the climacteric of naturally ripening bananas and in fruits treated with 1-MCP. However, the fruits treated with 1-MCP were long delayed in relation to those allowed to ripen naturally, as ripening took place only after the increase of endogenous ethylene synthesis.

Using a quantitative proteomic approach, in the same work, the authors have identified 18 proteins related to starch degradation in protein extracts isolated from starch granules of unripe and ripe bananas. Among them, MaGWD1, MaPWD1, MaSEX4, MaLSF1, MaBAM4, MaBAM7, MaAMY2B, MaAMY2C, MaAMY3, and MaISA3 had higher levels in the extracts isolated from ripe than those from unripe fruits. A similar trend was observed in their transcript levels, as confirmed for the MaGWD1 protein by Western blotting using an anti-GWD1 antibody. Moreover, MaGWD1 accumulation was accelerated in ethylene-treated bananas and delayed after 1-MCP treatment.

The ethylene dependence of the gene expression of enzymes related to starch degradation was also observed in cold stored cv. Nanicão bananas, as the activity of BMY was reduced in fruits of that cultivar that had low levels of ethylene (Peroni-Okita et al., 2013). Thus, there is little doubt that ethylene is a hormone directly related to the activation of the starch mobilization system during banana ripening. In addition, the hormone appears to stimulate the activity of enzymes related to the sucrose metabolism, such as SuSy, SPS, and acid and neutral INV (Nascimento et al., 1997a; Choudhury et al., 2008; Li et al., 2011).

Although the evidence points to a significant role for ethylene in the regulation of starch mobilization in bananas, there is a lack of systematic analysis of ethylene response elements in the promoter regions of genes associated with starch metabolism in the *Musa* genome. Miao et al. (2017) have identified response elements to diverse hormonal classes in the upstream regions of 16 *MaBAM* genes. Interestingly, only *MaBAM3c*, which was highly expressed in the fruit after the climacteric peak, showed a single element of ethylene response (ERE) in the promoter region. This gene also had elements of response to auxin, abscisic acid, and methyl jasmonate in the promoter region, suggesting a multi-hormonal regulation of expression.

The analysis of promoter regions of other genes may also contribute to the understanding of the mechanism by which ethylene regulates starch mobilization in bananas. More than 200 AP2/EREBP transcription factors (TF) were identified in the *Musa* genome (D'Hont et al., 2012), indicating a high number of candidates for downstream effectors of ethylene signaling regarding the expression of starch-related enzymes, such as the TF named ethylene response factors (ERF). Ethylene-signaling activation starts with its recognition by a family of five ethylene receptors (ETR1, ETR2, ERS1, ERS2, and EIN4), which regulate downstream proteins such as CTR1, EIN2, EIN3, and EIN5. EIN3 (ethylene insensitive 3) is a nuclear-localized protein that acts as a TF by activating the ERF through binding to primary ERE (Liu et al., 2015). Mbeguie-A-Mbeguie et al. (2008) have isolated five *EIN3-like* genes from bananas, named *MaEIL* (*Musa acuminata ethylene insensitive 3-like*) from 1 to 5. All of them were differentially expressed during ripening with *MaEIL2* being the exclusive gene induced after ethylene treatment (**Figure 3A**). These TF regulate banana ERF, culminating in the expression of ripening-related genes such as the ones involved in starch-to-sucrose metabolism (**Figure 3B**).

A study of 15 banana *ERF* genes in ethylene- and 1-MCP-treated fruits found that *MaERF9* was upregulated, and *MaERF11* was downregulated in peel and pulp during ripening or after ethylene treatment (Yu et al., 2013). MaERF9 protein was considered to disrupt ethylene burst during banana ripening (with high starch degradation), while MaERF11 was a repressor of banana ripening (with low starch degradation) through direct and indirect mechanisms of action (Han et al., 2016). It can be deduced that the direct effect was caused by MaERF11 binding to the promoters of several ripening-related genes inhibiting their expression. The indirect effect was due to the recruitment of

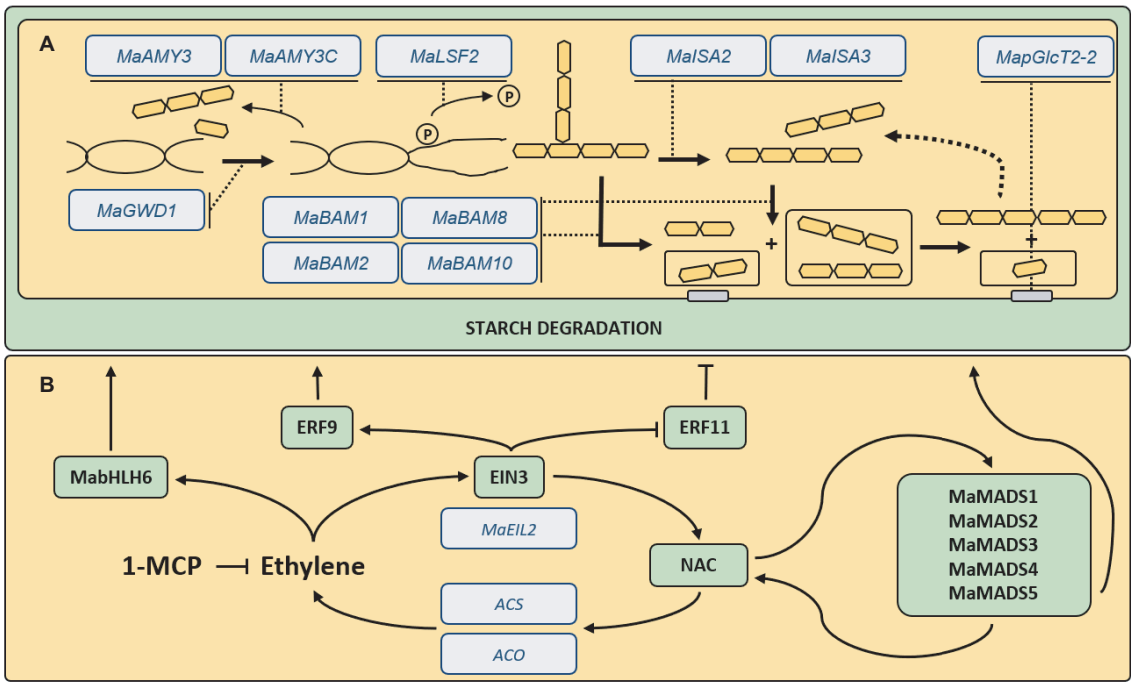

FIGURE 3 | Ethylene-induced regulation of starch degradation. **(A)** A dual feedback loop of MADS-box sequences (MaMADS) and no apical meristem (NAM), *Arabidopsis* transcription activation factor 1/2 (ATAF), and cup-shaped cotyledon (CUC) (NAC) transcription factors appear to regulate ethylene responses in bananas. This results in the **(B)** induction of several genes related to starch degradation during fruit ripening including a glucan, water dikinase (Ma*GWD1*), six α- and β-amylases (*MaAMY3*, *MaAMY3c*, *MaBAM1*, *MaBAM2*, *MaBAM8*, and *MaBAM10*), two isoamylases (*MaISA2* and *MaISA3*), and a plastidic glucose translocator (*MapGlcT2-2*).

a banana histone deacetylase (MaHDA1) that alters the H3 and H4 histone acetylation levels during fruit ripening, reinforcing the repression of the ripening-related genes, until *MaERF11* expression decreases at the onset of ripening, triggering all the downstream events (Han et al., 2016).

Xiao et al. (2018) have found binding elements of bHLH (basic helix-loop-helix) TF in the upstream regions of 27 genes related to the starch mobilization in bananas. A transcription factor designated MabHLH6 demonstrated binding capacity to the promoter region of 11 genes and activated the transient expression of the luciferin reporter gene in tobacco leaves. These findings indicate that MabHLH6 is a direct regulator of several genes encoding enzymes from the starch mobilization pathway. In naturally ripened fruits, both transcript and MabHLH6 protein accumulated after the climacteric. Ethylene-treated bananas showed that MabHLH6 expression was hastened, and treatment with 1-MCP delayed the induction of MabHLH6 expression. This suggests that MabHLH6 regulation is ethylene-dependent, reinforcing the hypothesis that starch degradation in bananas could be mediated by TF from families other than the AP2/EREBP (Xiao et al., 2018).

Although they are still poorly explored, there are indications that auxins and gibberellins also exert an influence on starch-to-sugar metabolism in bananas. In fact, the role of plant hormones other than ethylene on the regulation of gene expression in climacteric and non-climacteric fruits shows that ripening is a net result of hormonal inputs in response to developmental factors as well as environmental signals (Seymour et al., 2013). It has been demonstrated, for instance, that tomato SlARF4, an auxin response factor, negatively regulates starch synthesis during tomato development (Sagar et al., 2013). Regarding banana fruit, previous studies (Purgatto et al., 2001) have indicated that auxin treatment delays starch mobilization with impact on sugar synthesis, and such observations were related to the downregulation of a BAM isoform. Interestingly, auxin treatment negatively affected beta-amylolytic activity without affecting sucrose synthetic activity, indicating that the delay on sugar accumulation is mostly due to impairment in starch mobilization (Purgatto et al., 2001).

Treatment with gibberellic acid also promoted a delay on starch degradation in bananas (Rossetto et al., 2003), although the mechanism is apparently not the same as for auxin. Studies with other hormones such as abscisic acid and methyl jasmonate have influenced banana ripening in other aspects, such as cell wall metabolism (Lohani et al., 2004) and carotenoid synthesis (Kaur et al., 2017). However, more research needs to be carried out to fully understand the cross talk of these plant hormones in starch-to-sucrose metabolism during banana ripening.

Another class of TF involved in starch metabolism in bananas is the MADS-box sequences (Mini-chromosome maintenance deficient 1–MCM1, AGAMOUS, DEFICIENS, and Serum response factor–SRF). The *MaMADS1* and *MaMADS2* genes, which were overexpressed during ripening of cv. Grand Nain (Cavendish, genotype group AAA), shared 49 and 54% homology

to the *LeRIN-MADS* (*RIPENING INHIBITOR*), a MADS gene from the tomato, respectively (Friedman et al., 2007). Elitzur et al. (2010) have further observed that the expression of *MaMADS2*, *3*, *4*, and *5* genes increased before ethylene peak, while *MaMADS1* expression was observed together with the ethylene peak. *MaMADS3*, *4,* and *5* expressions were induced by ethylene treatment. Intriguingly, 1-MCP treatment at the onset of the ethylene burst also increased the expression of *MaMADS4 and MaMADS1,* suggesting two independent programs occurring throughout banana ripening with special roles for *MaMADS1 and MaMADS2* (Elitzur et al., 2010). According to Roy Choudhury et al. (2012), MaMADS5 protein interacts with the promoters of banana ripening genes, such as *MaSPS,* stimulating their expression. A bioinformatics study has shown that MaMADS24 and MaMADS49 proteins can interact with several other *MaMADS* genes and with genes directly related to banana ripening, such as the hormone-response and ethylene-signaling genes, as well as starch degradation-related genes (Liu et al., 2017). Recently, Lü et al. (2018) have demonstrated that the banana tree has undergone genome duplications that were responsible for a unique TF dual-positive feedback loop circuit for regulating fruit ripening and, consequently, starch degradation. The circuit was derived from a no apical meristem (NAM), *Arabidopsis* transcription activation factor 1/2 (ATAF), and cup-shaped cotyledon (CUC) (NAC) TF and the three *MADS* transcription factors described above (*MaMADS* 1, 2, and 5).

The effects of hormones and TF on the starch-to-sugar metabolism in bananas continue to be a field for further research. Further studies may add more layers of complexity to the understanding of the regulation of this pivotal metabolism in the physiology of the fruit, in addition to enabling the enhancement of its commercial quality.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript.

AUTHOR CONTRIBUTIONS

All authors equally collected literature data, wrote the manuscript and revised the article.

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Fast and Furious: Ethylene-Triggered Changes in the Metabolism of Papaya Fruit During Ripening

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Papaya is a climacteric fleshy fruit characterized by fast ripening after harvest. During the relatively short postharvest period, papaya fruit undergoes several changes in metabolism that result in pulp softening and sweetening, as well as the development of a characteristic aroma. Since papaya is one of the most cultivated and appreciated tropical fruit crops worldwide, extensive research has been conducted to not only understand the formation of the quality and nutritional attributes of ripe fruit but also to develop methods for controlling the ripening process. However, most strategies to postpone papaya ripening, and therefore to increase shelf life, have failed to maintain fruit quality. Ethylene blockage precludes carotenoid biosynthesis, while cold storage can induce chilling injury and negatively affect the volatile profile of papaya. As a climacteric fruit, the fast ripening of papaya is triggered by ethylene biosynthesis. The generation of the climacteric ethylene positive feedback loop is elicited by the expression of a specific transcription factor that leads to an up-regulation of 1-*aminocyclopropane-1-carboxylic acid* (ACC) *synthase* (ACS) and *ACC-oxidase* (ACO) expression, triggering the system II ethylene biosynthesis. The ethylene burst occurs about 3 to 4 days after harvest and induces pectinase expression. The disassembling of the papaya cell wall appears to help in fruit sweetness, while glucose and fructose are also produced by acidic invertases. The increase in ethylene production also results in carotenoid accumulation due to the induction of cyclases and hydroxylases, leading to yellow and red/orange-colored pulp phenotypes. Moreover, the production of volatile terpene linalool, an important biological marker for papaya's sensorial quality, is also induced by ethylene. All these mentioned processes are related to papaya's sensorial and nutritional quality. We describe the understanding of ethylene-triggered events that influence papaya quality and nutritional traits, as these characteristics are a consequence of an accelerated metabolism during fruit ripening.

Keywords: papaya, climacteric fruits, ethylene, fruit metabolism, cell wall, fruit quality

INTRODUCTION

Papaya (*Carica papaya* L.) is a typical climacteric fleshy fruit that is appreciated worldwide because of the sweetness and characteristic flavor of its soft yellow or orange/red pulp (Fabi et al., 2007, 2010b). Tropical countries from Asia are the main producers of papaya, accounting for 56% of worldwide production. However, countries from South America (16%), Africa (10%), and Central America (9%) are also important producers of papaya (Food and Agriculture Organization of the United Nations (FAOSTAT), 2017). As papayas have a relatively short shelf life compared to other fruits, maintaining fruit quality during transport from producing countries to consumer centers (e.g., USA and Europe) is a challenge. In 2016, the main countries that produced papayas for exportation were Mexico (47%), Guatemala (14%), and Brazil (11%, Food and Agriculture Organization of the United Nations (FAOSTAT), 2017), with Mexico being the main supplier to the United States and Brazil the main supplier to Europe (Evans and Ballen, 2012).

European recommendations for papaya exporting countries take into account fruit softening as a determinant factor in fruit shelf life (CBI Ministry of Foreign Affairs, 2018), since the fast softening during papaya ripening facilitates physical injury during handling and transportation. Thus, as the susceptibility of papayas to disease increases proportionally with softening (Manrique and Lajolo, 2004), the recommendation for exportation is to maintain the fruit at 10°C during shipping to prevent overripening due to heat (CBI Ministry of Foreign Affairs, 2018). However, as will be discussed later, low temperatures negatively impact some fruit quality attributes of ripe papayas.

The ripening of fleshy fruits is a physiological process that alters appearance, texture, flavor, and aroma. These changes function to attract seed-dispersing organisms (Giovannoni, 2004). In climacteric fruit, such as tomatoes, bananas, and papayas, the onset of ripening coincides with an increase in respiration and ethylene production, the latter being essential to induce molecular mechanisms responsible for accelerating senescence and for the physiological changes that occur during ripening (Abeles et al., 1992; Gapper et al., 2013). The ripening process in climacteric fruits induces changes in both sensorial and nutritional qualities that are essential for consumer acceptability. Some climacteric fruits are harvested unripe and treated with exogenous ethylene or ethylene-derived molecules to precipitate ripening. Thus, ethylene appears to be the main hormone responsible for regulating the molecular pathways that influence the development of the sensorial and nutritional attributes of climacteric fruits (Lü et al., 2018). It has long been known that the safe and effective control of ethylene-mediated responses could extend the postharvest shelf life of climacteric fruits (Wills et al., 1981). However, interfering with natural ethylene-mediated responses during ripening could also negatively impact fruit quality.

While the mechanism by which ethylene is involved in fruit ripening has been thoroughly studied, efforts are still needed to fully understand this process. The ethylene burst in climacteric fruit is controlled by an autocatalytic mechanism, named system II, that synthesizes ethylene (McMurchie et al., 1972; McManus, 2012). Ethylene synthesis involves the conversion of S-adenosyl methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) by the action of 1-amino cyclopropane-1-carboxylic acid synthase (ACS), in which ACC is converted to ethylene by ACC oxidase (ACO) (Yang and Hoffman, 1984). ACS and ACO enzymes have already been identified in papayas, and their responses are increased with ethylene production and reduced when ethylene is blocked (Razali et al., 2013). A decrease in ACS and ACO occurs in papayas stored at low temperatures, but levels are restored after exogenous ethylene treatment (Zou et al., 2014). The ethylene downstream cascade involves multiple transcription factors, including ethylene response factors (ERFs), that are involved in the control of plant growth, defense, responses to the environment, and plant hormones (Xie et al., 2016), including those involved in the papaya ripening process (Li et al., 2013). Transcription factors of the MADS-box, NAC, and AP2/ERF gene families are also involved in the control of papaya ripening (Fabi et al., 2012). More recently, a NAC transcription factor, rather than MADS transcription factors, was found to regulate ACS and ACO expression during papaya ripening (Lü et al., 2018). Papaya has not undergone whole-genome duplication, unlike other climacteric fruits where this process has been utilized to duplicate the MADS transcription factors that form the ripening circuits (Périn et al., 2002; Lee et al., 2013). NAC is one of the largest plant-specific transcription factor families, with members involved in many developmental processes such as senescence, stress, cell wall formation, and embryo development (Lü et al., 2018). Lü et al. (2018) have suggested that instead of neofunctionalization of the duplicated MADS genes, plants without whole-genome duplication may have repurposed their carpel senescence NAC to generate a positive feedback loop where ethylene regulates ripening, as is the case with papayas. They also suggested that ethylene generated by this feedback loop is autocatalytic. A NAC transcription factor expressed in climacteric fruits, such as papayas and peaches, binds to the promoter regions of some of the key ripening-related genes stimulating their expression in pigment accumulation, volatile secondary metabolite production, cell wall softening, and sugar accumulation (Lü et al., 2018).

Therefore, the ethylene-mediated effects in fruit metabolism that influence the softening, sweetness, flavor, and color of papaya pulp during ripening will be further discussed.

PULP SOFTENING IS THE MAIN BIOCHEMICAL MODIFICATION THAT OCCURS DURING PAPAYA RIPENING

In climacteric fleshy fruits, researches and producers give special attention to ethylene-induced textural changes during ripening, as changes in peel and pulp not only influence softening, crispness, and juiciness (Chaïb et al., 2007) but also increase postharvest losses (Manrique and Lajolo, 2004). In fact, textural changes in most of the fleshy fruits result from complex

mechanisms that primarily influence plant cell wall architecture, whose breakdown is considered as the major factor responsible for the pulp-softening process (Brummell, 2006).

The cell wall architecture of fleshy fruits is comprised of complex polysaccharides, such as pectin, hemicellulose, and cellulose, as well as minor components including proteins and phenolic compounds (Carpita and Gibeaut, 1993). Cellulose is comprised of long, rigid, and inextensible microfibrils of 1,4-β-D-glucose (Glc) residues, which are bound tightly together by hydrogen bonds (Brummell, 2006). Hemicelluloses represent a diverse range of structural polymers that constitute the plant cell wall within fruit pulp (Scheller and Ulvskov, 2010). In dicotyledonous plants, such as papayas, xyloglucan (XYL) is the major hemicellulose (Tucker et al., 2017). As with cellulose, XYL consists of a backbone of 1,4-β-D-Glc residues such as cellulose, but smaller and substituted with 1-6-α-D-xylose (Xyl) side chains. Furthermore, these Xyl side chains can be substituted at the O-2 position with β-galactose (Gal) or α-arabionse (Ara; Scheller and Ulvskov, 2010). Pectin is a complex and heterogeneous polysaccharide that is mainly comprised of α-1,4-D-galacturonic acid (GalA) residues that have varying degrees of acetyl and methyl esterification, and these residues are called homogalacturonan (HG). Xylosylation may further modify HG into xylogalacturonans (XGs). Pectin also contains structures made up of repeating units of intercalated GalA (1,4-α-D-GalpA) and rhamnose (1,2-α-L-Rhap) called rhamnogalacturonan type I (RG-I). These structures have side groups of arabinose (arabinan), galactose (galactan), and type I arabinogalactan at the O-4 position of the Rha residues (Mohnen, 2008; Maxwell et al., 2012). Rhamnogalacturonan type II (RG-II) structures are less common in papayas and are composed of HG molecules with side groups of up to 13 different sugars and more than 20 types of glycosidic linkages (Mohnen, 2008; Bar-Peled et al., 2012). The firmness of fleshy fruits results from turgor pressure maintenance by the cell wall while also maintaining cellular adhesion (Wang et al., 2018). Pulp softening occurs by the water dissolution of the majority of these polysaccharides from the primary cell wall and middle lamella, with pectin being the main one (Brummell and Harpster, 2001).

Structural changes that occur in the cell wall during ripening are regulated by hydrolases responsible for degrading cell wall polysaccharides (Gapper et al., 2013; Balic et al., 2014), whose expression is generally regulated by ethylene production (Tucker et al., 2017). Fruit softening is a complex event that involves several enzymes including pectinases and hemicellulases; however, pectinases, such as polygalacturonases (PGs), pectate lyases (PLs), and pectin methyl esterases (PMEs) appear to be the major enzymes that act on fleshy fruit softening. Polygalacturonases remove the galacturosyl residues from pectin (Atkinson et al., 1998), PLs cleave de-esterified pectin (Marín-Rodríguez et al., 2002), and PMEs hydrolyze methyl-groups of esterified polyuronides (Wakabayashi et al., 2003). Furthermore, side chains of pectin can be degraded by other glycosidases, such as β-galactosidases, which remove the galactosyl residues from pectin and from XYL (Smith et al., 2014); α-arabinofuranosidases, which remove arabinosyl from pectin (Sozzi et al., 2002; Itai et al., 2003); and rhamnogalacturonases, which remove α-1,2 linkages between galacturonosyl and rhamnosyl residues (Wong, 2008).

Despite multiple glycoside hydrolases seeming to be responsible for papaya softening, the main enzymes that play a central role in pulp softening are the PGs (Fabi et al., 2014). Some contribution of hemicellulose degradation to pulp softening appears to occur as an increase in endoxylanase expression occurs during papaya ripening (Huerta-Ocampo et al., 2012). Furthermore, β-galactanases are also related to papaya pulp softening through the hydrolysis of both the pectic and the hemicellulosic fractions (Lazan et al., 2004; Fabi et al., 2014). In order to understand the role of ethylene in the expression of cell wall-degrading enzymes, researchers have treated papayas with 1-methylcyclopropene (1-MCP), an ethylene antagonist. As expected, this had a strong effect on pulp softening (Fabi et al., 2007). The pulp firmness of 1-MCP-treated papayas decreased marginally during ripening, although not enough to reach an edible state, and there was no detectable PG activity. Notably, 1-MCP-treated papayas were unable to soften at the same rate as untreated papayas (Fabi et al., 2007, 2009; Sañudo-Barajas et al., 2009). Treatment with 1-MCP also reduced endoxylanase protein levels (Huerta-Ocampo et al., 2012).

To confirm that ethylene affects PG activity and, therefore, pulp softening during papaya ripening, Fabi et al. (2009) found that treatment with exogenous ethylene had induced PG expression with a concomitant increase in pulp softening. Furthermore, agroinfiltration of PG1 in 1-MCP-treated papayas significantly enhanced pulp softening compared with 1-MCP-treated papayas that were agroinfiltrated with an empty vector (Fabi et al., 2014).

Papaya cell wall structural changes during ripening involve pectin with the solubilization of long chains of galacturonans and a decrease in the molecular weight of polysaccharides (Lazan et al., 1995; Manrique and Lajolo, 2004; Shiga et al., 2009). Polygalacturonases act on papaya pulp softening by mobilizing high-molecular weight pectin from less soluble to more soluble cell wall fractions, especially pectin that is tightly bound to cellulose/hemicellulose, and pectins that are bound to each other by calcium bridges (do Prado et al., 2016). Furthermore, the degree of methyl esterification in papaya pectin changes during ripening since unripe papaya pectin has a lower degree of methyl esterification compared to ripe papaya pectin (Manrique and Lajolo, 2002, 2004; do Prado et al., 2017; Prado et al., 2019). This variation during papaya ripening was first associated to higher PME activity (Manrique and Lajolo, 2004). However, no increase in gene expression of PME appears to occur during papaya ripening (Fabi et al., 2010a, 2012, 2014; do Prado et al., 2016), and the activity of PG does not require the simultaneous removal of methyl-esterified groups from pectin (Fabi et al., 2014). Therefore, recent studies support the hypothesis that the increase in the degree of methyl esterification during papaya ripening is a result of the enrichment of the water-soluble pectin fraction that comes from the insoluble fraction due to the massive action of PG rather than an association with increased PME activity (**Figure 1**; Fabi et al., 2014; do Prado et al., 2016, 2017). The resulted high-methylated low-molecular pectin found in

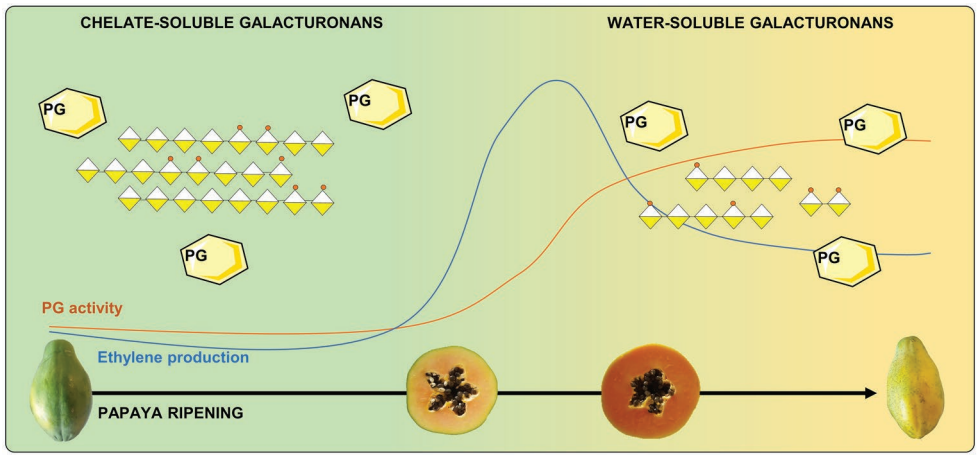

FIGURE 1 | Ethylene production and PG activity during papaya ripening: papaya pectin cell wall solubilization. Ethylene triggers PGs that massively solubilize high-molecular weight pectin by action in the non-methylated areas and releasing the low-molecular weight fractions that will be enriched in methylated fractions due to the lower activity of PME in ripe papayas. PG, polygalacturonase.

ripe papayas showed anticancer effects in diverse *in vitro* tests (do Prado et al., 2017; Prado et al., 2019).

Although the use of MCP-1 is useful in gaining further insight into the role of ethylene in papaya softening, cold storage is another way to decrease ethylene action after harvesting. This latter approach is useful as a postharvest technique as it decreases fruit ripening rates and, therefore, pulp softening (Gomes et al., 2016). The storage of "Golden" papaya at 10°C for 10 days had been found to be effective in reducing ethylene production and fruit ripening. Notably, after a 10-day cold storage, fruits can be stored at room temperature to restore ethylene production and pulp softening (Gomes et al., 2016). However, when cold storage occurs for a longer period (e.g., 20 days at 11°C), ethylene production did not recover when the fruit was subsequently stored at ambient temperatures (Bron and Jacomino, 2009). It seems that the prolonged inhibition of ethylene, either by the inhibition of receptor sites (1-MCP) or by prolonged storage at low temperatures, strongly affects the recovery of the ethylene-mediated response, which negatively influences the pulp softening that is crucial to the quality of the ripe fruit.

PULP SWEETNESS AS A RESULT OF PAPAYA PRIMARY METABOLISM

The qualitative and quantitative composition of primary soluble sugars is crucial to papaya sweetness, although fruit firmness also plays a role as there is a correlation between pulp softening and the perception of sweetness during consumption (Gomez et al., 2002). Thus, it is necessary to understand the key regulatory enzymes involved in the metabolism of soluble sugars, as well as the endogenous and exogenous factors that influence these biochemical pathways, so as to improve both preharvest management and postharvest handling to increase the final sensorial quality of ripe papayas. In papayas, the increment in soluble sugars occurs mainly during fruit growth while still attached to the plant (Zhou and Paull, 2001).

In most fleshy fruits, there are three main enzymes that have a key regulatory role in the accumulation of soluble sugars: sucrose phosphate synthase (SPS), sucrose synthase (SS), and acid invertase (AI; Zhou and Paull, 2001). In papayas, sugar accumulation begins after seed maturation and is accompanied by increased activity of SS during fruit development. Acid invertase also appears to increase throughout papaya development (Zhou and Paull, 2001), and its expression is reduced in harvested unripe papayas. Another increase in AI expression has also been observed after the onset of ethylene production during ripening (Gomez et al., 1999). Sucrose phosphate synthase activity remains low throughout papaya development however (Zhou and Paull, 2001). After harvesting, SPS activity follows the tendency of sucrose formation, since the ratio between SPS activity and sucrose content is constant throughout the papaya ripening process (Gomez et al., 2002). SPS is a highly conserved glycosyltransferase in dicots that catalyzes the transfer of glucose from uridine diphosphate glucose (UDP-Glc) to D-fructose-6-phosphate, thereby forming D-sucrose-6-phosphate (Castleden et al., 2004). As SPS also catalyzes the reversible reaction, it is considered as a key control point of sucrose biosynthesis in both monocots and dicots (Huber and Huber, 1996). Sucrose synthase is also a glycosyltransferase, but it catalyzes the reversible formation of UDP-Glc and D-fructose from UDP and D-sucrose (Zheng et al., 2011). Although SS could act in Glc linked to other nucleotide diphosphate sugars than UDP, such as adenosine diphosphate glucose (ADP-Glc), UDP is the preferred substrate in plants (Kleczkowski et al., 2004). Finally, AI can control the balance between sucrose, glucose, and fructose in fleshy climacteric fruits by an irreversible reaction that cleaves sucrose (Moriguchi et al., 1992).

Climacteric fruits, such as bananas, commonly increase soluble sugars content after harvesting through starch degradation, which directly correlates with pulp sweetening (Shiga et al., 2011; Aquino et al., 2016). Since unripe papayas have low starch content (less than 3% by fresh weight; Oboh et al., 2015), most of the soluble sugars in papayas accumulate during fruit development. However, there is also an increase in sucrose, glucose, and fructose, as well as a pattern of expression and activity of both AI and SPS during ripening (Gomez et al., 1999, 2002). These results suggest a possible role for ethylene-mediated effects on soluble sugar accumulation in ripe papayas. This hypothesis was confirmed by a previous study of our group (Fabi et al., 2007), which demonstrated that 1-MCP-treated papayas have a distinct pattern of sucrose synthesis during ripening compared to untreated papayas. More recently, Shen et al. (2017) showed that other genes related to soluble sugar metabolism, including *UDP-galactose transporter 3* (*UTR3*), *sugar transporter* (*STP*), and *β-fructofuranosidase* (*BFF*), were induced during the ripening of ethylene-treated papaya and reduced in 1-MCP-treated papaya. However, despite ethylene appearing to be important in enhancing *UTR3*, *STP*, and *BFF* expression, it is unknown whether ethylene-induced changes in the expression pattern of these enzymes affect soluble sugar metabolism during ripening and, therefore, the sensorial quality of papaya.

During papaya ripening, the sucrose content appears to reduce after the onset of ethylene production, which is in agreement with the increase in AI expression (Gomez et al., 2002; Nogueira et al., 2012). In contrast, 1-MCP-treated papayas have been found to have a 10-fold higher level of sucrose compared to untreated ripe fruit (Fabi et al., 2007). Thus, as AI activity appears to be strongly regulated by ethylene during papaya ripening (**Figure 2**), exogenous treatments or conditions that affect ethylene production may affect the ratios between sucrose, glucose, and fructose, thereby influencing pulp sweetness.

The use of gamma irradiation in fleshly fruits such as guavas (Zhao et al., 2017) and tomatoes (Guerreiro et al., 2016) could represent an effective method for fruit decontamination, thus reducing postharvest losses (Farkas and Mohácsi-Farkas, 2011). Depending on the intensity of the applied gamma irradiation, the sensorial quality of fruits could be negatively affected because of irradiation-induced changes in fruit metabolism. In papaya, the application of standard irradiation intensities between 0.5 and 1.0 kGy in unripe fruit did not appear to negatively influence fruit ripening (Paull, 1992). However, analysis of fruit metabolism revealed that these gamma irradiation intensities could reduce soluble sugars content in ripe papayas. This reduction appears to be related to a decrease in AI activity, and these changes are associated with reduced ethylene production throughout the ripening of the irradiated fruit (Gomez et al., 1999).

In addition to gamma irradiation, ozone application has been proposed as a method for fruit decontamination. Furthermore, ozone treatment is used to extend shelf life by reducing oxygen concentrations during fruit storage and shipping, thereby delaying the ripening of climacteric fruits (Chrysargyris and Tzortzakis, 2017). Thus, as ozone influences fruit respiration and therefore the onset of ethylene production in climacteric fruits, it is expected that this postharvest treatment will also affect soluble sugar metabolism during papaya ripening. Although a previous study did not report significant differences between the total soluble solid content of ozone-treated and untreated papayas (Bataller et al., 2012), the soluble sugars ratio between sucrose and glucose/fructose in ripe fruits could be altered. Recently, the treatment of unripe papaya with plant extracts, such as Neem (*Azadirachta indica* Juss), has been proposed as an alternative for maintaining food quality for a longer postharvest period (Freitas et al., 2018). However, as with ozone treatment, the observation of fruit quality maintenance for a longer period was not accompanied by an evaluation of soluble sugar metabolism. Therefore, further studies are needed to confirm the effects of

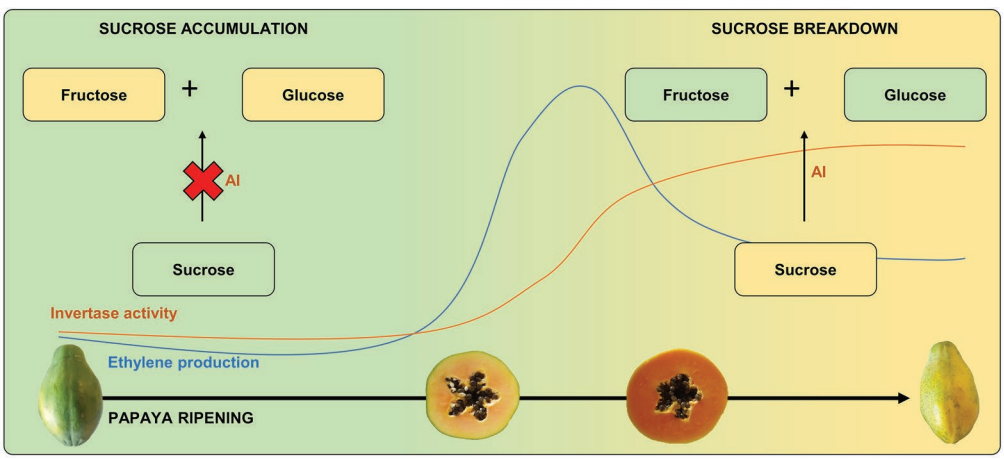

FIGURE 2 | Ethylene production and invertase activity during papaya ripening: papaya sucrose breakdown. Invertase activity is regulated by ethylene burst since sucrose is higher in unripe papayas or in papayas in which ethylene perception is blocked, with a subsequent increase in fructose and glucose after ripening/ethylene production. AI, acid invertase.

ozone as well as other postharvest treatments that may affect ethylene production, since there is a clear role of ethylene on enzymes that orchestrate the metabolism of soluble sugars during papaya ripening.

CLIMACTERIC ALTERATION OF PAPAYA FLAVOR

Papayas have a characteristic sweet flavor that has been studied for more than half a century (Katague and Kirch, 1965; Flath and Forrey, 1977). The volatile profile of papaya consists of a mixture of compounds including esters, terpenes, alcohols, and ketones (Pino et al., 2003; Fuggate et al., 2010; Pino, 2014; Jing et al., 2015; Kelebek et al., 2015). Although there is great heterogeneity among the volatile profiles of distinct papaya varieties (Ulrich and Wijawa, 2010; Jing et al., 2015; Kelebek et al., 2015), some compounds appear to be characteristic of the papaya aroma. In this context, linalool and their oxidative derivatives are generally regarded as the main volatile compounds in most of the distinct cultivars of papaya (Devitt et al., 2006; Gomes et al., 2016; Lieb et al., 2018) along with low-molecular weight esters, including ethyl butanoate and methyl butanoate (Almora et al., 2004; Balbontín et al., 2007; Pino, 2014). Considering that the increase in volatile esters is significantly higher in harvested papayas compared to fruit that is still attached to the plant (Fuggate et al., 2010), and considering the magnitude of difference between the volatile profiles of unripe and ripe papayas (Fuggate et al., 2010; Gomes et al., 2016), it appears that ethylene plays an important role in the development of flavor during papaya ripening.

Balbontín et al. (2007) suggested that most of the volatile esters synthesized during papaya ripening are derived from primary and secondary metabolism compounds, such as fatty acids and amino acid. The release of these compounds is stimulated by ethylene treatment (Defilippi et al., 2005; Li et al., 2016). Ethyl acetate, ethyl octanoate, and methyl hexanoate were also found to not be induced in 1-MCP-treated papayas, whereas ethylene-induced papayas increased the amounts of these volatile esters throughout ripening (Balbontín et al., 2007). Interestingly, volatile esters with a higher molecular weight, including butyl hexanoate and octyl acetate, reached higher values in 1-MCP-treated papayas compared to both untreated and ethylene-treated papayas. These results suggests that only the synthesis of the main esters related to aroma quality in ripe papaya—which are those volatile compounds with lower molecular weight produced from C1 and C2 alcohols and C6 and C8 acyl-coenzyme A—were enhanced during the onset of ethylene production (Balbontín et al., 2007).

The volatile profile of ripe papayas also consists of branched-chain volatiles (Rocha et al., 2017; Lieb et al., 2018) derived mainly from the amino acid precursors isoleucine and valine, which are responsible for the formation of ethyl-2-methyl and butyl-2-methyl esters. The synthesis of these branched-chain volatiles also appears to be regulated by ethylene, as 1-MCP-treated papayas have reduced ethyl-2-methyl butanoate levels (Balbontín et al., 2007).

The abovementioned results regarding the synthesis of volatile compounds during ripening provide insights into the development of aroma in ripe papayas. However, little is known about the relationships among the metabolism of these volatile compounds and the sensorial quality of the ripe fruit. In this context, a recent study applied a gas chromatography-olfactometry (GC-O)-assisted approach to optimize the extraction and detection of the main volatile compounds responsible for the aroma of ripe papayas (Rocha et al., 2017). In GC-O, a panel of human assessors describes the aroma of each of the volatile compounds from a sample that has been previously separated through gas chromatography, allowing the identification of the main peaks responsible for the overall aroma of the sample (Brattoli et al., 2011). In summary, GC-O refers to the use of human assessors as sensitive and selective detectors of odor-active compounds (Delahunty et al., 2006), and it is a useful tool to assess the contribution of each volatile compound to a fruit's aroma. Studies have successfully applied GC-O-assisted approaches or aroma dilution analysis to assess the volatile profile of papayas (Jirovetz et al., 2003; Pino, 2014; Rocha et al., 2017). Jirovetz et al. (2003) and Pino (2014) found linalool as the major compound in papaya flavor. However, the major compounds considered as odor-active and contributors to the typical papaya aroma found in other studies were δ-octalactone (sweet and herbal), benzyl isothiocyanate (papaya), methyl butanoate (fruity), and ethyl butanoate (fruity; Pino, 2014; Rocha et al., 2017).

Gomes et al. (2016) explored the volatile profile of papayas in response to cold storage, which clearly affects ethylene production (Bron and Jacomino, 2009). The authors explored if the cold storage of papayas at temperatures in which the fruit is resistant to cold injury influenced the volatile profile in ripe papayas. The authors found that when papayas were left at 10°C for 10 days and then subsequently at ambient temperature to complete the ripening process, the fruits were able to restore ethylene production, as well as the development of the loss of green color and the increase in pulp softening to a similar extent to that of fruit stored at ambient temperature, but the process was postponed by a few days. However, there were striking differences between the volatile profiles of the two groups. Interestingly, the synthesis of linalool, regarded in GC-O as one of the main volatile compounds in papaya, was affected by cold storage. These reduced linalool levels in cold-stored papayas appeared to be related to the down-regulation of *linalool synthase* (*LIS*) expression (Gomes et al., 2016). Façanha (2016) also found reduced levels of linalool throughout the ripening of 1-MCP-treated papayas and increased levels of this volatile compound in ethylene-treated papayas. Thus, as LIS uses geranyl diphosphate (GPP) to synthesize linalool in a single-step reaction (Gutensohn et al., 2013), the reduced *LIS* expression, and therefore reduced levels of linalool in both cold storage papayas and in 1-MCP-treated papayas, strongly suggests a possible role of ethylene in linalool biosynthesis through modulation of *LIS* expression.

GPP originates from the plastid-localized 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, which is important

not only in the biosynthesis of linalool and other volatile compounds, including β-ionone and 6-methyl-5-hepten-2-one, but also in carotenoid biosynthesis and in the development of the characteristic of pulp color in ripe papayas.

PULP COLOR CHANGES IN RIPENING PAPAYAS AS A CONSEQUENCE OF CAROTENOID SYNTHESIS

The characteristic color of ripe papaya pulp (yellow or orange/red) is due to different types of carotenoids. Carotenoids are molecules with a general structure that consists of a 40-carbon acyclic polyene chain containing 9–11 conjugated double bonds and with or without terminating rings, and they are classified as carotenes (hydrocarbons) or as xanthophylls (oxygenated derivatives; Khoo et al., 2011). Distinct papaya varieties have different pulp colors depending mainly on their carotenoid metabolism during ripening. In general, orange/red varieties have relatively high amounts of lycopene, which is a central compound in the metabolism of carotenoids during papaya ripening and is responsible for the red color not only in papayas (Barreto et al., 2011) but also in tomatoes (Arias et al., 2000), guavas (Rojas-Garbanzo et al., 2017), and watermelons (Perkins-Veazie et al., 2006).

Most of the over 600 naturally occurring carotenoids (Sigurdson et al., 2017) originate from the MEP pathway (**Figure 3A**), which starts with a reaction between pyruvate and glyceraldehyde-3-phosphate, resulting in the downstream production of isopentenyl diphosphate (IPP) and dimethyl allyl diphosphate (DMAPP; Ruiz-Sola and Rodríguez-Concepción, 2012; Yang and Guo, 2014). Then, three IPP molecules and one DMAPP molecule are used as substrates by geranyl-geranyl diphosphate (GGPP) synthase for the synthesis of GGPP, a 20-carbon molecule (Majer et al., 2017). In addition to the presence of relatively high levels of lycopene, orange/red papayas present lower amounts of carotenoids that are synthesized downstream to lycopene in the MEP pathway, such as β-carotene, β-cryptoxanthin, and zeaxanthin (Schweiggert et al., 2011). For both papaya cultivars "Golden" and "Sunrise Solo," all-trans-lycopene was the main carotenoid in early stages and all-trans-β-cryptoxanthin was the main carotenoid in overripe fruits (Martins et al., 2016).

Yellow pulp varieties are characterized by the presence of these last carotenoids with very low to no detectable levels of lycopene (Shen et al., 2019). As the metabolism of papaya carotenoids starts from phytoene and occurs in a well-known cascade process (**Figure 3B**), it is possible to establish a relationship between the pattern of enzymes that acts downstream to phytoene and the color of papaya pulp during ripening. Geranyl-geranyl diphosphate is the precursor of chlorophylls, ubiquinones, and tocopherols. Phytoene synthase (PSY) uses two molecules of GGPP to produce phytoene, a colorless 40-carbon acyclic polyene molecule, which is the first step in carotenoid biosynthesis in the MEP pathway. Phytoene can be further used as a substrate by phytoene desaturase (PDS) to produce ζ-carotene, which can be a substrate for ζ-carotene desaturase (ZDS) for the synthesis of lycopene, a bright red carotenoid widely found in the pulp of orange/red papaya (Nisar et al., 2015). In yellow papayas, there is no significant accumulation of lycopene because of the conversion of phytoene by PDS and ZDS and by both lycopene β-cyclases (LCY-β) and carotene hydroxylases (CHYB). These enzymes rapidly convert lycopene into xanthophylls and β-carotene (Blas et al., 2010; Shen et al., 2019). In orange/red papayas, the initial stages of ripening are characterized mainly by the presence of xanthophylls, including β-cryptoxanthin, which are synthesized from lycopene downstream by lycopene β-cyclase (LCY-β; Blas et al., 2010; Schweiggert et al., 2011). However, after the onset of ethylene production in red/orange papayas, the conversion of lycopene into cyclic carotenoids appears to be strongly decreased due to lycopene accumulation in pulp (Barreto et al., 2011; Shen et al., 2019). The accumulation of lycopene in orange/red papayas compared to yellow papayas seems to occur both by a frame shift mutation in the *LCY-β2* gene, which results in a dysfunctional enzyme phenotype, and by other LCY genes (e.g., *LCY-β* and *LCY-ε*) that are

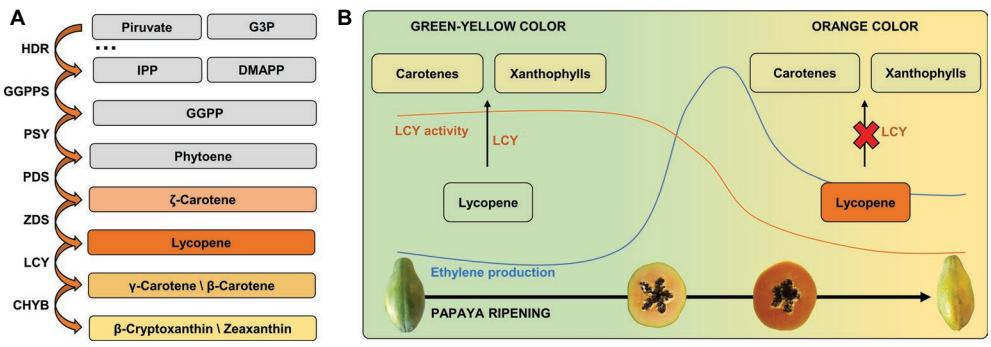

FIGURE 3 | Ethylene production and carotenoids accumulation (LCY activity) during papaya ripening: papaya's green/yellow color changing to orange/red color. **(A)** Carotenoids derivated from MEP pathway. **(B)** Papaya LCY activity during ripening drives the lycopene accumulation and pulp color changes through the decreased conversion of lycopene in carotenes and xanthophylls. G3P, glyceraldehyde-3-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethyl allyl diphosphate; GGPP, geranyl-geranyl diphosphate; HDR, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; GGPPS, geranyl-geranyl diphosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; LCY, lycopene cyclase; CHYB, carotene hydroxylase.

down-regulated during orange/red papaya ripening (Shen et al., 2017). The ζ-carotene desaturase enzyme responsible for converting phytoene into lycopene shows a different pattern of expression during ripening and also between the cultivars "Golden" and "Sunrise Solo," while the lycopene β-cyclase gene, responsible for converting lycopene to β-carotene, is up-regulated in both cultivars (Martins et al., 2016).

Interestingly, both ethylene- and 1-MCP-treated papayas had lower levels of minor carotenoids as compared to those of untreated papaya, similar to what was previously reported for the major carotenoids (Fabi et al., 2007; Barreto et al., 2011). Furthermore, the treatment of distinct papaya varieties with 1-MCP significantly reduced the carotenoid content in fruit pulp throughout ripening (Moya-León et al., 2004; Fabi et al., 2007; Barreto et al., 2011). Barreto et al. (2011) suggested that the impairment on carotenoid accumulation in papaya pulp by 1-MCP could occur either by the consumption of early carotenoid precursors including GGPP, or by inhibiting PSY or PDS activity. The latter hypothesis was confirmed by Fu et al. (2016), who revealed that a transcription factor (CpNAC1) induced by ethylene enhances the expression of PDS genes (e.g., *CpPDS2* and *CpPDS4*). Recently, Fu et al. (2017) provided new insights into the role of other transcription factors that regulate ethylene responses and are involved in the regulation of several genes related to carotenoid biosynthesis. Therefore, as with pulp softening, sweetness, and the development of flavor, the carotenoid content in papayas is also regulated by ethylene-mediated responses during fruit ripening. Thus, while further studies are needed to define the specific genes whose expression relates to changes in the carotenoid content in papaya pulp, it is known that the reduction of ethylene production at low temperatures influences the composition of carotenoids in ripe papaya pulp (Rivera-Pastrana et al., 2010).

CONCLUSIONS

Changes in the primary and secondary metabolism of papaya are mainly dependent on ethylene, whose onset burst occurs 2–3 days after the harvest of unripe fruit. Ethylene-triggered events during papaya ripening include an increase in PG and AI expression that are related to pulp softening and sweetening, respectively, as well as changes in carotenoid metabolism that influence both aroma and color, thereby leading to the formation of the expected quality attributes in ripe papaya. As ethylene-triggered events clearly affect the final quality of ripe papayas, studies have investigated the regulatory mechanisms that regulate ethylene function in papaya. Despite recent findings that highlight the ethylene-triggered events during papaya ripening, more efforts are needed to fully understand the key downstream regulators of ethylene in papaya pulp to better develop pre- and postharvest practices to extend papaya shelf life without resulting in losses in quality and nutritional aspects.

AUTHOR CONTRIBUTIONS

SP provided illustrations. Both SP and JF wrote the manuscript, contributed to critical review of the manuscript, and revised and approved the manuscript.

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Central Metabolism Is Tuned to the Availability of Oxygen in Developing Melon Fruit

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Respiration of bulky plant organs such as fleshy fruits depends on oxygen (O_2) availability and often decreases with O_2 concentration to avoid anoxia, but the relationship between O_2 diffusional resistance and metabolic adjustments remains unclear. Melon fruit (*Cucumis melo* L.) was used to study relationships between O_2 availability and metabolism in fleshy fruits. Enzyme activities, primary metabolites and O_2 partial pressure were quantified from the periphery to the inner fruit mesocarp, at three stages of development. Hypoxia was gradually established during fruit development, but there was no strong oxygen gradient between the outer- and the inner mesocarp. These trends were confirmed by a mathematical modeling approach combining O_2 diffusion equations and O_2 demand estimates of the mesocarp tissue. A multivariate analysis of metabolites, enzyme activities, O_2 demand and concentration reveals that metabolite gradients and enzyme capacities observed in melon fruits reflect continuous metabolic adjustments thus ensuring a timely maturation of the mesocarp. The present results suggest that the metabolic adjustments, especially the tuning of the capacity of cytochrome c oxidase (COX) to O_2-availability that occurs during growth development, contribute to optimizing the O_2-demand and avoiding the establishment of an O_2 gradient within the flesh.

Keywords: fruit, *Cucumis melo*, hypoxia, metabolism, modeling, cytochrome c oxidase

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INTRODUCTION

Bulky organs such as fruits, tubers or roots are prone to hypoxia and are known to decrease respiration with O_2 concentration to avoid anoxia (Geigenberger, 2003). Their respiration usually follows Michealis-Menten kinetics but the involved mechanisms are still unclear (Ho et al., 2011). There is also the widespread idea that bulky organs are characterized by up to strong O_2 gradients, as for example in potato tubers (Geigenberger, 2003). On the one hand, diffusional resistance in bulky organs has been invoked as explaining the occurrence of O_2 gradients, which result in limited availability of O_2 to respiration (Ho et al., 2011). On the other hand the recent finding of an O_2 sensor in Arabidopsis (Licausi et al., 2011) suggests that plant tissues are capable of metabolic adjustments limiting respiration, which occur well above the K_m value for O_2 of cytochrome c

oxidase (COX). Strikingly, whereas the K_m value of COX is often invoked in literature dealing with hypoxia, its capacity is rarely taken into account, although its modulation is a further and obvious control point for respiration. The picture is further complicated by the fact that hypoxia is actually needed in a range of processes associated to ripening. Thus, the production of a range of volatiles involves alcohol dehydrogenases (ADHs) and further component of fermentation (Manriquez et al., 2006). Due to its large size and easily accessible flesh, melon (*Cucumis melo* L.) is an interesting model to study relationships between oxygen availability and metabolism as influencing fruit growth and quality. Indeed, the balance between respiration and fermentation is at the heart of fruit development and the establishment of their quality during ripening (Pesis, 2005). Besides, melon is an economically important crop with an expanding world production situated around 32 million tons in 2012[1]. It is a member of the Cucurbitaceae family, which represents a yearly world market of hundreds of billions of dollars and 50 kg per person, with watermelon alone being the second most produced fruit in the world[1].

Whilst fruit flavor quality is largely the result of volatile compounds and their contribution to aroma, sweetness depends upon sugar concentration and the ratio between sugars and acids. Thus, melon fruits at commercial maturity are characterized by high contents of sucrose, glucose and fructose, as well as by a low organic acid content and the absence of starch (Lingle and Dunlap, 1987; Schaffer et al., 1987; Hubbard et al., 1989; Burger et al., 2000). The accumulation of these three soluble sugars appears to be controlled by carbohydrate metabolism in the fruit sink itself. Indeed, in the Curcubitaceae family, sucrose and the galactosyl-sucrose oligosaccharides, raffinose and stachyose, are synthetized in the leaf source and translocated to the fruit sink (Mitchell et al., 1992; Fiehn, 2003). Following which, raffinose and stachyose do not accumulate in the fruit, thus indicating a rapid metabolic conversion. Melon is a highly polymorphic species, in which variations in fruit sugar and acid contents are not just under genetic control, but are also influenced by the environment (Burger et al., 2000; Burger and Schaffer, 2007; Tang et al., 2012; Cohen et al., 2014). Numerous studies of fruit growth and ripening have shown that melon fruits undergo dramatic metabolic transitions. In particular, the accumulation of sucrose occurring at ripening has been associated with the loss of soluble acid invertase (AI) and a concomitant increase in sucrose phosphate synthase (SPS) (McCollum et al., 1988; Hubbard et al., 1989; Iwatsubo et al., 1992; Lester et al., 2001; Burger and Schaffer, 2007). However, both sucrose synthase (SuSy) and neutral invertase (NI) also show increased activities at ripening (Schaffer et al., 1987; Hubbard et al., 1989).

Recently, metabolomics approaches have been developed to identify key metabolites and their variations in fruits of various melon cultivars, including spatial and developmental measurements (Biais et al., 2009; Moing et al., 2011; Bernillon et al., 2013). Data analysis revealed several gradients of metabolites in fruit flesh at maturity such as sucrose, alanine, valine, γ-aminobutyric acid (GABA) and ethanol (Biais et al., 2010), which can be related with differences in metabolism. A decrease in the ATP/ADP ratio was also found to be associated with changes in alanine, GABA and ethanol, and was interpreted as being the result of acclimation to oxygen limitation as operated via alcoholic fermentation.

In the present study, metabolic changes occurring from the periphery to the fruit center and across three stages of fruit development were investigated using a multilevel approach. Thus major primary metabolites were determined by proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and gas chromatography coupled to mass spectrometry (GC-EI-TOF/MS) to investigate how and when metabolic gradients take place during melon fruit development. Enzyme activities of sucrose metabolism, tricarboxylic acid (TCA) cycle and glycolysis were measured using robotized assays and a microplate-based assay for COX capacity was developed to investigate its involvement in fruit central metabolism. Oxygen partial pressure was measured by using an oxygen-sensitive optical glass-sensor and a model was built to calculate steady state O_2 concentrations at any position within the mesocarp based on O_2 diffusion and consumption. The calculation of O_2 demand using a construction cost model took in account the rates of biomass production at different depths within the mesocarp. By combining these approaches we investigated the relationship between O_2 diffusional resistance and metabolic adjustments occurring in melon fruit development.

MATERIALS AND METHODS

Melon Growth and Sample Handling

Melon plants (*Cucumis melo* L. var. Cantalupensis group Charentais cv. Escrito) were grown in an open field (9200 plants ha^{-1}) in Moissac (France, 44° 6′ 17″ N, 1° 5′ 6″ E) between April and August 2008 and Sainte-Livrade (France, 44° 23′ 56″ N, 0° 35′ 25″ E) between May and September 2011. Irrigation, fertilization and pathogen-pest control were performed according to standard commercial practices (Bernillon et al., 2013). Melon fruits were harvested at four stages of development (**Figure 1A**). Stage 1 corresponds to developing fruits with a diameter of 80 mm, Stage 2 to developing fruits with a diameter of 100–110 mm just before the appearance of the suberized net on the skin, Stage 3 to early ripening fruits with a fruit diameter of 130–135 mm and Stage 4 to ripening fruits at the beginning of abscission (commercial maturity). For O_2 tension measurements in melon mesocarp, 32 melons were harvested from Stages 1–3 and kept at laboratory temperature during the measurements. For ^{1}H-NMR analysis of polar compounds and enzyme assays, stages 2–4 were used. Nine melons were selected to make three homogeneous lots (biological replicates) of three fruits each. Two slices of 1 cm thickness were cut in the equatorial plane of each fruit. The skin and seeds were removed and five concentric mesocarp rings of flesh (7 ± 2 mm width) were taken from the periphery (outer mesocarp, named location 1) to the centre (inner mescocarp, named location 5) as explained in Biais et al. (2010) and **Figure 1B**. The flesh rings of a given position taken from a given fruit lot were pooled and

[1]http://faostat.fao.org/

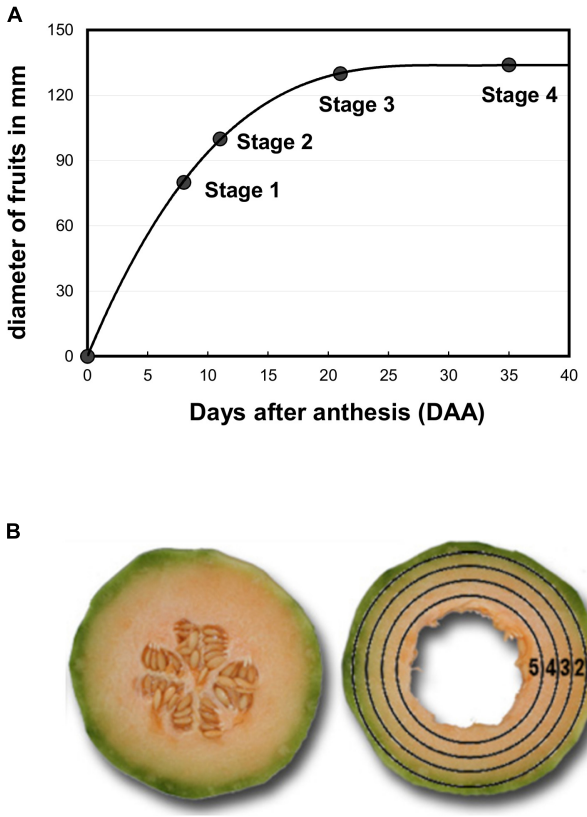

FIGURE 1 | (A) Growth curve of melon fruits (*Cucumis melo* L. var Cantalupensis group Charentais cv. Escrito). The diameter of harvested fruits was measured at each stage of development and was used to assess the growth curve. Melon fruits at stages 1, 2, 3, and 4 correspond to fruits harvested at 8, 11, 20, and 34 days after anthesis (DAA). **(B)** Sample preparation for biochemical analyses. A slice (7 ± 2 mm width) was first cut at the equator of the fruit. The seeds and placenta were removed, and then five concentric rings were cut in the slice and immediately frozen in liquid nitrogen. Position 1 corresponds to both the epicarp and green mesocarp, and positions 2–5 to the orange mesocarp.

immediately frozen in liquid nitrogen, before then being stored at −80°C. These were subsequently ground in liquid nitrogen and the 45 powdered samples (three stages × three biological replicates × five flesh positions) were further stored at -80°C until further processing. For ^{1}H-NMR analysis an aliquot of each sample was lyophilized. In parallel, dry matter content was determined using 250 mg FW powder aliquots dried in a 70°C oven.

Chemicals

All chemicals and substrates used for chemical and biochemical analyses were purchased from Sigma-Aldrich Ltd. (Gillingham, United Kingdom), except for acetyl coenzyme A, adenosine-5′-triphosphate (ATP), dithiothreitol, *n*-dodecyl β-D maltopyranoside, leupeptin, nicotinamide adenine dinucleotide (NAD), NADH, nicotinamide adenine dinucleotide phosphate (NADP), NADPH and phosphoenolpyruvate, that were purchased from Roche Applied Science (Meylan, F). All enzymes were purchased from Roche Applied Science (Meylan, F) except aldolase (from rabbit muscle), glycerokinase (from *E. coli*) and triose phosphate isomerase (from rabbit muscle) that were purchased from Sigma-Aldrich Ltd. (Gillingham, United Kingdom). Bradford reagent was purchased from Bio-Rad (Marnes-la-Coquette, F). Reagents used for mRNA quantification were purchased from Fisher Scientific (Illkirch, F), Qiagen (Courtaboeuf, F), Bio-Rad (Mitry-Mory, F), and Promega (Charbonnières-les-Bains, F).

^{1}H NMR and GC-EI-TOF/MS Analyses of Polar Metabolites of Ground Flesh Samples

Polar metabolites were extracted from ground melon samples corresponding to the five concentric mesocarp rings of flesh. For NMR analyses the frozen powdered samples were lyophilized and polar metabolites were extracted from 50 mg of lyophilized powder successively with 2 ml of ethanol/water mixtures: 80/20, 50/50 (v/v) and pure water (4 ml) for 15 min at 80°C. The supernatants were combined, dried under vacuum and lyophilized. The pellet was kept for the determination of protein content. Two technical replicates were prepared per biological sample. The lyophilized extracts were mixed with 500 µl of 400 mM potassium phosphate buffer solution pH 6.0, 1 mM ethylene diamine tetraacetic acid disodium salt (EDTA), in D_2O. The pH was the adjusted to 6 with KOD when necessary, and lyophilized again. The lyophilized extracts were stored in darkness under vacuum at room temperature, before ^{1}H-NMR analysis which was completed within 1 week. For ^{1}H-NMR analysis, each dried pH-adjusted extract was solubilized in 0.5 ml of D_2O with (trimethylsilyl) propionic-2,2,3,3-d4 acid (TSP) sodium salt (0.01% final concentration for chemical shift calibration), centrifuged at 10,000 g for 5 min and transferred into an 5 mm NMR tube. Quantitative ^{1}H-NMR spectra were recorded at 500.162 MHz and 300 K on a Bruker Avance spectrometer (Wissembourg, France), using a 5 mm inverse probe and an electronic reference for quantification as described previously (Mounet et al., 2007; Biais et al., 2009; Moing et al., 2011). For GC-EI-TOF/MS, 100 mg aliquots (± 2 mg) of frozen ground samples were extracted with 1 ml chloroform/methanol/water (1:2.5:1), the polar metabolite fraction was obtained by further addition of 0.5 ml of water and dried by speed vacuum concentration. The extracts were derivatized and analyzed by GC-EI-TOF/MS on an Agilent 6890N gas chromatograph (Stockport, United Kingdom), coupled to a Leco Pegasus III mass spectrometer (St Joseph, United States). Chromatographic deconvolution was performed within the LECO ChromaTof v2.15 software, the extracted peak areas for each of the deconvolved metabolite features were normalized against the succinic-*d4* acid internal standard. The detailed procedures for extraction, derivatization, sample analysis, mass spectral deconvolution and metabolite identification, have been described in detail previously (Allwood et al., 2009; Biais et al., 2009; Moing et al., 2011). The full dataset can be found at http://www.cbib.u-bordeaux2.fr/MERYB/public/PublicREF.php?REF=M08002.

Protein Content

The pellet was resuspended in 1 ml 100 mM NaOH and heated for 30 min at 95°C. After cooling to room temperature and centrifugation at 5,000 g, the protein content of the remaining supernatant was determined according to Bradford (1976).

Enzyme Activity Measurements

Aliquots of 50 mg fresh weight (FW) were extracted by vigorous shaking with 500 µl of extraction buffer composed of 20% (v/v) glycerol, 0.25% (w/v) bovine serum albumin, 1% (v/v) Triton-X100, 50 mM HEPES/KOH pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM ε-aminocaproic acid, 1 mM benzamidine, 10 mM leupeptin, 0.5 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride, which was added just prior to extraction. The following enzyme activities (SPS, SUSY, acid invertase, PFP, PFK, MDH, PK, PEPC, CS, IDH, see **Figure 3** legend for the full enzyme name) were assayed using a robotized platform as described in Gibon et al. (2004, 2006, 2009), Studart-Guimaräes et al. (2005), and Steinhauser et al. (2010).

Enolase was assayed in the direction of phospho*enol*pyruvate production as described previously (Burell et al., 1994). The assay consisted of 10 µl desalted extract in 100 mM HEPES/NaOH (pH 7.5), 10 mM $MgCl_2$, 0.2 mM NADH, 2.7 mM ADP, 5 units.ml^{-1} pyruvate kinase and 6 units.ml^{-1} lactate dehydrogenase. The reaction was initiated by the addition of 2-phosphoglycerate to a final concentration of 0.5 mM.

NADP dependent malic enzyme activity (NADP-ME) was assayed using a protocol adapted from Wheeler et al. (2005). The assay was performed with 2 µl of extract in 100 mM HEPES/KOH (pH 7.5), 10 mM $MgCl_2$, 1 mM $NADP^+$ and 0.05% (v/v) Triton X100. The reaction was initiated by addition of malate to a final concentration of 10 mM and a final volume of 20 µl. After 40 min of incubation at 25°C, the reaction was stopped by the addition of 20 µl of HCl 0.5 M/Tricine/KOH 0.1 M (pH 9). Following sample mixing and a 10 min delay, the acid was neutralized by the addition of 20 µl of 0.5 M NaOH. The quantification of phospho*enol*pyruvate was then performed by adding 100 mM HEPES/KOH (pH 7.5), 10 mM $MgCl_2$, 0.05% (v/v) Triton X100 and 1 mM NADH in a final volume of 110 µl. Absorbance at 340 nm was recorded until stabilized, then 2 µl of lactate dehydrogenase 100 units.ml^{-1} were added to start the reaction and absorbance was again recorded until stabilized.

Alcohol dehydrogenase (ADH) activity was assayed using 5 µl of extract in 100 mM HEPES/KOH (pH 7.5), 0.1 mM EDTA, 1.2 mM NAD^+, 1 mM thiazolyl blue tetrazolium bromide (MTT), 0.2 mM phenazine ethosulfate and 0.05% (v/v) Triton X100. The reaction was initiated by the addition of ethanol to a final concentration of 25 mM and a final volume of 100 µl. Absorbance was read at 600 nm until rate stabilized.

The respiratory chain enzymes, i.e., cytochrome *c* oxidase (COX) and succinate dehydrogenase (SDH), were assayed as described in Rustin et al. (1994) and Vinogradov et al. (1980), respectively, using a modified extraction procedure adapted to plant tissues. Briefly, aliquots of 100 mg FW powder were extracted at 4°C by vortexing with 500 µL of potassium phosphate buffer solution (50 mM potassium phosphate pH 7.2, 1 mM EDTA) supplemented with 1% (w/v) *n*-dodecyl β-D maltopyranoside. To eliminate low molecular weight reducing compounds and to decrease the detergent concentration, the homogenate was subsequently filtrated through a 2 ml column of Sephadex G25 coarse medium (Sigma-Aldrich, Lyon, France), equilibrated at 4°C with the phosphate buffer solution supplemented with 0.1% (w/v) *n*-dodecyl β-D maltopyranoside. Sephadex columns were then centrifuged at 4°C for 2 min at 2400 *g* and the filtrate stored on ice. The enzyme recovery was checked using isolated mitochondria for which COX and SDH activities can be measured before and after filtration. SDH was assayed at 25°C using the phenazine methosulfate-(PMS) mediated reduction of dichloroindolphosphate (DCIP) monitored at 600 nm with a spectrophotometer. The SDH assay consisted of 10–50 µL of filtrate in a final volume of 1 ml of phosphate buffer solution (pH 7.2) containing 0.1% (w/v) *n*-dodecyl β-D maltopyranoside, 5 mM succinate, 0.1 mM KCN and 0.05 mM DCIP. The reaction was initiated by the addition of PMS to a final concentration of 1.6 mM. COX was assayed at 25°C by monitoring the oxidation of reduced cytochrome *c* at 550 nm. The COX assay consisted of 10–50 µL of filtrate in a final volume of 1 ml of phosphate buffer solution (pH 7.2) containing 0.1% (w/v) *n*-dodecyl β-D maltopyranoside. The reaction was initiated by the addition of chemically-reduced cytochrome *c* at a final concentration of 50 µM. The specificity of the assay was checked by addition of KCN.

O_2 Measurements Within the Melon Mesocarp

The O_2 tension (expressed in kPa) was measured by using an oxygen-sensitive optical glass-sensor (microsensor, PreSens, Neuburg, DE) connected to a fiber optic oxygen meter (MicroX TX3 PreSens, Neuburg, DE), and was based upon dynamic fluorescence quenching. The O_2 microprobe tip has a diameter of 140 µm and is protected by a hypodermic needle linked to a syringe. In contrast to oxygen microelectrodes, this oxygen microprobe does not consume oxygen and shows no stirring dependence of the signal, and thus prevents the establishment of an artificial oxygen sink within the measured tissue. The microsensor was calibrated in water that had been well equilibrated with ambient air (21 kPa O_2) and also in water that had been depleted of oxygen with Na_2SO_3. The electrode signal was stable for at least 4 h. The fruit was first placed on a support and fixed. Subsequently, the microsensor was positioned on the fruit surface and driven into the fruit by a micromanipulator at 5 mm intervals. Just after the insertion of the needle, the microsensor occupied the small hole in the tissue at the needles entry point, without being in contact with the outside. At each position the sensor was paused for approximately 120 s to allow equilibration and to obtain a continuous measurement. The mean of 100 measurements with a standard error of less than 5% represents a single data point in the subsequently presented figures. From 32 melons harvested at stages 1–4, 16 were used within 48 h to measure O_2 tension at three different depths (7.5, 12.5, and 17.5 mm) in the mesocarp and at three different equatorial positions. Only in mature fruits (stage 4), the O_2

tension was measured deeper into the mesocarp (22.5, 27.5, and 32.5 mm). After the measurements were performed, the melon fruits were sliced at the level of the measurement transect to verify the exact position of the sensor tip within the distinct zones of the mesocarp.

Modeling of the Oxygen Gradient Within the Melon Mesocarp

The oxygen concentration within the melon was modeled assuming that the steady state oxygen concentration at any position within the mesocarp is a function of the oxygen diffusion and consumption within the tissue. This was achieved by solving the time-dependent diffusion equation in spherical co-ordinates with spherical symmetry (McElwain, 1978):

$$\frac{\partial C}{\partial t} = D\left(\frac{\partial^2 C}{\partial R^2} + \frac{2}{R}\frac{\partial C}{\partial R} - \frac{\alpha C}{C + K_\mathrm{m}}\right)$$

with the following dimension-less variables:

$$C = \frac{P}{P_0}\ ;\quad R = \frac{r}{r_0}\ ;\ \alpha = \frac{V_\mathrm{oxygen} r_0^2}{P_0 D};\ K_\mathrm{m} = \frac{k_\mathrm{m}}{P_0}$$

Where: R is the relative distance from the centre, r_0, the radius of the melon, D, the diffusion coefficient within the mesocarp (D = 2.4 10^{-5} cm^2 s^{-1}) and P_0, the oxygen tension outside the melon (21 kPa, at 25°C). K_m is the half saturation constant of the terminal oxidase of the respiratory chain. It was equal to either 0.108 or 0.134 kPa, for the COX or the alternative oxidase, respectively (Armstrong and Beckett, 2011). These values were assumed to be constant as a function of the depth (i.e., as a function of the respiratory rate). V_oxygen is the O_2 consumption rate of the tissue, which was estimated using a construction cost model (see below) and was computed as a depth-dependent polynomial function (see **Supplemental Figures S1**, **S2**).

The steady state problem ($\partial C/\partial t = 0$) was solved using the PDEX4 function in Matlab 2007 and the following initial and boundary conditions:

at $t = 0$, $C = 0$

at $r = 0.5\ r_0$, $\frac{dC}{dR} = 0$

at $r = r_0$, $\frac{dC}{dR} = \frac{hr_0}{D}(1 - C)$

where h is the permeability coefficient of the melon skin, expressed in cm s^{-1}.

The Matlab script is provided within the **Supplementary Material** (Note 1 in **Supplemental Data**).

For each developmental stage, an h-value was obtained by fitting the O_2 measurements and minimizing an *Obj* score, i.e., the sum of the squared residuals weighed by the standard deviation of each measurement, according to the following equation:

$$Obj = \sum_{\mathrm{i}=1}^{n}\left(\frac{[\mathrm{O_2}]_\mathrm{i}\, cal - [\mathrm{O_2}]_\mathrm{i}\, exp}{\sigma_\mathrm{i} exp}\right)^2$$

where n is the number of depths, $[O_2]_\mathrm{i}cal$, and $[O_2]_\mathrm{i}exp$, the calculated and experimental values of O_2 concentration at a given depth and $\sigma_\mathrm{i}exp$, the standard deviation of the measures.

Calculation of the Oxygen Demand Using a Construction Cost Model

Rates of biomass production at different depths within the mesocarp were calculated by expressing biomass as a function of time for five layers of equal thickness. For this, considering that the fruit is a sphere, the volumes of the six corresponding layers nested into one another's spheres, were calculated throughout development, based on estimates of the changes in fruit diameter, and subtracted to obtain the volumes of the sectors (see **Supplemental Figures S1**, **S2**). Volumes were then converted to biomass using density estimates and fitted polynomials that were integrated to obtain the rates of biomass production.

The flux of oxygen consumption was calculated at each stage and for each sector as the sum of the growth-linked and maintenance respiration, according to the following equation (Heuvelink, 1995; Liu et al., 2007):

$$\frac{dC_\mathrm{respiration}}{dt} = q_\mathrm{growth} * \frac{dDW}{dt} + q_\mathrm{maintenance} * DW * Q_{10}^{(t^\circ - 20)/10} \quad (1)$$

where: $dC_\mathrm{respiration}/dt$ is the respiration-linked carbon consumption of the sector (g of C day^{-1} g^{-1} DW); dDW/dt and DW, the growth rate and the dry weight content of the sector, respectively, Q_{10}, the temperature-dependent coefficient for maintenance respiration (Q_{10} = 2) and $t°$, the growth temperature (averaged temperature of the culture was 25.4°C). q_growth, the carbon requirement for growth-linked respiration, is well known for a variety of fleshy fruits and is equal to 0.1 g C g^{-1} DW at 20°C (Heuvelink, 1995; Liu et al., 2007; Dai et al., 2010, and references therein). In contrast, $q_\mathrm{maintenance}$, the carbon requirement for cell maintenance, is hardly available for fruits and needs to be estimated. It was calculated assuming that the maintenance-linked respiration of aerobically grown cells is proportional to the amount of respiratory complexes and accounts for 10–20% of the complex IV (i.e., COX) activity (Devin et al., 2006, and references therein). Finally, the oxygen consumption rate (expressed in µmol O_2 min^{-1}.ml^{-1}) was calculated by using the sector volume, the fresh-to-dry weight ratio, assuming that the tissue density is equal to 1 g FW per ml and considering that the coefficient of respiration equals 1, i.e., neglecting the ethanol production vs. oxidative phosphorylation.

Total RNA Extraction and qRT-PCR Analysis

Total RNA was extracted from melon mesocarp tissues using Trizol reagent (Fisher scientific). One hundred milligrams of frozen mesocarp powder were homogenized with 1 ml of Trizol reagent and spiked with a mix of the five artificial poly(A+) RNAs (ArrayControl^{TM} Spots and Spikes, Fisher Scientific) to achieve absolute quantification of transcripts. Concentration of RNA spikes were 2.4 × 10^{11}, 4.8 × 10^{10}, 2.4 × 10^9, 2.5 × 10^7, and 4.0 × 10^6 copies per gram FW. Total RNA was purified with the RNeasy kit (Qiagen), then treated with the Turbo DNase

(Fisher Scientific). cDNA was synthesized from 500 ng total RNA using the iScript cDNA synthesis kit (Bio-Rad) and was diluted 5 times with nuclease free water. qRT-PCR reactions and data analysis were performed as described by Piques et al. (2009) with slight modifications. The 20 µl PCR mixture contained 2 µl of the diluted cDNA template, 10 µl of 2 × GoTaq® qPCR Master Mix (Promega), and 0.2 µM of the forward and reverse primers for each gene. The primers used to amplify melon genes were designed using primer3 software[2]. The primers for the RNA spike-in controls were designed as described in Pyl et al. (2012). All primer sequences are available in **Supplemental Table S2**. PCR reactions were run on the CFX96™ Real-Time PCR Detection System (Bio-Rad) under the following conditions: 95°C for 2 min, followed by 40 cycles at 95°C for 3 s, at the annealing temperature of 60°C for 30 s, and a dissociation curve analysis, 65–95°C with temperature increment of 0.5°C every 5 s. For each sample, cycle threshold values and copy numbers for the 5 spike-in controls were used to generate a standard curve. All standard curves, derived from the five spike-in controls, had R^2-values higher than 0.99 and were used to calculate the concentration of the mRNA as copy number g^{-1} FW and copy number mg^{-1} protein.

Statistical Analysis

Principal component analysis (PCA) and correlation analysis (Pearson coefficient) were performed using R[3] with the package FactomineR (Lê et al., 2008).

RESULTS

Melon fruits (*Cucumis melo* var. Cantalupensis group Charentais cv. Escrito) were grown in the open field under common agricultural practices; the fruits were ripe within 45 days post anthesis. The Escrito cultivar, which is widely cultivated in France, is considered as average regarding aromaticity and shelf-life (Allwood et al., 2014). To study the metabolic changes that occur during ripening, samples were taken during fruit growth (stage 2), at early fruit ripening (stage 3) and at fruit maturity (stage 4) (**Figure 1A**). For each stage, nine fruits were harvested and cut into five concentric mesocarp rings, the concentric tissue sections were pooled for three fruits and homogenized, thus creating three replicate samples each made up of three pooled fruits, prior to extraction and metabolite quantification (**Figure 1B**). For oxygen pressure measurements, an additional stage corresponding to fruit growth (stage 1) was used because data obtained at stage 2 appeared more scattered than at stages 3 and 4.

Maturation of the Melon Mesocarp Is Centrifugal

The changes in metabolites that occurred during fruit development in the five concentric mesocarp rings of flesh taken from the periphery (outer mesocarp, sector 1) to the centre (inner mesocarp, sector 5) are presented in **Figure 2** (see also **Supplemental Table S1**). Soluble sugars were the most abundant metabolites throughout fruit development. Whilst sugars such as stachyose and galactose exhibited a steady and very low content in the fleshy mesocarp at the three development stages (0.15 and 0.5 $\mu mol.g^{-1}$ FW, respectively), Suc, glucose (Glc), and fructose (Fru) (the major soluble sugars in melon) revealed different patterns. Suc exhibited a steady and relatively low content in the fleshy mesocarp around 20–40 $\mu mol.g^{-1}$ FW during fruit development (stage 2) and early fruit ripening (stage 3), whilst at fruit maturity (stage 4) a strong Suc gradient was observed from the periphery to the center of the fruit. Suc concentration increased regularly from the periphery (50 $\mu mol.g^{-1}$ FW, position 1) to the center of the fruit (200 $\mu mol.g^{-1}$ FW, position 5). In contrast, Glc and Fru concentrations were highest during fruit development, ranging between 80 and 110 $\mu mol.g^{-1}$ FW at stages 2 and 3 and decreased at fruit maturity (stage 4), with 60 and 80 $\mu mol.g^{-1}$ FW for Glc and Fru, respectively. The two hexoses exhibited a small gradient between outer and inner mesocarp at stages 2 and 3. The Glc and Fru concentrations increased regularly from the periphery (80 $\mu mol.g^{-1}$ FW, position 1) to the center of the fruit (110 $\mu mol.g^{-1}$ FW, position 4 and 5). At maturity the Glc gradient disappeared and the Fru gradient was inverted.

Among the five detected organic acids, pyruvate, fumarate and succinate showed the same pattern. During fruit development (stage 2) and at the early ripening stage (stage 3), they were almost undetectable, but increased dramatically at fruit maturity, where a marked gradient was also observed from the periphery to the center of the fruit. Malate and citrate had different patterns. With the exception of the outer mesocarp (position 1), malate levels remained low and stable throughout the rest of the fleshy mesocarp (less than 5 $\mu mol.g^{-1}$ FW), whilst a small gradient was apparent for citrate between the outer and inner mesocarp (15–20 $\mu mol.g^{-1}$ FW) during stages 2 and 3. At maturity, the citrate gradient was still apparent in the fleshy sectors, with slightly higher concentrations (20–25 $\mu mol.g^{-1}$ FW) than observed during the earlier developmental stages, further a strong increase of citrate was observed in the peripheral sector at full maturity.

Eleven and nine amino acids were measured by GC-EI-TOF/MS and ^{1}H-NMR, respectively. Among the eight major amino acids observed (**Figure 2**), five were found at concentrations greater than 2 $\mu mol.g^{-1}$ FW in the fleshy mesocarp at maturity (Ala, Gln, Glu, Asp, and GABA). All these amino acids, as well as the three aromatic amino acids, showed the same pattern of accumulation. Whilst no strong gradients were observed for amino acids during development, marked gradients were observed from the periphery to the center of the fruit for almost all amino acids measured at full maturity in this study. Interestingly, the fact that at ripening the protein content did not decrease while amino acids were strongly increased indicates that the latter increase was not due to proteolysis (**Supplemental Figure S3**). In conclusion, when comparing the metabolic shifts that occur throughout the different developmental stages of the melon fruit, characteristic changes in patterns of metabolite

[2]http://primer3.ut.ee/

[3]http://www.r-project.org/

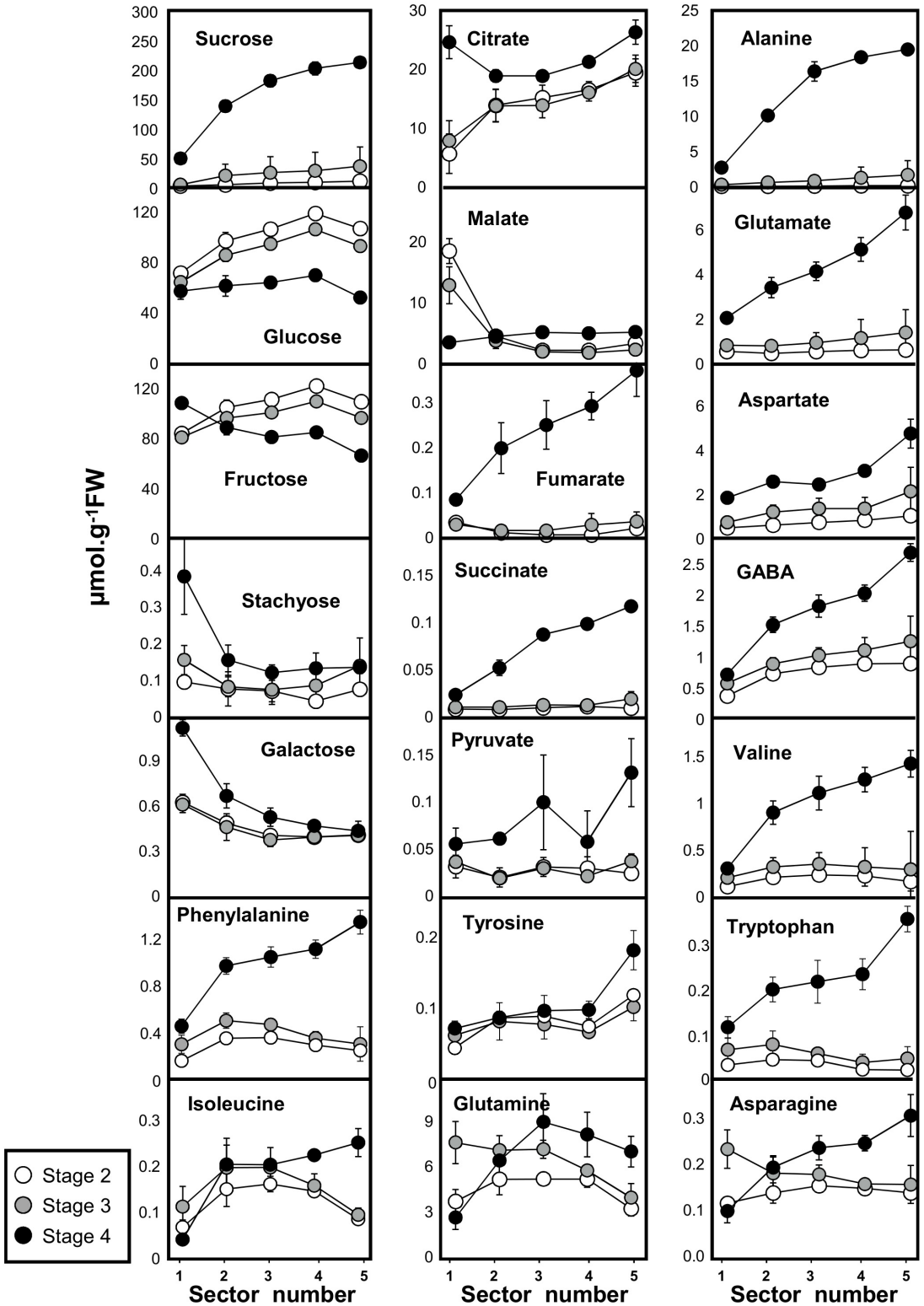

FIGURE 2 | Absolute concentrations in 18 primary metabolites measured by quantitative ^{1}H NMR spectroscopy (16 metabolites) and GC-EI-TOF/MS (pyruvate and succinate) in developing melon (*Cucumis melo* L. var Cantalupensis group Charentais cv. Escrito) fruit harvested at three developmental stages. Sector 1, 2, 3, 4, and 5 correspond to five concentric mesocarp rings taken from the periphery (outer epicarp + green mesocarp, named sector 1) to the fruit centre (inner mescocarp, named sector 5). Concentrations are given in μmol.g^{-1}FW. The results are the means of 9 measurements (3 biological replicates × 3 technical replicates), bars represent standard error (n = 3). The data are available in **Supplemental Table S1**.

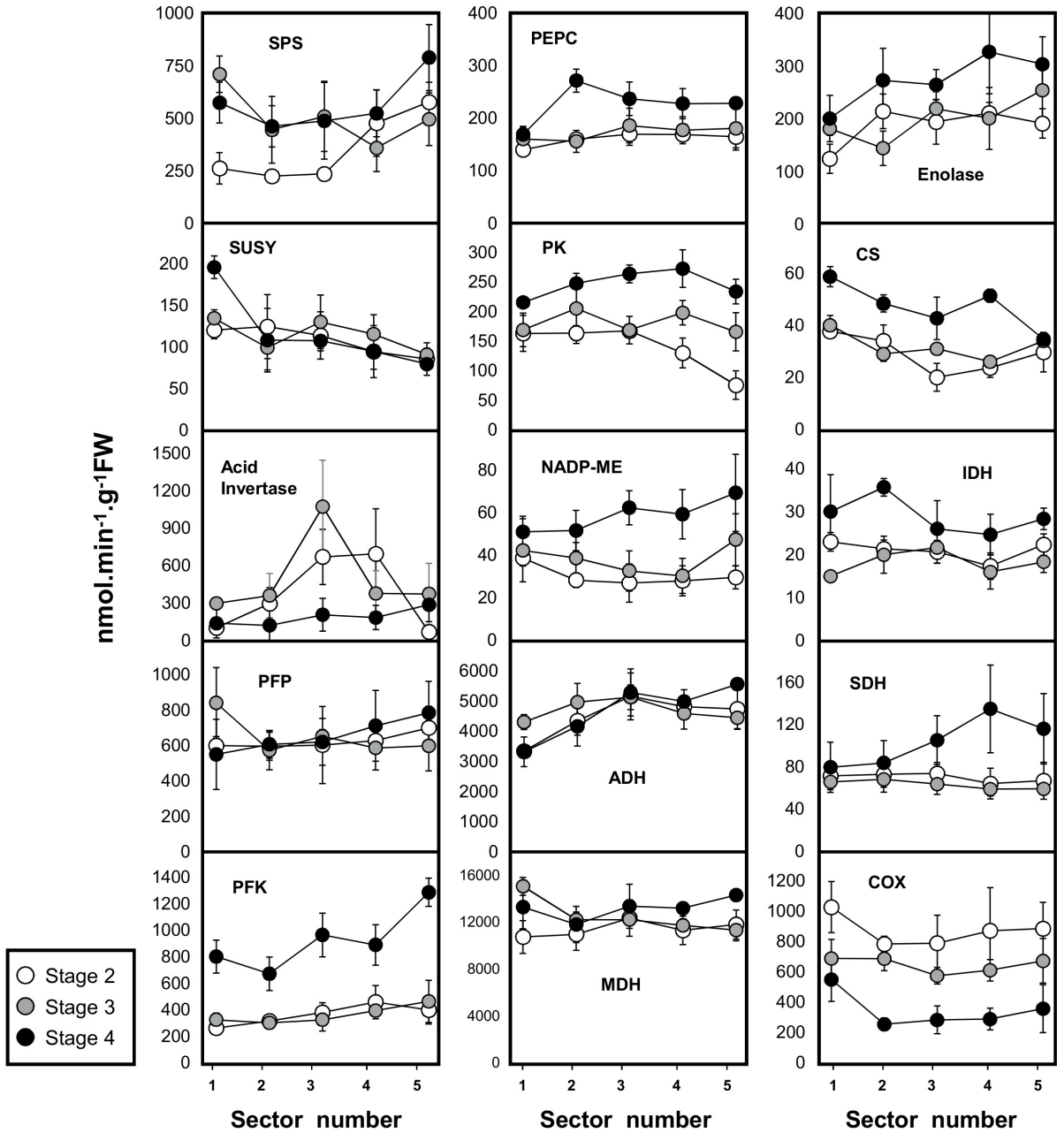

FIGURE 3 | Activities of 15 enzymes from central metabolism during the development of melon (*Cucumis melo* L. var Cantalupensis group Charentais cv. Escrito) fruit harvested at 3 developmental stages. Sector 1, 2, 3, 4, and 5 correspond to five concentric mesocarp rings taken from the periphery (outer epicarp + green mesocarp, named sector 1) to the fruit center (inner mescocarp, named sector 5). Three enzymes involved in sugar metabolism [sucrose synthase (SUSY), acid invertase, sucrose phosphate synthase (SPS)], enolase, 4 glycolytic enzymes [ATP-dependent phosphofructokinase (PFK), pyrophosphate dependent phosphofructokinase (PFP), pyruvate kinase (PK)], 4 enzymes of the tricarboxylic acid cycle [NAD-dependent malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), citrate synthase (CS), succinate dehydrogenase (SDH)], 2 anaplerotic enzymes [phosphoenolpyruvate carboxylase (PEPC) and NADP malic enzyme (NADP-ME)], 1 enzyme of the respiratory chain [cytochrome c oxidase (COX)] and 1 enzyme involved in fermentation [alcohol dehydrogenase (ADH)]. Enzyme activities are expressed as nmol.g^{-1}FW.min^{-1}. The results are the means of 3 × 3 measurements (3 biological replicates × 3 technical replicates), bars represent standard error (n = 3). The data are available in **Supplemental Table S1**.

accumulation were revealed to be common between amino acids, organic acids and Suc. The gradients observed at different time points and from the periphery to the center of the fruit for amino acids (Ala, Val, Glu, GABA, Asp, Asn, Ile, Phe, Tyr, Trp), organic acids (succinate, fumarate, pyruvate, and citrate) and Suc indicates that the process of maturation starts from the inner part of the fruit. We next therefore investigated whether these metabolic changes were

associated with changes in the activities of enzymes involved in central metabolism.

Most Enzyme Capacities Show Marked Spatial and/or Developmental Gradients

In order to follow metabolic changes in mesocarp tissue during melon fruit development, we profiled capacities (i.e., maximal measurable catalytic activities) of 15 enzymes from central metabolism (**Figure 3**, see also **Supplemental Table S1**). The developmental pattern of Suc accumulation at fruit maturity (stage 4) and the concomitant decrease in Glc and Fru contents were associated to a reduction in acid invertase activity (600–187 nmol.min^{-1}. g^{-1} FW, average of the whole mesocarp at stages 2 and 4). The clear pattern observed for acid invertase was not consistently observed for the two other enzymes involved in Suc metabolism, i.e., SPS and SuSy. A moderate increase in SPS at fruit maturity (stage 4) compared to developing fruit (stage 2) (356–568 nmol.min^{-1}. g^{-1} FW, average of the whole mesocarp at stages 2 and 4) was observed while for SuSy there was a small gradient between the outer and inner mesocarp (sectors 1–5) at all developmental stages (196–80 nmol.min^{-1}. g^{-1} FW, stage 4) (**Figure 3**).

We next explored variations in the capacities of glycolytic enzymes such as ATP-dependent phosphofructokinase (PFK), PPi-dependent phosphofructokinase (PFP), enolase and pyruvate kinase (PK). These four glycolytic enzymes exhibited comparable profiles throughout fruit development. Their activities remained low during stages 2 and 3 while their highest activities were observed at fruit maturity (stage 4). For the four glycolytic enzymes (PFK, PFP, enolase, PK), a net gradient could be observed between sector 1, which corresponds to the green mesocarp below the epicarp, and sectors 2–5, which correspond to the orange mesocarp at full maturity.

Next, the activities of TCA-cycle enzymes, i.e., citrate synthase (CS), NAD-isocitrate dehydrogenase (IDH), SDH and NAD-malate dehydrogenase (MDH), as well as two further enzymes involved in the metabolism of organic acids, i.e., phospho*enol*pyruvate carboxylase (PEPC) and NADP-malic enzyme (ME), were investigated (**Figure 3**). These six activities had comparable profiles during the development of the melon fruit, and peaked at maturity (stage 4). No strong gradient could be observed for these enzymes involved in TCA and organic acid metabolism between the outer and inner mesocarp (except for CS). Conversely, the COX activity of the respiratory chain was relatively high in the mesocarp during stage 1 (871 nmol.min^{-1}. g^{-1} FW average for the whole mesocarp) and, regardless of the location within the tissue, decreased throughout development (down to 346 nmol.min^{-1}. g^{-1} FW average for the whole mesocarp at maturity). A gradient of the COX capacity was also observed between sector 1 corresponding to the green mesocarp, and the orange sectors (from 549 to 259 nmol.min^{-1}.g^{-1} FW at stage 4) (**Figure 3**). Considering that fermentation is particularly important in fruit, ADH was also investigated. ADH activity was high at all three developmental stages and surprisingly high in the external layer of the mesocarp where it reached 3,320 nmol.min^{-1}.g^{-1} FW at stage 2. At maturity, ADH showed the same gradient pattern as the activities of the glycolytic enzymes (PFP, PFK, Enolase, and PK; **Figure 3**).

To summarize, among the 15 tested enzyme activities expressed on a FW basis, only the COX activity of the respiratory chain and acid invertase activities showed a clear decrease at maturity while the other enzymes were stable or tended to slightly increase. The intriguing results obtained with enzymes involved in fermentation (high ADH activity at the periphery and before ripening) and respiration (strong decrease of the COX activity at maturity) prompted us to investigate O_2 availability, as lowering oxygen within the mesocarp might lead to hypoxia and could influence the energy status, which can affect primary metabolism.

Hypoxia Is Gradually Established During the Development of the Melon Fruit Although There Is No Strong Oxygen Gradient Between the Outer- and the Inner Mesocarp

Fine glass microsensors (tip diameter 140 μm) were used to measure O_2 concentrations at three to five different depths in the mesocarp (from 7.5 to 32.5 mm) from three different equatorial positions of fruits harvested across the developmental stages 1–4. The microsensor was introduced into the mesocarp on the equator axis and was driven toward the center of the fruit (**Figure 4A**). Regardless of the developmental status, the O_2 level immediately declined just below the external layer of the mesocarp, from 21 kPa (atmospheric O_2 level) to 16.3 kPa at stage 1 or even to 10.5 kPa at stage 4 (**Figure 4A**). Then, within fruits harvested at stage 1 (developing fruits with a diameter of 80–90 mm), O_2 decreased slightly from 16.3 kPa in the outer layer to 15.5 kPa at 17.5 mm depth. At this developmental stage, all investigated fruits showed similar O_2 profiles. In developing fruits with a diameter of 100–110 mm that are characterized by the absence of the suberized net on the skin (stage 2), the O_2 concentration seemed to be stable regardless of the position on the fruit where it was recorded, but there was a relatively large variability between fruits. Indeed, some fruits at stage 2 showed comparable O_2 concentrations to fruits at stage 1 (around 16.3 kPa), while others showed lower O_2 concentrations (down to 15.5 kPa). These results suggest that fruits selected on the basis of their diameter for stage 2 can in fact be at different developmental stages. The lowest O_2 levels were always detected in early ripening fruits (stage 3), i.e., in fruits with diameter of 130–135 mm (down to 9 kPa). In mature fruit (stage 4), the O_2 concentration was very stable across all tissue sections and types (9.9 kPa). The results show that in the mesocarp O_2 concentrations decrease throughout fruit development and that the O_2 concentration was consistent throughout the whole mesocarp regardless of tissue depth. The relationship between mean O_2 tensions measured in melon fruits harvested at different developmental stages is given in **Figure 4B**. A steady decrease in O_2 was found during the development of melon fruit from stages 1 to 3 and the O_2 tensions remained very stable at around 9.9 ± 0.4 kPa in the whole mesocarp without there being an apparent O_2 gradient in the tissue.

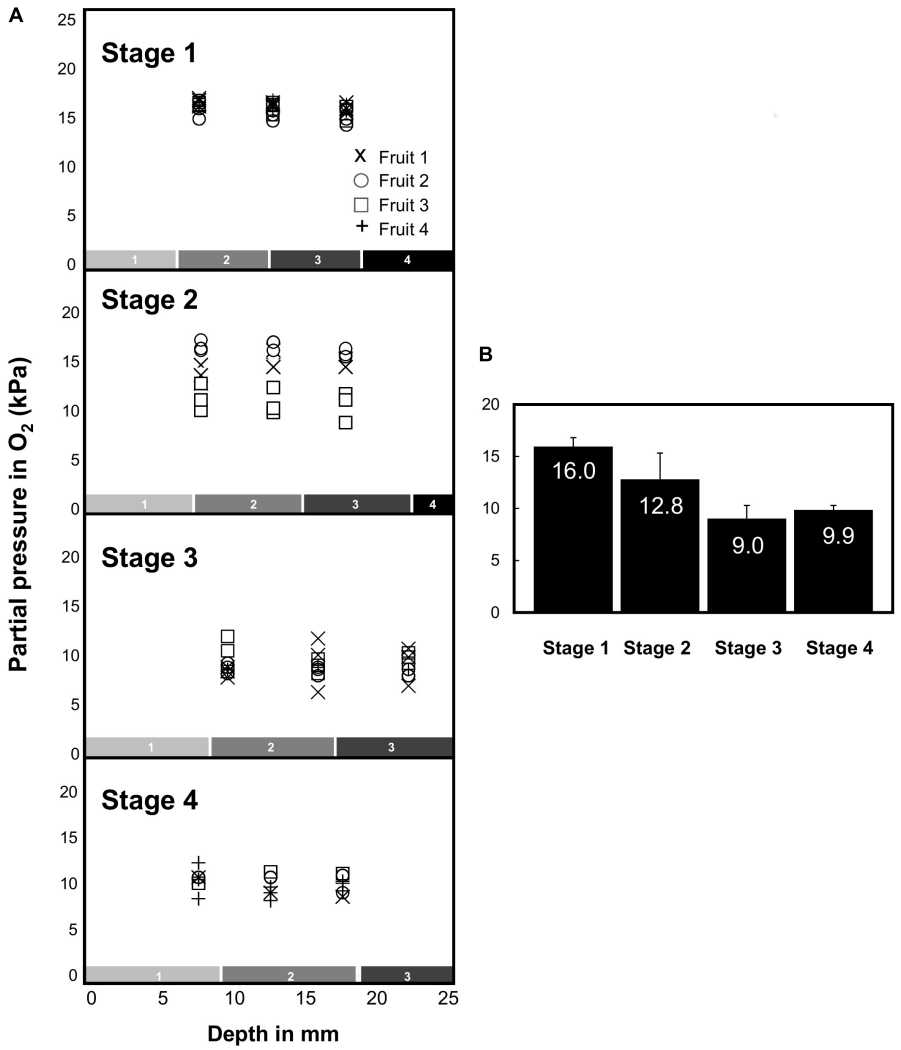

FIGURE 4 | Oxygen tension (expressed in kPa) measured in the mesocarp of 16 melon (*Cucumis melo* L. var Cantalupensis group Charentais cv. Escrito) fruits harvested at stages 1, 2, 3, and 4 with an oxygen-sensitive optical glass-sensor connected to a fiber optic oxygen meter (MicroX TX3 PreSens) based on dynamic fluorescence quenching. **(A)** O_2 tension measured at 3 depths (7.5, 12.5, and 17.5 mm) in the mesocarp and at three different equatorial positions. In mature fruits (stage 4), O_2 tension was measured deeper in the mesocarp (22.5, 27.5, and 32.5 mm). The numbered boxes (gray to black, 1–4) indicate the evolution of the size of the harvested concentric mesocarp ring at stages 1–4. **(B)** Mean O_2 tensions measured in melon fruits harvested at developmental stages 1–4.

Given the intriguing absence of marked gradients in the oxygen concentrations of the mesocarp, it was logical to develop a simple model to predict and analyze the oxygen demand, diffusion, and concentration across the melon flesh.

Oxygen Demand Meets Oxygen Diffusion

Firstly, oxygen demand was calculated from estimates of the rates of biomass production at different depths within the mesocarp (see Materials and Methods). This was first performed by expressing biomass as a function of time for each of the five layers analyzed above (**Supplemental Figure S1**). Fitted polynomials were then integrated to obtain the rates of biomass production and respiration was finally estimated by using a fruit construction cost (process-based) model previously applied to other fleshy fruits, such as tomato, kiwi, peach and grape berry (Liu et al., 2007; Dai et al., 2010, and references therein). Respiratory activity was calculated using either a low or a high estimate of the maintenance-linked respiration (**Supplemental Figure S2**). Next, the oxygen concentration was modeled assuming that a steady state O_2 concentration at any position within the mesocarp is reached when O_2 delivery (diffusion) equals O_2 consumption (respiration). The complete mathematical formulation of this problem has already been described by McElwain (1978) for spherical biological objects. Briefly, the melon was modeled as a homogeneous sphere (i.e., without gas phase in the intercellular space) whose radius varied from stage to stage. Moreover, no O_2 diffusion constraint was supposed to occur outside the melon. In contrast, a constrained diffusion of O_2 across the skin (i.e., h coefficient) and within the mesocarp (i.e., D coefficient) was considered.

Finally, the O_2 gradient was assumed to vanish at the inner limit of the mesocarp, at half of the melon radius. Even though the measured O_2 concentrations within the mesocarp largely exceeded the half saturation constant of the COX, the later parameter was taken into account in our calculations, as in Armstrong and Beckett (2011).

Figure 5 represents the calculated and measured O_2 tensions as a function of the position within the mesocarp and of the developmental stage. Several general trends can be drawn from the simulations. Firstly, the O_2 gradient profile depends on the presence (unbroken line) or not (dashed line) of a gradient of respiratory activity within the tissue. Indeed, parameterizing the growth- and maintenance-dependent O_2 consuming activity gradient with the COX enzymatic activity makes the O_2 gradient flatter regardless of the melon stage. Secondly, the O_2 concentration within the tissue depends on the external O_2 availability, i.e., on the occurrence of diffusion constraints at the melon surface with (blue line) and without (red line) the permeability barrier on the surface of the melon). Indeed, setting a high *h*-value just makes the O_2 concentrations higher within the tissue, the shape of the gradient being unchanged (**Figure 5**).

For each stage, the numerical solutions were compared with the corresponding analytical results (open circles in **Figure 5**). Good agreement between simulations and measurements were obtained when considering both a decrease in the O_2 consumption rate from the surface to the center of the melon and a diffusion constraint at the surface of the melon. A least-square fit of the measured O_2 concentrations gave the highest *h*-values in the earlier stages ($3.7\ 10^{-5}$ and $1.4\ 10^{-5}$ $cm.s^{-1}$ for melons harvested at stages 1 and 2) whereas this permeability coefficient value drastically decreased during later stages, to reach $0.23\ 10^{-5}$ $cm.s^{-1}$ at stage 4 (**Figure 5**). The latter analysis was performed using a high estimate of the maintenance-linked respiration. It should be stressed that similar trends were observed with a low estimate (**Supplemental Figure S4**).

Even though respiratory activity is probably under the control of the major oxidase (i.e., COX), the modeling approach was repeated using the K_m value of alternative oxidase (AOX), which is one order of magnitude higher (0.134 vs. 0.0108 kPa, Armstrong and Beckett, 2011). It is worth mentioning that the O_2 gradient profiles obtained using these values were very similar (**Supplemental Figure S5**). This tends to demonstrate that the affinity of the terminal oxidase of the respiratory chain does not greatly influence the balance between oxygen demand and diffusion throughout melon development (see also Armstrong and Beckett, 2011).

Overall, our modeling approach tends to demonstrate that the melon skin exerts increasing O_2 diffusion constraints during fruit development which, in turn, may favor the establishment of hypoxia within the mesocarp tissue (see **Supplemental Figure S4**). However, the O_2 consumption of this tissue undergoes a spatiotemporal decrease due to a concomitant decrease in the growth rate and the respiratory chain capacity. Therefore, melon development is characterized by a concomitant decrease in both the O_2 demand and

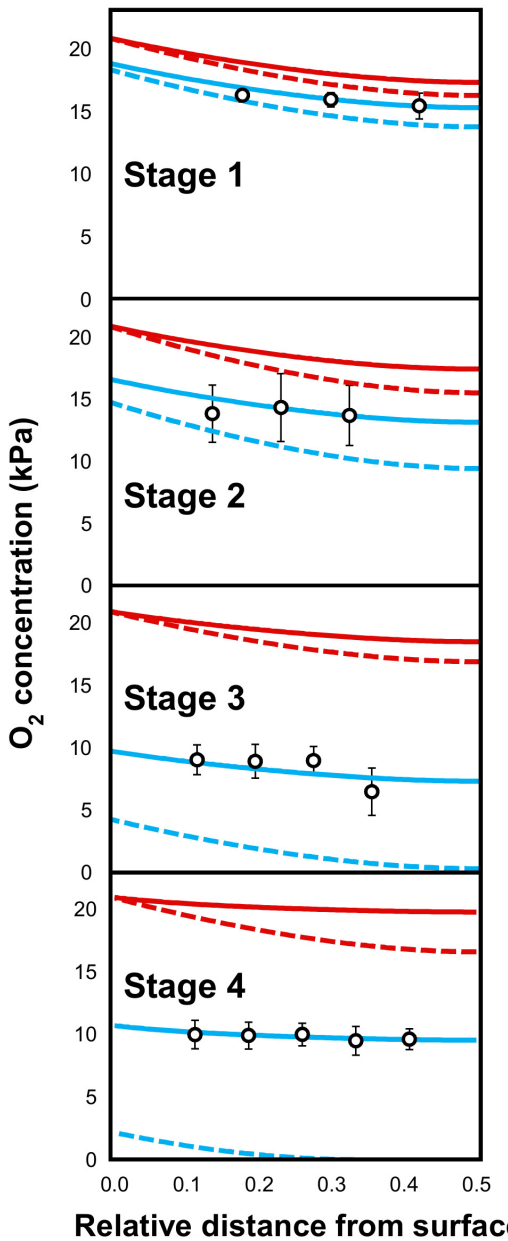

FIGURE 5 | Comparison of measured (open circles) and calculated (red and blue unbroken or dashed lines) O_2 tensions in the mesocarp of melon (*Cucumis melo* L. var Cantalupensis group Charentais cv. Escrito) fruit harvested at stages 1, 2, 3, and 4. O_2 concentration was modeled assuming that a steady state O_2 concentration is reached at any position within the mesocarp when O_2 delivery by diffusion) equals O_2 consumption by respiration. At each stage, a depth-dependent function of the respiratory activity was defined (see **Supplemental Figure S2A**) and the *h*-value (i.e., the permeability coefficient of the melon skin) was optimized to fit the measured O_2 concentrations. Unbroken or dashed lines represent the O_2 gradient profile when a gradient of respiratory activity within the tissue is present or not, respectively. Blue and red lines represent the O_2 concentration within the tissue when a permeability barrier on the surface of the melon occurs (*h*-value is equal to 3.7×10^{-5}, 1.4×10^{-5}, 0.31×10^{-5}, and 0.23×10^{-5} $cm.s^{-1}$ at stages 1, 2, 3, and 4, respectively) or not ($h = 10^{-3}$ $cm.s^{-1}$), respectively. Oxygen concentration is expressed in kPa. A relative distance of 0 corresponds to the melon surface and 0.5, to the inner limit of the mesocarp.

availability, thus ending with O_2 partial pressure values above 40% regardless of the developmental stage and the section of the mesocarp.

Integration of Metabolite Profiles, Enzyme Activities, and O_2 Variables Reveals That the Cytochrome c Oxidase Capacity Is Tuned to Oxygen Availability

Principal component analysis (PCA) was used to integrate metabolite and enzyme data with O_2 demand and concentrations (**Figure 6**). The data for oxygen concentration obtained with the fine glass microsensors did not correspond to the samples used for biochemical analysis and could therefore not be used directly to perform the PCA. Thus, the model described above was used instead, in order to obtain estimates of the oxygen concentration in the different sectors of the mesocarp and at the different developmental stages within which metabolites and enzymes had been measured. Finally, both O_2 demand and O_2 concentration estimates were included within the variables. The PCA was performed with averaged data of estimated oxygen-demand and -concentration, 18 metabolites measured by quantitative ^{1}H NMR spectroscopy and GC-EI-TOF/MS, and 15 enzyme capacities from central metabolism in the five radial sections of the mesocarp expressed on a FW basis (**Supplemental Figure S6**) and on a protein basis (**Figure 6**). The latter gave the best separation of both samples and variables but the general trends were the same for both expression bases. The score plots (**Figures 6A,B**) indicate that the first principal component (PC1), which explains 40% of the total variance, separates the developmental stages whereas PC2, which explains 32% of the total variance, separates the sectors. The scores plots also indicate that the deeper the sector, the larger the amplitude of metabolic change over time. The corresponding loadings plot (**Figure 6C**) highlights four groups of variables, which were clearly associated to growth, ripening, the outer sector 1 (green outer mesocarp) and the inner sectors 2–5. The latter two groups of variables only responded weakly to development (low loadings along PC1). However, in the green outer mesocarp, galactose was closely associated with growth, suggesting that a decrease in carbon demand would lead to a small imbalance between the import and degradation of stachyose. The variable group attributed to growth was also associated with O_2 demand and O_2 concentration (**Figure 6C**). Strikingly, the latter two variables were well correlated with each other ($p = 2.7 \times 10^{-5}$) and with the capacities of the COX ($p = 1.5 \times 10^{-3}$ and 1.6×10^{-6}, respectively) and to a lesser extent SuSy ($p = 3.0 \times 10^{-2}$ and 1.2×10^{-2}, respectively). PFP was also significantly correlated to O_2 concentration ($p = 8.0 \times 10^{-4}$). Glc and Fru, which were highly correlated with each other ($p = 1.6 \times 10^{-12}$), were also positively correlated (p-values ranging from 2.0×10^{-2} to 2.1×10^{-6}) with the previous variables, whereas they were negatively correlated with Suc (p-values ranging from 5.9×10^{-3} to 1.4×10^{-4}). Furthermore, Suc was strongly correlated with pyruvate, Ala, Glu, succinate and fumarate ($p < 3.0 \times 10^{-4}$). ME was also correlated to this group of metabolites. In contrast and as already seen above, changes in ADH were moderately associated to growth, and not to ripening, which implies that the rise in ADH was early during fruit development and not a response to the climacteric crisis.

Tuning of Cytochrome c Oxidase Capacity to Oxygen Availability Is Not Under Transcriptional Control

Because of the strong correlation found between COX capacity on the one hand and O_2 concentration or O_2 demand on the other hand, the expression of genes coding for COX subunits was studied to check whether there is a transcriptional regulation in COX capacity in response to O_2. Given the supramolecular organization of COX, subunits were selected according to their origin (nucleus vs. mitochondrial DNA encoded), their known function (catalysis vs. assembly) and their gene expression change – sometime in opposite directions – in response to oxygen availability (Mansilla et al., 2018). Finally, expression of COX1, COX5b, COX5c, COX6, COX11, and COX15 genes was studied by measuring the concentrations of the corresponding transcripts (**Supplemental Figure S7**). When transcripts were expressed on a protein basis, no significant correlations were found with COX capacity, O_2 concentration or O_2 demand. When transcripts were expressed on a FW basis, negative correlations were found between COX capacity and cox1 ($R^2 = 0.66$, $p = 2.5 \times 10^{-4}$) as well as cox15 ($R^2 = 0.35$, $p = 2 \times 10^{-2}$). O_2 and O_2-demand were also weakly and negatively correlated to cox1 ($R^2 = 0.34$, $p = 0.02$, and $R^2 = 0.42$, $p = 0.009$, respectively). Thus, the link found between COX capacity and O_2 could not be attributed to an adjustment of the expression of these encoding genes.

DISCUSSION

Metabolite Gradients Observed in Melon Fruit Reflect Metabolic Adjustments

The present study confirms that raffinose and stachyose, which are translocated via the phloem in the range of hundreds of mM (Haritatos et al., 1996; Knop et al., 2001), do not accumulate in the fruit flesh, thus indicating a rapid metabolism of these sugars within the fruit. The fact that stachyose and its degradation product galactose sharply decreased from the periphery to the inner mesocarp suggests that carbon import into the mesocarp is mostly centripetal. During fruit growth, glucose, fructose and to a lesser extent citrate were the most abundant metabolites found in the mesocarp. Contrary to stachyose and galactose, these metabolites were increasingly abundant toward the inner part of the mesocarp. This gradient may reflect a spatial difference in sugar metabolism that is perhaps linked to decreasing growth rate and thus decreasing carbon demand when going deeper into the mesocarp.

At ripening, the strong accumulation of a range of metabolites, i.e., sucrose, pyruvate, fumarate, succinate, alanine, glutamate, aspartate, asparagine, valine, and GABA (see also Moing et al.,

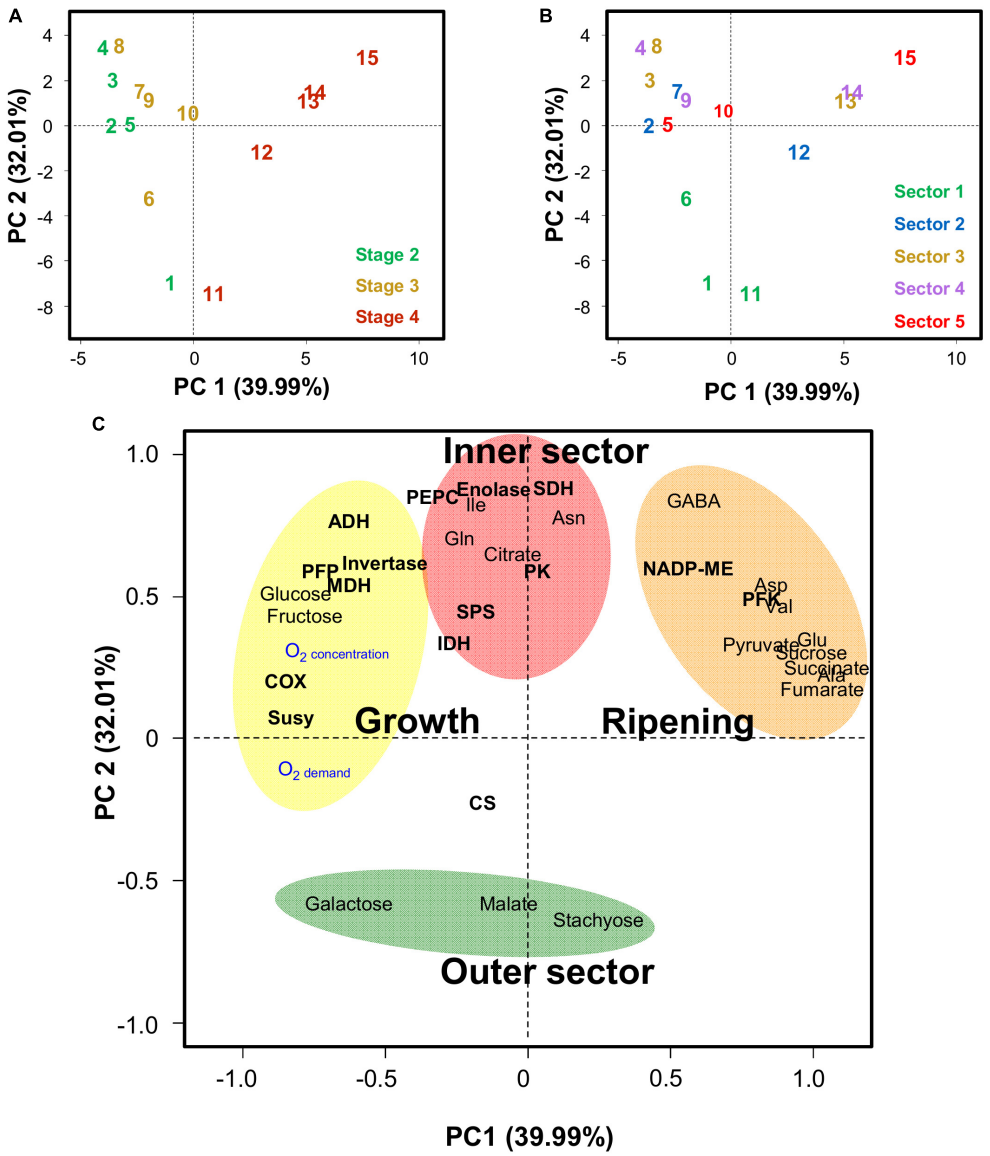

FIGURE 6 | Principal component analysis (PCA) of estimated oxygen-demand and -concentration, 18 metabolites measured by quantitative ^{1}H NMR spectroscopy and GC-EI-TOF/MS, and 15 enzyme capacities from central metabolism in five radial sections of the mesocarp (sector 1, to epicarp + green mesocarp; 5 inner orange mesocarp, see **Figure 1**) of melon (*Cucumis melo* L. var Cantalupensis group Charentais cv. Escrito) fruit at three stages of development, stages 1, 2, and 3. PCA was performed with averaged data expressed on a protein basis and on a FW basis (**Supplemental Figure S6**). **(A)** PCA scores plot of the first two principal components (PC1 and PC2) showing the distribution of the samples at three stages of development. **(B)** Same PCA scores plot of the first two principal components (PC1 and PC2) showing the distribution of the samples from the 5 radial sections of the mesocarp. **(C)** PCA loadings plot showing three different areas. Abbreviations for enzymes are given in the legend of **Figure 3**.

2011), was probably enabled by the arrest of growth that occurred at around growth stage 3. Indeed, assuming that the import and processing of carbon and nitrogen would continue, a decrease in carbon demand would likely result in the accumulation of sugars and organic acids, and a decrease in protein synthesis resulting in the accumulation of amino acids. This accumulation also coincided with a relatively strong drop in the oxygen concentration (on average, from >60% to <50% of the atmospheric concentration), which was probably linked to a drop in the O_2-permeability of the skin (**Figure 5**). Certainly, with the exception of fumarate, these metabolites, which are also precursors of a range of volatiles (Manriquez et al., 2006), are often accumulated in plant tissues under hypoxia (Menegus et al., 1989; Edwards et al., 1998; Roessner et al., 2001; Miyashita et al., 2007; Narsai et al., 2011). Among the enzymes studied here, only PFK and ME continued to accumulate during ripening (**Figures 3**, **6**). Interestingly, the activation of ME under hypoxia has been shown to lead to the accumulation of pyruvate and Ala (Edwards et al., 1998). Besides, the metabolites that accumulated

during ripening, were first seen to accumulate in the inner part of the fruit while oxygen gradients became flatter (they were more marked during growth stages 1–2), indicating that a more marked hypoxia was not the cause of their more pronounced accumulation. The fact that the capacities of most enzymes, in particular ADH and enzymes involved in glycolysis, were already higher in the inner part of the melon at the early growth stages could have potentiated such an accumulation, for example by favoring higher fluxes. Such "priming" phenomenon evokes the increased capacities of a range of enzymes involved in carbon metabolism (including PFP, PFK, Enolase, PK, and ADH), as observed in maize root tips pre-treated by hypoxia and that have been associated with improved survival under subsequent anoxia (Xia et al., 1995; Bouny and Saglio, 1996).

Sucrose Accumulation Is Linked to Fermentation

The large increase in sucrose relative to glucose and fructose that is typically observed in melon has been attributed to the loss of soluble AI and the maintenance of SPS activity during ripening (Schaffer et al., 1987; Hubbard et al., 1989). However, in the present study AI activity was decreased at ripening but not lost, while SPS was maintained or even slightly decreased (**Figure 3**). This is actually in line with a recent study showing that whilst the mRNA of the only expressed gene encoding soluble invertase detected vanished in ripening melon fruits, invertase activity was still detected (Dai et al., 2011). The significantly negative correlation found between sucrose and SuSy suggests that decreasing SuSy may also be necessary for sucrose accumulation. Indeed, the highest SuSy activity was found in the outer mesocarp where sucrose, like stachyose, is probably unloaded from the phloem. Finally, the fact that sucrose was strongly correlated to a group of metabolites known to accumulate under hypoxia raises the question of a possible additional regulation of sucrose turnover by oxygen availability. In line with this, it has been reported that sucrose degradation is quickly inhibited in slices of potato tubers exposed to mild hypoxia (Geigenberger, 2003). Furthermore, in the roots and hypocotyl of soybean seedlings under hypoxia, sucrose has also been found to be accumulated despite maintained acid and alkaline invertase activities (Nanjo et al., 2010).

Melon Fruit Achieves the Avoidance of Oxygen Gradient Within the Mesocarp

Oxygen gradients linked to oxygen diffusion have been reported for a range of plant systems. For example, potato tubers (Bologa et al., 2003) or soybean developing seeds (Borisjuk and Rolletschek, 2009) show dramatic O_2 gradients when under optimal growth conditions, with the root nodules of legumes even achieving anoxic conditions within their center, an absolute requirement for N-fixation (Ott et al., 2005). Surprisingly, there were no such gradients in the mesocarp of melon fruit, even though hypoxia takes place and despite the fact that melons are relatively fast growing fruits that reach remarkably large sizes. A simple explanation is that the spherical form of the fruit guarantees that the O_2 demand decreases from the outside to the inside, simply because there is considerably less biomass being produced in the inside than at the periphery. The model integrating O_2 diffusion and demand presented here confirms this hypothesis.

Additionally, it is likely that mechanisms avoiding the waste of O_2 taking place across the developmental stages studied here were also modulating the consumption of O_2. Thus, SuSy and PFP, which were associated to the outer mesocarp where O_2-demand was highest, are both considered as energy saving enzymes. The breakdown of Suc into hexose phosphates requires only one PPi when initiated via SuSy and two ATP when initiated via invertase (Geigenberger, 2003). Similarly, the phosphorylation of fructose-6P costs one PPi via PFP and one ATP via PFK, which again saves energy, especially in growing tissues where PPi is produced at high rates (Stitt, 1998). It is also striking that PFK activity increased quite dramatically at ripening, although O_2-availability was decreased. It suggests that ATP-usage was no longer critical and/or that it was required to replace PPi-dependent reactions because PPi production had dropped, both events being consequences of arrested growth. This is in line with a recent study carried out on tomato pericarp which demonstrated that the energy-saving priority of fruit growth vanishes at the end of development thus leading to the onset of the climacteric crisis (Colombie et al., 2017).

Finally, the most remarkable metabolic adjustment to fluctuating O_2 discovered by this study was the tuning of the capacity of COX to O_2-availability. It is worth mentioning that such hypotheses are strongly debated in the literature (Armstrong and Beckett, 2011; Nikoloski and van Dongen, 2011). In plant cells, the local O_2 tension results in a balance between oxygen diffusivity within the tissue and the activity of the oxygen consuming enzymes, especially COX, which is involved in the mitochondrial oxidative phosphorylation pathway. In dense, metabolically active and growing tissues such as seeds, seedlings, tubers and fleshy fruits, the lack of systems for O_2 distribution leads to the limitation of oxygen diffusion, which may lead to a fall in the internal O_2 concentrations (Borisjuk and Rolletschek, 2009). Admittedly, a falling internal O_2 concentration results in a restriction of metabolic activity (Geigenberger, 2003). Within potato tubers (Geigenberger et al., 2000), pea and bean seeds (Rolletschek et al., 2002), it is paralleled by a severe decrease in the ATP/ADP ratio and adenylate energy charge (AEC), indicating that respiration is being inhibited. In melon fruits, despite a drastic decrease in ATP/ADP, from 31 (outer mesocarp) to 6 (inner mesocarp), the AEC is only slightly decreased, from 0.97 in the outer mesocarp to 0.83 in the inner mesocarp (Biais et al., 2009). As reported by Pradet and Raymond (1983), small variations in AEC with high values (between 0.85 and 0.95) imply significant variations in the ATP/ADP and ATP/AMP ratios and may thus conceal quite different regulatory situations for enzymatic ATP-utilizing processes. In a developing melon fruit, relatively high AEC and low ATP/ADP suggest

that ATP-generating pathways (i.e., glycolysis and oxidative phosphorylation) are fitting the energy supply to the demands of the processes involved in growth and maintenance. Changes in both ATP/ADP and AEC have been observed *in vitro* and *in vivo* in potato tubers (Geigenberger et al., 2000) and tomato pericarp (Menu et al., 2004) under conditions of artificially low atmospheric oxygen pressure. Most importantly, the present study indicates that in melon fruit placed under atmospheric oxygen tension the decrease in the ATP/ADP ratio observed from the periphery to the inner mesocarp is not the consequence of an oxygen gradient within the tissue but rather a decrease in the capacity of the respiratory chain (i.e., the COX activity). Importantly, the affinity of COX for O_2 corresponds to O_2 concentrations that are much lower than those reported here, which suggests that regulatory events involving oxygen-sensing (Licausi et al., 2011; Weits et al., 2014) are taking place. The modulation of the capacity of COX therefore appears as a potent metabolic adjustment avoiding hypoxia. COX is usually considered as the rate-controlling step of the oxidative phosphorylation pathway. In various aerobically growing cells, down-regulation of COX activity participates to a fine-tuning of the respiratory capacity to changes in ATP demand, thus leading to energy status homeostasis (Devin et al., 2006). Here, our measurements provide evidence that the modulation of COX capacity (V_{max}), rather than its apparent affinity for O_2 (K_m), can explain, at least in part, the spatiotemporal changes in the O_2 concentration that are observed in bulky growing plant organs such as fleshy fruits. Transcriptional regulation is unlikely to occur in melon fruit since the transcript levels of nuclear- and mitochondria-encoded COX genes, especially COX5b and COX5c which are known to be downregulated by O_2 deprivation in rice (Tsuji et al., 2000) sunflower (Curi et al., 2002) and Arabidopsis thaliana (Mansilla et al., 2018), were not positively correlated to the COX activity nor to the local O_2 concentration. Therefore, mechanisms underlying the down-regulation of COX activity remain to be elucidated. However, it is clear that these changes do not result from a down-modulation of the mitochondria biogenesis *per se* (i.e., mitochondria content) since the evolution patterns of COX did not parallel with those of other mitochondrial enzymes (i.e., SDH, CS, and IDH). However, a modulation of the COX activity by means of post-translational modifications of regulatory subunits (Mansilla et al., 2018) is not to be excluded under our conditions.

Skin as a Key Factor Controlling Hypoxia-Driven Maturation of the Melon Fruit

Oxygen deprivation is usually seen as leading to a serious disruption of plant metabolism, which is essentially an aerobic process (Geigenberger, 2003). As discussed above, melon fruit appears to be capable of maintaining a low-slope gradient of O_2 concentrations throughout the mesocarp and at different developmental stages, especially during ripening where O_2 concentrations were lowest (nearly 9 kPa corresponding to 40% of atmospheric O_2). However, despite these mechanisms, the entire mesocarp became hypoxic quite early during fruit development (at stage 1, the average O_2 concentration was already decreased by 24%). The modeling approach developed in the present study suggests that this was due to changes in skin permeability to O_2. These computations are in line with gas transport measurements performed on pear and apple tissues showing that skin has a 20–50-fold lower O_2 diffusibility than flesh (Ho et al., 2006, 2011). So, why would melon fruit build a skin that provokes hypoxia? Firstly, in many fruit species fermentative metabolic features are essential for the production of aroma volatiles during ripening (Manriquez et al., 2006). Hypoxia and/or products of fermentation such as ethanol and acetaldehyde are also triggers of fruit maturation (Pesis, 2005). Secondly, oxygen- and ethylene sensing mechanisms are known to interact at the molecular level within plants (Bailey-Serres et al., 2012), as well as fruits (Min et al., 2012), and oxygen promotes ripening in climacteric fruits by stimulating the ethylene response via a mechanism that obeys Michaelis–Menten kinetics (Beaudry, 1999). Therefore, decreasing internal O_2 concentrations can also be seen as a way of controlling the rate of ripening.

CONCLUSION

Taken together our results show that the entire mesocarp becomes hypoxic quite early during fruit development and suggest that in developing melon fruits O_2 supply and O_2 consumption are finely tuned by a combination of factors to ensure a timely maturation of the mesocarp. The spherical form of the fruit combined with the metabolic adjustments, especially the tuning of the capacity of COX to O_2 availability that occurs during growth contribute to optimizing the O_2 demand and avoiding the establishment of an O_2 gradient within the flesh. The fact that such "controlled" hypoxia occurs relatively early in the fruit's development would favor the increased activity of a range of enzymes involved in glycolysis and fermentation and that are essential for maturation. In addition the modeling approach developed in the present study suggests that the decrease in the skin permeability to O_2 that coincides with the climacteric peak would moderate ripening while favoring fermentation throughout the mesocarp and almost at the same time, thus ensuring uniform maturation of the melon flesh. Skin permeability for oxygen therefore appears as a particularly interesting trait to investigate, especially in relation to metabolism, in further melon varieties and fruit species, as it could lead to improvements of fruit quality.

The assay for COX developed here is relatively easy to perform, thus providing an opportunity to investigate bulky organs in other species to verify whether COX adjustment to oxygen availability is a generic mechanism. The fact that the assay can also be performed in high throughput could then open a range of reverse and forward genetic approaches to investigate and manipulate hypoxia in plants. It will indeed be particularly

interesting to try to investigate the regulation of respiration in relation to oxygen availability.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available because the data are provided in the **Supplementary Data** of the present manuscript. Requests to access the datasets should be directed to yves.gibon@inra.fr.

AUTHOR CONTRIBUTIONS

KM, BPB, DR, AM, and YG planned the experiments. KM, BPB, BB, MC, JA, CD, MM, RG, CC, and AM performed the experiments. KM, BPB, DR, and YG interpreted the data and wrote the manuscript. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00594/full#supplementary-material

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REVIEW
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Primary Metabolites, Anthocyanins, and Hydrolyzable Tannins in the Pomegranate Fruit

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Pomegranate (*Punica granatum* L.) is an important and interesting fruit tree that is cultivated in many parts of the world. In recent years, along with the increase in its cultivation and consumption there has been a dramatic increase in the scientific interest in its biology, methods of cultivation, adaptation to environmental cues and its health-promoting properties. Quite a large proportion of the various metabolites produced in the pomegranate were determined and their content in the bark, roots, leaves, and fruit was reported. Many reviews on polyphenolic compound content, antioxidant activity and health-promoting compounds were published recently. However, only very few recent reports were dedicated to primary metabolites, despite the fact that much work was done on organic acids, sugars, proteins, lipids, and amino acids of the pomegranate fruit. In this review, a special effort was made to present these recent studies and the review is devoted to primary metabolites. The reported data show high variation in the content of primary metabolites within the pomegranate fruit; therefore the data is presented (whenever possible) according to fruit tissues (peel, arils, and seeds), developmental stages of the fruit, environmental and climatic conditions, and genetic background. Most of the data on pomegranate is based on metabolic content and contains no genetic or molecular analysis except for work done on anthocyanins and hydrolyzable tannins. In those cases, gene assignment and genetic control studies were pointed out in the review. The recent publication of the genome sequences from several pomegranate varieties and transcriptomic data from fruits, flowers, and leaves is expected to facilitate the understanding of genetic control of metabolites in pomegranate.

Keywords: pomegranate, fruit, lipids, sugars, polyphenols, proteins, organic acids, metabolites

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INTRODUCTION

Pomegranate (*Punica granatum* L.) is a fruit tree grown today in a wide range of subtropical and tropical geographical locations spread all over the globe; these locations include many countries in Asia, Europe, South and North America, Africa, and Australia (Holland et al., 2009). Pomegranate is considered a minor fruit and is far from the top of the list of consumed fruits, such as apple, banana, grapes, and citrus; however, it is one of the most interesting fruits in terms of cultural, traditional, and potential therapeutic usage.

The pomegranate fruit is a fleshy berry with a nearly round shape, crowned by a prominent calyx. Its relatively thick peel has an outer colored skin and the fruit's inner structure contains multi-arils chambers separated by membranous walls (Holland et al., 2009). The edible part of the pomegranate fruit, the arils, contains seeds and a special layer of cells (juice cells) that are of epidermal origin and protrude from the outer epidermal cells of the seed (Fahan, 1976; Holland et al., 2009). The external fruit color ranges from yellow, green or pink overlaid with pink to deep red or deep purple. The color of the juicy layer can vary from white to deep red (Holland et al., 2009). Various parts of the pomegranate fruit were traditionally used as treatments against various ailments including stomachaches and bacterial infections (Holland and Bar-Ya'akov, 2014). The traditional usages were strengthened by modern scientific studies focused on health beneficial metabolites and their therapeutic effects and mechanisms of action on human and animal health. These studies were thoroughly reviewed in recent years. Most of the therapeutic effects of the pomegranate fruit were attributed to its secondary and primary metabolites, such as polyphenols, including flavonoids, anthocynains and hydrolizable tannins, fatty acids, and lipids (Seeram et al., 2006a; Lansky and Newman, 2007; Jurenka, 2008; Viuda-Martos et al., 2010; Teixeira da Silva et al., 2013; Holland and Bar-Ya'akov, 2014; Wu and Tian, 2017). These metabolites were found in all fruit parts, including the fruit peel (ellagitannis, flavonoids, anthocyanins), arils (ellagitannis, flavonoids, anthocyanins), seeds (fatty acids, lipids), and membranous walls (mostly ellagitannins). Anthocyanin biosynthesis occurs in parallel in the arils and in the fruit peel. These two tissues are not necessarily correlated in their activity with respect to color production, and often, the two tissues display different colors (Holland et al., 2009; Dafny-Yalin et al., 2010). The same situation could appear in other biochemical pathways responsible for other important metabolites.

High variability was reported for pomegranate fruit that manifests, among other phenomena, considerable differences in size, shape, color, date of ripening, and taste. This external variability is interesting in view of the fact that the only edible species among the *Punica*, which include only two species, is the cultivated pomegranate (*P. granatum* L.). The only other pomegranate known to science is the non-edible species *Punica protopunica*, endemic to Socotra (Holland et al., 2009). The fruit of this species is small and not colorful and no biochemical, genetic, or molecular studies of its fruit were published. This high variability is also reflected in the content of primary and secondary metabolites. Quite substantial work has been devoted in recent years to determining primary metabolites in the pomegranate fruit. These efforts include studies of sugar, organic acids, protein, amino acids, and lipid content and composition. In general, the pomegranate fruit consists of 50% peel, 40% arils, and 10% seeds (per weight). The arils contain 85% water, 10% total sugars, 1.5% metabolites and bioactive compounds such as organic acids, phenolics, and flavonoids (Tezcan et al., 2009). The seeds are a rich source of lipids; pomegranate seed oil comprises 12–20% of total seed weight (Viuda-Martos et al., 2010). It appears that primary and secondary metabolites showed extensive variability due to the fact that the fruit used for the various studies originated from different varieties and highly variable climatic conditions and taken from trees grown under different agro-technical methods. While pomegranate reviews published up until now focused mainly on secondary metabolites (e.g., polyphenols, anthocyanins), there are only few that focused on primary metabolites, despite their great importance to taste attributes and to the nutritional index of the fruit. In this review, we focus on primary metabolites and on secondary metabolites, anthocyanins and hydrolizable tannins, with special attention to the variability of their content and composition. A special effort was aimed at the developmental, genetic, and environmental effects on the content and composition of primary metabolites. Whenever available, primary metabolites in each of the fruit organ, peel, arils, and seeds, were specified.

PRIMARY METABOLITES

Sugars

The pomegranate fruit is a rich source of sugars. The level of the sugars in pomegranate juice is highly correlated with the level of total soluble solids (TSS). Shwartz et al. (2009) and Dafny-Yalin et al. (2010) calculated a value of $R^2 = 0.89$, $P < 0.01$. The TSS level in the juice ranges from 4.2 to 8.5 g/100 g depending on cultivars, climatic conditions, and cultural techniques (reviewed by Kalaycioglu and Erim (2017); Amir et al. (2018)). Pomegranate juice contains a high amount of polyphenols such as flavonoids, ellgitannins, and the color molecules anthocyanins. A substantial fraction of these molecules are known to be conjugated to sugars, mostly glucose. The taste of arils from various pomegranate varieties is significantly variable, ranging from sour to sweet (Holland et al., 2009; Amir et al., 2018). Sugar content is an important parameter influencing taste, although it is highly influenced by organic acid content as well. Many studies examined the sugars in the pomegranate fruit, mainly in the juice, revealing glucose, and fructose as the main component of the juice sugars (**Figure 1**). Sugars found in the fruit peel were in some controversy among studies from different countries. It should be noted that those studies were done for different purposes and therefore followed different procedures of extraction and detection that might explain this disagreement. Some of the studies indicated glucose and fructose as the main sugars while others found that xylose and arabinose are the main sugars (Hasnaoui et al., 2014).

Differences Among Varieties

Composition of sugars in the juice

Arils are a rich source of sugars. Studies obtained from different countries have shown that the composition of sugars among pomegranate varieties might differ. Analyses of the sugars in pomegranate aril juice from 29 worldwide varieties grown in Israel and 19 cultivars from Spain have shown that fructose and glucose were the major sugars found in the arils, while sucrose and maltose were detected in lesser amounts. In some varieties, these two former sugars are the only sugars that were detected (Melgarejo et al., 2000; Dafny-Yalin et al., 2010). In many studies the levels of fructose were similar to those of glucose in pomegranate juices, and both varied in different

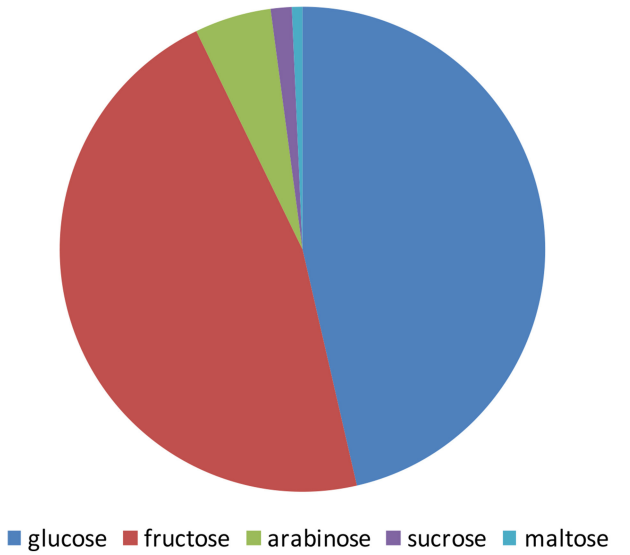

FIGURE 1 | Schematic illustration of the main sugars content in pomegranate juice from different varieties grown in Tunis, Turkey, Spain, and Israel. The values presented are average values of the percentage of each sugar of the total amount of sugars measured by Melgarejo et al. (2000); Tzulker et al. (2007); Hasnaoui et al. (2011); Caliskan and Bayazit (2012).

varieties by a factor of up to two-fold ranging from 4.2 to 8.5 g/100 g (Kalaycioglu and Erim, 2017; Amir et al., 2018). In 76 Turkish varieties glucose levels showed a range of 4.2–8.3 g/100 g juice (Caliskan and Bayazit, 2012). Forty Spanish varieties showed a range of 5.5–7.8 g/100 g (Melgarejo et al., 2000), 29 Israeli varieties showed a range of 4.8–6.6 g/100 g (Tzulker et al., 2007), and 30 Tunisian varieties showed a range of 5.7–8.5 g/100 g (Hasnaoui et al., 2011). In addition to the main fructose and glucose sugars, some other sugars (arabinose, sucrose, and maltose) were also detected in several varieties at a relatively negligible level (**Figure 1**). Examination of 30 Tunisian varieties that showed low concentrations of arabinose and sucrose (9- and 23-fold lower than glucose, respectively; Hasnaoui et al., 2011). Determination of sugar contents in 6 Spanish varieties has shown that they all contain maltose and sucrose, but possess 45- and 70-fold lower quantities of glucose, respectively (Legua et al., 2012). Sucrose was also found in an about 33-fold lower quantity than glucose in 6 Turkish varieties (Ozgen et al., 2008) and an up to 13-fold lower quantity in 53 out of 76 varieties (Caliskan and Bayazit, 2012). Maltose was only detected in one of 29 Israeli varieties (Dafny-Yalin et al., 2010).

In line with the sugar measurements, the aril juice TSS have shown a relatively narrow range as reported in different publications from different varieties in diverse pomegranate collections (reviewed by Kalaycioglu and Erim (2017); Amir et al. (2018)). Examination of 20 varieties from Iran and Spain, 10 from Morocco, 29 from Israel, and 9 from Italy have shown ranges of 11.4–15.1; 15.1–17.7; 15.2–17.6; 13.7–17.8, and 13.6–18.5%, respectively (Tzulker et al., 2007; Tehranifara et al., 2010; Legua et al., 2012; Ferrara et al., 2014; Alcaraz-Mármola et al., 2017).

Composition of sugars in the peel

Several studies specifically measured the level of sugars and TSS in the fruit peel (Dafny-Yalin et al., 2010; Orak et al., 2012; Hasnaoui et al., 2014; Ahmadi Gavlighi et al., 2018). Notably, the major sugars that were detected in the peels differed between the varieties grown in Tunisia, Iran and Israel. In the 12 Tunisian varieties, xylose and arabinose represented more than 60% of the total content, followed by galactose (14%), glucose (∼10%), mannose (∼5%), rhamnose (∼4%), and fucose (∼1.5%) (Hasnaoui et al., 2014). Peels from one Iranian variety showed that the main sugar is glucose (44.9–68.1%), followed by galactose (14.6–19.4%), mannose (3.4–18.1%), arabinose (3.1–18.1%), and rhamnose (3.5–6.0%) (Ahmadi Gavlighi et al., 2018). However, in the 29 worldwide varieties grown in Israel, the major sugars were glucose and fructose. The level of glucose varied in the range of 0.9–4.8 g/100 g (5.3-fold), and that of fructose in 0.9–6.6 g/100 g (6.6-fold). The level of fructose was higher than that of glucose in most of the varieties. Maltose was found at an about 50-fold lower concentration than that of glucose and fructose, in a range of 0.8–48.9 mg/100 g, while sucrose was detected in only 6 varieties at relatively low levels (up to 3.1 mg/100 g). Mannitol was also detected in all the varieties, ranging from 10 to 300 mg/100 g (Dafny-Yalin et al., 2010).

As expected from these results, the TSS varied between the different collections. In 12 Tunisian cultivars, it ranged from 16.8 to 19.6 g/100 g (Hasnaoui et al., 2014), which was more than the range of three Indian varieties that show a range of 13.7–14.5 g/100 g (Dzugan et al., 2018). However, these values were much higher than the results reported for 4 Turkish varieties that ranged from 3.8 to 6.4 g/100 g (Orak et al., 2012), and the 29 varieties grown in Israel, which showed a range of 5.2–11.3 g/100 g. In these varieties, the peels had a 2- to 3-fold lower level of TSS compared with the aril juice (Dafny-Yalin et al., 2010).

Differences During Fruit Development

Several studies followed the changes in the levels of sugars and TSS in aril juice during fruit development. The results taken from three cultivars (American and Indian) in South Africa (Fawole and Opara, 2013c; Mphahlele et al., 2016), and two cultivars (Israeli and American) from Israel (Shwartz et al., 2009) have shown that the level of TSS rose during the development process in accordance with the levels of glucose and fructose. The increase shown in the two Israeli grown varieties and in "Wonderful" in South Africa was significant (Schwartz et al., 2009; Mphahlele et al., 2016). The presented data indicate that the developmental stage of the pomegranate fruit is associated with sugar accumulation.

Climate and Geographic Influence

Analysis of TSS and sugar content in different collections revealed that their values depended on climate and growth conditions. To gain more knowledge on the effect of the environmental conditions on the levels of sugars, 11 varieties from the Israeli collection in the Jezreel Valley (Mediterranean climate) were planted in Israel's southern Arava Valley (hot-dry desert climate). Arils from both habitats were analyzed. The varieties grown in Mediterranean climate showed significantly

higher levels of glucose and fructose in the juice than those grown in a hotter habitat (Schwartz et al., 2009). Similar results were also reported from the analyses of the 10 Chinese cultivars that grew in four different habitats (Li et al., 2015). "Wonderful," which was grown in Israel in two habitats (Ben-Arie et al., 1984), as well as in three habitats in South Africa (Mphahlele et al., 2016), showed that relatively higher temperatures can decrease sugar content, whereas cooler temperatures apparently promoted the increase in glucose and fructose. Thus, temperatures appear to play an important role in the sugar content of pomegranate juice.

Genetics

Only one study concerning the genetic control of sugar content in pomegranate was reported. Sugar content expressed as TSS in aril juice was mapped using an F2 population. Two QTLs were detected on linkage group 2 of the genetic map with a LOD score of about 6 and separated by a distance of 20 cM (Harel-Beja et al., 2015).

Organic Acids

Analyses of the organic acids of pomegranate aril juice have shown that citric acid is generally the predominant organic acid and its content can reach up to 3.76 g/100 g in the juice. In addition, it contains significantly lower levels of malic, oxalic, succinic, tartaric, and ascorbic acids (**Table 1**). In the fruit peel, citric acid is the predominant organic acid and its content can reach up to 1.68 g/100 g. Smaller amounts of malic, succinic, and oxalic acid were also detected in peels.

Differences Among Varieties

Differences in organic acid composition in aril juice as well as in peels were detected among pomegranate varieties.

Composition of organic acids in the juice

The citric acid's level varies significantly between the different varieties. A range of 0.4–31.4 g/L, 78-fold difference in citric acid content was found among 12 Tunisian varieties (Hasnaoui et al., 2011); 0.08–0.25 g/100 g, 30.7-fold variance among 40 Spanish varieties (Melgarejo et al., 2000); 0.33–8.96 g/L, 27-fold among 13 Turkish varieties (Poyrazolua et al., 2002); and 0.2–2.0 g/100g, 22-fold among 29 Israeli varieties (Dafny-Yalin et al., 2010) (**Table 1**). In addition to citric acid, other organic acids were detected in the juice of pomegranates. These include malic, succinic, and oxalic acids (**Table 1**). However, the levels of these organic acids were relatively low compared to those of the citric acid. In addition, traces of ascorbic, acetic, tartaric, quinic, fumaric, maleic, and lactic acids were detected in some accessions (Melgarejo et al., 2000; Poyrazolua et al., 2002; Aarabi et al., 2008). This suggests that citric acid is a major component of acidic taste in pomegranate fruits. Indeed, the level of citric acid shows a strong and positive correlation with total titratable acidity ($R^2 = 0.91$, $P < 0.01$) as measured in several collections (e.g., Poyrazolua et al., 2002; Tzulker et al., 2007; Hasnaoui et al., 2011). This value significantly changes between the different varieties. The values were about 15-fold in a study of 29 varieties in Israel (Dafny-Yalin et al., 2010), 10 varieties from Morocco and 40 from Tunisia (Hasnaoui et al., 2011; Legua et al., 2012).

Since the level of sugars does not change much and that of acidity differs considerably among the varieties, acidity level is considered to be the main factor that determines the variability of taste in arils (Ben-Arie et al., 1984; Lobit et al., 2003). The ratio of TSS to total acidity values ranged significantly from 6.1 to 64.6 among 29 Israeli-grown varieties when the low values stand for the sour pomegranates and the high for the sweet fruits (Dafny-Yalin et al., 2010). Measurements of 40 Spanish varieties revealed that in sour taste fruits, this ratio ranged from 32 to 96, and in fruits with a sour-sweet taste the values were 17–28 (Gil et al., 1995b). In sweet and sour varieties in Italy the TSS to total acidity ratio ranged from 6.6 to 35.2 (Ferrara et al., 2011).

Composition of organic acids in the peel

As in the arils, the major organic acid in the peel is citric acid, but its level was about 3- to 5-fold lower compared to its level in the arils. The citric acid levels vary from 11 to 390 mg/100 g (13-fold) among the 29 cultivars (Dafny-Yalin et al., 2010). Besides citric acid, malic acid (1.5–32 mg/100 g) and succinic acid (2.5–14 mg/100 g) were also detected in the peel. Eight out of 12 cultivars containing oxalic acid in their arils also contained oxalic acid in their peel (6–31 mg/100 g) (Dafny-Yalin et al., 2010). Citric acid was also the dominant organic acid (507–1,678 mg/100 g) in the peels of six cultivars grown in Georgia, followed by malic (93–116 mg/100 g) and succinic acids (13–16.5 mg/100 g), while oxalic acid was found at a lower level (7.4–9.3 mg/100 g) (Pande and Akoh, 2009). Total acidity values in peels ranged in four Turkish cultivars from 1.48 to 3.66% (Orak et al., 2012), from 0.27 to 1.23% in 29 Israeli varieties (Dafny-Yalin et al., 2010), from 0.97 to 1.39% in five Turkish cultivars (Gözlekçi et al., 2011), and from 0.36 to 0.40% in three Indian cultivars (Dzugan et al., 2018). The level of organic acids in pomegranate peel appears to be highly variable. The reasons for this could be manifold and depend on genetic background, methods of extraction and fruit ripening stage at harvest time. It appears that the developmental stage of the pomegranate fruit in sour cultivars is associated with reduction in total acidity.

Differences During Fruit Development

Several studies followed the levels of organic acids and titratable acidity in aril juice during fruit development. Indian and Tunisian cultivars grown in India ("Ganesh" and "Taifi") (Kulkarni and Aradhya, 2005), and Israeli and American cultivars grown in Israel ("Rosh Hapered" and "Wonderful") (Shwartz et al., 2009) showed that total acidity decreased during fruit maturation. Respectively, the TSS to total acidity ratio and the pH increased across all the tested cultivars.

During 10 weeks of the development and ripening of two cultivars grown in Israel ("Wonderful" and "Rosh Hapered"), the level of citric acid as well as that of total acidity in "Wonderful" decreased significantly. The content of citric acid in the sweet "Rosh Hapered" was the lowest compared to malic, succinic and oxalic acids, and was not significantly correlated with total acidity in this cultivar. The levels of malic and ascorbic acids increased in both cultivars during fruit development (Shwartz et al., 2009). Unlike the results obtained from two different studies done in Israel with "Wonderful" (Ben-Arie et al., 1984;

TABLE 1 | The levels of organic acids (g/100 g juice) and total titratable acidity (%) in aril juices of different varieties from different collections grown in different countries.

Variety no.	Growing country	Citric acid	Malic acid	Oxalic acid	Succinic acid	Tartaric acid	Ascorbic acid	Titratable acidity (%)	References
10	Morocco	0.00–3.20	0.30–1.50	nd	0.03–0.37	nd	nd	2.4–37.5 (15.6)	Legua et al., 2012
15	Spain	0.06–1.85	0.09–0.14	nd	nd	0.02–0.04	nd	1.9–14.3 (7.5)	Mena et al., 2011
40	Spain	0.08–0.25	0.08–0.21	0.01–0.07	nd	0.00–0.01	nd	2.1–12.4 (5.9)	Melgarejo et al., 2000
29	Israel	0.20–2.00	0.02–0.60	0.00–0.42	0.00–0.26	nd	0.06–0.12	0.2–3 (15)	Dafny-Yalin et al., 2010
30	Tunisia	0.04–3.14	0.72–2.04	0.03–0.65	0.14–0.89	0.00–0.18	nd	0.2–3.4 (16.7)	Hasnaoui et al., 2011
20	Spain	0.04–1.90	0.35–1.20	nd	nd	nd	nd	1.4–19.2 (13.7)	Alcaraz-Mármola et al., 2017
13	Turkey	0.03–0.90	0.06–0.69	0.00–0.67	0.00–0.15	0.03–0.28	nd	4.6–17.3 (3.8)	Poyrazolua et al., 2002
7	Turkey	0.39–1.31	0.03–0.24	nd	nd	nd	nd	nd	Tezcan et al., 2009
6	Turkey	0.20–3.20	0.09–0.15	nd	nd	nd	0.01–0.06	0.5–3.8 (7.6)	Ozgen et al., 2008
25	Iran	0.00–3.76	0.02–0.37	0.01–0.06	0.00–0.13	0.03–0.11	0.00–0.01	nd	Aarabi et al., 2008

nd, not detected; different units used in different studies were converted (1 g juice is equivalent to 1 ml juice); Numbers in bracket are the fold change between the values.

Shwartz et al., 2009), in South Africa, citric acid and succinic acid increased during the development of "Wonderful" while malic acid did not significantly change (Mphahlele et al., 2016). This increase in citric acid was observed despite the fact that total acidity decreased from 2.1 to 1.1 g/100 mL. Analysis of the levels of ascorbic acid (Vitamin C) reveals that its level decreased significantly during the development of Tunisian and Indian cultivars (Al-Maiman and Ahmad, 2002; Kulkarni and Aradhya, 2005). However, in the two cultivars that were grown in Israel, the content of ascorbic acid increased (Shwartz et al., 2009) during development. The differences in the content of citric acid in "Wonderful" between South Africa and other countries are intriguing in view of the fact that citric acid is a major organic acid that contributes to acidity and that total acidity decreased in all studies, including the one from South Africa.

Climate and Geographic Influence

Total titratable acidity values were shown to be affected by climate and growth conditions. Arils from 11 varieties grown in the Jezreel Valley (Mediterranean climate) and in the Southern Arava Valley (hot-dry desert climate) were analyzed to study the effect of environmental and climatic conditions on the arils' acid content. The cultivars grown in Mediterranean climate had higher acidity levels compared to the acidity levels found in desert climate. This was in accordance with the higher contents of citric and malic acids, the two main organic acids in the arils (Schwartz et al., 2009).

Generally sour cultivars are mostly grown in northern cold regions, while sweet cultivars with low acidity values are mostly found in regions having hot dry conditions. In Southern Spain and North Africa most of the commercialized cultivars have a sweet taste (Al-Kahtani, 1992), while in North Turkey and Russia sour cultivars are commercialized (Gabbasova and Abdurazakova, 1969; Mayuoni-Kirshinbaum and Porat, 2014; Alcaraz-Mármola et al., 2017). In northern regions such as Russia, Macedonia, Georgia, and Turkey, the total acidity ranged from 0.5 to 2.3% (Gabbasova and Abdurazakova, 1969), 0.6 to 2.2% (Pande and Akoh, 2009), 0.4 to 2.8% (Veres, 1977), and 1.7 to 4.6% (Poyrazolua et al., 2002), respectively. However, in hot climates such as in India, Egypt, and Saudi Arabia, total acidity values dropped to 0.12–0.13% (Al-Maiman and Ahmad, 2002), 0.03–0.10%, and 0.02–0.14%, respectively (Al-Kahtani, 1992).

Amino Acids

Amino acids are organic compounds that among other functions have an important role in protein biosynthesis and secondary metabolite syntheses. In addition to their role as building blocks of proteins, amino acids function as precursors or intermediates in biosynthetic pathways such as production of color molecules and volatiles in fruits, energy release through degradation, signaling processes in plant metabolism regulation and plant stress response (Creighton, 1993; Tatjana et al., 2015; Li et al., 2017). Plants are a nutritional source for these elements and hence the importance of amino acid availability in fruits. There are very limited data and only a handful of research publications concerning amino acid in the pomegranate fruit.

Composition of Amino Acids in the Juice

There are just two studies involving amino acid profile in pomegranate juice and they are incomparable (**Figure 2**). In this context, it should be pointed out that different detection methods might result in different amino acid compositions. Thus, one cannot conclude in general the composition of amino acids in pomegranate juices excluding the fact that serine is found at high percentages in all juices studied.

Li et al. (2017) studied amino acids in juices of separated arils of six Chinese cultivars from two regions. Glutamine, serine, aspartate, and alanine are the most abundant amino acids in the juice, while glycine was not detected. All the essential amino acids exist but in smaller portions (**Figure 2A**). Tezcan et al. (2013) analyzed fresh squeezed juice from three pomegranate fruits and three commercial pomegranate juices obtained from local markets (claimed to be 100% pomegranate). The authors did not specify if the juice was squeezed from separated arils or from the intact arils. In addition to the L-amino acids that were identified, D-proline was detected in all the juices and D-leucine in one of the commercial juices. L-serine, L-proline, and L-alanine are the

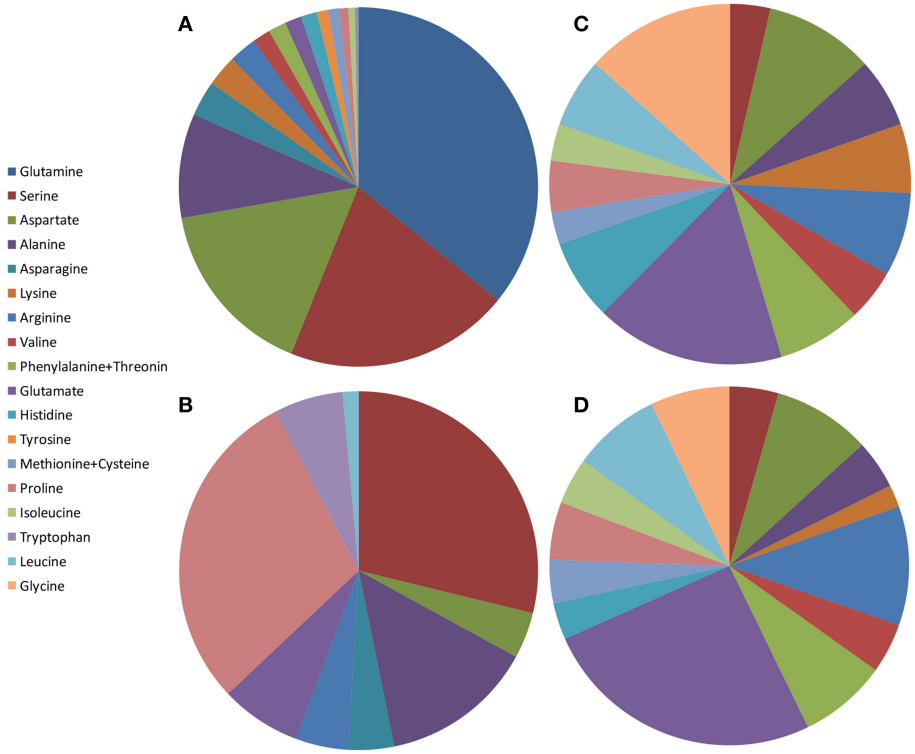

FIGURE 2 | Schematic illustration of amino acid content in pomegranate fruit tissues from different varieties and countries. The values presented are average values of the percentage of each amino acid of the total amount of amino acids measured: **(A)** juice from China by Li et al. (2017); **(B)** juice from Turkey by Tezcan et al. (2013); **(C)** peel powder from Egypt by Rowayshed et al. (2013); **(D)** seed powder from Tunisia and Egypt by Elfalleh et al. (2011) and Rowayshed et al. (2013).

most abundant amino acids in these pomegranates juices, while D-leucine was not detected (**Figure 2B**).

Composition of Amino Acids in the Peel

Rowayshed et al. (2013) studied the peel powder of local Egyptian fruit obtained from the market. They found that glutamine (0.52 g/100 g), glycine (0.41 g/100 g), and aspartate (0.3 g/100 g) are the most abundant amino acids in the pomegranate peel powder studied. Tryptophan was not detected but all the other essential amino acids exist (**Figure 2C**). Asparagine, glutamine, and tyrosine were not measured in this study. The peel powder studied contained an exceptionally higher content of lysine, isoleucine, methionine, and cysteine than the reference protein pattern of FAO/WHO (Rowayshed et al., 2013).

Composition of Amino Acids in the Seeds

There are only two studies involving amino acid profiling in pomegranate seeds (Elfalleh et al., 2011; Rowayshed et al., 2013). Elfalleh et al. (2011) studied amino acids in seeds of two Tunisian commercial pomegranate cultivars and Rowayshed et al. (2013) studied local Egyptian varieties. Both revealed high proportions of glutamate (3.5 g/100 g), arginine (1.9 and 1.47 g/100 g respectively), and aspartate (1.9 and 1.21 g/100 g, respectively) in dry seeds. **Figure 2D** shows the average results of these studies as percent of each amino acid of the total amino acids measured. Notably, the amino acid composition in seed powder was very similar to those found in peels of local Egyptian fruit (Rowayshed et al., 2013).

The two groups (Elfalleh et al., 2011; Rowayshed et al., 2013) found that essential amino acid content is much higher than the requirement of FAO/WHO for adults. Since these essential amino acids are usually deficient in most foods, the authors suggested that these tissues can serve as food supplements.

Differences Among Varieties

A study on juices of six pomegranate cultivars grown in two regions in China revealed that the genotype of the pomegranate had a significant effect on the amino acid profile and content (Li et al., 2017). Clustering analysis relying on amino acid content showed segregation of amino acids in these pomegranate cultivar juices. This difference was mainly attributed to the differences in the content of cysteine, ornithine, aspartate, serine, methionine, and leucine. Analysis of proline in three Turkish cultivars from three regions in two successive years show that proline varied among pomegranate cultivars (Halilova and Yildiz, 2009) although the data published show insignificant difference between the cultivars (i.e., 65 ± 31, 60 ± 30, and 59 ± 33 mg/L). Elfalleh et al. (2011) studied amino acids in seeds of two Tunisian commercial pomegranate cultivars. No significant differences were found between "Jebali" and "Gabsi" cultivars but significant differences ($p < 0.05$) were observed in the levels

of glycine, cysteine, methionine, histidine, arginine, and proline (Elfalleh et al., 2011).

Differences During Fruit Development

No information is available regarding amino acid content changes through fruit development except for the information given by Nuncio-Jáuregui et al. (2014) for proline. This group evaluated the effects of fruit maturation stage on pomegranate juices' chemical structure. A positive relationship between fruit maturation stage and proline content was reported in different cultivars, showing that proline increased from 32 to 84 mg/L in "Mollar de Elche" juices (Nuncio-Jáuregui et al., 2014).

Climate and Geographic Influence

Li et al. (2017) studied six pomegranate cultivars from Shandong (near the ocean) and Xinjiang (Eurasian continental climate) regions in China. The pomegranate juices from separated arils from Shandong had higher levels of total amino acids and of essential amino acids than those from Xinjiang. The total glutamate-, aspartate-, pyruvate-, and serine-related amino acids were higher in the Shandong juices, while the total aromatic amino acid contents were higher in the Xinjiang juices.

Halilova and Yildiz (2009) quantified proline content in freshly pressed juice of three Turkish cultivars from three regions in 2 successive years. The authors found that the average proline content was 30 mg/L in the first year and 93 mg/L in the second, which was drier and hotter than the first year. The authors claim that this 3-fold increase in the second year indicates that climatic change affects proline accumulation (Halilova and Yildiz, 2009).

According to the researches, it can be concluded that amino acid content in pomegranate fruits is influenced by environmental conditions, particularly temperature and water availability.

Proteins

The data on proteins in pomegranate fruits are limited and mainly concern total protein content in various tissues. Most of the studies do not report specific protein functions with the exception of storage proteins in the seeds and lipid transfer proteins in the arils. In general, the percent of total proteins in pomegranate juice is usually low, from <1.0 to 1.1%, which is quite a narrow range. Diversely, the percent of total proteins in pomegranate seeds varies from 4.1 to 16.9%, which is quite a wide range. In this tissue, the presence of the storage proteins, globulins, albumins, glutelins, and prolamins, is prominent and the first two are the major proteins found in most of the studies.

Differences Among Varieties

Composition of proteins in aril flesh and juice

Elfalleh et al. (2011) studied proteins in the juice and pulp of two Tunisian commercial pomegranate cultivars. Protein content in the juice was 7.95 ± 0.89 g/L and differed significantly between the two cultivars (Elfalleh et al., 2011). Elfalleh et al. (2009) studied fresh pomegranate juices (from arils only) and dry pulps of fully mature fruits from six local Tunisian ecotypes. The juice protein content was about 6.67 ± 2.26 g/L and the dry pulp protein content was 22.9%. The juice protein contents were significantly different, varying from 9.93 ± 1.90 g/L in "Chetoui" to 4.13 ± 1.20 g/L in "Gabsi 2." The authors state that pomegranate is highly proteinic (∼0.66%) compared to red wine (0.04%) and raw apple juice (0.27%) (Elfalleh et al., 2009). Al-Maiman and Ahmad (2002) studied total protein amounts in the aril juice of Saudi Arabian pomegranate "Taifi." Fully-ripe fruits contained 1.05% protein in the juice (Al-Maiman and Ahmad, 2002). Kulkarni and Aradhya (2005) report total protein conteny of about 85 mg/100 g in the juice of ripe Indian "Ganesh" (100 days after fruit set).

Composition of proteins in the seeds

El-Nemr et al. (1990) determined crude protein in fully ripened Egyptian pomegranate fruits obtained from the local market. The analysis discovered that seeds, but not juice, contain protein and that 13.2% of the constituents measured in the dry seeds were unidentified proteins (El-Nemr et al., 1990). Al-Maiman and Ahmad (2002) studied total protein amounts in seeds of Saudi Arabian pomegranate "Taifi." Fully-ripe fruits contained 4.06% of protein in the seeds (Al-Maiman and Ahmad, 2002). Elfalleh et al. (2011) studied proteins in the seeds of two Tunisian commercial pomegranate cultivars. Seed storage protein content was 167.8 ± 8.9 mg/g dry weight, which constitutes 16.9% of the seeds' dry weight. Globulins (62.4 mg/g) and albumins (54.1 mg/g) are its major fractions, followed by glutelins (33.2 mg/g) and prolamins (18.1 mg/g). No significant difference in the content of albumins and glutelins was found between the cultivars (Elfalleh et al., 2011). Elfalleh et al. (2010) studied storage proteins in seeds of mature pomegranate fruits of eight different Tunisian cultivars from five Tunisian regions. The authors reported that the seeds contain 16.8% proteins (per dry weight). The pomegranate seeds accumulated mainly globulins (43%) and albumins (32%). Glutelins constituted 16% and prolamins only 9% of the proteins found. Significant differences between the cultivars in the total amount of storage proteins, ranging from 15.4% in "Beldi" to 20.1% in "Rafrafi" was found, as well as differences for each fraction's content (Elfalleh et al., 2010). Zang (2011) determined protein content in pomegranate seeds oil originating from Xinjiang. The average content of crude protein in pomegranate seeds was 14.3%, in which glutelins and residual protein constituted more than 80% of total protein content, and the contents of globulins, albumins, and prolamins were lower (Zang, 2011).

In summary, it appears that there are differences in total protein content between pomegranate varieties. There is also variability in the content of the different storage proteins in the seeds. This variation may be connected to their genetic background but also to different analysis methods or environmental conditions.

Differences During Fruit Development

Al-Maiman and Ahmad (2002) studied total protein amounts in aril juice and seeds of Saudi Arabian pomegranate "Taifi" and compared unripe, half-ripe, and fully-ripe fruits. Protein concentration in seeds was found to be about four times higher than in the juice (an average of 4.06 vs. 1.05%). No significant changes were observed in protein concentration

during fruit development in the seeds. The juice of unripe fruits contained significantly less proteins than the quantities in half-ripe and fully-ripe fruits (Al-Maiman and Ahmad, 2002). Kulkarni and Aradhya (2005) reported total protein content in squeezed separated arils of the Indian "Ganesh" at seven fruit developmental stages (20, 40, 60, 80, 100, 120, and 140 days from fruit set). The study revealed significant changes in total protein content in the juice during fruit development. The highest total protein (209 mg/100 g) occurred 20 days after fruit set with a rapid decrease (66.9%) toward 80 days after fruit set. An increase (58.7%) occurred from 80 to 120 days and a significant slight decrease (6.3%) in total protein content occurred after 120 days (Kulkarni and Aradhya, 2005).

These two studies indicated that total protein content in the juice changes during pomegranate fruit development, but this does not happen in the seeds.

Lipids

Lipids are a group of small hydrophobic molecules that include fatty acids, waxes, sterols, fat-soluble vitamins, phospholipids, mono-, di-, and triglycerides. Primary and secondary lipids have diverse functions in living organisms, including energy storage, cell signaling, nutrition (fats and vitamins), hormones, transport, and structural components of cell membranes. The most lipid-rich fraction in pomegranates is the seeds, which contribute 10% to fruit weight. Generally, seed oil constitutes 6–20% of seed weight and contains a large quantity of lipids (Viuda-Martos et al., 2010; Ferrara et al., 2011). The chain length of the lipids is divided to three classes: Medium- (C6–C12), long- (C14–C20), and very long (C22 and C24). The total lipid (the term refers to primary and secondary lipids) percentage in seeds varies from 4.4 to 27.2% (El-Nemr et al., 1990; Pande and Akoh, 2009; Jing et al., 2012; Ferrara et al., 2014; Verardo et al., 2014; Fernandes et al., 2015a). The list of lipids found in pomegranate fruit tissues is presented in **Table 2**.

Punicic acid is the most abundant fatty acid in seed oil, constituting over 60% of the fatty acids, mostly followed by oleic acid, linoleic acid, and palmitic acid in a variable order (**Figure 3A**, Pande and Akoh, 2009; Jing et al., 2012; Ferrara et al., 2014; Verardo et al., 2014; Wu and Tian, 2017). Triterpenoids and phytosterols have been found in pomegranate seed and fruit peel (Seeram et al., 2006b; Verardo et al., 2014; Wu and Tian, 2017). The major phytosterol detected in seed oil is sitosterol (Kaufman and Wiesman, 2007; Pande and Akoh, 2009; Verardo et al., 2014). Most of the lipids were identified in the seeds, small amounts of lipids were also detected in aril juice and fruit peel. It should be mentioned that the presence of some of the lipids such as human steroid hormones is disputed (Choi et al., 2006).

Differences Among Varieties

Composition of lipids in the seed oil

Verardo et al. (2014) studied the lipid composition (fatty acids, sterols, tocopherols, and phospholipids) of pomegranate seed oil from 17 varieties: (4 Israeli, 3 Spanish, 1 Turkish, 1 Iranian, 2 Tunisian, 6 Italian). The total lipid content (primary and secondary) of the pomegranate seeds varied from 7.6 to 16.2%.

TABLE 2 | Lipids including fatty acids, sterols, and triterpens identified in pomegranate fruit peel, aril juice, and seed tissues; (+) reported presence; (–) presence not yet reported.

Lipid group	Lipid molecule	Fruit peel	Aril juice	Seed
Fatty acid	Arachidic acid	+[a]	+[a]	+
	Behenic acid	–	–	+
	Capric acid	–	+	–
	Caproic acid	–	+	–
	Caprylic acid	–	+	+
	Catalpic acid	–	–	+
	Docosadienoic acid	–	–	+
	Eicosenoic acid	–	–	+
	Eicosapentaenoic acid	–	–	+
	α-Eleostearic acid	–	–	+
	β-Eleostearic acid	–	–	+
	Erucic acid	–	–	+
	Gadoleic acid	–	–	+
	Gondoic acid	–	–	+
	Lauric acid	–	–	+
	Lignoceric acid	–	–	+
	Linoleic acid	+[a]	+[a]	+
	Linolelaidic acid	–	–	+
	α-Linolenic acid	+[a]	+[a]	+
	γ-Linolenic acid	+[a]	+[a]	+
	Margaric acid	–	–	+
	Myristic acid	+[a]	+[a]	+
	Myristoleic acid	–	–	+
	Nervonic acid	+[a]	+[a]	+
	Oleic acid	+[a]	+[a]	+
	Palmitic acid	+[a]	+[a]	+
	Palmitoleic acid	+[a]	+[a]	+
	Pentadecylic acid	–	–	+
	Punicic acid	+[a]	+[a]	+
	Stearic acid	+[a]	+[a]	+
	cis-Vaccenic acid	–	–	+
	Triacylglycerols, 3-O-octadec-2-enoic acid	–	–	+
	Tricosylic acid	–	–	+
	9Z, 11E, 13Z-Octadecatrienoic acid	+	–	+
	8Z, 11Z, 13E-Octadecatrienoic acid	+	–	+
Sterol	Campesterol	–	–	+
	Cholesterol	–	–	+
	Citrostadienol	–	–	+
	Daucosterol	–	–	+
	Estradiol	–	–	+
	Estrone	–	–	+
	Estriol	–	–	+
	β –Sitosterol	–	–	+
	β-Sitosterol laurate	+	–	–
	β-Sitosterol myristate	+	–	–

(Continued)

TABLE 2 | Continued

Lipid group	Lipid molecule	Fruit peel	Aril juice	Seed
	Stigmasterol	−	−	+
	Testosterone	−	−	+
	Δ5-Avenasterol	−	−	+
Triterpene	Asiatic acid	−	−	+
	Betulinic acid	−	−	+
	Cycloartnol	−	−	+
	Punicanolic acid	+	−	−
	Squalene	−	−	+
	Ursolic acid	−	−	+
Glycosphingolipid	Glycosphingolipid N-palmitoyl cerebroside	−	−	+
	N-Palmitoyl cerebroside	−	−	+
Glycerolipid	1-*O*-Isopentyl-3-*O*-octadec-2-enoyl glycerol	+	−	+
	1-*O*-Octadecatrienoyl glycerol	−	−	+
	Di-*O*-Punicyl-*O*-octadeca-trienylglycerol	−	−	+
	Tri-*O*-Punicylglycerol	−	−	+
Phospholipid	Phosphatidylethanolamine	−	−	+
	Phosphatidylcholine	+[a]	+[a]	+
Tocopherol	α-Tocopherol	−	−	+
	γ-Tocopherol	−	−	+

[a] *The molecule was detected in a mix of juice and peels.*

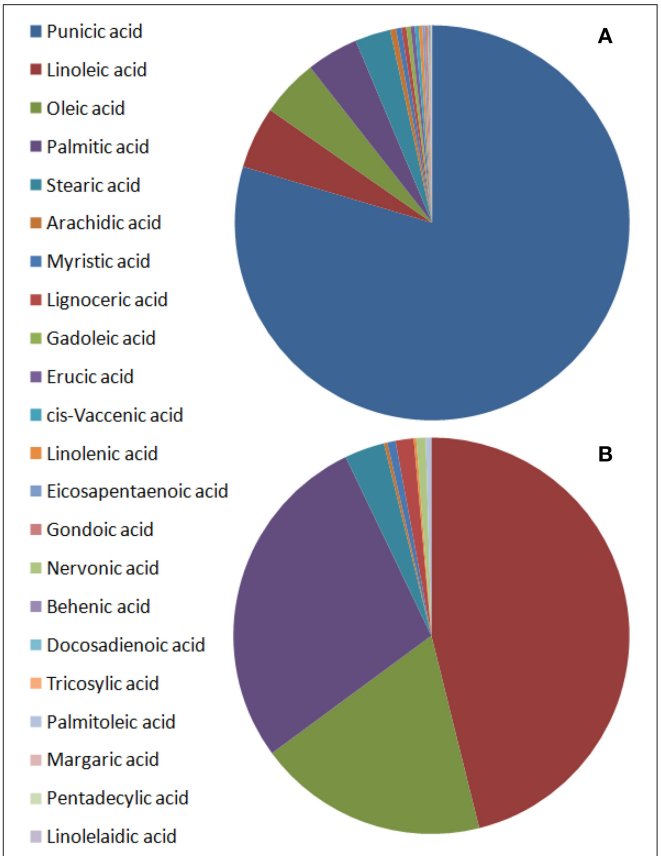

FIGURE 3 | Schematic illustration of fatty acid content in pomegranate fruit tissues from varieties of different origin. The values presented are average values of the percentage of each fatty acid of the total fatty acids measured. **(A)** Seed oils by Pande and Akoh (2009), Ferrara et al. (2014), and Verardo et al. (2014); **(B)** non-seed fruit tissues by Pande and Akoh (2009).

The oil consisted of 65–80% conjugated fatty acids of which punicic acid constituted 74–85%. Other major fatty acids were oleic, linoleic and palmitic acid. Polyunsaturated fatty acids constituted 87.2% of the total seed oil, while monounsaturated fatty acids constituted 7.1% and saturated fatty acids constituted 5.7% of the total amount of fatty acids in the seed oil. Total sterol content varied between 7.5 and 16.4 mg/g of oil. The major phytosterols detected were campesterol, stigmasterol, sitosterol, Δ5-avenasterol, and citrostadienol. Sitosterol constituted 65–74% of the total sterols. Triterpene compounds cycloartenol and squalene constituted 0.8–2.4 mg/g oil (45.1%) and 0.7–3.2 mg/g (42.5%), respectively. Phospholipids were 0.4–2.3% of the total lipids and phosphatidylethanolamine was the main compound, constituting 56–86% of total phospholipids. In addition, total tocopherol content ranged between 678.3 and 2627.4 μg/g of oil, and γ-tocopherol, was 91% of the total tocopherols. There were significant differences between the varieties in fatty acids, sterols, phospholipids, and tocopherols. Differences were also found between variants of the same variety. Such are for example differences that were found between the landraces "Wonderful" and "Wonderful 1" in the content of oleic acid (17.34 and 32.07%, respectively), and squelene (0.91 and 3.18 mg/g oil, respectively) (Verardo et al., 2014).

Jing et al. (2012) studied the lipid composition of extracted seed oil from four Chinese cultivars from Shanxi. Oil content in the seeds ranged from 114.2 to 147.9 mg/g and was significantly different between cultivars. Seed oil was rich in polyunsaturated fatty acids (87–92% of fatty acids), which were significantly different in the four cultivars. Punicic acid was the dominant fatty acid (73.5–78.8 g/100 g fatty acids). Total tocopherols ranged from 2,188 to 4,947 μg/g. The four cultivars were significantly different in their lipid content (Jing et al., 2012). Another study in China examined the content and composition of fatty acid in the seed oil of pomegranates from Xinjiang. The oil content was 18.2%, and unsaturated fatty acids were more than 70% of total fatty acid (Zang, 2011).

Ferrara et al. (2014) studied the oil content and fatty acid composition of 13 sweet and sour pomegranate genotypes from Puglia region in Southeastern Italy, of which 3 were of Israeli origin. The oil extracted from the dried seeds and the content of total lipids were significantly variable among these genotypes, ranging from 10.7 to 26.8% in sweet genotypes and from 4.9 to 17.4% in sour genotypes. Sixteen fatty acids were identified in this study, among which punicic acid was the major fatty acid in all genotypes. Punicic acid content exceeded 74.9%, followed by palmitic, linoleic, stearic, and oleic acids. There was low variability of fatty acid

composition between the genotypes. Unsaturated fatty acids in the seed oils constituted between 86.7 and 91.2% with saturated/unsaturated ratios ranging between 0.10 and 0.15 (Ferrara et al., 2014).

Fadavi et al. (2006) determined the fatty acid composition of seed oil from 25 Iranian pomegranate varieties. Oil was extracted from dry seeds of commercially ripe fresh fruits from Markazi and Yazd provinces in Iran. Oil content ranged from 6.6 to 19.3% (W/W). The pre-dominant fatty acid was linolenic acid–31.8–86.6%—followed by linoleic acid, oleic acid, stearic acid, and palmitic acid (Fadavi et al., 2006). Worth noticing is the fact that punicic acid was not detected in this study. This fatty acid was found to be the predominant acid in pomegranates in many other studies but was not detected in these varieties. Since punicic and linolenic acids are isomers, this might be a result of different classification or misinterpretation of the molecule's nature. Differences between the varieties were significant. Moreover, differences were found in the lipid contents of sweet, sour, and sour sweet varieties while the lowest was in sweet varieties and the highest in sour sweet.

The profile of fatty acids and phytosterols in pomegranate seed oil from four varieties grown in Israel was determined. Results showed linolenic acid to be the predominant fatty acid (64–83%). The linolenic acid fraction was composed of four different chromatographically separate peaks that are assumed to be attributed to different isomers of conjugated linolenic acid, and punicic acid was the major isomer. Phytosterols were found at quite a high concentration (4,089–6,205 mg/kg) with a wide variety of components, and the major phytosterols were β-sitosterol, campesterol, and stigmasterol (Kaufman and Wiesman, 2007). Fatty acid and tocopherol composition in the seed oil of nine worldwide pomegranate varieties that were grown in Spain was analyzed resulting in 4.4–12.0% oil content. Over 86% of the oil were unsaturated fatty acids, mainly punicic acid, ranging between 77.3 and 83.6% of total fatty acids. Total tocopherols ranged from 174.5 to 627.3 mg/100 g oil, mainly γ-tocopherol. There were significant differences between the cultivars (Fernandes et al., 2015a).

Lipids were also studied in local varieties from some other countries. Fatty acid content was studied in seeds of fully ripened local market Egyptian pomegranate fruits. Total lipids were 27.2% with saturated fatty acids being 83.6% of the total fatty acids. The predominant acid was caprylic acid (36.3%), followed by stearic acid, oleic and linoleic acids out of 11 fatty acids that were identified (El-Nemr et al., 1990). Punicic acid or linolenic acid were not detected. The oil content and fatty acid composition of the seed oil of seven Spanish sweet pomegranate varieties was 6.3–12.2%, of which 73.4–95.8% were unsaturated fatty acids. The predominant fatty acid was linolenic acid (43.4–88.2%), followed by linoleic acid, oleic acid, and palmitoleic acid. Differences in fatty acid composition were found among the varieties studied (Melgarejo and Artes, 2000). Fatty acid composition in the seed oil of 25 pomegranates varieties from two different regions of India showed oil content of 6.6–19.3%, most of it unsaturated fatty acids. The predominant fatty acid was linolenic acid (31.8–86.6%), followed by linoleic acid, oleic acid, stearic acid and palmitoleic acid. The varieties studied had similar but not identical fatty acid composition Parashar et al., 2010).

Significant differences were found in studies conducted with different varieties in different regions of the world, indicating that genetic background influences this trait. Nevertheless, the general structure of lipids, mainly in seed oil, is very similar (**Figure 3A**). The vast majority of the fatty acids are unsaturated fatty acids and punicic acid is by far the main fatty acid. Sitosterol is the most abundant phytosterol in pomegranate seed oil.

Composition of lipids in juice and peel

Pande and Akoh (2009) investigated the lipid profiles of six Georgia (USA) pomegranate varieties. Ripe fruits were used for the preparation of two fractions: seed tissue and non-seed tissues (peels and juice). Total lipid content in the seeds was 18.1–21.5% and 0.2–0.3% in the non-seed fraction. Punicic acid was the predominant fatty acid in the seed lipids and linoleic acid was the major fatty acid in the non-seed fraction. All the varieties had the same saturated/unsaturated fatty acids ratio of 0.1 for the seeds and 0.5 for non-seed tissues. Linoleic acid, palmitic acid, and oleic acid are most abundant in aril juice and peel and are secondary in abundance in seeds; however, punicic acid was not detected in the non-seed tissues. Pomegranate seed had a high content of α- and γ-tocopherol (167.3 and 84.6 mg/100 g, respectively). The most abundant phytosterol was β-sitosterol, ranging from 32.7 to 345.8 mg/100 g. There were more phospholipids in seeds than in aril juice and peel. Phosphatidylcholine content varied from 5.8 to 23.1 mg/100 g and phosphatidylethanolamine ranged from 10.2 to 74.2 mg/100 g in all the seed varieties. Significant differences were found between the four cultivars in their different lipid components (Pande and Akoh, 2009). **Figure 3B** illustrates a representative picture of the relative content of fatty acids in pomegranate non-seed fruit tissues (based on Pande and Akoh, 2009).

Differences During Fruit Development

Only one publication describes the changes that occur during seed development. Al-Maiman and Ahmad (2002) studied unripe, half-ripe, and fully-ripe fruits of Saudi Arabian pomegranate "Taifi." Lipid concentration in seeds during fruit maturation was 0.2, 0.01, and 0.25%, respectively and unsaturated fatty acids constituted 81.6, 82.1, and 84.6% of all fatty acids. No significant changes were observed in lipid concentration and fatty acid classes in the seeds during fruit development (Al-Maiman and Ahmad, 2002).

Climate and Geographic Influence

To our best knowledge, only one study compared the content of seed oil among the same varieties under different climates. However, no study that deals with climate or geographical influence on fruit lipid composition was published. Seed oil content of several pomegranate varieties was measured for trees grown in a Mediterranean climate (Newe Ya'ar) and desert climate (Arava desert). Oil content ranged from 7.76 to 17.96 g/100 g of dried seeds, varying for the different accessions. Four accessions (P.G.114-15, P.G.116-17, P.G.128-29, and P.G.130-31)

exhibited significantly higher oil contents when grown in the southern Arava compared to Newe Ya'ar. These results suggest that fruits grown in a hot dry climate may have higher oil content in their seeds (Schwartz et al., 2009).

SECONDARY METABOLITES

Anthocyanins

Anthocyanins are the key color molecules of pomegranate present in various parts of the pomegranate trees, including leaves, flowers, and fruits. The pomegranate fruit is a rich source of anthocyanins and produces several derivatives of anthocyanins. These secondary metabolites accumulate in all fruit tissues and mainly in the edible part of the fruit, the arils, and in the fruit peel (Gil et al., 1995b; Hernandez et al., 1999; Tzulker et al., 2007). Six anthocyanin molecules were identified in pomegranate fruit, including mono- and di-glucosides of cyanidin (red pigments), delphinidin (purple pigments), and pelargonidin (orange pigments) (Du et al., 1975; Gil et al., 1995b). All six anthocyanin pigments were detected in pomegranate cultivars from different geographical regions, which include Israeli, Turkish, Spanish, Californian, Tunisian, Italian, and Chinese pomegranates (Gil et al., 1995a,b; Ben-Simhon et al., 2011; Turkyilmaz, 2013; Zhao et al., 2013). However, differences in the relative amounts of anthocyanins were found, depending on variety, climatic, and cultural variables (Gil et al., 1995a; Ben-Simhon et al., 2011; Borochov-Neori et al., 2011). Some unusual anthocyanin molecules were reported by Fischer et al. (2011a,b), who detected cyanidin pentoside in pomegranate peel and juice and cyanidin rutinoside and cyanidin pentoside-hexoside in the juice. Zhao et al. (2013) reported that peonidin hexoside and myricetin hexoside were detected in the peel of a dark red Chinese cultivar. These findings suggest that the pigment profile of pomegranates may be much more diverse.

The function of anthocyanin in the biology of the pomegranate tree is not yet fully understood. The tree of the "white" phenotype pomegranate varieties, which do not produce any anthocyanin (Ben-Simhon et al., 2015), is vigorous and fertile. It seems, however, that the white flowers and anthocyanin-less fruits are more susceptible to browning and radiation damages (personal communication). The accumulation of anthocyanin in young pomegranate leaves also suggests that it acts to protect the tissues from abiotic and biotic stresses during leaf development.

Differences Among Varieties

Composition of anthocyanins in the peel

In an attempt to determine the color variability among pomegranate varieties, 29 varieties that represent most of the phenotypic variability in the Israeli pomegranate collection were assayed. Total anthocyanin levels were measured for both peel and aril extracts. The content of total anthocyanins in the peel varied between 0.2 and 8.0×10^2 mg/L, while the anthocyanin content in the aril juice varied between 0.2 and 3.5×10^2 mg/L (Tzulker et al., 2007). The high variation that was detected in peel total anthocyanin content is also observed by the naked eye. Thus, fruit from various varieties in the Israeli collection display colors which range from purple to dark red to green (Tzulker et al., 2007; Holland and Bar-Ya'akov, 2008; Holland et al., 2009, **Figure 4**). Three pomegranate cultivars with different colors were also studied in China for their anthocyanin content in fruit peel and juice. Significant differences were found in anthocyanin concentration among different cultivars in their fruit peel (up to 344 mg/100 g) and in their juice (up to 364 mg/100 g) (Zhu et al., 2015).

No correlation was observed between the content of anthocyanin in the peel and in the arils. The predominant color of the fruit peel is mostly the outcome of accumulation of cyanidin derivatives. Cyanidine derivatives constitute about 85% of the anthocynins in ripened pomegranate fruit, while pelargonidin derivatives constitute about 15% in ripened fruit (Ben-Simhon et al., 2011). Only low or undetectable levels of delphinidins were usually found in the fruit skin (Du et al., 1975; Gil et al., 1995b; Ben-Simhon et al., 2011). However, in some dark red Chinese cultivars, large amounts of cyanidin mono-glycoside and delphinidin mono-glycoside (over 100 mg/100 g) were found in the skin (Zhao et al., 2013).

Composition of anthocyanins in the juice

While most Israeli and Mediterranean cultivars displayed negligible levels of delphinidines in their skins, delphinidins, and cyanidins were the major anthocyanins in their aril juice (Gil et al., 1995b; Seeram et al., 2006a; Borochov-Neori et al., 2011). In Israeli cultivars delphinidine derivative content could reach about 40% of the total anthocyanin content of the aril juice and cyanidines could reach about 60% of the total anthocyanins in the aril juice. Similar data was also reported for "Mollar" in Spain, where delphinidine derivatives constituted about 50% and cyanidine constituted about 45% of the aril juice anthocynins (Gil et al., 1995b). Juices from fruits of 30 varieties grown in Tunisia were studied for their anthocyanin content. The total anthocyanin content was different among varieties and ranged from 9 to 115 mg/L juice (Hasnaoui et al., 2011). Aligourchi et al. (2008) measured the amounts of total anthocyanins in the juice of 15 pomegranate varieties obtained from Yazd province in Iran. There was significant difference in total anthocyanin levels among varieties ranging from 15.0 to 252.2 mg/L juice.

From these studies of different varieties originating from several regions in the world and from many others not reported here, it is evident that there are significant quantitative and qualitative differences in the anthocyanin content of peel and juice between pomegranate varieties. These differences can be attributed to the diverse genetic background of the fruits tested.

Differences During Fruit Development

The differences found in the composition and quantity of anthocyanin between the peel and the arils suggest that anthocyanin accumulation in these tissues reflects differential genetic control of anthocyanin production. This assumption is further supported by the different dynamics of anthocyanin accumulation in the peel and arils during fruit development (Ben-Simhon et al., 2011; Holland and Bar-Ya'akov, 2014). This tissue- specific differential

FIGURE 4 | Fruit peel and arils of various varieties in the Israeli pomegranate collection display a wide range of colors.

accumulation of anthocyanins is one of the main difficulties in determining the ripening time of pomegranate fruit by external phenotypic parameters.

Composition of anthocyanins in the juice

Kulkarni and Aradhya (2005) reported total anthocyanin content in squeezed arils of the Indian "Ganesh" at 20, 40, 60, 80, 100, 120, and 140 days after fruit set. A 100% increase in the anthocyanin concentration was observed between 20 and 80 days after fruit set. The highest concentration (138 mg/100 g) was recorded after 100 days, followed by a slight significant decrease (9.3%) up to 140 days of fruit development (Kulkarni and Aradhya, 2005). Shwartz et al. (2009) investigated changes in total anthocyanin content in the arils of two Israeli varieties during fruit maturation. The anthocyanin levels in the aril juice increased significantly during maturation in "Wonderful," which had red aril color (from 165 to 328 mg/L), but not in "Rosh Hapered," which had light pink aril color. Hernandez et al. (1999) studied changes in the quantity and quality of anthocyanins in the juice of five Spanish pomegranate varieties during ripening. Juice from the fruit 26–34 weeks after flower set (immature fruits to commercially mature fruits) was extracted and analyzed. Generally, there was an increase in juice pigmentation during fruit ripening, but total anthocyanin during fruit maturation differed among varieties. Three varieties did not show any change in the first 4 weeks of fruit development and then, the anthocyanin concentration increased rapidly until ripening. The two other varieties already showed an increase in anthocyanin content at very early stages. In the early fruit development stages, delphinidin di-glucoside was the main pigment, followed by cyanidin di-glucoside, while in the later stages, the mono-glucoside derivatives of cyanidin and delphinidin increased considerably. The pelargonidin derivatives were always present in small amounts. The authors found that fruits located in the north side of the trees showed an earlier increase in anthocyanin pigmentation, which was explained by lower temperatures during the night in this niche (Hernandez et al., 1999). The pattern of di-glucosides in the first stages of fruit development and mono-glucosides anthocyanin prevailing in the latter stage was also shown in another study done by Gil et al. (1995b) in Spain.

Composition of anthocyanins in the peel

Zhao et al. (2013) studied three Chinese cultivars of pomegranates with fruit peel colors ranging from green to dark red. The fruit skin was analyzed for anthocyanin content and composition during fruit development at 10-day intervals until full ripening. They found that cultivars' peel color and fruit developmental stage significantly influenced the profile of the anthocyanins and their content. The pigment content generally increased toward ripening and the relative amounts of the six primary anthocyanin molecules changed (Zhao et al., 2013). Shwartz et al. (2009) also studied the changes in anthocyanin content in the peel of two pomegranate cultivars. Peel color significantly changed during maturation in both varieties. Total anthocyanin was significantly correlated with color index in both accessions, indicating that the total anthocyanin content contributed significantly to the peel's skin color (Schwartz et al., 2009).

Climate and Geographic Influence

One of the most interesting aspects of pomegranate color from academic and practical point of view is the influence of environmental conditions on color accumulation. It is well-known that pomegranate fruit color, like that of other anthocyanin-accumulating plants, such as grapes, red orange, and roses, is sensitive to high temperatures (Lo Piero et al., 2005; Ubi et al., 2006; Mori et al., 2007; Ferrara et al., 2015). When fruits of evergreen pomegranates that can produce all year round were tested in the Arava desert in Israel during winter and summer, it was found that the content of anthocyanin in the aril juice was inversely related to the sum of heat units accumulated during fruit ripening (Borochov-Neori et al., 2009). Moreover, it was found that in addition to their effects on the content of anthocyanins, the change of season influenced the level and composition of the anthocyanin derivatives in the juice. Thus, cyanidine molecules accumulated in the hotter

season and delphinidin derivatives accumulated in the cooler season (Borochov-Neori et al., 2011). It was also noticed that di-glycosidic derivatives mostly accumulated in the hot season, while mono-glycosidic derivatives were mostly accumulated during the cooler season, suggesting that di-glycosidic conjugates of anthocyanins are more stable in higher temperatures (Borochov-Neori et al., 2011). The effect of temperature on anthocyanin accumulation was also demonstrated when the content of anthocyanin in 11 different cultivars grown in the Arava desert in Israel was compared to the content of anthocyanin of the same cultivars grown accumulation in aril juice and peel was suggested (Schwartz et al., 2009).

Anthocyanin content in the arils and peel of pomegranate fruit is also sensitive to salt stress (Borochov-Neori et al., 2013). When two different pomegranate cultivars were irrigated with saline water it was found that increased salinity had a positive influence on anthocyanin accumulation in the pomegranate fruit peel (Borochov-Neori et al., 2014). The increase in anthocyanin accumulation corroborates the proposed function of anthocyanins in plant response to environmental stress conditions (Chalker-Scott, 1999; Hatier and Gould, 2009; Steyn, 2009). The magnitude of the effect of increased salinity concentrations from 1 to 6 dS^{m-1} was about 4-fold (from 20 to 80 mg/Kg) of anthocyanin for the highly colorful "Wonderful," and about 8-fold (from 5 to 40 mg/Kg) for the pale color "SP-2." These findings differ from those obtained for the anthocyanin in the arils (Borochov-Neori et al., 2014), where salinity had an adverse effect on anthocyanin accumulation, especially in "Wonderful." As for exposure to different temperatures, exposure to salinity affected the level of anthocyanin derivatives. At elevated salinity levels, "Wonderful" fruit peel accumulated purple delphinidins in addition to the major pigment types, cyanidins and pelargonidins, whereas in "SP-2" the proportion of the orange color pelargonidins increased.

The significance of these data to the physiology of the fruit and trees is not yet understood. However, it showed that anthocyanin content is dynamic and depends on environmental conditions, water quality and the genetic background of the trees. This understanding is important for commercial perspectives, as it determines the choice of cultivars in different environmental conditions and geographical locations. It also influences the quality and suitability of the fruit for medical or nutritional consumption.

Genetics

The high variability in color of the skin and arils of pomegranate suggest a strong genetic control of anthocyanin production in pomegranate. Several expressed genes that are highly correlated with anthocyanin accumulation during fruit development were first identified by Ben-Simhon et al. (2011). These genes were initially isolated on the basis of their homology to known genes involved in the production of flavonoids and anthocyanins. They included the structural genes: *PgLDOX* (*ANS*), *PgDFR*, and *PgCHS* and the regulatory genes: *PgTTG1* (*WD40*), *PgAN1* (*BHLH*), and *PgAn2* (*Myb*). Up until now the only genes from pomegranate for which a confirmed function in anthocyanin production was reported are the genes which encode for the enzyme leucoanthocyanidin oxidase *PgLDOX* (Ben-Simhon et al., 2015) and for the *WD40* type of transcription factor *PgTTG1* (Ben-Simhon et al., 2011). The function of the pomegranate gene *PgTTG1* was shown by complementing the TTG1 mutant of arabidopsis with the pomegranate *PgTTG1* homolog (Ben-Simhon et al., 2011). In this case, the *pgTTG1* function was demonstrated for both the ability to regulate anthocyanin production and for regulating trichome formation. The function of *PgLDOX* was confirmed by identifying the site of the recessive mutation within its coding sequence located between positions 90–91 downstream of the ATG initiation codon. The mutation disrupts the gene in the anthocyanin-less pomegranate mutant. This mutation abolishes the expression of *LDOX* in all the pomegranate tissues and prevents the accumulation of anthocyanin (Ben-Simhon et al., 2015). The clear linkage of the mutation to the inability to produce anthocyanins was accomplished by genetic mapping, using segregating F2 populations for a white phenotype mutant that does not produce anthocyanins in its fruit and leaf tissues (Ben-Simhon et al., 2015). The identification of *PgLDOX* as the gene responsible for the anthocyanin-less pomegranate phenotype was supported by Zhang et al. (2009), who showed that the anthocyanin-less mutant does not express the *PgLDOX* gene. These authors cloned several additional candidate genes from white and red pomegranate cultivars related to anthocyanin synthesis and studied their expression (Zhao et al., 2015). The recent determination of the pomegranate genome and expression analysis of candidate genes combined with the accumulation of anthocyanins in flowers and the outer seed coats during development provide a more comprehensive list of putative genes involved in the anthocyanin synthesis pathway (Qin et al., 2017).

Hydrolyzable Tannins

In addition to anthocyanins, pomegranate is also a rich source of hydrolyzable tannins. Hydrolyzable tannins are further divided into gallotannins and ellagitannins according to the phenolic groups that are esterified to the hydroxyl groups of glucose: gallic acid in gallotannins and hexahydroxydiphenic acid (HHDP) in ellagitannins. More than 60 hydrolyzable tannins have been (tentatively) identified in pomegranate, of which over 30 are reportedly present in fruit peel, aril juice, and seed (Fischer et al., 2011a; Mena et al., 2012; Ito et al., 2014; Ambigaipalan et al., 2017; Wu and Tian, 2017; Liu and Seeram, 2018) (**Table 3**).

In pomegranate fruit peels, punicalagin α and β isomers (designated punicalagins) are the predominant form of hydrolyzable tannins accounting for over 85% of total tannins (Seeram et al., 2005). Other major hydrolyzable tannins in fruit peels include punicalin, ellagic acid, gallagic acid, and ellagic acid glycosides. In most cases, hydrolyzable tannins are present in both fruit peel and aril juice (**Table 3**). There are only a few reports on hydrolyzable tannins in pomegranate seeds; interestingly, 3,3′-di-*O*-methylellagic acid and 3,3′,4′-tri-*O*-methylellagic acid have been identified in seeds, but not in fruit peels or aril juice (**Table 3**). It is worth noting that metabolite identification depends on the pomegranate accessions that are being analyzed. For example, a castalagin derivative and a galloyl-bis-HHDP-hexoside (casuarinin) derivative were

TABLE 3 | Hydrolyzable tannins (tentatively) identified in pomegranate fruit peel, aril juice, and seed tissues. HHDP, hexahydroxydiphenic acid; (+) reported presence; (–) presence not yet reported.

Hydrolizable tannin	Fruit peel	Aril juice	Seed
3,3′-Di-O-methylellagic acid	–	–	+
3,3′,4′-Tri-O-methylellagic acid	–	–	+
Brevifolin carboxylic acid	+	+	–
Casuarinin	+	–	–
Dehydro-galloyl-HHDP-hexoside	–	+	–
Di-HHDP-galloylglucose-pentoside	–	+	–
Digalloyl-gallagyl-hexoside	–	+	–
Digalloyl-triHHDP-diglucoside (sanguiin H10)	–	+	–
Digalloyl hexoside	+	+	+
Digalloyl-HHDP-glucoside (punigluconin)	+	–	–
Ellagic acid	+	+	+
Ellagic acid pentoside	+	+	+
Ellagic acid deoxyhexoside	+	+	+
Ellagic acid hexoside	+	+	+
Eucalbanin B	–	+	–
Eucarpanin T1	–	+	–
Gallagic acid	+	–	–
Gallagyl hexoside	+	–	–
Galloyl-HHDP-glucuronide	+	–	–
Galloyl-gallagyl-hexoside	+	–	–
Galloyl hexoside	+	+	–
Galloyl-HHDP-hexoside (Corilagin)	+	+	+
Granatin A	+	–	–
Granatin B	+	+	-
HHDP hexoside	+	+	+
Lagerstannin B	+	–	–
Lagerstannin C	+	+	–
Oenothein B	–	–	–
Pedunculagin I	+	+	–
Pedunculagin II	+	+	–
Pomegraniin A	–	+	–
Pomegraniin B	–	+	–
Punicacortein C	+	–	–
Punicalagin α	+	+	–
Punicalagin β	+	+	–
Punicalin α	+	+	–
Punicalin β	+	+	–
Tellimagrandin I	+	–	–
Trisgalloyl hexoside	+	+	–
Valoneic acid dilactone	+	+	+

only detectable in fruit peels of "Acide," but not in the other three Tunisian pomegranate accessions ("Gabsi," "Nebli," and "Tounsi") (Abid et al., 2017). Conversely, punicalagins, galloyl-HHDP-hexoside, galloyl-HHDP-DHHDP-hexoside (granatin B), and digalloyl-HHDP-hexoside (pedunculagin II) were present in fruit peels of all four accessions, and most abundant in "Acide" (Abid et al., 2017).

Efficient and effective metabolite extraction methods are also a key to understanding the composition and content of hydrolyzable tannins of pomegranates and their different tissues.

Differences Among Varieties

Although there is a wide variety of pomegranate accessions worldwide (Holland et al., 2009), only a small portion of these accessions have been analyzed in detail for hydrolyzable tannins.

Composition of hydrolyzable tannins in the juice

Pomegranates have traditionally been consumed for fresh aril juice; therefore, several studies focused on quantification of hydrolyzable tannins in this tissue. Aril juices of 12 commercial pomegranate varieties and 5 non-commercial varieties grown and harvested in different regions (Israel, Turkey, Spain, Iran, Tunisia, and Italy) contained 139.7–473.4 mg/L of ellagic acid and 300–810 mg/L of total phenolic acids and hydrolyzable tannins (Gómez-Caravaca et al., 2013). Ellagic acid levels in the aril juices of eight Iranian cultivars were evaluated and ranged from 7 to 160 mg/L. Interestingly, total tannins, ranging from 15 to 32 mg/100 g, showed an inverse correlation with ellagic acid concentrations in these cultivars (Mousavinejad et al., 2009).

In recent years, industrial procedures have been established that press juice from whole pomegranate fruits. Therefore, the commercial pomegranate juices contain hydrolyzable tannins from aril juice as well as other parts of the fruit. For example, the commercial juices of "Wonderful" contained 1,500–1,900 mg/L of punicalagins, about 100-fold higher than those present in aril juice (Gil et al., 2000). Similarly, punicalagins were in the range of 31–607 mg/L in aril juices, and 156–1,169 mg/L in whole fruit juices of 10 Iranian pomegranate cultivars (Akhavan et al., 2015). However, the levels of punicalagins in the aril juices of some cultivars were particularly high, e.g., the punicalagin level in the aril juice of "JPGRT" (607 mg/L) was significantly higher than those in whole fruit juices of cultivars "PSY" (156 mg/L), "VKT" (286 mg/L), "MY" (338 mg/L), "SRAB" (549 mg/L), and "TML" (569 mg/L) (Akhavan et al., 2015).

Four major hydrolyzable tannins, including punicalagins, punicalins, gallagic acid, and ellagic acid, were quantified from whole fruits and aril juices of 29 local and domesticated Israeli accessions (Tzulker et al., 2007). In addition to variations in the relative abundance of the four hydrolyzable tannins (puncalagins on the scale of 10^5 mg/L, punicalins 10^4 mg/L, gallic acid 10^3 mg/L, and ellagic acid 10^2 mg/L in whole fruit extracts), the accessions analyzed were largely different in the concentrations of each hydrolyzable tannin in whole fruits and aril juices. Furthermore, the hydrolyzable tannins were a thousand fold less concentrated in aril juices than in whole fruits (Tzulker et al., 2007).

Composition of hydrolyzable tannins in the peel

Pomegranate fruit peels, though inedible, contribute to hydrolyzable tannins in commercial juice products and have drawn attention for being a rich source of valuable compounds. Total phenolics in fruit peels of four Tunisian cultivars were

studied. Not only varietal differences in total tannins were observed, there were also more tannins in the acetone than in the water or ethanol extracts (Abid et al., 2017).

Composition of hydrolyzable tannins in the seeds

In comparison with fruit peels and aril juices, hydrolyzable tannins are less abundant in seeds. Total tannins, including gallotannins, ellagic acid derivatives, and gallagyl tannins (mainly punicalagins and punicalins) were 4,792–6,894 mg/L in fruit peels of six cultivars grown in the southern United States, which were 50- to 60-fold and over 100-fold higher than those in aril juices and seeds, respectively (Pande and Akoh, 2009). Punicalagins, punicalins, gallic acid, and ellagic acid were quantified in fruit peels, aril juices, and seeds of five widely consumed pomegranate cultivars in China (Li et al., 2016). Punicalagins were found in fruit peels in the range of 61.75–125.23 mg/g dry weight. In all of the cultivars analyzed, fruit peels contained more punicalagins and punicalins than aril juices did, while these hydrolyzable tannins were not detected in seeds (Li et al., 2016).

Although hydrolyzable tannin composition and content cannot be directly compared among different studies due to the different extraction and quantification methods they employed, it can be concluded that hydrolyzable tannins vary in different pomegranate accessions grown in the same region, suggesting genetic contributions to hydrolyzable tannins. On the other hand, variations in hydrolyzable tannins were also observed for the same cultivar, such as "Wonderful," when grown in multiple locations in the world. This phenomenon can be due to the many landraces of "Wonderful" and additionally suggests that climate and cultivation have an effect on hydrolyzable tannins.

Differences During Fruit Development

Several studies have compared hydrolyzable tannin profiles in developing pomegranate fruits. However, the fruit developmental stages were defined by different standards, such as days after fruit set/full bloom, physico-chemical properties, or physiological attributes of the fruit (Fawole and Opara, 2013b). Developing fruits of two cultivars, "Wonderful" and "Rosh Hapered," grown in Israel were collected during a span of 8 or 10 weeks (Shwartz et al., 2009). Three major hydrolyzable tannins, gallagic acid, punicalin isomers (designated punicalins), and punicalagins, were quantified in water extracts of the developing fruits. All three hydrolyzable tannins showed decreased accumulation in developing fruit peels in both cultivars (Shwartz et al., 2009). Fruits of "Ruby" grown in South Africa were harvested at five stages according to days after full bloom (Fawole and Opara, 2013a). Total hydrolyzable tannins in aril juice declined during the progression of fruit maturation, and were accompanied by decreases in ellagic acid and gallic acid (Fawole and Opara, 2013a).

Relative amounts of hydrolyzable tannins in fruit peel, aril juice, and seed of developing pomegranate fruits were also investigated. Fruits of the Chinese cultivar "Taishanhong" were harvested at 10-day intervals for nine collections. Unicalagins, ellagic acid, and gallic acid were higher in fruit peel than aril juice and in seed; all three metabolites showed decreased accumulation in the three tissues during fruit development (Han et al., 2015). When quantified by absorption of the methanolic extracts at 550 nm, total hydrolyzable tannins gradually decreased in fruit peels at low, low-medium, medium, and medium-high (corresponding too early to late fruit development) stages of the Spanish cultivar "Mollar de Elche." In contrast, they were not detectable in aril juice at all stages. In seeds total hydrolizable tannins increased at medium and then decreased at medium-high stages (Fernandes et al., 2015b).

Overall, despite the differences in the genetic background, growth conditions, harvesting scheme, and extraction and quantification methods, there is a consistent trend of decreasing hydrolyzable tannin accumulation in fruit peels, aril juice, and seed through pomegranate fruit development.

Climate and Geographic Influence

To understand the impact of growth environment on hydrolyzable tannin profiles, fruit peel and aril juice hydrolyzable tannins were compared for 11 accessions grown in the

TABLE 4 | The main metabolites identified in pomegranate fruit peel, aril juice, and seed tissues.

Tissue	Sugars	Organic acids	Amino acids	Proteins	Fatty acids	Anthocyanins	Hydrolyzable tannins
Peel	Glucose Fructose Or Xylose Arabinose	Citric acid	Glutamate Glycine Aspartate	Unknown	Linoleic acid Palmitic acid Oleic acid	Cyanidin Pelargonidin	Punicalagin
Aril juice	Glucose Fructose	Citric acid	Glutamine Serine Aspartate Or Proline Serine Alanine	Unknown	Linoleic acid Palmitic acid Oleic acid	Cyanidin Pelargonidin Delphinidin	Ellagic acid
Seed	Unknown	Unknown	Glutamate Arginine Aspartate	Globulins Albumins	Punicic acid	Unknown	Unknown

Mediterranean or desert climate in Israel (Schwartz et al., 2009). Mediterranean climate promoted high levels of hydrolyzable tannins in aril juice in most of the accessions evaluated; in contrast, desert climate had a positive impact on hydrolyzable tannins in fruit peels (Schwartz et al., 2009). It was reported that the sweet/sour phenotype and environment interactions had the most influence (54.4%) on total tannin variations in aril juice of 10 commercial cultivars grown in four different regions in China, followed by the growth environment (45.6%). There were negative correlations of overall average temperature with total polyphenol, total tannin, and punicalagin concentrations. The sweet/sour phenotype only accounted for 0.06% of the variations in tannins among different cultivars (Li et al., 2015).

The quality of aril juice under deficit (i.e., reduced) irrigation was investigated in Spain (Mena et al., 2013). Three water regimes were applied to pomegranate trees at 75% evapotranspiration (ET_o, non-stressed control), 43% ET_o (moderate water stress), and 12% ET_o (severe water stress). Water stress drastically decreased punicalagins, causing 30 and 70% reduction in moderate and sever stresses, respectively, in aril juices of fruits harvested from the corresponding trees (Mena et al., 2013). This study provided valuable information on the implications of water stress on the hydrolyzable tannin metabolism and the nutritional value of aril juice.

Genetics

To allow functional assessment of hydrolyzable tannin metabolic and regulatory genes *in planta*, a pomegranate hairy root culture system was established that produces a substantial amount of hydrolyzable tannins and is easily transformable (Ono et al., 2012). The hairy root culture system has been successfully utilized for genetic characterization of candidate hydrolyzable tannin biosynthetic genes in pomegranate, such as *PgUGT84A23* and *PgUGT84A24* involved in β-glucogallin production (Ono et al., 2016). Together with the recently published reference genomes (Qin et al., 2017; Yuan et al., 2017), pomegranate hairy roots hold great potential for functional genomics in the hydrolyzable tannin pathway.

SUMMARY

In this review, we have made an effort to summarize the most updated data on primary metabolites and on the most notable secondary metabolites of pomegranate fruit. Along with this effort, it was important for us to reflect the variability of metabolite content and composition and its dependence on the genetic background and environmental conditions. Finally, we present a summary of the main metabolites identified in pomegranate fruit peel, aril juice, and seed tissues (**Table 4**).

AUTHOR CONTRIBUTIONS

The manuscript was written by DH, IB-Y, LT, and RA. All authors assisted in writing all the chapters. DH is the corresponding author and focused on anthocynins, lipids, proteins, and amino acids. LT focused on hydrolizable tannins. RA focused on sugars and organic acids. IB focused on lipids, proteins, amino acids together with DH.

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REVIEW
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Metabolic Dynamics During Loquat Fruit Ripening and Postharvest Technologies

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Loquat is an important fruit widely cultivated worldwide with high commercial value. During loquat fruit development, ripening, and storage, many important metabolites undergo dramatic changes, resulting in accumulation of a diverse mixture of nutrients. Given the value of loquat fruit, significant progresses have been achieved in understanding the metabolic changes during fruit ripening and storage, as well as postharvest technologies applied in loquat fruit in recent years. The objective of the present review is to summarize currently available knowledge and provide new references for improving loquat fruit quality.

Keywords: loquat fruit, metabolites, development, ripening, postharvest treatment

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INTRODUCTION

Loquat (*Eriobotrya japonica* Lindl.) is a subtropical evergreen fruit tree originated in south China. It has been cultivated for more than 2000 years in China and is now widely cultivated in over 30 countries around the world (Lin et al., 1999), and China is the largest producer with a growing area and production. Loquat blooms in autumn and early winter, and its fruit ripens in early summer when other fruits are in off-seasons. Loquat fruit is favored by consumers for its attractive appearance, juicy taste, and rich nutrients. Moreover, it is an important source of soluble fiber, vitamins, carotenoids, antioxidants, and minerals including calcium, potassium, phosphorus, and magnesium that are essential to human body (Tian et al., 2007). In addition, loquat has numerous medical functions previously recorded in "Compendium of Materia Medica," which plays important roles in regulating blood pressure; stimulating circulatory system; lowering risk of cancer; treating inflammation; preventing diabetes; soothing the respiratory system; improving immune system, digestion, skin health, and eye vision; and fighting against viruses (Kumar et al., 2014). Therefore, loquat is also considered as a health fruit.

Loquat fruit is classified as non-climacteric fruit, since there is no sudden rise in respiration rate and ethylene production during fruit ripening (Alos et al., 2017). Loquat fruit requires 4–5 months from blossom to fully ripe, which could be divided into five stages: immature green (IMG), mature green (MG), breaker (Br), orange (Or), and fully ripe (FR) (**Figure 1**), with reference to fruit development and ripening process in the model species, *Solanum lycopersicum*.

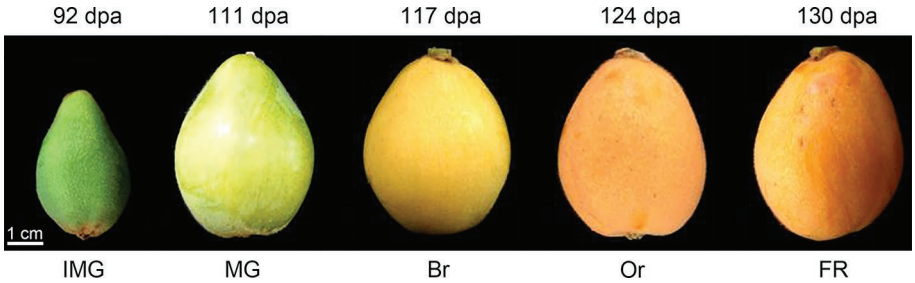

FIGURE 1 | Phenotypes of loquat fruit during different developmental stages. The whole development process can typically be divided into five stages, including immature green (IMG) at 92 days postanthesis (dpa), mature green (MG) at 111 dpa, breaker (Br) at 117 dpa, orange (Or) at 124 dpa, and fully ripe (FR) at 130 dpa. Bar = 1 cm.

Loquat fruit ripening is a complex and precisely regulated process involving numerous physiological and chemical changes in primary and specialized metabolites, including pigments, sugars, organic acids, and phenolic compounds (Tian et al., 2007). Therefore, the understanding toward metabolic changes in loquat fruit may be helpful for improving fruit quality and values. During the past several decades, many advances have been achieved in revealing the variations in metabolites during fruit development, ripening, and postharvest storage. This review will focus on variations in major metabolites during loquat fruit development ripening and postharvest storage and also summarize postharvest technologies currently available for loquat industry.

CHANGES IN METABOLITES OF LOQUAT DURING FRUIT DEVELOPMENT AND RIPENING

Carotenoid Metabolism

Carotenoids are important pigments associated with yellow-to-red color ranges. They play vital roles in determining fruit color, which is a critical factor for loquat fruit quality (Gross et al., 1973). According to the color of fruit fresh, loquat fruit can be divided into two groups: yellow-fleshed and white-fleshed (**Figure 2**). The composition and content of carotenoids accumulated in yellow- and white-fleshed loquat fruit are significantly different. Yellow-fleshed fruit showing orange or red color contain high levels of carotenoids, whereas white-fleshed fruit exhibiting white color have a low level. Zhou et al. (2007) found that 12 yellow-fleshed fruits had a higher content 214.50–475.22 μg/g dried weight (DW) than 11 white-flesh cultivars 91.52–202.28 μg/g DW. Reported that nine yellow-fleshed fruits had much higher level β-carotene 11.55–15.69 μg/g fresh weight (FW) than two white-fleshed fruits. Conversely, the percentage of lutein in yellow-fleshed fruits was much lower than that of white-fleshed fruit. Moreover, there are significant differences in content and composition of carotenoid between flesh and peel in loquat fruit. Beta-carotene and lutein are the most abundant carotenoids in the peel and accounted for about 60% of the total colored carotenoids in 23 yellow- and white-fleshed cultivars, whereas more than a half of the colored carotenoids in the flesh were beta-carotene (Zhou et al., 2007). Thirty-two types of carotenoids were reported in peel and only 18 were identified in flesh (Hadjipieri et al., 2017). In general, the most obvious changes in size and color occur during loquat fruit development and ripening. Immature and mature green loquat fruit contain a higher level of chlorophylls with a few carotenoids, which was similar to those in leaves. During fruit ripening, chlorophylls are rapidly degraded, whereas massive carotenoids are synthesized, resulting in changes in fruit color from green to yellow (**Figure 3A**). Total amount of carotenoids in yellow-fleshed cultivar and white-fleshed cultivar did not have difference at mature green stage, but increased dramatically during ripening, leading to a significant difference in the two cultivars (Zhang et al., 2016a). Levels of beta-carotene in peel and flesh of "Zaozhong 6" increased gradually and reached the peak levels at fully ripen stage (Zhang et al., 2016a). These data indicate significant changes in carotenoid content and compositions during loquat fruit development and ripening.

Plenty of researches have proven that the transcriptional regulations on the carotenoid biosynthesis pathway play crucial roles in the differential accumulation of carotenoids in plants. Over the past several decades, numerous attempts have been made to elucidate the mechanism for the differences between yellow-fleshed and white-fleshed loquat fruit, especially in the expression of carotenoid biosynthesis genes. Sun et al. (2013) reported that the transcriptional downregulation of β-carotene 3-hydroxylase gene (*HYB*) was the main reason for lacking of β-carotene in the white-fleshed mutant. Fu et al. (2011) proved that the lower expression of phytoene synthase 1 (*PSY1*), chromoplast-specific lycopene β-cyclase (*CYCB*), and β-carotene hydroxylase (*BCH*) was closely correlated with the lower level of carotenoid in white-fleshed "Baisha" fruit. Similarly, the expression of *PSY1*, ζ-carotene desaturase (*ZDS*), *CYCB*, and *BCH* in the peel and flesh of "Obusa" were upregulated during fruit ripening (Hadjipieri et al., 2017). These results suggest that the expression of *EjPSY*, *EjCYCB*, and *EjBCH* may play important roles in regulating carotenoids synthesis during loquat fruit ripening. PSY is a key enzyme well received as a "star player" in carotenoid biosynthesis. Numerous studies have been conducted

FIGURE 2 | Phenotypes of yellow-fleshed and white-fleshed loquat fruit at fully ripening. Bar = 1 cm.

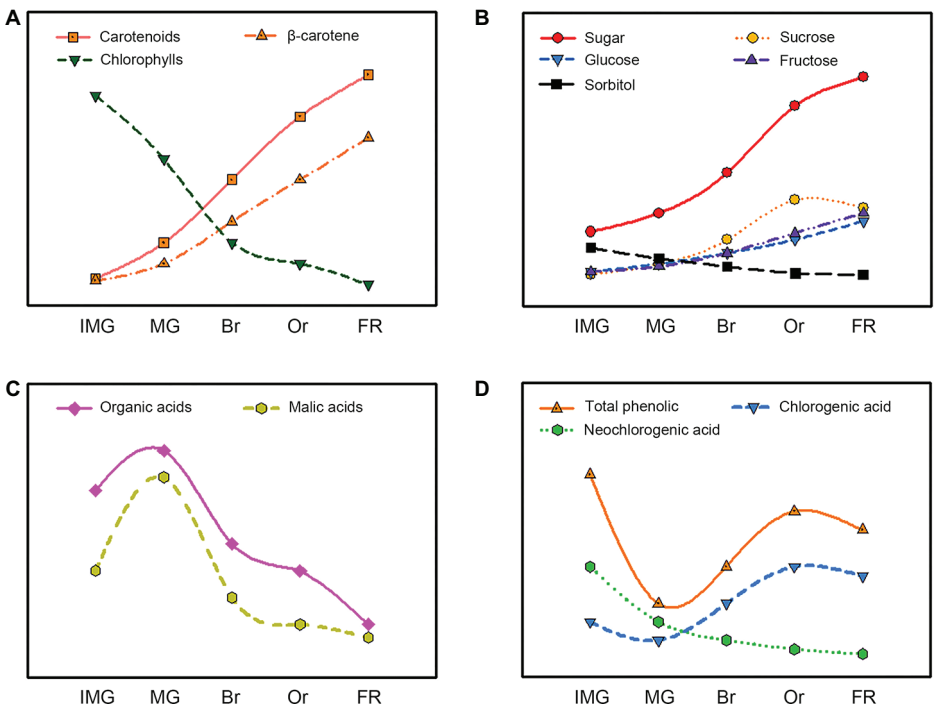

FIGURE 3 | Evolution of the carotenoids **(A)**, sugars **(B)**, organic acids **(C)**, and phenolics **(D)** content during loquat fruit development and ripening.

to regulate carotenoid content *via PSY* (Fraser et al., 2007; Fantini et al., 2013). In loquat, four *PSY* genes, *EjPSY1*, *EjPSY2A*, *EjPSY2B*, and *EjPSY3*, have been characterized, and they exhibited different expression patterns. *EjPSY1* mainly expressed in leaves, and the peel of loquat fruit, *EjPSY2A*, showed high expression in the peel and flesh during fruit ripening, whereas *EjPSY2B* only expressed in leaves and *EjPSY3* displayed a low expression in all the tissues examined (Fu et al., 2014). These results suggested that *EjPSY1* and *EjPSY2A* were responsible for carotenoid accumulation in peel and flesh, respectively. A mutant *EjPSY2Ad*, which loses the C-terminal catalytic domain and has no PSY catalytic activity, was found in all seven white-fleshed cultivars, explaining why white-fleshed fruit have lower level of carotenoids.

EjPSY3 contains alternatively spliced forms in "Luoyangqing" and "Baisha" and has no function in carotenoid accumulation (Fu et al., 2014). In addition, virus-induced gene silencing-mediated suppression of *EjPSY* resulted in decreased carotenoid content in loquat fruit (Hong et al., 2018), indicating that *PSY* gene positively regulated carotenoid accumulation in loquat fruit.

Carotenoids are synthesized and accumulated in plastid during fruit ripening, and it has been demonstrated that plastids play important roles in regulating carotenoid accumulation (Lu et al., 2006) and also undergo significant changes during the differentiation of chloroplasts into chromoplasts during loquat fruit ripening. Some studies showed that numerous chromoplasts existed in the peel and flesh of yellow-fleshed "Luoyangqing" fruit at fully ripe stage, while less chromoplasts in smaller size were detected in the peel of white-fleshed "Baisha" fruit and no chromoplast was found in the flesh of "Baisha" fruit (Fu et al., 2011). Further investigation revealed that carotenoids were mainly deposited in the lipid globules in chromoplasts of "Luoyangqing" and "Baisha" peel and presented in crystalline form in the flesh of "Luoyangqing" fruit (Fu et al., 2011). Therefore, abnormal chromoplast development may be responsible for the lack of carotenoid accumulation in white-fleshed loquat. In addition to these, genes involved in chloroplast differentiation or carotenoid sequestration also participated in carotenoid accumulation. ORANGE (*OR*) encodes a DnaJ protein and is considered to be a master regulator for normal differentiation of chromoplast in cauliflower and melon fruit (Lu et al., 2006; Tzuri et al., 2015). The *OR* homologous gene was isolated from loquat, and the transcript level of *EjOR* was slightly higher in "Luoyangqing" than that in "Baisha" during fruit ripening (Fu et al., 2011), indicating that *OR* gene might function in regulating carotenoid accumulation in loquat fruit.

Sugar Metabolism

Sugar and organic acid are major soluble components in ripe fruits and have vital roles in fruit taste and flavor, which are key indicators of fruit quality (Zhu et al., 2013; Liu et al., 2016). Sugar and acid content as well as the ratio of them are the important index of flavor of fruits. Numerous studies showed that fructose, sucrose, glucose, and sorbitol were major sugars in ripe loquat fruit, but the ratio of these ingredients varied among different cultivars. Hirai (1980) showed that sucrose was the most abundant sugar in the ripe "Tanaka" loquat, whereas sorbitol predominated in the young fruit. Ding et al. (1998a) reported that the major sugars in "Mogi" loquat fruit were fructose, sucrose, glucose, and sorbitol. Glucose and fructose serve as the dominant sugars and constitute at least 80% of the total sugar in 12 cultivars of loquat fruit, whereas sucrose and sorbitol were less abundant (Xu and Chen, 2011). Similarly, Toker et al. (2013) reported that glucose and fructose acted as the primary sugar compositions and accounted for more than 91.42% in 15 cultivars of loquat fruit in Turkey. These studies suggest that significant differences in the composition of sugars were observed among various cultivars, which may be attributed to different genetic backgrounds and cultivation environment. During loquat fruit development and ripening, sugar content is relatively slow at early stages but increases rapidly during fruit expanding period and further slows down at breaker stages (**Figure 3B**; Hirai, 1980; Serrano et al., 2003; Ni et al., 2011). Sorbitol, a primary sugar in young loquat fruit, decreases with fruit development, whereas sucrose, glucose, and fructose increase sharply with fruit ripening, and sucrose accumulates faster than glucose and fructose and then becomes the major sugar in ripe fruit (Serrano et al., 2003; Wu et al., 2015). Sucrose contents in yellow-fleshed "Dahongpao" and white-fleshed "Ninghaibai" are unchanged during fruit development, whereas sorbitol contents decreased rapidly with fruit development (Chen et al., 2010), indicating that fructose and glucose contents increase dramatically during ripening and become the dominant sugars in loquat fruits.

According to the types of production, sugar metabolism could be sorted into sucrose, sorbitol, and hexose metabolism in fruits (Kanayama, 2017). Sucrose phosphate synthase (SPS), sucrose synthase (SS), and invertase (IN) are key enzymes involved in sucrose metabolism (Bruneau et al., 1991; Sturm, 1999). Sorbitol-6-phosphate dehydrogenase (S6PDH), sorbitol dehydrogenase (SDH), and sorbitol oxidase (SOX) are key enzymes responsible for sorbitol metabolism (Sakanishi et al., 1998; Iida et al., 2004). All these enzymes play important roles in the sugar metabolism during fruit ripening. Abnasan-Bantog et al. (1999) found that the activities of SDH, SPS, SS, and AIV enzymes in "Mogi" fruit were low at early stages of fruit development but increased dramatically during fruit ripening stages simultaneously with sugar accumulation. However, the activity, protein abundance, and transcript level of S6PDH increased during loquat development and decreased during ripening in parallel with NAD^+-SDH (Abnasan-Bantog et al., 2000), indicating that sorbitol may be synthesized in loquat fruit. Ni et al. (2009) reported that the changes in the activities of SS and SPS were positively correlated with the dynamics in sucrose accumulation, and thus, they proposed that SS and SPP were key enzymes in the regulation of sucrose accumulation in "Qingzhong," "Bahong," and "Jidanbai" fruit. Chen et al. (2010) demonstrated that the activities of IN, SS, and SDH decreased at early stages of fruit development but increased at later stages of fruit ripening in white-fleshed cultivar "Ninghaibai" and yellow-fleshed cultivar "Dahongpao" and that the activities of IN and SS cleavage in yellow-fleshed fruit were lower than those in white-fleshed fruit at late stages of fruit development. Currently, most attempts focus on patterns of sugar accumulation and activities of related enzymes, and much less are known about the transcriptional changes of sugar metabolism genes. Up to now, the genes encoding SS, SPS, SDH, VIN, sorbitol transporter protein, and fructokinase have been cloned. Wang et al. (2015a) found that the transcript level of *EjVIN* was highest in the early stages of fruit development and ripe fruit and could be induced by exogenous fructose or glucose treatment. Further analyses showed that overexpression of loquat *EjVIN* in tobacco decreased the sucrose level, indicating that *EjVIN* played important role during early development stage of loquat fruit. Song et al. (2016) showed

that the transcript levels of *EjSPS1*, *EjSPS2*, and *EjSS-C* in "Jiefangzhong" fruit increased gradually during fruit development and reached the peaks at fully ripe stage, whereas the transcript level of *EjSS-S* was high at early stage and dramatically decreased with fruit development. These results indicated that *EjSPS1*, *EjSPS2*, and *EjSS-C* had vital roles in promoting sugar accumulation in "Jiefangzhong" loquat fruit. Li et al. (2016a,b) cloned *NAD*$^+$*-SDH* gene from the yellow-fleshed cultivar "Dongting" loquat, which is a white-fleshed bud mutant, and proved that three SNP loci were presented in the *NAD*$^+$*-SDH* gene from the mutant fruit, leading to excessive conversion of sorbitol into fructose at maturation stage. These data suggest that the sugar content in loquat fruit may also be influenced by cultivation measures and exogenous substances during fruit development and ripening.

Organic Acid Metabolism

Organic acids are also determiners in fruit flavor and can be used as substrates for respiration. Numerous researches showed that malic, tartaric, quinic, citric, succinic, fumaric, and oxalic acids were predominant organic acids in ripe loquat fruit, but their composition and contents in loquat fruit varied among cultivars (Toker et al., 2013). They found that malic acid was the primary acid, followed by tartaric, succinic, oxalic, and citric acids in ripe fruits of 12 loquat cultivars in Turkey. Organic acid in loquat fruit increased at the early stages of fruit growth, decreased with fruit ripening, and reached the lowest at full ripen (**Figure 3C**; Serrano et al., 2003; Chen et al., 2009). Likewise, malic acid was the predominant organic acid in unripe loquat fruit and exhibited a similar change as the total organic acid during loquat fruit development and ripening, whereas citric acid, succinic acid, and ascorbic acid contents were much lower than malic acid, which decreased to the lowest at the full ripening stages (Serrano et al., 2003). Chen et al. (2009) also reported that the malic acid and citric acid contents increased during the early stages of fruit development and decreased at the later stages in "Changhong 3" and "Jiefangzhong," but quinic acid was the abundant in loquat fruit flesh at the early stages of fruit development and decreased with the fruit development and ripening.

Organic acid metabolism is a complex process involving biosynthesis, transport, storage, and degradation of multiple organic acids, which keeps the contents of organic acids in balance. Given the importance and content of malic acid, significant progresses have been achieved in revealing malic acid metabolism in fruits. Malic acid content is positively correlated with NAD$^+$-dependent malate dehydrogenase (NAD-MDH) activity and negatively with NADP$^+$-dependent malic enzyme (NADP-ME) activity (Chen et al., 2009), suggesting that NAD-MDH and NADP-ME might play important roles in malate biosynthesis and degradation. In addition, genes encoding *EjPEPC*, *EjNADP-ME*, *EjcyNAD-MDH*, *EjmNAD-MDH*, *EjV-ATPase A*, and *EjV-PPiase* were cloned by Yang et al. (2011), and they found that the transcript level of *EjNADP-ME* in the high-acid cultivar was significantly higher than that in the low-acid cultivar, whereas the expression of *EjmNAD-MDH*, *EjV-ATPase A*, and *EjV-PPiase* displayed opposite patterns. The expression of *EjNADP-ME*, *EjmNAD-MDH*, *EjV-ATPase A*, and *EjV-PPiase* exhibited similar patterns in both cultivars, whereas the expression patterns of *EjPEPC* and *EjcyNAD-MAD* were different (Yang et al., 2011), indicating that these enzymes may play a vital role in regulating malic acid accumulation in loquat fruit.

Phenolic Metabolism

Phenolic compounds serve as important secondary metabolites, which are crucial for defense response, anti-oxidation, color formation, seed dormancy, programmed cell death, and responses to abiotic stresses (Vogt, 2010). Up to now, hydroxybenzoic acid, hydroxycinnamic acid, flavonol, flavanone, flavone, lignin, and other phenolic compounds have been identified in loquat fruit. However, the total amount and the compositions of phenolic compounds exhibited great differences in different tissues of loquat fruit (Koba et al., 2007). The highest total phenolic content was found in seeds, followed by peel and flesh (Pande and Akoh, 2010). The composition and content of phenolic compounds in ripe loquat fruit also vary greatly among different cultivars. Ding et al. (2001) analyzed the composition of phenolic compounds in seven Japanese loquat cultivars and found the contents of phenolic compounds varied from 81.8 to 173.8 mg/100 g FW. Ercisli et al. (2012) showed that the total phenolic contents of seven Turkish cultivars of loquat fruit ranged from 140 to 253 μg g^{-1} FW. Xu et al. (2014a) reported that chlorogenic acid, neochlorogenic acid, 4-*O*-caffeoylquinic acid, protocatechuic acid, and 4-hydroxybenzoic acid were the primary phenolic compounds in ripe loquat fruit. Chlorogenic acid was considered to be the major phenolic compound and accounted for at least 75% of total phenolic in the dried fruit of seven loquat cultivars in China (Zhang et al., 2015).

During loquat fruit development and ripening, the total phenolic contents first decreased sharply and then increased dramatically (**Figure 3D**; Ding et al., 2001). Neochlorogenic acid was the major phenolic in young fruit and its content decreased with fruit ripening, whereas chlorogenic acid content underwent similar changes and became the predominant phenolic in ripe fruit of "Mogi" and "Tanaka" (Ding et al., 1998b, 2001). The increases in total phenolic and chlorogenic acid can be recognized as indicators for loquat fruit ripening. Moreover, the composition of phenolics in young loquat fruit is more diversified than those in ripe fruit. More than 10 types of phenolics were detected in young "Mogi" fruit, whereas only 7 types were in ripe loquat (Ding et al., 2001). Given the scientific importance of chlorogenic acid, the activities of enzyme involved in chlorogenic acid biosynthesis were extensively investigated (Clifford et al., 2017). Chlorogenic acid is a product of phenylalanine metabolism and its biosynthesis pathways are well illustrated in plants (Niggeweg et al., 2004). Phenylalanine ammonia lyase (PAL), 4-coumarate: CoA ligase (CL), and hydroxycinnamoyl CoA: quinate hydroxycinnamoyl transferase (HQT) are key enzymes involved

in chlorogenic acid biosynthesis (Gramazio et al., 2014). The activities of PAL and HQT in loquat fruit was high initially, but decreased to the lowest levels, and then increased to the peaks at 1 week prior to harvest (Ding et al., 2001). The changes of these enzyme activities were well correlated with the variations in chlorogenic acid during development and ripening of loquat fruit.

CHANGES IN METABOLITES OF LOQUAT FRUIT DURING STORAGE

Loquat fruit is consumed largely as fresh fruit, which should be harvested at the eating-ripe stage and transported to consumers. After harvest, respiration is the most important physiological process in loquat fruit and causes the fruit nutrients to be gradually degraded, eventually leading to deterioration in fruit quality and decay (Tian et al., 2007; Pareek et al., 2014). Loquat belongs to a non-climacteric fruit and has ethylene production at a low level during postharvest storage (Blumenfeld, 1980). The respiration and ethylene production gradually declined after harvest, which can be dramatically suppressed at low temperature (Ding et al., 1998a; Alos et al., 2017). Ethylene has been shown to play vital roles in regulating climacteric fruit ripening *via* ethylene biosynthesis and signaling (Liu et al., 2015a). Although numerous studies showed that ethylene was not necessary for the ripening of non-climatic fruit, exogenous ethylene treatment could also influence ethylene emission of loquat fruit. Treatment with exogenous ethylene increased ethylene release of "Luoyangqing" fruit and enhanced fruit browning, whereas 1-methlcyclopropene (1-MCP) treatment inhibited browning by lowering lipoxygenase (LOX) and polyphenol oxidase (PPO) activities and reducing O^{2-} accumulation and oxidation of polyphenols (Cai et al., 2006a). Exogenous ethylene could also influence the ripening of loquat *via* ethylene signaling, which depends on loquat cultivars. Wang et al. (2009) showed that the transcript abundance of the genes involved in ethylene signaling decreased during fruit ripening, but ethylene treatment could strongly induce ethylene response sensor 1a (*EjERS1a*), ethylene response sensor 1a (*EjERS1b*), constitutive triple response 1 (*EjCTR1*), and ethylene response factor 3 (*EjERF3*) expression in "Luoyangqing." Alos et al. (2017) found that the transcript levels of 1-aminoacyclopropane 1-carboxylate synthase 1 (*EjACS1*), 1-aminoacyclopropane 1-carboxylate oxidase 1 (*EjACO1*), *EjCTR1*, and ethylene-insensitive 3-like 1 (*EjEIL1*) were significantly increased during storage periods, and exogenous ethylene treatment did not affect the expression of these genes involved in ethylene biosynthesis and signaling, whereas 1-MCP significantly inhibited the expression of *EjACS1*, *EjACO1*, *EjACO2*, *EjERS1a*, and *EjCTR1*.

Loquat fruit has a short postharvest life (no more than 10 days) at normal temperature and undergoes a series of physiological disorders, including internal browning, adherence of the peel and flesh, dry pulp tissue, and decay (Ding et al., 2002; Tian et al., 2007). In addition, many metabolites also show significant changes during storage. Total sugar decreased sharply, and the rate of declination was significantly negatively correlated with the storage temperature during storage. Sucrose, fructose, glucose, and sorbitol are the main sugars in the ripe loquat fruit. Among these sugars, sucrose declined rapidly, while fructose, glucose, and sorbitol showed slight changes during storage at 20°C (Ding et al., 1998a). Low temperature storage is effective to maintain loquat fruit quality. Wei et al. (2017) reported that "Jiefangzhong" loquat fruit stored at 0°C exhibited higher glucose and fructose content than those stored at 5°C, which should be attributed to the increases in the activities of AI, NI, SPS, and SS. Total acid content exhibited a decrease during storage. The primary organic acids in ripe loquat fruit is malic acid, which is represented about 90% of total acids. During storage, the concentration of malic acid declined dramatically and displayed a negative correlation with the storage temperature (Ding et al., 1998a). 1-MCP treatment could effectively maintain higher level of malic acid (Cao et al., 2011). In addition, citric acid and succinic acid were maintained relatively constant, while fumaric acid content in the fruit stored at 20°C was higher than that in the fruit stored at lower temperature (Ding et al., 1998a). The declining rate of organic acids was faster than that of sugar, which consequently elevated the ratio between sugars and acids, resulting in a flat flavor unsuitable for eating. Total carotenoids in loquat fruit increased steadily, and low temperature was not beneficial for the accumulation of carotenoids (Ding et al., 1998a). Chen et al. (2015) reported that lutein, β-cryptoxanthin, and β-carotene contents in "Zaozhong No.6" fruits stored at 25°C were higher than those at 4 and 8°C. Total phenolic content declined significantly during storage at 20°C. Ding et al. (1999) reported that the contents of chlorogenic acid, 5-feruloylquinic acid, neochlorogenic acid, and hydroxybenzoic acid significantly decreased during storage, whereas caffeic acid notably increased. Total flavonoids firstly increased and then decreased during cold storage (Cao et al., 2011).

Lignification is one of the important characteristics occurring naturally along with fruit ripening and senescence, which has been considered to be a major challenge confronted by loquat postharvest industry (Cai et al., 2006b), particularly in yellow-fleshed loquat. Low temperature storage promoted the incidence of flesh lignification, which was characterized by difficult peeling, browning, leathery, and juiceless pulp (Cai et al., 2006b; Li et al., 2017). Lignin is one of the ultimate metabolites produced by phenylpropanoid pathway, and its accumulation directly leads to lignification in plants (Zhao, 2016). Phenylpropanone is first deaminated, followed by a series of reactions, including hydroxylation, methylation, and reduction, and finally generates the lignin (Zhao, 2016). Genes encoding phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), cinnamyl alcohol dehydrogenase (CAD), peroxidase (POD), and other key enzymes involved in lignin biosynthesis had been almost exclusively identified in plants. In addition, the activities of PAL, CAD, and POD were closely related to the lignification in loquat fruit. Cai et al. (2006b) found that lignification led to an increase in firmness of loquat fruit during storage at 20°C, which was resulted from increases in PAL, CAD, and POD activities.

Shan et al. (2008) reported that the transcript abundance and the enzymatic activities of CAD and POD were strongly associated with lignification in loquat fruit. Moreover, the activity of CAD and the expression of *EjCAD1* were positively correlated with low temperature-induced lignification in loquat fruit. Additionally, other genes also displayed important roles in lignification of loquat. *EjCCoAOMT* was induced by low temperature, and this might be another reason for lignification stored at low temperature (Liu et al., 2015b). The activities of PAL, C4H, and 4CL were positively correlated with lignification of loquat fruit (Li et al., 2017). All results suggest that various enzymes involved in phenylpropanoid pathway contribute to lignin biosynthesis in loquat fruit during storage. Recently, several transcription factors have been proved to directly regulate lignin biosynthesis. EjMYB1 and EjMYB2 can competitively interact with the promoter of lignin biosynthetic genes to regulate loquat fruit lignification in chilling injury (Xu et al., 2014b). EjNAC1 could activate the expression of genes involved in lignin biosynthesis pathway (Xu et al., 2015). EjAP2–1 could interact with EjMYBs and function as an indirect transcriptional repressor on fruit lignification induced by chilling injury (Zeng et al., 2015). EjHSF3 could regulate loquat fruit lignification *via* interaction with lignin biosynthetic genes and the regulator EjAP2–1 (Zeng et al., 2016). EjMYB8 served as a regulator in chilling-induced lignification of loquat fruit by physically binding to *Ej4CL1* promoter, and transient overexpression of *EjMYB8* in tobacco and loquat leaves increased the lignification level (Wang et al., 2016). EjNAC3 could regulate chilling-induced lignification of loquat fruit *via* direct interaction with an atypical CAD-like gene (Ge et al., 2017). All of these transcription factors significantly modulated the expression of genes involved in phenylpropanoid pathway for lignification during loquat fruit development and low temperature storage.

POSTHARVEST TECHNOLOGIES OF LOQUAT FRUIT

Loquat fruit are perishable and have a short postharvest life at ambient temperature. Up to now, considerable strategies are currently available to reduce postharvest losses, maintain fruit quality, and prolong shelf life of harvested loquat fruit, which can be classified into three categories, namely physical, chemical, and biological technologies (**Figure 4**).

Physical Technologies

Low temperature and controlled atmospheres are main physical measures to maintain the quality of fresh fruits and vegetables during storage (Tian et al., 2005; Wang et al., 2005; Meng et al., 2009; Zhang and Tian, 2010). In addition, other promising technologies, including hot treatment (Zong et al., 2010), radiation and microwave (Casals et al., 2010), hyperbaric pressure (Goyette et al., 2012), and ultraviolet radiation (Fonseca and Rushing, 2008), could also effectively maintain the quality of fruits. Like other fruits, low temperature storage is a technique widely used in the storage, transport, and market of loquat fruit. Low temperature could effectively decrease the respiration rate and ethylene production, extend shelf life, and reduce decay of harvested loquat fruit (Zheng et al., 1993; Ding et al., 1998a; Gao et al., 2007). We found that loquat fruit kept in modified atmosphere packaging (MAP), in which the gas compositions can be adjusted by fruit and vegetables respiration, resulting in high-CO_2 and low-O_2 atmospheres, at 1°C showed more effectiveness in reducing fruit decay, off-flavor, and weight loss than that at 6°C (Ding et al., 2006). The combination of 1-MCP and MAP displayed a better effect on

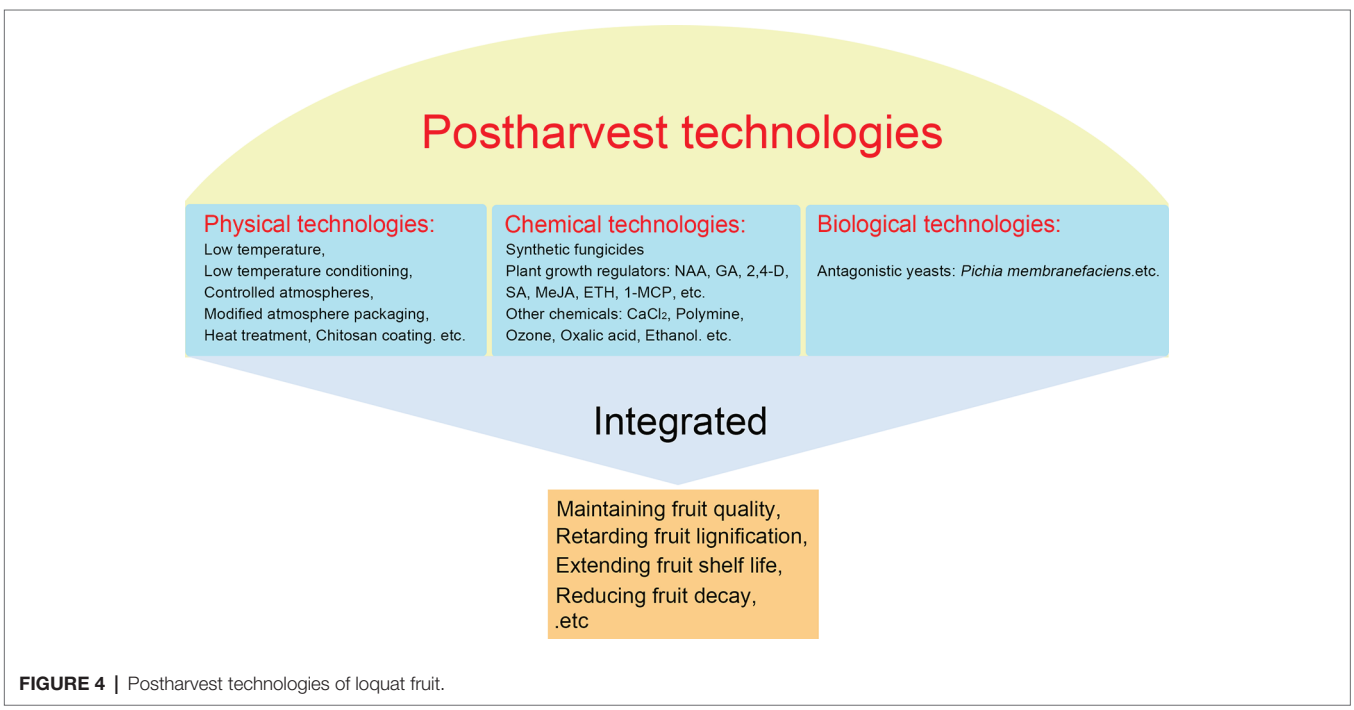

FIGURE 4 | Postharvest technologies of loquat fruit.

maintaining the fruit quality by decreasing softening, deterioration, and browning rate (Oz and Ulukanli, 2011). However, extremely low temperature may cause chilling injury, resulting in increased firmness and lignification during storage, which was characterized by stuck peel, leathery, and juiceless pulp. 1-MCP treatment could effectively alle*via*te loquat fruit chill injury by modifying the fatty acid and cell wall polysaccharide composition and inhibiting LOX and phospholipase activities (Cao et al., 2009b,c). Additionally, low temperature conditioning (LTC) serves as an alternative technique for significantly improving tolerance to low temperature (Wang, 1993). LTC treatment (pre-stored at 10°C for 6 days then transferred to 0°C storage) could effectively reduce incidence of chilling injury and browning index, increase the content of glycine betaine, and maintain the content of sugar and titratable acids (Jin et al., 2015). Moreover, LTC treatment (pre-stored at 5°C for 6 days then transferred to 0°C storage) could also suppress the expression of some positive transcription factors and key genes involved in lignification of loquat fruit (Xu et al., 2014b; Wang et al., 2016).

Controlled atmospheres (CA) combined with low temperature storage are a more effective approach to maintain the quality and prolong the shelf life of loquat fruit. Our previous results indicated that loquat fruit kept in CA with 10% O_2 plus 1% CO_2 at 1°C could be stored for more than 50 days with normal flavor and low decay index (Ding et al., 2006). Moreover, short-term high-O_2 treatment at the beginning of storage had little effect on fruit flavor, but stimulated ethanol accumulation in loquat fruit, and reduced activities of endo-polygalacturonase and exo-polygalacturonase (Ding et al., 2006). Treatment with high O_2 (90%) could effectively inhibit the respiration rate and the PPO activity, which had a better flavor than control fruits after storage for 35 days (Zheng et al., 2000a). CA conditions are more effective for reducing the activities of PPO and oxidative stress compared to other treatments, such as LTC or MAP, which may be the reason why loquat fruit stored in CA conditions had lower decay index than that kept in other conditions.

Compared to low temperature and controlled atmosphere storage, other physical technologies applied in loquat fruit are scarce. Heat air treatment with 38°C for 5 h could dramatically reduce the internal browning of "Jiefangzhong" loquat fruit in chilling injury *via* maintenance of the integrity of cell membrane and higher unsaturated/saturated fatty acid ratio (Rui et al., 2010). Chitosan coating could significantly reduce weight loss and flesh browning, maintain antioxidant capacity, and minimize the losses of total polyphenol, carotenoid, and ascorbic acid in loquat fruits during cold storage (Petriccione et al., 2015).

Chemical Technologies

Chemical technologies are still effective in controlling postharvest decay in fresh fruits and vegetables. In the early years, fungicides are widely applied in harvested loquat fruit to control postharvest diseases. Based on concerns about the safety of chemical fungicides, some plant growth regulators have been used to harvest loquat fruit. Treatment with salicylic acid (0.1 g/l) inhibited the rotten fruit, decreased the weight losses, and maintained higher titratable acid and ascorbic acid content of loquat fruit (Chen et al., 2008). Loquat fruit treated with methyl jasmonate (MeJA), which is a methyl ester widely distributed in plants, displayed significantly higher levels of sugars, organic acids, total phenolics, and carotenoids and maintained higher levels of antioxidants and antioxidant activity (Cao et al., 2009a). The mode of action of MeJA to alleviate chilling injury and enhance fruit resistance has been proved *via* enhancing the activities of antioxidant enzymes and PR-proteins expression (Cao et al., 2008a; Zhu and Tian, 2012; Jin et al., 2014). Exogenous 1-MCP treatment displayed longer shelf life and lower chilling injury by keeping lower respiration rate, PAL and LOX activities, and firmness (Ding et al., 2006; Cai et al., 2006a).

With the development of postharvest technologies, the role of many chemical agents applied in postharvest fruits and vegetables was further investigated in harvested loquat fruit. Calcium chloride treatment retained TSS and ascorbic acid content and decreased browning index, relative electrical conductivity, and weight loss (Babu et al., 2015). Ozone treatment at 150 mg/m^3 had the best effect on loquat fruit and 200 mg/m^3 treatment had obvious side effects with high decay (Zhang et al., 2011). Ethanol treatment could significantly reduce the disease incidence of anthracnose rot in harvested loquat by increasing the activities of PAL, POD, PPO, chitinase, and β-1,3-glucanase and inhibiting the growth of *Colletotrichum acutatum* (Wang et al., 2015b). However, chemical technologies have an obvious effect on extending the storage life of loquat fruit, and the regulation of chemicals has a concentration effect. The optimum concentration depended on loquat cultivars, fruit maturity, treatment time, and temperature of storage environment.

Biological Technologies

Biological technologies, serving as healthy and environmentally friendly method for controlling disease, have been widely considered as potential methods instead of chemical fungicides in postharvest field. Currently, antagonistic yeasts have been proved to effectively control postharvest decay of various fruits, such as tomato (Zong et al., 2010), peach (Xu et al., 2008), sweet cherry (Xu and Tian, 2008), loquat (Cao et al., 2008b), table grape (Meng and Tian, 2009), apple (Li et al., 2016a), and banana (Zhimo et al., 2017). Antagonistic yeasts have a wide range of advantages, including broad-spectrum antimicrobial, simply nutrition requirement, easy culture, strong resistance to biotic and abiotic stresses, and flexible and easy to combine with other methods (Liu et al., 2011). Cao et al. (2008b) found that treatment with *Pichia membranifaciens* at 1×10^8 CFU ml^{-1} significantly inhibited the incidence and progress of anthracnose rot in the harvested loquat fruit. However, application of antagonistic yeasts could not gain a satisfying result as fungicides. Many attempts have been made to increase the efficacy of antagonistic yeasts in controlling postharvest decay, especially combination with other treatments, such as calcium chloride (An et al., 2012), silicon (Qin and Tian, 2005), glycine betaine (Liu et al., 2011), salicylic acid (Qin et al., 2003), chitosan

(Meng and Tian, 2009), and MeJA (Yao and Tian, 2005). Application of MeJA (Cao et al., 2009c) and $CaCl_2$ (Cao et al., 2008b) could effectively improve the biocontrol activity of *P. membranifaciens* on anthracnose rot in harvested loquat fruit. These integrative techniques will be used to effectively control postharest diseases of loquat fruit.

AUTHOR CONTRIBUTIONS

JC, TC, ZZ, BL, GQ, and ST designed and wrote the manuscript.

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Peach Fruit Development: A Comparative Proteomic Study Between Endocarp and Mesocarp at Very Early Stages Underpins the Main Differential Biochemical Processes Between These Tissues

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Peach (*Prunus persica*) is an important economically temperate fruit. The development follows double sigmoid curve with four phases (S1–S4). We centered our work in the early development. In addition to S1, we studied the very early stage (E) characterized by the lag zone of the exponential growing phase S1, and the second stage (S2) when the pit starts hardening. "Dixiland" peach fruit were collected at 9 (E), 29 (S1), and 53 (S2) days after flowering (DAF) and endocarp and mesocarp were separated. There was a pronounced decrease in total protein content along development in both tissues. Quantitative proteomic allowed the identification of changes in protein profiles across development and revealed the main biochemical pathways sustaining tissue differentiation. Protein metabolism was the category most represented among differentially proteins in all tissues and stages. The decrease in protein synthesis machinery observed during development would be responsible of the protein fall, rather than a proteolytic process; and reduced protein synthesis during early development would reroute cell resources to lignin biosynthesis. These changes were accompanied by net decrease in total amino acids in E1–S1 and increase in S1–S2 transitions. Amino acid profiling, showed Asn parallels this trend. Concerted changes in Asn and in enzymes involved in its metabolism reveal that increased synthesis and decreased catabolism of Asn may conduct to an Asn increase during very early development and that the β-Cyano-Alanine synthase/β-Cyano-Alanine hydratase could be the pathway for Asn synthesis in "Dixiland" peach fruit. Additionally, photosynthetic machinery decays during early development in mesocarp and endocarp. Proteins related to photosynthesis are found to a higher extent in mesocarp than in endocarp. We conclude mesocarpic photosynthesis is possible to occur early on the development, first providing both carbon and reductive power and latter only reductive power. Together with proteomic, histological tests and anatomical analysis help to provide information about changes and

differences in cells and cell-walls in both tissues. Collectively, this work represents the first approach in building protein databases during peach fruit development focusing on endocarp and mesocarp tissues and provides novel insights into the biology of peach fruit development preceding pit hardening.

Keywords: *Prunus persica*, fruit development, lignification, endocarp, mesocarp, asparagine, β–cyanoalanine hydratase, β–cyanoalanine synthase

INTRODUCTION

Peach (*Prunus persica*) is a stone fruit of agricultural relevance, not only because of its economic value but also because of its relevance in human health as an important source of phenolic compounds, cyanogenic glucosides and phytoestrogens. In addition, peach has become the reference species for the Prunus family, which also encompasses other fruits such as berries, plums, apricots and almonds (Shulaev et al., 2008). Peach is a fleshy fruit consisting of a single seed surrounded by a pericarp. The pericarp is differentiated in three layers; the endocarp which is adjacent to the seed, the mesocarp consisting of the soft edible region of the fruit, and the exocarp or skin (Dardick and Callahan, 2014). Peach fruit is classified as a drupe, since during its development the endocarp undergoes a hardening process by secondary cell wall formation and lignin deposition.

Peach fruit development follows double sigmoid curve in which four phases can be defined (Tonutti et al., 1991), with growing occurring only during three of the stages and the interval corresponds to the stone formation (Callahan et al., 2009). The growth curve starts after pollination and fertilization. While the number of days of extension of each phase depends on the species, the typical features of each stage do not differ. The beginning is characterized by a rapid growth (exponential) and it is characterized by a high rate of cell division and elongation (S1). The extent of this phase is uniform along cultivars. During the second phase (S2), the endocarp starts becoming hardener to form the stone (Dardick et al., 2010). There is no net increase in fruit size at this stage and the duration is highly dependent on the cultivars, being shorter for early ripening varieties and longer for late ripening varieties (Bonghi et al., 2011). In the next step (S3), an exponential growth of the pericarp occurs again, which is the consequence of an increase in the cell division. In the last stage (S4), the fruit reaches its final size and ripening starts. S4 consist of S4-1, in which fruit gets its final size, and S4-2, when the fruit ripens in an ethylene dependent manner. S4-2 is the only phase that can take place even detached from the tree (Borsani et al., 2009).

The process of pit hardening has not been deeply studied (Dardick and Callahan, 2014). Early on, the presence of lignin in the stone was described by Ryugo (1961), as well as its biosynthetic intermediates (Ryugo, 1963). Later on, peroxidases and phenoloxidases were identified as enzymes involved in this process (Abeles and Biles, 1991; Alba et al., 1995, 2000). Hayama et al. (2006) identified cellulose synthase A1 as involved in cellulose synthesis in the endocarp during the hardening. Finally, Dardick et al. (2010) using the microarray technology, observed that certain genes linked to the phenylpropanoid pathway, lignin formation and flavonoid synthesis are transiently induced during lignification and subsequent stone hardening. They demonstrated that peach genes orthologous to *SHATTERPROOF*, *SEEDSTICK*, and *SECONDARY WALL THICKENING PROMOTING FACTOR 1* from *A. thaliana* are specifically expressed in the endocarp of the fruit, while the negative regulator *FRUITFUL* predominates in mesocarp and exocarp. They also revealed the coordination of the synthetic pathways of lignin and flavonoids during the early development of the fruit (Dardick et al., 2010). Later, Hu et al. (2011) showed that during development, while enzymes involved in lignin biosynthesis are up-regulated, enzymes like chalcone synthase, chalcone isomerase, anthocyanidin reductase, and leucoanthocyanidin dioxygenase, involved in the flavonoid pathways, are down-regulated in the endocarp at the beginning of S2.

Throughout the early stages of development, pericarp and seed/embryo are closely associated. When the pit is completely hard, this relationship becomes less strict (Ognjanov et al., 1995). Seed development and maturation has been earlier studied covering morphological aspects and biochemical (lipid and sugar contents) composition (Tukey, 1936; Ognjanov et al., 1995). More recently, Bonghi et al. (2011) performed transcriptomic analysis using seed and mesocarp from peach at S1 to S4 and identified marker genes for organ/tissue at each stage. Based on genes that respond to hormones, they proposed that auxin, cytokinins, and gibberellins are important signals for seed-mesocarp crosstalk during early development, while abscisic acid and ethylene act later.

In a previous work, by means of metabolomic studies and by analyzing the main regulatory enzymes of the identified metabolic processes, we analyzed the mesocarp pathways operating in the peach fruit mesocarp using "Dixiland" variety throughout development and maturation (Lombardo et al., 2011). At stage E, high levels of active polyphenols were detected, such as caffeoylquinic acids, which are substrates for the phenylpropanoid and lignin pathways during stone hardening. Sucrose levels showed a large increase during development (E1 to S4), mainly due to its translocation from the leaf. Interestingly, during early development, high levels of total proteins were observed in stage E, which decreased markedly in the mesocarp of S1 and S2. These results suggest that immature fruit store large amounts of protein, which could be later used to sustain the processes that are carried out in stages S1 and S2 (Lombardo et al., 2011). Therefore, the purpose of this research was to determine the reconfiguration of the proteome during the profound decrease in the protein levels that takes place at

early stages of fruit development (from E to S2) comparing the endocarp and mesocarp separately in order to find out the main biochemical pathways that sustain the differentiation of these tissues.

MATERIALS AND METHODS

Plant Material

Prunus persica (L.) Batsch cv "Dixiland" trees were grown at the Estación Experimental Agropecuaria INTA (33° 44′ 12.1″ south latitude and 59° 47′48.0″ west longitude). The orchard received routine horticultural care including winter and summer pruning, fruit thinning, fertilizing and pest control. Fruits were collected during the seasons 2015–2016, 2016–2017, and 2017–2018. Sampling was conducted as in Lombardo et al. (2011) as follows: 9 days after flowering DAF (E), 29 DAF (S1), and 53 DAF (S2).

Fresh fruit were manually pealed and dissected in mesocarp (m) and endocarp (e). Fresh material was used for histochemical procedures and weight measurements. The rest of the material was immediately frozen in liquid N_2 and stored at −80°C for further experiments.

Dry (DW) and fresh (FW) weight were determined using at least ten fruits. For DW measurements, fruits were incubated at 80°C until constant weight.

Free Amino Acid Analysis

The amino acid profile in peach fruit was assessed by Reverse Phase-HPLC and phenylisothiocyanate (PITC) derivatization as in Dhar et al. (2013). A C18 column (5 μm, 250 × 4.6 mm, LUNA Phenomenex) with a C18 guard security pre-column (4 × 3 mm) and an ÄKTA purifier equipment (GE Healthcare, Uppsala, Sweden) were used.

For amino acid extraction, 0.3 g of tissue were disaggregated in a mortar with 1 ml of 0.1 M HCl. After centrifugation at 14,000 g at 4°C, the supernatant was precipitated with 10% (v/v) TCA and maintained on ice during 30 min. After clarification, the amino acids were derivatized as follows. Fifty microliter of the supernatant were mixed with 50 μl of methanol/water/triethylamine (2:2:1, v/v) and dried immediately under vacuum. Then, PITC reagent (methanol/triethylamine/water/PITC, 7:1:1:1, v/v) was added and kept at room temperature for 20 min. After drying, the PITC derivatives were dissolved in 300 μl acetate buffer (mobile phase A).

HPLC was conducted as exactly described in Ruggieri et al. (2018). Mobile phase A consisted in sodium acetate trihydrate (pH 6.4) with 0.5 ml of triethylamine (TEA) and mobile phase B of acetonitrile: H_2O (6: 4, v/v). All solutions were filtered through a 0.22 mm Millipore membrane. One hundred microliters of sample or standard were injected. Running conditions: a gradient between phases A and B was used (**Supplementary Table 1A**). The column was kept at 39°C and a flux of 1 ml/min. Amino acids were detected by measuring the absorbance at 254 nm.

Calibration curves were prepared by duplicate as reported in Ruggieri et al. (2018) using cysteine, arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tyrosine, threonine, valine, alanine, aspartic acid, glutamic acid, glycine, proline, serine, asparagine, glutamine, cystine, ornithine, citrulline, and tryptophan as standards. Calibration equations are shown in **Supplementary Table 1B**. The amount of each amino acid in the samples was expressed as μmol per gram of fresh weight (μmol. gFW^{-1}).

RNA Extraction and cDNA Synthesis

RNA was extracted following the procedure described in Meisel et al. (2005) using 3 g of fresh tissue. Due to the small size of fruits at stage E, a pool of at least four fruits was used. Then total RNA was treated with DNase RQ1 (Promega). The quality of the extracted RNA was checked by electrophoresis and the concentration measured using the Take3^{TM} Micro-Volume Plate adaptor and a EPCOCH2 spectrophotometer (BioTek^{R}). Three μg of RNA was retro-transcribed using oligo(dT) and Mo-MLV reverse transcriptase (Promega), according to the manufacturer's instructions.

Quantitative Real-Time PCR (qRT-PCR)

Quantitative real-time PCR was conducted in an Mx3005P QPCR (Agilent technologies, Stratagene) cycler equipped with MxPro QPCR version 4.10 software.

Reactions were performed in a final volume of 20 μl containing 1X Taq activity buffer (Promega), 200 μM dNTPs, 1 mM $MgCl_2$, 0.8 U of GoTaq DNA polymerase enzyme (Promega), 0.5 μM of each primer, 0.5X SYBRGreen I (Invitrogen) and 1 μl of a fivefold dilution of each cDNA. Oligonucleotides primers were designed with the aid of Primer3 software[1]. Elongation factor 1 (ppa005702) was used as internal control (forward primer: 5′-TCCAGTTCTTGATTGCCACA-3′ and reverse primer 5′-CCATACCTGCATCTCCGTTC-3′). To amplify β–cyanoalanine hydratase (ppa008090) the following primers were used: 5′-CGCTGATTCCAGGGATGTAT-3′ (forward primer) and 5′-CCCATCATAATTGGGTCCAG-3′ (reverser primers).

The cycling parameters were as follows: an initial denaturation step at 94°C for 2 min; 40 cycles of 96°C for 10 s; 58°C for 15 s; 72°C for 1 min, and 77°C for 7 s to detect fluorescence, and final elongation step at 72°C for 4 min. Melting curves were generated by rising the temperature from 65 to 95°C. The resulting amplicons were separated in a 2% (w/v) agarose gel. Three biological and three technical replicates were conducted. Relative expression was estimated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Histochemical Staining Procedures

Samples were taken from fruits, cut in cubes of 3–4 mm side and fixed at 4°C in 50% (v/v) ethanol, 10% formaldehyde and 5% (v/v) acetic acid for 2 days (the solution was renewed once). The samples were dehydrated with a graded ethanol series and embedded in paraffin. Cross sections, 8 μm thick, were made with a rotary microtome (E. Leitz Wetzlar, New York) and placed onto gelatine-coated slides for microscopy.

[1] http://primer3.ut.ee/

Sections were dewaxed and rehydrated with xylene and then ethanol series following standard protocols and used for the staining procedures as follows.

Samples were stained with 17.5 mg. ml^{-1} Calcofluor white (Sigma) for 5 min for visualization of cellulose. Sections were washed with 1X Phosphate-Buffered Saline (PBS) (pH 7.4) and mounted with anti-fade solution (0.1% (w/v) p-phenylene-diamine and 50% (v/v) glycerol in 1X PBS).

Aniline blue was used to enhance the overall fluorescence of all plant cell walls (Smith and Mc Cully, 1978) and to follow modifications in the cell wall composition. The stained material was viewed with a microscope Nikon Eclipse TE-2000-E2 with confocal system Nikon C1Plus SiR using the following settings excitation = 405, 488, and 543 nm; emission = 450/435 nm (blue), 515/530 nm (green), and 605/675 nm (red). Images were acquired with the Nikon EZ-C1 software. For well width estimation, each image was then divided into nine square regions and five of them were analyzed. Three images were analyzed at each stage of development for each tissue. The following process was repeated until a stable value was found. Cell wall width was measured using the program "Image J"[2] in sections at 90° with respect to the perimeter of the wall.

For polysaccharides, dewaxed sections were incubated in periodic acid (1% w/v) for 30 min, washed and then incubated with Schiff's reagent (Biopur, Argentina) for 1 h. After rinsing, the sections were ready for observation with light microscopy. Images were acquired through a Nikon Labophot-2 Light microscope using a TV Lens C-0.45x Nikon digital camera Micrometrics SE (standard edition) Premium.

Lignin deposition was evidenced by the use of the Phloroglucinol staining using fresh fruit. A solution of 5% (w/v) phloroglucinol (Sigma) in 80% (v/v) methanol was applied to the fruit surface. After 5 min, some drops of HCL concentrated were added. The presence of lignified tissues was revealed as a red-violet coloration.

Proteomic Analysis Using High–Resolution Mass Spectrometry

Protein Extraction

Total proteins were extracted from 0.5 g of fresh tissue using a buffer containing 50 mM Tris-HCl, pH 7; 1 mM EDTA; 0.5% (v/v) Triton X-100; 10 mM β-mercaptoethanol; 10% (p/v) glycerol; 2 mM $MgCl_2$; 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and polyvinyl polypirrolidone (PVPP).

Protein Quantitation

Protein concentration was determined according to Bradford (1976) using the Protein Assay reagent from Bio-Rad and BSA as standard.

Protein Modification and Proteomics

Forty micrograms of proteins extracted from mesocarp and endocarp of peaches at stages E, S1, and S2 were precipitated with 1/5 volumes of 100% (w/v) TCA overnight at −20°C. The pellet was washed twice with cold acetone and proteins were finally resuspended in 50 µl 8 M Urea and reduced with 10 mM DTT for 45 min at 56°C. After alkylation with 20 mM iodoacetamide for 40 min, proteins were precipitated with 1/5 100% (w/v) TCA overnight, washed with cold acetone, dried and delivered to the Proteomics Core Facility CEQUIBIEM, Buenos Aires, Argentina. Proteins were resuspended in 50 mM NH_4HCO_3, pH 8 and digested overnight with sequencing-grade modified trypsin (Promega). Zip-Tip C18 (Merck Millipore) columns were used for desalting. Resulted peptides were separated in a nano-HPLC (EASY-nLC 1000, Thermo Fisher Scientific, Germany) coupled to a mass spectrometer with Orbitrap technology (Q-Exactive with High Collision Dissociation cell and Orbitrap analyzer, Thermo Fisher Scientific, Germany). Peptides were ionized by electrospray. Proteome Discoverer 2.1 software (ThermoScientific, Germany) and the peach reference proteome set from uniprot (*Prunus persica* (*Amygdalus persica*)-UP000006882-Uniprot) were used to identify peptides and proteins.

Differential Proteome Analysis

Statistical analysis of proteomics data was conducted using the Perseus software platform (Tyanova et al., 2016)[3]. Before analysis, data were normalized and subjected to manually missing-value imputation. Missing/zero values were replaced by the minimum value detected by the mass spectrometer (considered as the detection limit) when at least two of the three replicates were missing. Instead, when the peptide was detected in two of the three replicates the missing/zero values were left blank. LFQ protein intensities were log2 transformed.

Two-sample tests were conducted to compare proteomes of Ee vs. S1e; S1e vs. S2e; Em vs. S1m; S1m vs. S2m; Ee vs. Em; S1e vs. S1m; S2e vs. S2m; by applying the standard *t*-test statistic with a permutation-based false discovery rate of 0.05. A $q \leq 0.05$ and a fold change (FC) < 0.5 or > 2 were used as significance threshold parameters. Three biological replicates were used for each sample analyzed (Ee, S1e, S2e, Em, S1m or S2m). The *p*-value was set at 0.05. Volcano plots showing *q*-values (−log2) were used to assess differences in Ee vs. S1e; S1e vs. S2e; Em vs. S1m; S1m vs. S2m; Ee vs. Em; S1e vs. S1m; S2e vs. S2m.

Ontology annotations of significantly regulated proteins for "cellular component," "biological process," and "molecular function" were analyzed to assess common localizations and functions by using MapMan (Usadel et al., 2009).

Data Statistical Analysis and Representation

With the exception of proteomic analysis where Perseus software was used and of cell width data, data was analyzed using one way-ANOVA. Minimum significance differences were calculated by the Bonferroni or Fisher tests ($\alpha = 0.05$) using the Sigma Stat Package (Systat Software Inc., San Jose, CA, United States). The Kruskal-Wallis One Way Analysis of Variance on ranks followed by the non-parametric Dunn's test ($\alpha = 0.05$) was used for comparison of cell width measurements in each type of tissue and the Mann–Whitney *U*-test ($\alpha = 0.05$) was used to compare the width of cell walls between endocarp and mesocarp, at each stage.

[2]http://mev.tm4.org

[3]http://www.perseus-framework.org

Principal component analysis (PCA) was conducted using the XLSTAT software (Microsoft Excel) and amino acid quantification data. In the case of proteins, PCA was conducted using Clustvis (Metsalu and Vilo, 2015).

For data visualization, MultiExperiment Viewer software was used (MeVv5.1.1, Saeed et al., 2003)[4].

RESULTS

"Dixiland" Peach Growing Curve

The first step in our analysis was the establishment of a growing curve in order to identify the different phases of the process. **Supplementary Figure 1A** shows the fresh weight of "Dixiland" peach fruit vs. the days after flowering (DAF). As shown in Lombardo et al. (2011) this cultivar exhibits the peach fruit typical growing curve. In addition to weight, volume, calculated using the formula for the volume of an elliptical spheroid, was used as indicative of fruit size. **Supplementary Figure 1B** shows the dramatic increase in fruit volume from E to S1 (42.3-fold) and a threefold thereafter.

Lignin deposition, detected by phloroglucinol–HCl staining, was followed as a way of "stage check." No coloration in the endocarp or the mesocarp was observed during E stage, and only positive reaction was observed in the exocarp (**Figure 1A**). During S1, in addition to exocarp, vascular bundles are stained in the endocarp and the mesocarp. In S2, lignin deposition is clearly observed in the endocarp as large regions of red coloration with a clear perimeter of lignin deposition surrounding the seed. In this stage, vascular bundles in the mesocarp are also stained.

Collected E, S1, and S2 fruit were dissected manually by separating the endocarp from the mesocarp and used for further analysis. Fresh and dry weight curves reveal the same trend of increase of weight evolution in each tissue (**Figure 1B**). Total protein content decreased from E to S2 in both tissues, with endocarp showing a reduction of 3.6-times from Ee to S1e and a similar trend of decrease from S1e to S2e. While a similar tendency was observed in the mesocarp during the S1m to S2m transition (2.9-fold), the decrease in total protein was more pronounced in the Em to S1m transition (11.4-fold) (**Figure 1C**). In addition, chlorophyll content was lower in endocarp with respect to the mesocarp. At all stages analyzed, chlorophyll a was around 20-fold higher in the mesocarp than in the endocarp and chlorophyll b was 10-times greater in mesocarp than the endocarp. Both chlorophylls tend to decrease from E to S2 in both tissues (**Figure 1D**).

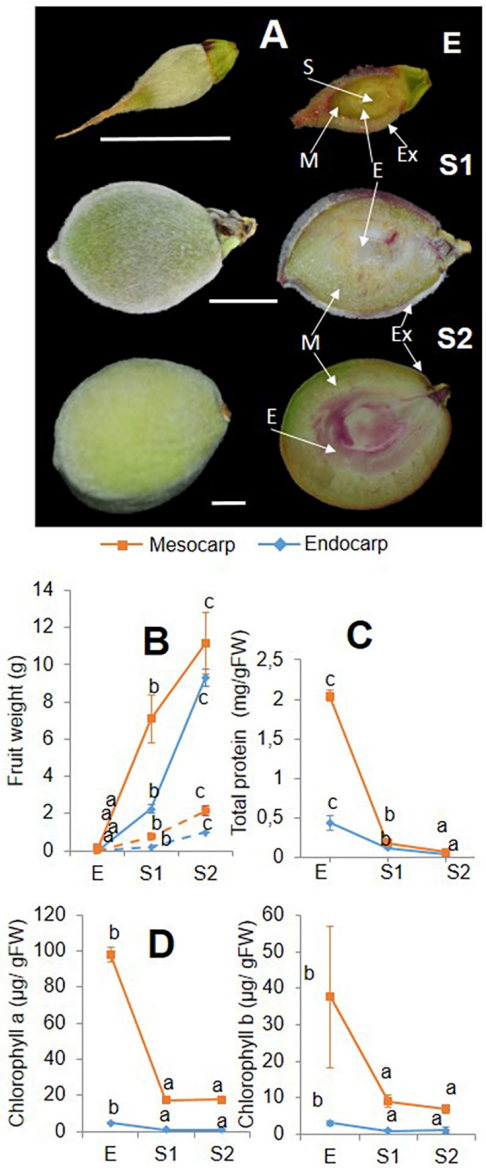

FIGURE 1 | (A) Lignin staining during early developing of "Dixiland" peach fruit. Peach fruit were collected after 9 (E), 29 (S1) and 53 (S2) DAF and subjected to phloroglucinol–HCl staining to detect lignin deposition. Scale bars: 1 cm. S: seed; E: Endocarp; M: mesocarp; Ex: exocarp. Weight **(B)**, protein quantification **(C)** and chlorophyll analysis **(D)** were conducted in mesocarp (orange) and endocarp (blue) from peach fruit at E, S1, and S2 stages. Whole and dotted lines, respectively, represent fresh and dry weight curves. Total protein and chlorophylls are expressed in a fresh weight basis. Within each tissues, values with different letters are statically significant different ($p < 0.05$).

Fruit Proteomics During Early Development in Endocarp and Mesocarp

Quantitative proteomics was conducted using label-free based LC-MS in the endocarp and mesocarp of peach fruit at developmental stages E (Ee and Em), S1 (S1e and S1m), and S2 (S2e and S2m). The entire dataset of protein identification of each sample is presented in **Supplementary Table 2**. The amount of total identified proteins varied between tissues and stages. In endocarp, the number of different proteins was 654, 929, and 988, for Ee, S1e, and S2e, respectively. In contrast, the number of detected proteins in mesocarp tended to decrease from E to S2 (1154, 996, and 917, for Em, S1m, and S2m, respectively). Proteins detected ranged between 4 and 239.7 kDa and from 3.87 to 11.81 pI (**Supplementary Table 2**).

[4]http://www.tm4.org/

Principal component analysis (PCA) was conducted with all proteome data obtained (**Supplementary Figure 2**). First principal component (PC1) explained 31.1% of the variation, while the second (PC2) and the third (PC3) principal components accumulated a total variation of 53.2 and 73.1%, respectively. The plots show that the protein profiling at E, S1, and S2 in endocarp and mesocarp is unique for each tissue at each stage. The profiles of the mesocarp (Em, S1m, and S2m) are more closely related than those of endocarp (Ee, S1e, and S2e) throughout the period analyzed (**Supplementary Figures 2B,C**). On the other hand, proteomes of both tissues at S2 are the closest related since S2m and S2e group together in PC2 vs. PC1, PC3 vs. PC1, and PC3 vs. PC2 (**Supplementary Figure 2**). In the other stages (E and S1), the proteomes of mesocarp and endocarp seem to be more divergent (**Supplementary Figure 2**).

To identify the proteins with differential abundance (PDA) in the tissues and stages, the Perseus software platform was used. Comparisons were conducted by using *t*-test ($P < 0.05$, Student's *t*-test). Increases in more than twofold and less than a half were considered of biological relevance. The results are first presented showing PDA along development in endocarp and mesocarp; and secondly, PDA between endocarp and mesocarp at each developmental stage.

An initial analysis was the evolution of the protein profile in endocarp and mesocarp during development. From 569 proteins statistically determined to be differentially abundant in S1e with respect to Ee, 457 increased in S1 with respect to E, and 112 decreased. Five hundred and one proteins differed in abundance in S2e with respect to S1e, of which 236 were increased in S2 with respect to S1, and 265 decreased. In mesocarp the number of differentially proteins was lower, with 423 proteins varying between Em and S1m, and 335 between S1m and S2m. While 177 proteins were increased in S1m with respect to Em, 171 proteins were increased in S2m with respect to S1m.

With the aid of MapMan software (**Supplementary Table 3**) the distribution of PDA according to their functional category in endocarp (**Figure 2A**) and mesocarp (**Figure 2B**) was assessed. In both tissues and during the E to S1 and S1 to S2 transitions protein metabolism was the most represented functional category accounting between a 18 and 30% of all PDA. In all cases, not assigned and miscellaneous were the second and third over-represented categories. Amino acid metabolism, signaling, cell wall, cellular and stress were highly represented categories in the comparisons, as well. Particularly, during the transition from S1e to S2e, secondary metabolism represented a 4% of the total PDA. RNA metabolism represented the 5 and 6%, during the Em to S1m and S1m to S2m transitions, respectively. Notably, photosynthesis and lipid metabolism represented the 7 and 4% of proteins during S1m to S2m shift.

Figure 3 depicts an overview of the PDA involved in metabolic pathways modulated in mesocarp and endocarp during early "Dixiland" peach fruit development using MapMan program (Usadel et al., 2009). As it can be observed, during peach fruit development there is an important protein composition remodeling in both mesocarp and endocarp, with changes in the relative amount of proteins involved in cell wall, lipid, amino acid, carbohydrate, photosynthesis, energy, antioxidant, nucleotide, tetrapyrrole, N and S metabolisms (**Figure 3**).

Considering that protein metabolism was the GO term most represented, the distribution of proteins within the subclasses was analyzed and shown in **Figure 4**. As expected, within all transitions protein synthesis and degradation were the most represented subgroups (**Figure 4A**). It is very interesting to note that within proteins involved in protein synthesis, those constituting the ribosome were the ones that varied the most, being the majority repressed in S1 with respect to E and in S2 with respect to S1 in both mesocarp and endocarp (**Figure 4B**). With respect to protein degradation, subtilases and serine proteases were the most represented, with subtilases being induced in the E to S1 transition and further repressed during S1–S2, in both endocarp and mesocarp (**Figure 4C**). While subunits of proteasome were repressed in E to S1 transition, they were induced in the transition from S1 to S2 (**Figure 4C**) in both endocarp and mesocarp. On the other hand, the different ubiquitins showed variable response in the different tissues and stages.

Further analysis focused on PDA between mesocarp and endocarp at each developmental stage. At stage E, 568 PDA were detected between endocarp and mesocarp. While 501 are increased in Em with respect to Ee, 61 were decreased. At S1, 341 proteins differed in their abundance, with 184 and 157 are increased and decreased, respectively, in mesocarp with respect to endocarp. Finally, 367 proteins vary in their amount between mesocarp and endocarp at S2. Of these, 163 are increased in mesocarp with respect of endocarp, and the rest are decreased. **Figure 5** represents the distribution of PDA between mesocarp and endocarp according to the biological function. In general, a similar distribution not only with respect to the function but also to the proportion of increase and decrease is observed at stages S1 and S2. In contrast, at E a great number of proteins belonging to many functional categories (such as signaling, cellular, hormone, nucleotide, RNA, amino acid and lipid metabolism) are present in a greater extent in the mesocarp than in the endocarp. On the other hand, irrespectively of the stage of development, an overview of the PDA between tissues indicates that many proteins related to photosynthesis like those acting as structural components or binding chlorophyll in the photosystems, participating in the transport of electrons, in ATP synthesis, in the carbon reduction cycle and in photorespiration occur in a higher extent in the mesocarp with respect to the endocarp. A mean of 2000-fold of increase was detected for the different photosynthetic proteins. **Supplementary Figure 3** shows the magnitude of variation of the photosynthetic-related proteins. In addition, enzymes involved in tetrapyrrole synthesis such as glutamate-1-semialdehyde 2,1-aminomutase (ppa005146m), porphobilinogen synthase (ppa006219m), protochlorophyllide reductase (ppa006788m), and magnesium chelatase (ppa006200m) were between 1000- and 6000-fold higher in the mesocarp than in the endocarp (**Supplementary Tables 3E,F,G**), in high agreement with chlorophyll measurements (**Figure 1D**). To clearly visualize the variable proteins between endocarp and mesocarp, at each

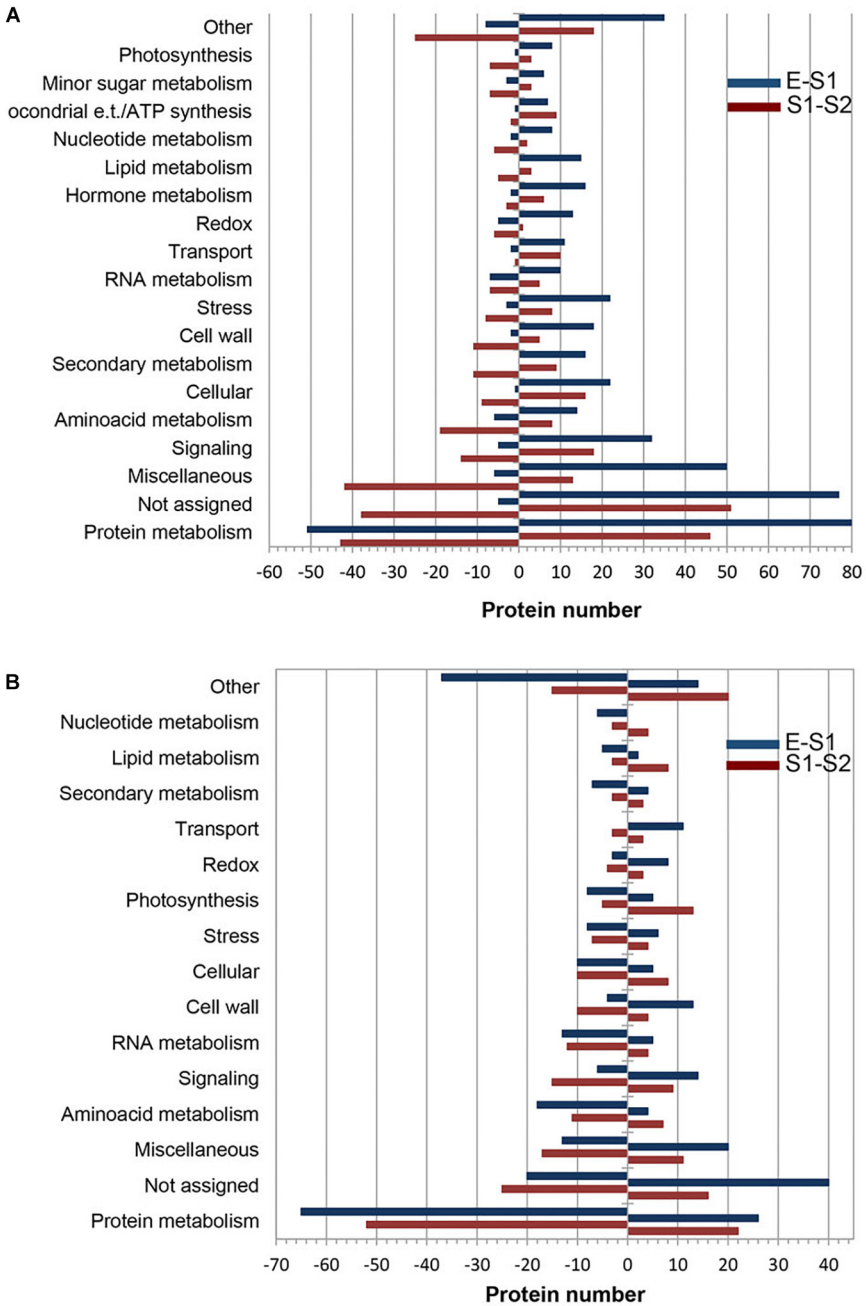

FIGURE 2 | Functional classification of differentially expressed proteins over very early stages of peach fruit development. Proteins from endocarp **(A)** and mesocarp **(B)** tissues were analyzed at E, S1, and S2. Blue bars correspond to proteins increased (positive values) and decreased (negative values) in S1 with respect to E. Red bars represent the number of proteins increased (positive values) and decreased (negative values) in S2 with respect to S1.

stage, schemes representing metabolic pathways are shown in **Supplementary Figure 4**.

Amino Acid Profiling at Early Peach Development

Considering that PDA involved in protein metabolism was the most represented category in endocarp and mesocarp during fruit development, amino acid profiling, conducted by phenylisothiocyanate (PITC) derivatization followed by HPLC, was analyzed in these tissues over fruit development. Not only relative amounts of each amino acid were revealed by this approach but also their absolute amounts due to the aid of calibrations curves. Amounts of each amino acid identified are shown in **Supplementary Table 4** and expressed in μg per gram of fresh tissue. PCA of the data reveals that three PC explain the

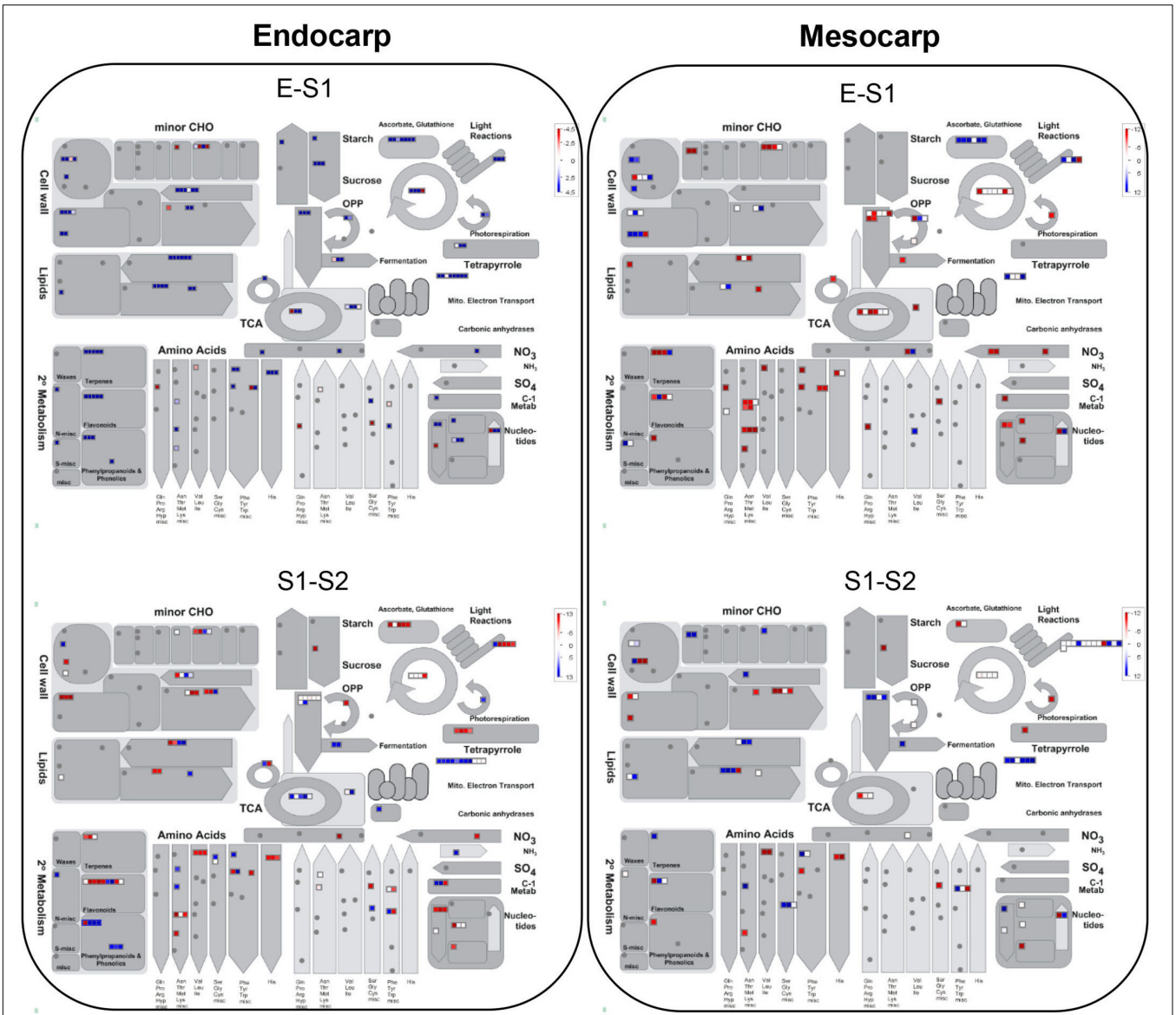

FIGURE 3 | Overview of PDA in endocarp and mesocarp during the transitions from E to S1 and from S1 to S2 in relation to their correspondent metabolic pathways. Each square corresponds to a protein. Red and blue indicate lower and higher expression in the earlier stage of development, respectively, in a log2 basis. Scale bar is indicated at the top right of each figure. Images were generated using MapMan program (Usadel et al., 2009).

84.7% of the variation (**Supplementary Figure 5**). The first PC explains a 40.2% of the variation, the second one the 29.4% and the third one the 15.1%. As it is the case of the proteome analysis, amino acid profiling of S2m and S2e group together in PC2 vs. PC1 and are closely related to S1e and S1m. In addition, it is clearly visualized that the profiles of E (either Ee or Em) appear in the plots distant from the other samples and of each other.

In order to have a clear picture of the relevance of the changes in amino acid composition, the total amount of free amino acids was calculated (**Figure 6A**). In both tissues, the total amount decrease from E to S1 and increase thereafter restoring the initial levels in mesocarp and exceeding the amounts at E in endocarp.

Figure 6B represents the percentage of each amino acid in a weight basis in mesocarp and endocarp at each developmental stage. The amount of each amino acid at the different developmental stages in endocarp and mesocarp is shown as a heat map (**Figure 6C**). Asparagine not only is the most abundant amino acid in the fruit under study but also it increases over development (**Figure 7**). On the other hand, other key amino acids involved in N metabolism, such as Gln, Asp, and Glu show a decline in mesocarp and endocarp. Serine, which is another abundant amino acid of the fruit, also tend to increase from E to S1 in both tissues. Neither the precursor in phenylpropanoid metabolism Phe nor its closely related amino acid Tyr exhibited

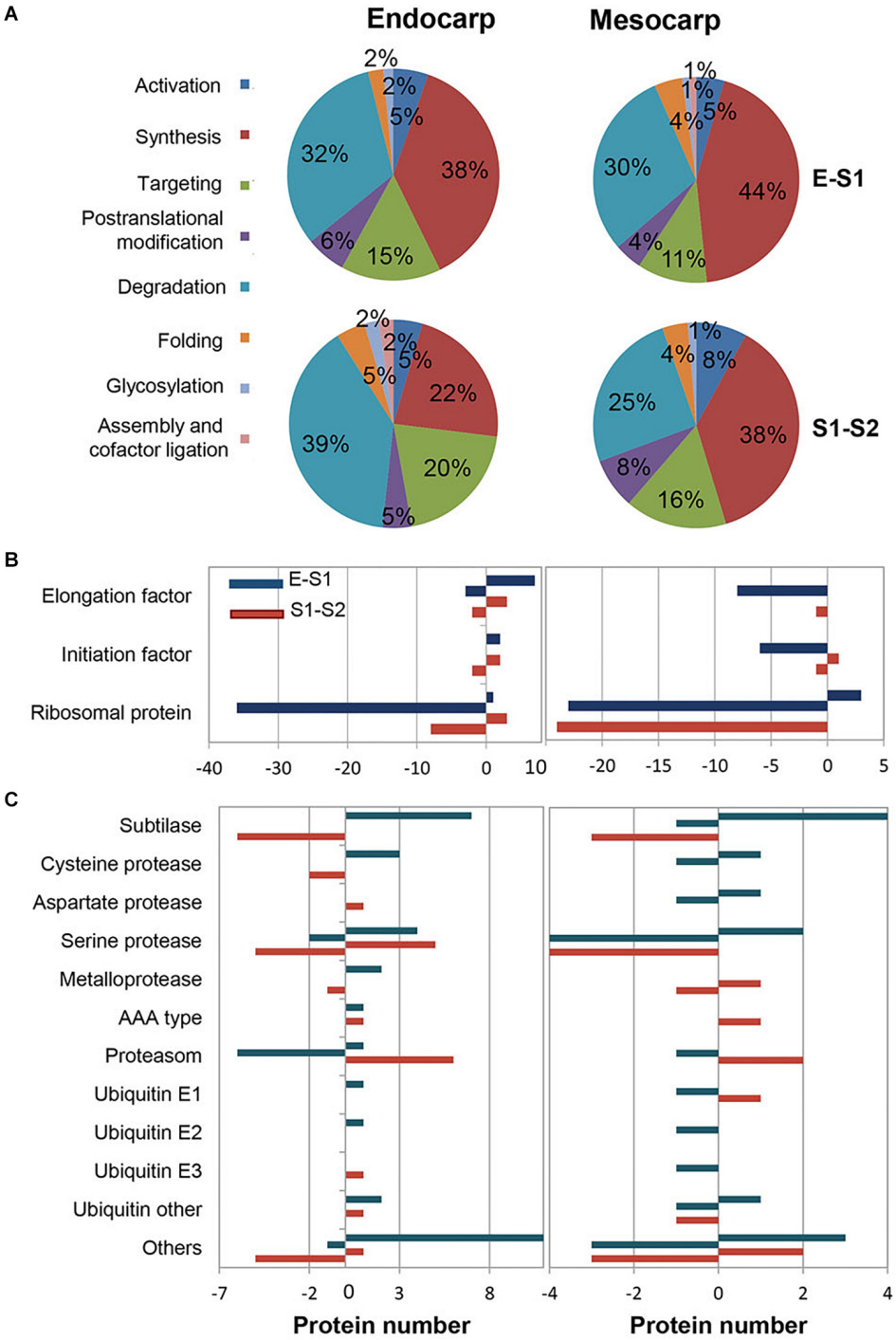

FIGURE 4 | Distribution of variable proteins across very early stages of development within protein metabolism functional category. E1 to S1 and S1 to S2 transitions were analyzed in both endocarp and mesocarp. **(A)** Pie charts representing the total number of PDA distributed within "protein metabolism" GO terms subcategories. **(B)** Classification of PDA involved in protein biosynthesis in endocarp (left graph) and mesocarp (right graph). **(C)** Distribution of PDA participating in protein degradation in endocarp (left graph) and mesocarp (right graph). Blue bars correspond to proteins increased (positive values) and decreased (negative values) in S1 with respect to E. Red bars represent the number of proteins increased (positive values) and decreased (negative values) in S2 with respect to S1.

changes in their amounts during development in mesocarp (**Supplementary Table 4**). Interestingly, Tyr increased and Phe decreased in S2 in endocarp.

Taking into account the highly abundance of Asn in peach fruit, the metabolic pathways in which it is involved were explored. For this purpose, the genes encoding the enzymes

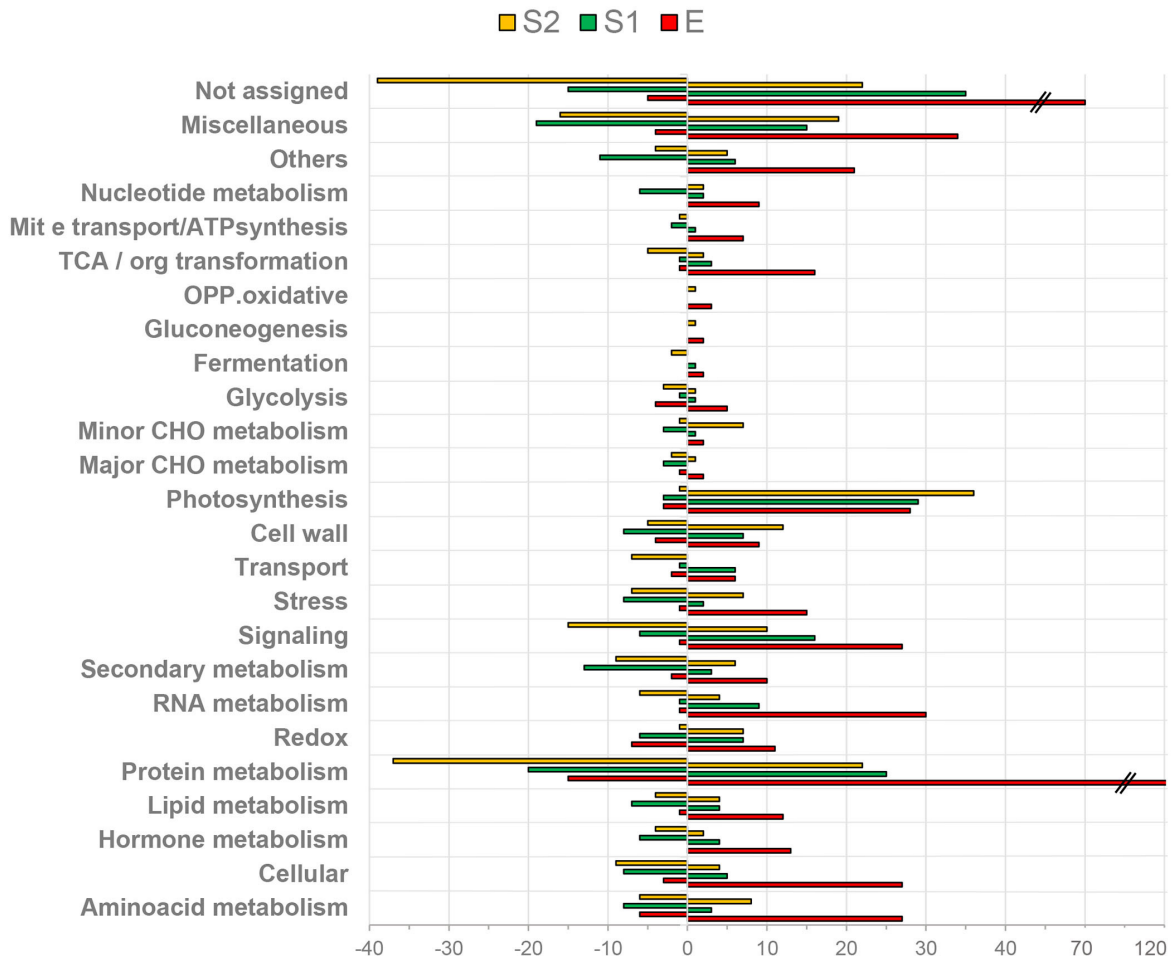

FIGURE 5 | Functional classification of PDA in endocarp and in mesocarp. Positive and negative values represent the number of proteins increased and decreased, respectively, in mesocarp with respect to endocarp when proteomes of fruits at E (red bars), S1 (green bars) and S2 (yellow bars) were analyzed.

catalyzing its synthesis and degradation were explored in the peach genome[5] based on known pathways. Once identified, the corresponding proteins were identified based on their uniprot accession number (**Supplementary Table 5**). These numbers were used to search the presence of these proteins in peach fruit proteome over development (**Supplementary Table 2**). To our surprise, none of the Asparagine synthetases identified in peach genome were found in peach proteome during very early development. In contrast, two Asparaginases (M5WUV5 and M5X0K4) were detected in both mesocarp and endocarp (**Figure 7** and **Supplementary Tables 2**, **3**). Both isoforms exhibited a similar trend of variation during the early development, which is opposite to that of the Asn profile. Moreover, neither Asp, nor Glu or Gln followed the trend of Asn variation during development.

Conversely, β–cyanoalanine synthase (β–CAS) and β–cyanoalanine hydratase producing L-Ans from L-Cys and hydrogencyanide were detected (**Figure 7**). The corresponding protein profiles are shown in **Figure 7**. In addition, as means of validation of these results, the transcript profile of β–CAS was also explored by qRT-PCR. Transcript profile agrees with that of the protein (**Figure 7**). In contrast, β–cyanoalanine nitrilase was not found in the proteome (**Supplementary Table 2**).

Microscopic Confocal Analysis of Mesocarpic and Endocarpic Cells and Cell Walls During Development

A combination of histological tests, anatomical analysis and the use of confocal microscopy was used to provide information about the cell sizes and the cell walls of the endocarp and mesocarp during early development.

Figure 8 shows that at each stage analyzed, the sizes of the cells from the endocarpic tissue (**Figure 8A**) are always smaller than those of the mesocarp (**Figure 8B**). Bright field images on transition zone between endocarp and mesocarp allow the visualization of the different cells (**Figure 8C**). In addition, the

[5]https://phytozome.jgi.doe.gov/pz/portal.htmlhttps://phytozome.jgi.doe.gov/pz/portal.html

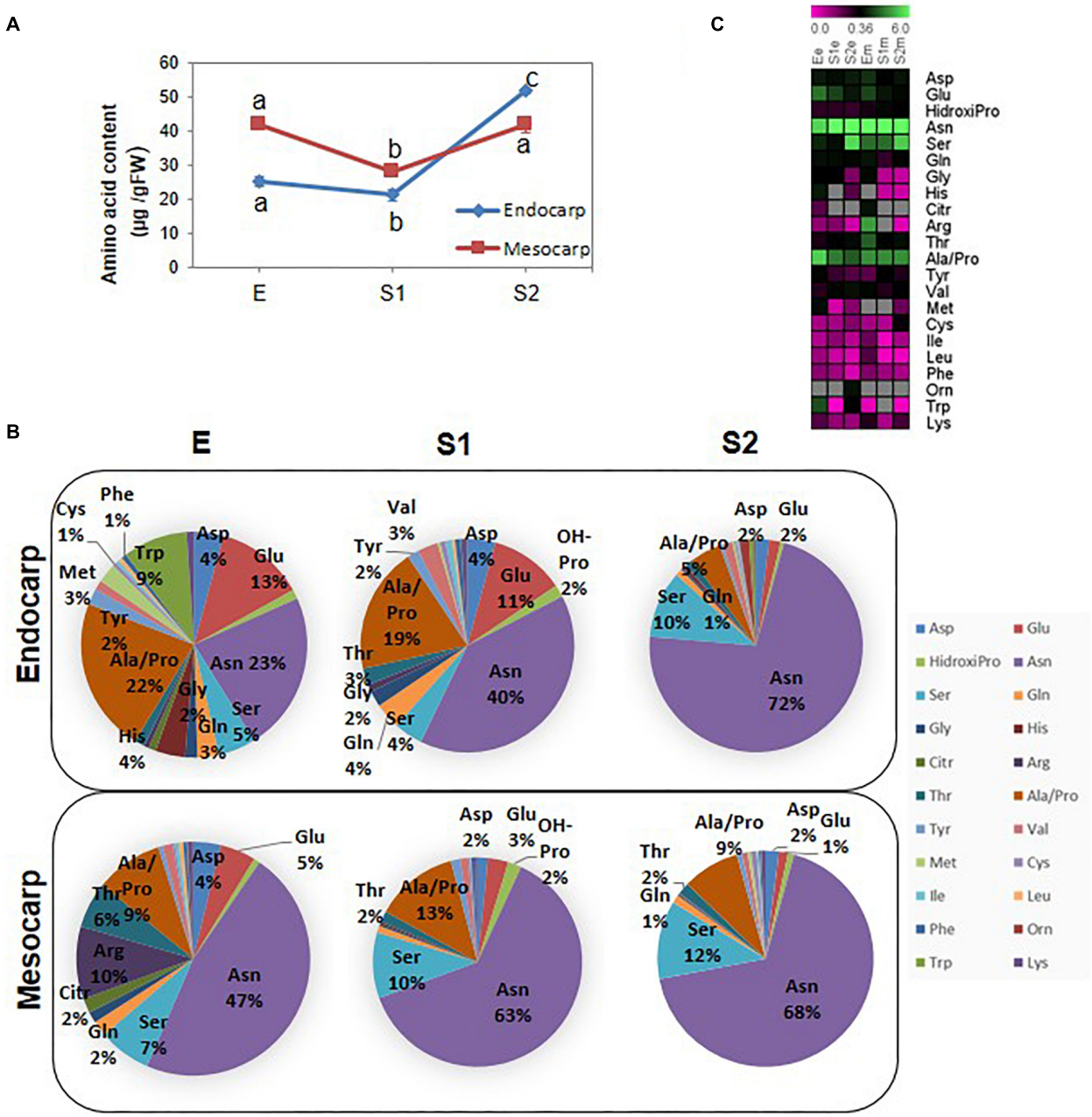

FIGURE 6 | Amino acid profiling during early peach fruit development. **(A)** Total amino acid quantification in endocarp and mesocarp. Values represent the mean of six independent determinations. Error bars represent the standard deviation. Bars with at least one same letter are not statistically different within the same tissue ($p < 0.001$). **(B)** Pie charts showing the proportion of each amino acid in the endocarp and the mesocarp at the developmental stages E, S1, and S2. **(C)** Heat map showing the amount of each amino acid during development in the endocarp (e) and the mesocarp (m). The scale bar at the top of the figure represents the amount of each amino acid expressed in µg/GFW. Gray boxes indicate that the amino acid was not detected.

size of both endocarpic and mesocarpic cells increases from E to S1, in agreement with increase in fruit size at this stage. Only a slight increase in cell dimension is observed in the transition from S1 to S2. To provide quantitative data on cell sizes, the number of cells per images of 51,042 μm^2 collected with 60X magnification was counted at each stage and tissue and shown in **Supplementary Figure 6**. The number of cells per field at each stage is always smaller in the mesocarp than in the endocarp, denoting bigger sizes for cells in the mesocarp. Moreover, in each tissue, the number of cells per area is higher at E than at S1. There are no statically significant differences between measurements at S1 and S2 within each tissue (**Supplementary Figure 6**). Moreover, it is clearly observed the lower degree of calcofluor fluorescence, used to reveal cellulose, in both endocarp

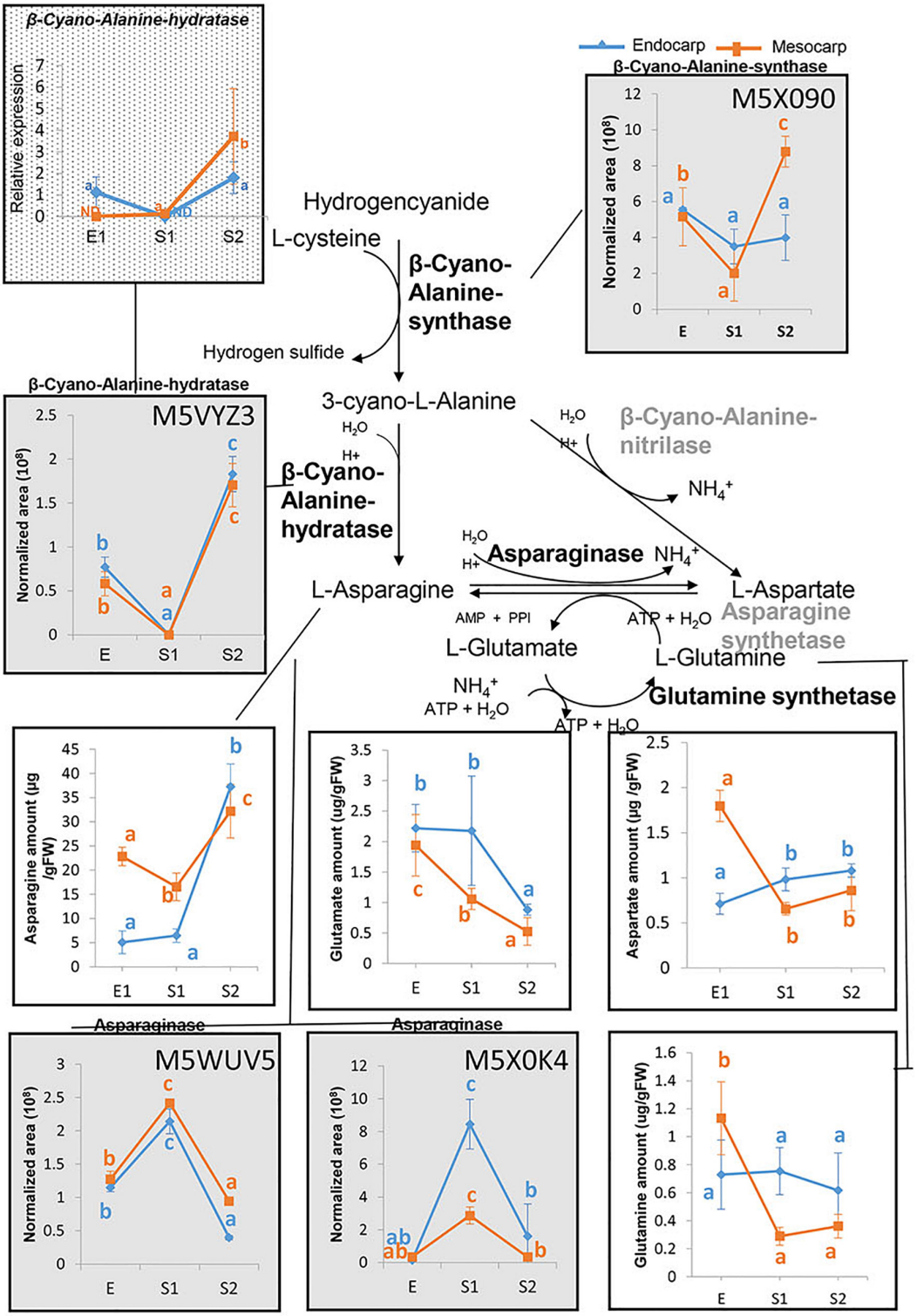

FIGURE 7 | Asparagine metabolism in peach fruit. An overview of the metabolic pathways conducting to asparagine biosynthesis and metabolism is shown. Graphs in gray background represent the proteins profiles as assessed by nanoHPLC-MS (within each box the uniprot accession number of the protein is presented), graphs with a white background show the amounts of amino acids determined by PICT-HPLC and the graph with dotted background displays the relative expression of the transcript encoding β–Cyano-Alanine-hydratase analyzed by qRT-PCR. For each parameter and tissue, values with at least one same letter are not statistically different. Error bars represent the standard deviation. Enzymes in gray were not detected in the proteome of "Dixiland" peach fruit during the very early development. ND, not detected.

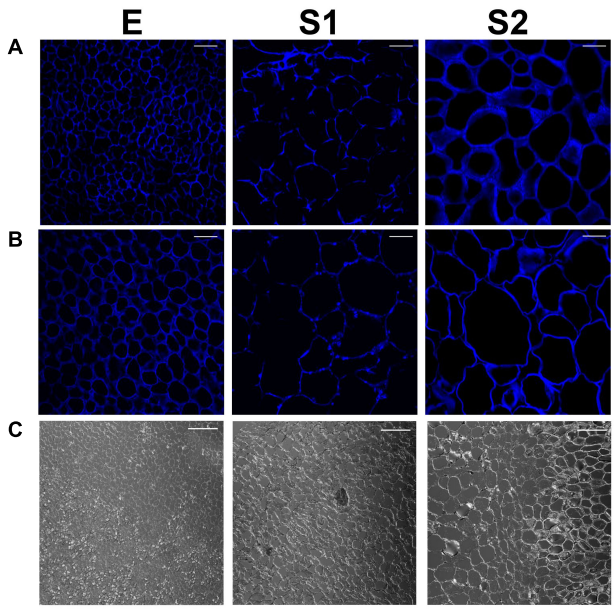

FIGURE 8 | Calcofluor white staining of endocarpic **(A)** and mesocarpic **(B)** cells at E, S1, and S2. Magnification used: 60X. Scale bars: 25 μm. Bright field images of the interphases between endocarp and mesocarp **(C)**. Magnification used: 20X.

and mesocarp of fruit at S1 (**Figures 8A,B**). Therefore, lower amounts of cellulose are deposited in the cell walls at S1.

Confocal laser scanning microscopy images of aniline blue-stained sections (**Supplementary Figure 7**) show clear differences between endocarp and mesocarp. In addition, it is particularly notorious, the higher red fluorescence in the endocarp at S2 with respect to S1, in agreement with lignin staining (**Figure 1A**). Therefore, the staining was useful to reveal the differences in cell wall composition along fruit development in both endocarp and mesocarp. As control, autofluorescence was recorded in the absence of aniline blue to reveal, by comparing with stained images, the enhancement of endogenous fluorescence by the fluorochrome (**Supplementary Figure 8**).

In addition, the single wall thickness of endocarpic and mesocarpic cell walls in sections of fruit at E, S1, and S2 stages were measured using the blue channel of images shown in **Supplementary Figure 7** (**Figure 9A**). In the endocarp, cell wall width was increased from stage E to S2. On the other hand, in mesocarp, the thickness of the wall was increased in S1 with respect to E, and remained constant at S2. Moreover, wall width was always different for cells located at the endocarp, with respect to those at the mesocarp at stages E ($p < 0.005$) and S1 ($p < 0.034$). Since cell walls in the endocarp start becoming lignified at S2, images from the red channel were also used to measure the wall thickness. In this way, cell walls were found to be statically significant thicker ($p < 0.001$) in the endocarp than in the mesocarp at S2 (**Figure 9B**).

To aid in the analysis of cell walls during early development, changes in abundance of proteins related to the cell wall were investigated within data on proteomic analysis (**Supplementary Table 3**) and shown in **Table 1**. In general, and in agreement with an expansion in cell during the transition from E to S1, the enzymes involved in the synthesis of wall precursors are highly up-regulated in endocarp. Moreover, an

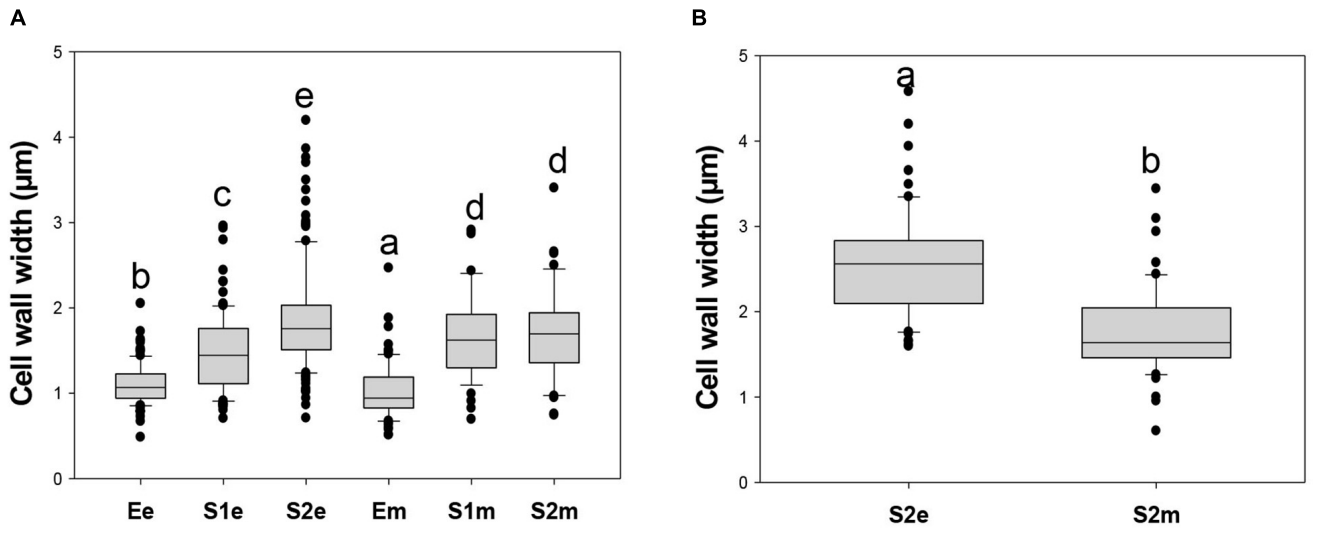

FIGURE 9 | Box plots of cell wall thickness of endocarpic (e) and mesocarpic (m) cell walls in sections of fruit at E, S1, and S2 stages. **(A)** Cell wall width was measured using confocal laser scanning microscopy images of aniline blue-stained sections collected in the blue channel. Cell wall width was measured using the program "Image J" (http://imagej.nih.gov/ij) in sections at 90° with respect to the perimeter of the wall. Plots were constructed with Sigma Plot Software. Since the distribution of the data is not parametric, the Kruskal-Wallis One Way Analysis of Variance on ranks was applied followed by the Dunn's Method for All Pairwise Multiple Comparison Procedure. **(B)** Cell wall width measured using images collected in the red channel from the endocarp and mesocarp of fruits collected at S2. Cell walls from S2e are thicker than from S2m ($p < 0.001$, Mann–Whitney *t*-test). Boxes with different letters are statistically different.

TABLE 1 | Changes in proteins abundances involved in cell wall metabolism during very early devolvement of peach fruit.

Acc. No.	Protein description	Fold change (log2)			
		Endocarp		Mesocarp	
		E–S1	S1–S2	E–S1	S1–S2
Cell wall precursor synthesis					
ppa004903m	UDP-N-acetylglucosamine pyrophosphorylase-related AT1G31070	8.53			11.07
ppa004485m	ADP-glucose pyrophosphorylase family protein AT1G74910	11.65			
ppa007618m	Mannose-1-phosphate guanylyltransferaseAT2G39770	10.98			
ppa008032m	UDP-D-glucose/UDP-D-galactose 4-epimerase 5 AT4G10960		−10.50		
ppa006917m	UDP-glucuronate decarboxylase AT1G08200	1.05			
ppa005045m	UDP-glucose 6-dehydrogenase AT5G15490		−1.33		
ppa004626m	UDP-glucuronate decarboxylase 1 AT3G62830	8.33	4.10		
ppa005905m	UDP-GLUCURONIC ACID DECARBOXYLASE 1 AT3G53520		11.00		
ppa007623m	GHMP kinase family protein AT3G01640	10.70			
Cell wall proteins: AGP, LRR and RGP					
ppa007675m	Fasciclin-like arabinogalactan 2 AT4G12730	12.40		10.83	1.05
ppa023463m	Fasciclin-like arabinogalactan protein 8 precursor AT3G46550	11.40		8.34	4.18
ppa006726m	Fasciclin-like arabinogalactan protein 8 precursor AT5G55730	−1.91			
ppa010321m	Fasciclin-like arabinogalactan protein 7 precursor AT2G04780	11.22			
ppa023379m	Fasciclin-like arabinogalactan protein 7 precursor AT5G60490		12.23		
ppa006630m	Leucine-rich repeat family protein/extensin family protein AT4G29240	11.08		2.10	
ppa024854m	Leucine-rich repeat family protein/extensin family protein AT3G22800		−11.14	1.55	−12.53
ppa000722m	Leucine-rich repeat family protein/extensin family protein AT2G15880			−10.30	
ppa004109m	Leucine-rich repeat family protein AT1G49750			10.95	
ppa023082m	Leucine-rich repeat family protein /Receptor-like protein kinase AT4G06744				11.69
ppa014775m	Leucine-rich repeat family protein Receptor-like protein kinase AT1G49750				−12.23
ppa007588m	Alpha-1,4-glucan-protein synthase AT3G08900		1.23		
ppa007760m	Alpha-1,4-glucan-protein synthase AT3G08900			9.92	
Cell wall degradation					
ppa002559m	Glycosyl hydrolase family 3 protein AT5G04885	−3.12		1.17	
ppa015037m	Beta-D-xylosidase AT3G19620	12.51		12.83	
ppa005849m	(1-4)-beta-mannan endohydrolase AT5G01930	12.50	−12.50		
ppa001692m	Xylan 1,4-beta-xylosidase AT5G64570		−1.37		
ppa001675m	Beta-D-xylosidase AT1G78060		−11.70		−9.38
ppa001583m	Beta-D-xylosidase AT5G10560			−1.67	
ppa004996m	Polygalacturonase AT4G23500		−10.66		
ppa005599m	Polygalacturonase (pectinase) AT5G49215		−11.93		
ppa004793m	Polygalacturonase (pectinase) AT1G19170		11.88		
ppa005960m	Polygalacturonase (pectinase) AT3G57790				−2.16
ppa018224m	Polygalacturonase (pectinase) AT3G6149				−10.23
ppa005535m	Protein dehydration-induced protein RD22-like protein 2 AT5G25610				−12.37
ppa004101m	Protein dehydration-induced protein RD22-like protein 2 AT5G25610				−12.15
Cell wall modification					
ppa010171m	Expansin-like A1 precursor AT3G45970	10.49		11.25	−11.25
ppa009472m	Xyloglucan endotransglycosylase AT5G65730	10.52		10.97	
ppa009610m	Xyloglucan endotransglucosylase 6 AT4G25810			9.63	
ppa009387m	Endoxyloglucan transferase A4 AT5G13870			−10.93	
Cell wall pectin esterases					
ppa003697m	Pectinesterase-3 precursor AT1G11580	12.87	−12.87		
ppa004300m	Pectinesterase PPE8B precursor AT4G33220			−1.42	
ppa003307m	Pectinesterase 1 AT1G53840	12.13	−12.13		
ppa006668m	Pectinacetylesterase AT4G19420	12.54	−12.86	10.32	−10.32
ppa006718m	Pectinacetylesterase AT4G19420	1.58		1.24	−1.59

The fold change in a log2 is presented. A positive FC in E–S1 and S1–S2 comparisons indicates an increase in the amount of protein in S1 and S2, respectively. Conversely, a negative value indicates a decrease. Prunus persica database (Phytozome) accession numbers are indicated. In addition, the Arabidopsis thaliana accession number is also provided with protein description. A blank indicates that there is no change registered in the protein in a specified comparison (the protein may remained unchanged or it could be undetected in both of the stages that are compared).

upregulation of enzymes involved in cell wall modification (expansin, xyloglucan endotransglycosilases, and endoxyloglucan transferase), remodelation (pectinesterases), and degradation, as well as the incorporation of proteins targeted to the cell wall (fasciclin-like arabinogalactans and leucine-rich repeat family proteins/extensins) is observed in the endocarp and in the mesocarp. In contrast, when comparing S2 with respect to S1 a decrease in proteins under the mentioned categories is observed, denoting a cease in cell wall expansion at stage 2 (**Table 1**).

Finally, PAS staining was conducted to reveal the presence of starch granules in plastids over development. While abundant starch grains were observed in mesocarp at E and S1, barely a few granules were observed at S2 (**Supplementary Figures 9C,D**). In contrast, granules were not observed in endocarp at S1 and S2 (**Supplementary Figures 9A,B**) but they were observed in E.

DISCUSSION

Overall Endocarp and Mesocarp Proteome Reconfiguration During Peach Fruit Development

Peach fruit development shows a double sigmoidal curve (**Supplementary Figure 1**, Tonutti et al., 1997), with four phases (S1–S4). The first stage (S1) is characterized by an exponential growth of the fruit, as accounted by increases in fruit size and weight (**Supplementary Figures 1A,B** and **Figure 1B**) and lasting until 45 DAF. This phase has an initial lag period, which is named here as E. Results obtained show significant differences between E and S1, which include chlorophyll levels (**Figure 1**), protein content and profiling (**Figures 1**, **2**, **4**, **7**), amino acid content and profiling (**Figure 6**) and starch content (**Supplementary Figure 9**); and thus, confirm the importance of the fractionated exploration of the E period. Another particular feature that our work includes is the dissection of the endocarp from the mesocarp during very early fruit development. In this sense, this proteomic approach reveals the uniqueness of the proteome of peach fruit at a stage very early after pollination (stage E), especially that of the endocarp (Ee), distantly in PCA plots from the proteomes of other stages and tissues (**Supplementary Figure 2**) and with numerous proteins occurring in a minor magnitude than in Em (**Figure 5**).

In addition, the proteome of the endocarp undergoes more pronounced remodeling over the development than that of the mesocarp; as it is shown by the closer association of mesocarpic samples (Em, S1m, and S2m) than the endocarpic profiles (Ee, S1e, and S2e) (**Supplementary Figure 2B**). Therefore, besides the mesocarp exhibits a decrease in total proteins of higher magnitude than that of the endocarp over early development, the changes in the protein profiling are less severe (**Figure 1C**). Moreover, despite the decrease in the net amount of proteins, the number of individually proteins identified tended to increase in the endocarp revealing changes in both protein quantity and quality (**Supplementary Table 2**). Previous high-throughput transcriptomic studies identified main genes and signaling pathways that regulate endocarp and mesocarp differentiation (Dardick and Callahan, 2014). Nevertheless, these studies are not enough to predict the resultant protein occurrences, since protein levels are also controlled by other mechanisms in addition to transcript levels. Thus, the work presented here represents the first approach in building protein databases during peach fruit development focusing on endocarp and mesocarp tissues.

A Decrease in the Protein Synthesis Machinery During Early Development Conducts to a Fall in the Protein Content

In agreement with previous works the presence of lignin is observed in endocarp at S2 (**Figure 1C**; Dardick et al., 2010). This process is accompanied by an important number of PDA involved in secondary metabolism being present in the endocarp during the transition from S1 to S2 (**Figure 2B** and **Supplementary Table 3**) and in cellulose deposition (**Figure 8**). Lignin synthesis is a costly process, which has a great demand on reductive power and hydroxycinnamyl alcohols (or monolignols). Monolignols derive from the phenylpropanoid pathway that uses Phe as substrate (Vanholme et al., 2010). Thus, during very early peach development not only growth but also stone formation have a great demand on substrates. In the past few years, significant progress has been made in understanding seed development (Bonghi et al., 2011), stone formation (Dardick et al., 2010; Hu et al., 2011) and the pericarp growth (Bonghi et al., 2011; Lombardo et al., 2011). Proteins initially accumulated in peach have been proposed as a resource for lignification (Lombardo et al., 2011). Here, we have shown that the great decrease in protein content occurs not only in the endocarp but also in the mesocarp, with a different extent of variation (**Figure 1C**).

To get insight into the nature of total protein change over development (**Figure 1C**) we conducted quantitative proteomics. Protein metabolism was the category most represented among PDA in all tissues and stages analyzed (**Figure 2**). A repression in the ribosomal proteins reveals that a decrease in protein synthesis is a key component in the fall of total proteins during early development (**Figure 4B**), especially in the mesocarp during the Em to S1m transition (**Figure 1C**), where there is also a decrease in initiation and elongation factors involved in protein synthesis. Thus, given the contribution of ribosomes to cell weight, the decrease in the ribosomal proteins *per se* may significant contribute to the net drop in total proteins during the transition from E to S1. Besides, at this stage there is a reduction in the components of the ubiquitin-proteasome system indicating that proteins would not increase their decay through this pathway and would instead occur through other proteases (**Figure 4C**). In relation, the amount of amino acids does not increase in the E to S1 transition; suggesting that the protein mobilization to

render amino acids as source of respiratory substrates (Araújo et al., 2011) is unlikely here. Considering that lignin synthesis starts very early during peach fruit development (Dardick et al., 2010; **Figure 1A**), it seems that protein synthesis would be reduced at this stage with the aim to reroute the resources to lignin biosynthesis. Similarly, in Arabidopsis the demands on basic metabolism and energy of the protein synthesis and degradation directly influences the cell growth (Piques et al., 2009).

Protein homeostasis depends on process of protein synthesis and degradation; and protein degradation plays a key role in plant growth, development and death (Palma et al., 2002). In contrast, our results show that in peach fruit during very early development the protein turnover could instead be more exerted at protein synthesis level. In this sense, ribosome modifications were found during development of Arabidopsis and bean leaves (Makrides and Goldthwaite, 1981; Schippers and Mueller-Roeber, 2010).

Following the E stage, in the S1–S2 transition, the system of ubiquitin-proteosome seems to be activated, as accounted by increases in the abundance of members of the proteasome 26S and ubiquitination process (**Figure 4C**) and in the increase in the content of total amino acids (**Figure 6A**). Nevertheless, the amino acid profiling shows that in each tissue the amino acid distribution at S1 and S2 are quite similar (**Figures 6A,B**), with the net increase in amino acid content mainly at expenses of increases in Asn (**Figure 6C**), ruling out the hypothesis of an increase of amino acids due to massive proteolysis; and, instead, more linked to Asn metabolism. In this sense, the occurrence and amount of enzymes involved in Asn metabolism reveals that the level of this amino acid, on one hand, is in parallel with the amount of β-CAS and β-cyanoalanine hydratase involved in Asn biosynthesis (**Figure 7**). On the other hand, the relative amounts of two Asparaginases (M5WUV5 and M5X0K4) are opposite to that of the amino acid (**Figure 7**). Thus, increased synthesis and decreased catabolism of Asn may conduct to an increase in this amino acid during very early development of peach fruit. In addition, the lack of detection of any asparagine synthase within the proteome (**Supplementary Table 2**), also support the hypothesis that the β-CAS-β-Cyano-Alanine hydratase could be a pathway for Asn synthesis in "Dixiland" peach fruit.

β-CAS is a key enzyme in cyanide detoxification (Blumenthal et al., 1968) and also the first step toward the synthesis of L-Asn in many species in the reaction catalyzed by the β-Cyano-Alanine hydratase (Castric et al., 1972; Machingura et al., 2016). The activity of these enzymes are present in fruit and flowers and increases during maturing process (Machingura et al., 2016). Hu et al. (2011) detected β-CAS in peach between 28 and 59 DAF. In agreement with our results, the levels of the protein increased in mesocarp during the S1–S2 transition. In endocarp, they detected a decrease from S1 to S2, while we sensed that decreased in the E to S1 transition. Asn together with Ala/Tyr, Asp, Gln, Glu, and γ-amino-butyrate are the amino acids transported at higher concentration by Prunus phloem (Douglas, 1993). Taken together, Asn synthesized in both mesocarp and endocarp could contribute to the Asn pool, which is also fed by import from the phloem. Further biochemical characterization of the enzymes of the β-CAS pathway in peach fruit is needed to reveal the importance of this pathway for fruit development. Considering the results presented here, together with previous work (Lombardo et al., 2011), we propose that Asn accumulated during very early peach development is further metabolized by Asparaginase during late development and ripening to provide skeletons for organic acids accumulation in the mesocarp.

Photosynthetic Machinery Decays During Early Development in Both Mesocarp and Endocarp

It is generally accepted that sink organs as fruit and root rely on photosynthetic organs (mainly leaves) to growth and develop (Cocaliadis et al., 2014). Sugars and sugar alcohols such as sucrose and sorbitol are the main photosynthates imported to peach fruit from the phloem (Moing et al., 1997; Lombardo et al., 2011) which are further metabolized to render hexoses. In peach, fructose, glucose, sorbitol and sucrose increase as the fruit develops, mainly after S3 (Lombardo et al., 2011). In agreement, the activity of invertases and sorbitol dehydrogenase also increase over the development of peach fruit (Lombardo et al., 2011).

The occurrence of photosynthesis in fruit has been largely explored in tomato, including different aspects such as chloroplast to chromoplast conversion, the regulation of the expression of the photosynthetic components and the importance of photosynthesis during very early development (reviewed in Cocaliadis et al., 2014). The contribution of photosynthesis to total carbon of tomato fruit has been estimated to be up to 20% (Hetherington et al., 1998), but argued by others (Carrara et al., 2001). In comparison, our knowledge on the occurrence of photosynthesis in peach fruit is null. Proteins involved in light-harvesting complexes, electron transfer, Calvin cycle, photorespiration reactions and chlorophyll synthesis have been detected in peach fruit here by a massive proteomic approach (**Supplementary Tables 2**, **3**). Chlorophylls have been detected as well (**Figure 1D**). On one hand, the higher levels of these proteins and chlorophylls in the mesocarp compared with the endocarp (**Supplementary Figure 3** and **Figure 1D**) are in agreement with the outer location of the mesocarp and thus, its closer proximity to the light, suggesting that the system would probably be operating at least in the light capture phase. In addition, the presence of abundant starch grains in mesocarp (**Supplementary Figure 9**), although not necessary indicates that the carbon derives from carbon fixation, it shows enough carbon resources to be stored. During the transition from E to S1, and in concert with the fall in chlorophylls in the mesocarp, there is a decrease in the PSII light harvesting subunits, in Rubisco Small subunit and in other Calvin cycle enzymes (**Supplementary Table 3**). Nevertheless, abundant starch granules are still observed (**Supplementary Figure 9**). Further,

in the transition from S1 to S2, an increase in the PSII light harvesting and polypeptide subunits, a decrease in key Calvin Cycle enzymes (sedoheptulose-bisphosphatase and phosphoglycerate kinase) and in starch, together with an increase in 6-phosphogluconate dehydrogenase oxidative pentose pathway, denote a demand on reductive power at stage S2 rather than on carbon fixation. In this line, at stage S2 the fruit almost does not increase in size. Taken together, these results suggest that mesocarp photosynthesis is possible to occur very early on the development, first providing both carbon and reductive power and latter only reductive power.

In the endocarp, the panorama is less clear at E and S1 as there are lower levels of chlorophyll and of proteins involved in photosynthesis compared with the mesocarp, in addition to the internal location in the pericarp (**Figure 1D** and **Supplementary Table 3**). In the transition from S1 to S2, PSII LHC and polypeptides, electron carriers, Rubisco small subunit and other Calvin enzymes decrease (**Supplementary Table 3**). Considering that at S2 the endocarp starts the lignification process it is highly probable that photosynthesis has no role at all. However, in tomato, it has been pointed out that fruit photosynthesis is critical for accurately timed seed development (Lytovchenko et al., 2011). Similarly, in endocarp of peach fruit photosynthesis may have a role in the seed development.

Cell Wall Modifications Over the Early Development of Peach Fruit: Identification of Key Proteins Involved

The cell wall is an essential plant structure involved in numerous important developmental processes, like growth and cell division and fruit ripening (Cosgrove, 2005). Several studies have been undertaken to elucidate the cell wall changes during peach fruit ripening and softening (Brummell et al., 2004) and on how alterations in the cell wall structure affect the shelf life (Brummell and Harpster, 2001). It is widely documented that a solubilization or depolymerization of pectin and matrix glycans of the cell wall by the action of exo- and endo-polygalacturonases, endo-β-1,4-mannanase, α-L-arabinofuranosidase and β-galactosidase goes with the process of softening (Callahan et al., 1992; Trainotti et al., 2003; Brummell et al., 2004; Bustamante et al., 2012; Genero et al., 2016). On the contrary, a comprehensive research of the cell wall biosynthetic enzymes and proteins occurrence during peach development is still missing.

During very early peach development, the fruit undergo a burst of cell division and elongation at E and S1, with a pause in S2, as accounted by modifications in the fruit volume, cell size and cell wall width and cellulose and aniline blue staining (**Figures 8**, **9** and **Supplementary Figures 1B**, **6**). In addition, a decrease in the level of proteins involved in cell division is detected in endocarp and mesocarp at S2 (ppa005822m, ppa009766m, ppa015773m; **Supplementary Table 3**). Cell expansion involves modifications in cell wall structure. The increases in XETs, FLAs and leucine-rich repeat family proteins/extensins (LRX) in the endocarp and in the mesocarp in the transition from E to S1 (**Table 1**) are in agreement with modifications in cell wall. Through cell enlargement and elongation, the structure of the cell wall is relaxed and then strengthened. In peach, a decrease in cellulose deposition is observed at S1, in agreement with an increase in cellulase activity at this stage described earlier by Bonghi et al. (1998). XETs modulate cell wall strength, flexibility and porosity, and cell expansion by linking xyloglucans with cellulose, and xyloglucans with (1,3; 1,4)-β-D-glucans (Eckardt, 2004; Nishikubo et al., 2011). LRXs are also cell wall-localized proteins involved in the regulation of plant growth (Draeger et al., 2015). In addition, FLAs are a subfamily of arabinogalactan proteins that participate in cell expansion and adhesion (Johnson et al., 2003). Thus, in "Dixiland" peach fruit, these cell wall proteins might have a key participation in the fast growth of the very early stage as reported for XETs and LRX in watermelon (Guo et al., 2011). Other proteins involved in the synthesis of cell wall precursors were also identified as highly induced in the transition from E to S1 in the endocarp (**Table 1** and **Supplementary Table 3**), like mannose-1-phosphate guanylyltransferase that provides GDP-mannose that is used to add mannose residues to cell wall molecules and as well as a precursor of GDP-fucose for the addition of fucose residues in the cell wall (Lukowitz et al., 2001). Later, in the transition from S1 to S2, and in agreement with the cease in growth of peach fruit at S2, a decrease in the levels of many isoforms of Polygalacturonase and Beta-D-xylosidase, key enzymes during fruit ripening, in both endocarp and mesocarp is observed (**Table 1**). In agreement, the decrease in the transcript encoding a Beta-D-xylosidase was observed in the transition from S1 to S2 in peach fruit (Di Santo et al., 2009).

Proteins Involved in Signaling and RNA Metabolism Vary Spatial and Temporally During Very Early of Peach Development

Processes of cell division and expansion, and tissue differentiation require tight regulation both at the level of gene activity and translation. These events are, in addition, coupled to phytohormone levels. Changes in the levels of auxins, gibberellins and cytokinins are key signals during early fruit development (Bonghi et al., 2011). Here, we have shown that changes in protein metabolism are key to fruit development. In addition, as it is show in **Figures 2**, **5**, the functional categories signaling and RNA metabolism are well represented among proteins changing across development in both mesocarp and endocarp, and also between endocarp and mesocarp. In consequence, it is not surprising the observation of variable proteins involved in RNA processing, RNA binding, regulation of transcription, calcium signaling, or participating in the signaling mediated by protein G, receptor kinases, phosphoinositides, MAP kinases and 14-3-3 proteins (**Supplementary Table 3**) during fruit development. Future studies could get more insight into these responses.

CONCLUSION

Fruit yield relies on a set of developmental processes, which include flower initiation and differentiation, fertilization, fruit set and development (Hanke et al., 2007). Each aspect may limit fruit production. Peach is an important fruit crop and have been turned into a valuable model, together with tomato, for the research of climacteric fruits. Here we got insight into the early stages of fruit development with distinction of the events in endocarp and mesocarp. In this respect, we provide valuable information on the nature and abundance of the proteins present in these tissues very early after pollination. This information, coupled with profiles of metabolites and transcripts available, provides novel insights into the biology of peach fruit development preceding pit hardening.

AUTHOR CONTRIBUTIONS

CR, CAB, COB, and GM conducted the experiments. CAB, MD, and ML conceived the project. MD and ML wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00715/full#supplementary-material

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REVIEW
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Primary Metabolism in Avocado Fruit

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Avocado (*Persea americana* Mill) is rich in a variety of essential nutrients and phytochemicals; thus, consumption has drastically increased in the last 10 years. Avocado unlike other fruit is characterized by oil accumulation during growth and development and presents a unique carbohydrate pattern. There are few previous and current studies related to primary metabolism. The fruit is also quite unique since it contains large amounts of C_7 sugars (mannoheptulose and perseitol) acting as transportable and storage sugars and as potential regulators of fruit ripening. These C_7 sugars play a central role during fruit growth and development, but still confirmation is needed regarding the biosynthetic routes and the physiological function during growth and development of avocado fruit. Relatively recent transcriptome studies on avocado mesocarp during development and ripening have revealed that most of the oil is synthesized during early stages of development and that oil synthesis is halted when the fruit is harvested (pre-climacteric stage). Most of the oil is accumulated in the form of triacylglycerol (TAG) representing 60–70% in dry basis of the mesocarp tissue. During early stages of fruit development, high expression of transcripts related to fatty acid and TAG biosynthesis has been reported and downregulation of same genes in more advanced stages but without cessation of the process until harvest. The increased expression of fatty acid key genes and regulators such as *PaWRI1*, *PaACP4-2,* and *PapPK-β-1* has also been reported to be consistent with the total fatty acid increase and fatty acid composition during avocado fruit development. During postharvest, there is minimal change in the fatty acid composition of the fruit. Almost inexistent information regarding the role of organic acid and amino acid metabolism during growth, development, and ripening of avocado is available. Cell wall metabolism understanding in avocado, even though crucial in terms of fruit quality, still presents severe gaps regarding the interactions between cell wall remodeling, fruit development, and postharvest modifications.

Keywords: *Persea americana*, mannoheptulose, perseitol, oil, fatty acids, amino acids, cell wall

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INTRODUCTION

Avocado (*Persea americana* Mill) is a rich oil fruit of high economic importance in the international trade. Its nutritional value has been lately highlighted: it is a rich source of monounsaturated and polyunsaturated fatty acids associated with a decreased risk of cardiovascular diseases (Mendez and Hernandez, 2007; Rodriguez-Sanchez et al., 2015). It is also an important source

of vitamins A, B, and C and minerals such as potassium, phosphorus, magnesium, iron, and a rich source of fiber and antioxidants (Villa-Rodriguez et al., 2011; Dreher and Davenport, 2013; Bill et al., 2014).

Persea americana Mill is quite unique not only from a compositional point of view but also shows a very peculiar and complex physiology compared to other fruit. For example, flowering period can last up to 3 months; thus, a broad range of fruit physiological ages can be on the same tree, which will be evident during postharvest storage and management (Lewis, 1978). Fruit set in addition is extremely low (less than 0.1%). Fruit can hang on the tree for more than 12 months, time far beyond needed to reach physiological maturity to be able to ripen when detached (Hernández et al., 2016).

What turns avocados unique compared to other fruit is the presence of C_7 sugars (e.g., mannoheptulose and perseitol) instead of C_6 sugars as main phloem transported sugars and as respiratory substrates (Liu et al., 1999b, 2002; Bertling and Bower, 2005). These C_7 sugars have been reported as the "tree factor" that inhibits the ripening process of the fruit on the tree (Liu et al., 1999b, 2002; Bertling and Bower, 2005; Landahl et al., 2009; Blakey et al., 2010) and possibly associated with the differences in ripening speed of fruit postharvest (Landahl et al., 2009).

Primary metabolism in avocado, even though an essential component of growth and major component of fruit quality, has been mainly focused on carbon and oil metabolism and to a much lesser extend to amino acids and organic acids. In the decades of the 1960s on, several studies on the role of C_7 sugars and starch during growth and development of avocado were reported (Bean et al., 1962; Liu et al., 1999a,b; Richings et al., 2000; Cowan, 2004, 2017; Tesfay et al., 2010). Unlike other fruit, avocado accumulates oil instead of sugars, thus oil metabolism during growth, development, and ripening has been of high interest (Salas et al., 2000; Ozdemir et al., 2004; Blakey et al., 2012; Ibarra-Laclette et al., 2015). With the advance in post-genomics tools (e.g., transcriptomics, proteomics, and metabolomics) more integrative studies related to carbohydrate and oil fruit metabolism during growth, development and ripening in avocado have been reported (Hurtado-Fernández et al., 2011, 2015a; Pedreschi et al., 2014; Ibarra-Laclette et al., 2015; Kilaru et al., 2015; Fuentealba et al., 2017; Rodríguez-López et al., 2017) but almost inexistent literature related to organic acid and amino acid metabolism.

Primary metabolism in avocado and other oily fruit undergoes large changes during growth, development, and ripening; thus, the reaction networks or metabolic pathways involved are quite similar, still the set of regulations that directly affect the metabolic fluxes to different destinations remains largely unknown (Beauvoit et al., 2018). Thus, in this review, we aim to: (1) delve into the importance of primary metabolism on quality impact for avocado and (2) provide a deep review of avocado fruit primary metabolism (cell wall, carbohydrates, oil, amino acids, and organic acids) with a main emphasis on studies on carbohydrate and oil metabolism.

Avocado Origin, Botany, and Commercial Importance

Avocado (*Persea americana* Mill) belongs to the Lauraceae family composed of 50 genera and approximately 2,500–3,000 species (Rohwer, 1993; Chanderbali et al., 2008). Three well recognized horticultural races exist: (1) the Mexican race (*P. americana* var. *drymifolia*) adapted to the tropical highlands; (2) the Guatemalan race (*P. americana* var. guatemalensis, L.O. Williams) adapted preferentially to medium elevations in the tropics; and (3) the West-Indian race (*P. americana* var. *americana*) cultivated in the lowland humid tropics (Chanderbali et al., 2008; Ibarra-Laclette et al., 2015).

Avocado (*Persea americana* Mill) is cultivated throughout the tropical and subtropical regions of the world. A total of 71.9% of the world production come from Americas and Mexico is currently by far the top producer in the world accounting for about 28% of the world production (FAOSTAT, 2018). Commercial avocado production is based on grafting cultivars onto rootstocks mainly of the Mexican and Guatemalan races. In subtropical climates, the Guatemalan genotypes represent the dominant horticultural race (Ibarra-Laclette et al., 2015). *Persea americana* Mill cv. Hass is the most important avocado cultivar worldwide representing 95% of the total volume commercialized. Its international trade has doubled in the span of 6 years (FruitTrop, 2015).

Avocado Fruit Development: Special Features of *Persea americana*

Avocado fruit follows a single sigmoid curve. Independent of the cultivar (early or late maturing cultivars), the early period of growth is characterized by a rapid cell division (Barmore, 1977). Cell division in the fruit mesocarp goes beyond the initial period of growth but continues during fruit development and continues even in the mature fruit attached to the tree (Van den Dool and Wolstenholme, 1983; Bower and Cutting, 1988). Avocado is a climacteric fruit characterized by an increase in the respiration rate and ethylene production at the onset of ripening. Compared with other climacteric fruits, avocado produces high amounts of ethylene (80–100 $\mu l\ kg^{-1}\ h^{-1}$ at 20°C) at the climacteric peak (Seymour and Tucker, 1993). Fruit does not ripen on the tree, which has been previously attributed to a possible role of C_7 sugars (mannoheptulose and perseitol) acting as inhibitors and controlling the ripening process (Liu et al., 1999b, 2002; Landahl et al., 2009) and to the low levels of ACC (1-aminocyclopropane-1-carboxylic acid) of fruit attached to the tree (Sitrit et al., 1986), but other studies indicate that additional metabolites might be involved (Pedreschi et al., 2014; Fuentealba et al., 2017).

Avocado fruit development period is quite extensive, between 6 and >12 months from flowering to maturity (Scora et al., 2002). Unlike other fruits that accumulate carbohydrates during growth and development, avocado accumulates mostly oil. Avocado mesocarp fruit starts oil accumulation a few weeks after the fruit sets and oil accumulation continues during growth and development until maturation (Ozdemir et al., 2004).

Based on dry weight, approximately, the mesocarp accumulates 60–70% oil and 10% carbohydrates. Oil is stored in the form of triacylglycerol (TAG) and mainly composed of oleic acid (Ozdemir et al., 2004); however, the fatty acid profile of the oil can be largely affected by environmental and geographical conditions (Donetti and Terry, 2014; Pedreschi et al., 2016). Sixty percent of the total carbohydrates, as previously mentioned, is composed of C_7 sugars (mannoheptulose and perseitol) (Liu et al., 2002).

General Overview on Primary Metabolism: Primary Pathways and Their Relationship With Fruit Quality

The most integrative studies on primary metabolism comprising information at different levels of cellular control (e.g., genes, proteins and metabolites) orchestrated by hormones have been carried out with tomato as considered as "model fruit." Major carbon source for fruit is usually sucrose, which is imported from the leaves to the phloem; however, avocado is unusual in which high contents of C_7 sugars (mannoheptulose and perseitol) are present. Fruit growth and ripening are based on central carbon metabolism, which provides the energy and biosynthetic precursors. These pathways involve sucrose, starch, major organic acids, respiration (Beauvoit et al., 2018), and fatty acid metabolism for a rich fat fruit such as avocado. Unlike other fruit in which the balance of sugars and organic acids is determinant of fruit quality, for avocado, the content of oil and profile of fatty acids is determinant of fruit quality.

Amino acid metabolism is important not only for providing the building blocks for protein synthesis but also for the synthesis of other metabolites (Gonda et al., 2010). Even though amino acids and derivatives can influence fruit quality and taste, very few studies have been carried out with avocado fruit regarding the behavior of different amino acids during fruit growth, development, and ripening.

Firmness is a very important quality parameter of avocado and as such is highly influenced by the composition and architecture of the cell wall. During growth, the cell wall protects and shapes the fruit, and then during ripening its disassembly has strong implications in quality perception and shelf-life. It has been reported that partial cell wall degradation at the ripe stage can provide sugars (e.g., uronic acids), which are phosphorylated and recycled *via* the UDP-sugar pyrophosphorylase, thus providing building blocks for other biosynthetic processes (e.g., protein synthesis, sugar accumulation) (Geserick and Tenhaken, 2013; Beauvoit et al., 2018).

Hormones are crucial during fruit growth and development. Cytokinins and gibberellins are involved in early events after pollination. Fruit growth and development are controlled by coordination of different plant hormones. Up to date, except for a recent study on tomato (Li et al., 2019), information is very limited related to the link between hormonal signals and primary metabolite changes during fruit development and ripening. Auxins and gibberellins promote cell division and expansion, thus influence fruit size. At fruit set, auxins and gibberellins impact the genes involved in primary metabolism (Ruiu et al., 2015). Ethylene and abscisic acid become important for fruit maturation since they tightly control fruit ripening in climacteric fruit. The role of these hormones in avocado ripening has been extensively reported.

CARBOHYDRATE METABOLISM DURING AVOCADO FRUIT DEVELOPMENT AND RIPENING

Monosaccharides play a key role in primary metabolism by linking energy derivation by photosynthesis to the demands of anabolic and catabolic processes. Monosaccharides are the substrates for sucrose biosynthesis, the major transportable sugar in most vascular plants (Cowan, 2017). However, avocado is quite unique, because C_7 sugars (mannoheptulose and perseitol) have been reported as the predominant transportable and storage sugars in leaves and fruit of avocado. Still, despite the importance of these C_7 sugars, there is only sparse information about the synthesis, metabolism, transport, and physiology of C_7 sugars not only in avocado but also in other species where they occur (Cowan, 2004, 2017). Roles of mannoheptulose and other C_7 sugars in the regulation of carbon flux and protection against oxidative damage are based on published studies (Cowan, 2004, 2017).

C_7 Sugars Occurrence, Biosynthesis, Transport and Compartmentalization in Avocado

Mannoheptulose from avocado fruit was first isolated and identified by LaForge (1916). Mannoheptulose and perseitol have been reported as the major soluble sugars in the phloem sap, leaf petiole exudates, seed, and mesocarp of avocado (Cowan, 2004). Based on perseitol concentrations of 2.5-fold higher in the sap from the trunk of the avocado tree than the sap from the petioles, different sites of synthesis for D-mannoheptulose and perseitol are assumed (Cowan, 2004). A possible route for D-mannoheptulose biosynthesis in avocado fruit involving D-arabinose-5-phosphate ↔ D-ribose-5-phosphate being both substrates converted to hexose 6-phosphate and triose phosphates *via* intermediates that include D-mannoheptulose 7-phosphate and D-altro-heptulose 1,7-bisphosphate has been postulated by Cowan (2004). Similarly, to the pathway of sedoheptulose biosynthesis, D-arabinose-5-phosphate and D-ribose-5-phosphate are then the substrates for transketolase activity and formation of D-manno-heptulose-7-phosphate and 1,7 biphosphate. Arabinose has been reported in substantial amounts in avocado (Jeong and Huber, 2004). By phosphate activity then, D-mannoheptulose would be formed. Cowan (2004) sustains that D-mannoheptulose is a potent inhibitor of respiration and prevents entry of glucose into glycolysis acting as a competitive inhibitor of hexokinase, thus altering the fate of sugar metabolism. Thus, to sustain fruit growth and

development, it becomes crucial the effects described above of D-mannoheptulose in order to continue sugar export and phloem loading.

In avocado leaves, Liu et al. (1999a, 2002) reported D-mannoheptulose and perseitol as products of CO_2 assimilation. D-mannoheptulose is formed in leaves as a phosphorylated product of sedoheptulose 1,7 biphosphate *via* condensation of dihydroxyacetone phosphate and erythrose-4-phosphate (Liu et al., 1999a, 2002). Previously, Häfliger et al. (1999) mentioned that C_7 sugars can be formed by three well-known enzymatic reactions that form C_7 intermediates: (1) erythrose-4-P + dihydroxyacetone-P sedoheptulose 1,7-bis P corresponding to an aldose reaction; (2) xylulose-5-P – ribose-5-P sedoheptulose-7-P-glyceraldehyde-3-P corresponding to a transketolase reaction; and (3) a transaldolase reaction – fructose-6-P + erythrose-4-P sedoheptulose-7-P + glyceraldehyde 3-P. All three pathways require NADPH/$NADP^+$. Following, Tesfay et al. (2012) reported conversion of D-mannoheptulose to perseitol and vice versa in dry seed, cotyledons, and mesocarp of avocado together with detection of aldolase enzymes possibly a transaldolase present in different avocado tissue types and potentially involved in the conversion of the C_7 sugar storage form (perseitol) into the C_7 sugar transport form D-mannoheptulose. But still, as reported by Cowan (2017), the origin of sedoheptulose 7-P and mannoheptulose 7-P as precursors of heptoses and heptitols in plants remains to be elucidated. Both sedoheptulose and mannoheptulose together with sucrose are products of the reductive pentose phosphate pathway in mature source leaves being C_7 sugar formation stimulated during photosynthesis having as evidence that mature leaves of avocado incorporated CO_2 into mannoheptulose-7-P, mannoheptulose, sedoheptulose 7-P, sucrose, and perseitol (Cowan, 2017).

Regarding C_7 sugars transport in avocado trees, previous studies carried out with sap collected from the petioles of mature leaves and sap collected from girdled branches (Liu et al., 1999a,b, 2002; Tesfay et al., 2012) appear to indicate that mannoheptulose and perseitol are the most important and major C_7 mobile sugars in the tree and readily available for growth and development. Different studies suggest that mannoheptulose is a precursor of perseitol being perseitol conversion independent of photosynthesis (Cowan, 2004, 2017). From studies not only on avocado but also on other C_7 containing species, Cowan (2017) proposed a C_7 biosynthesis pathway. C_7 sugars seem to arrive from CO_2 in photosynthetic organisms, and there is evidence supporting that sedoheptulose 7-P and mannoheptulose 7-P are precursors of sedoheptulose and mannoheptulose; however, the origin of the phosphorylated precursors is not known. The potential routes include transaldolase catalyzed condensation of dihydroxyacetone from D-fructose-6-P with the aldose D-erythrose-4-P to form sedoheptulose 7-P and then a transketolase catalyzed transfer of an α-hydroxy-acetyl from D-fructose-6-P to the aldopentose D-arabinose-5-P for mannoheptulose 7-P formation (Cowan, 2017). Thus, the proposed hypothesis states that C_7 sugars originate from fructose-6-P generated from hydrolysis of sucrose *via* a transketolase-dependent-heptulose shunt. Relatively recently, Kilaru et al. (2015) reported a complete transcriptome analysis of developing avocado fruit and found complete glycolytic pathways in cytosol and plastids being sucrose synthase and invertases highly expressed at different fruit developmental stages indicating that sucrose synthase besides being high abundant in the cytosol might be the major actor in the generation of hexoses necessary later for pyruvate synthesis. These authors also reported high expression of orthologs of transketolases and transaldolases in fruit mesocarp plastids suggesting also a possibility for their synthesis in the mesocarp. So possibly, sucrose synthase and invertases provide the supply of fructose for C_7 sugar biosynthesis (Cowan, 2017).

Soluble Sugar Metabolism During Avocado Growth, Development, and Ripening

During early stages of growth and development, more than 40% of the mesocarp weight is composed of sugars. Sugars continue to increase and accompany the rapid fruit growth period and then start to decline concomitant with oil accumulation (Kilaru et al., 2015). Liu et al. (2002) reported high total sugar concentrations (up to 44%, 40 and 22% of total dry weight) in the flesh, seed, and peel of *Persea americana* cv. Hass after 2 months of fruit set. Five different soluble sugars were followed during growth and development: sucrose, fructose, glucose, mannoheptulose, and perseitol. These five sugars constituted 98% of the total soluble sugar content of the fruit (Liu et al., 2002). In general, sucrose levels were low in all tissues (seed, peel, and flesh) and did not fluctuate much. For very young fruitlets the predominant sugars in seeds, peel and flesh of avocado corresponded to glucose, fructose, D-mannoheptulose, and perseitol but as fruit rapid expansion phase culminated then decreased levels of fructose and glucose were reported (Liu et al., 2002). In general, the most abundant sugars in avocado corresponded to C_7 sugars (mannoheptulose and perseitol). During early stages of growth, mannoheptulose corresponded to 12, 19, and 12% of the total soluble sugars in the peel, flesh, and seed, respectively, and perseitol corresponded to 9, 15, and 10% of the total soluble sugars in the peel, flesh and seed, respectively (Liu et al., 2002). Once, surpassed the rapid expansion phase, then mannoheptulose concentrations decreased in seed and flesh but still remained high in the peel (Liu et al., 2002). However, Cowan (2004) reported higher contents of mannoheptulose in avocado cv. Hass mesocarp from 2 months after fruit set on (210 days after fruit set, 78 mg g^{-1}) and the contents of perseitol remained stable during early stages of growth and during development (around 55 mg g^{-1}). Perseitol instead decreased in the peel and flesh. Legal minimum maturity for Hass avocado in California corresponds to 20.8% (dry matter content) and as fruit matured, D-mannoheptulose decreased but perseitol varied very little. For starch, the peel and flesh of avocado accumulated very little content of starch and did not vary during early stages of development. Instead, for early stages of development the seed accumulated low amounts of starch (<1%) but as it started to increase its weight, starch content

in the seed corresponded to 30% of its dry weight (Liu et al., 2002). Cessation of sugar accumulation coincides with oil accumulation. Kilaru et al. (2015) state that the carbon available for oil biosynthesis in avocado fruit seems atypical. High expression of different genes associated with glycolysis as potential source of pyruvate for fatty acid synthesis was reported by Kilaru et al. (2015).

C_7 sugars have been postulated as the ripening inhibitors of *Persea americana* fruit, while attached to the tree (Liu et al., 1999a,b, 2002) based on findings that C_7 sugars are phloem mobile, second mannoheptulose is a very potent inhibitor of respiratory processes performing as an hexokinase inhibitor, thus impeding the input of glucose into glycolysis (Board et al., 1995; Liu et al., 2002) and finally based on the inhibition of the ripening process of the fruit until C_7 sugars are metabolized to below a certain level in the fruit (Liu et al., 2002; Landahl et al., 2009). C_7 sugars (mannoheptulose and perseitol) may control/trigger the ripening process (Landahl et al., 2009; Meyer and Terry, 2010; Blakey et al., 2012), but other studies indicate that additional players are involved (Pedreschi et al., 2014; Fuentealba et al., 2017). Fuentealba et al. (2017) reported higher expression of a chloroplastic transketolase enzyme in the mesocarp tissue of slow ripening Hass avocados. Previous works have reported that C_7 sugars together with sucrose and C_6 sugars are used during the ripening process (Liu et al., 1999b; Landahl et al., 2009). Liu et al. (2002) reported for mature but unripened fruit, perseitol as the main form of carbohydrate (~30 mg g^{-1} dry weight) and greatly exceeded the levels of sucrose, fructose, and glucose and starch, while mannoheptulose levels remained low during the ripening process. This ripening process was accompanied by increases in C_6 sugars, while contents of perseitol and starch substantially decreased (Liu et al., 2002). In fully ripe fruit, perseitol declined to levels below those of sucrose and hexoses (Liu et al., 2002). Similarly, after harvest and during ripening of avocado cv. Hass allowed to ripen at room temperature, Blakey et al. (2012) reported a decline in the mesocarp content of mannoheptulose and perseitol in fruit from three different locations and increases and decreases for glucose, fructose and sucrose during ripening for the fruit from these three different locations. The increase of free glucose during avocado fruit ripening was associated to the high amounts of cellulose produced by avocado fruit during ripening possibly stimulating the synthesis of abscisic acid and ripening. The low correlations found between C_7 sugar concentrations and respiration or total protein content were attributed to the fact that C_7 sugars are used in several cellular processes (Blakey et al., 2012).

Avocado cv. Hass dominates the international trade with more than 95% participation. Depending on the origin country and distance to the market, avocados are transported at low temperature and with controlled atmosphere conditions. Under these transport or storage conditions, the content and role of C_7 sugars and total soluble sugars of the fruit are key to reach such markets with excellent fruit quality. These C_7 sugars have also been reported to act as antioxidants in the mesocarp of avocado and thus its initial content at harvest correlated to the storability of the fruit conferring not only carbohydrates to sustain respiration but also stress-protection agents (Tesfay et al., 2010).

METABOLISM OF ORGANIC ACIDS DURING AVOCADO FRUIT GROWTH, DEVELOPMENT, AND RIPENING

Organic acids in fruit can act as intermediate in diverse metabolic pathways, as precursors for the synthesis of amino acids, plant hormones, fatty acids, secondary metabolites, and cell wall components (Walker and Famiani, 2018). The most known organic acids malic, citric, isocitric, galacturonic, oxalic, and tartaric are quite abundant in some fruit, and the phenolic acids and ascorbic acid are present in all fruit. In fruit in general, most of the malate and citrate of the pulp is localized in the vacuole, and most of the organic acids present in the pulp are not imported but synthesized in the pulp from transported sugars (Etienne et al., 2013; Famiani et al., 2015).

Not much work up to our knowledge has been reported regarding the most known organic acids evolution during growth, development, and ripening of *Persea americana* possibly due to its high oil content that significantly contributes to fruit quality and taste instead of other fruit in which organic acids such as malate, citrate, tartaric, etc. play a significant role in fruit quality and taste. Avocado is classified as a non-acid fruit and compared to other fruit, and it contains very low amounts of citric and malic acid being the tartaric acid the predominant organic acid (Duckworth, 1966; Ahmed et al., 2010; Viña et al., 2013; Defilippi et al., 2015). Defilippi et al. (2015) reported the organic acid profile of Hass avocado at harvest and during ripening at 20°C up to 15 days. Total acids (sum of tartaric, malic, citric and ascorbic) tended to decrease as ripening advanced and correlated to the decrease of titrable acidity during this period. This observed decrease was mainly governed by the drastic decrease of malic acid. Tartaric acid remained constant during this period and the contribution of ascorbic acid and citric acid was low and remained relatively constant during this period (Defilippi et al., 2015). Ahmed et al. (2010) reported for avocado cv. Fuerte, a mild decrease of titrable acidity during ripening as well as a mild but significant decrease of ascorbic acid during this period. In addition, Arias et al. (2012) reported higher acidity of the seed than the peel and mesocarp in avocado cv. Hass from Algarve, but the acidity of the mesocarp was higher (0.04% citric acid) than the value reported for avocados cv. Hass from Mexico. At harvest, Fuentealba et al. (2017) reported at the protein level differences in expression of enzymes involved in the metabolism of organic acids such ATP dependent citrate synthase, cytosolic NAD malate dehydrogenase and for Hass avocado mesocarp displaying differences in ripening speed suggesting that the speed of ripening is dependent on primary metabolism. Citrate, isocitrate, and malate are intermediates of the Krebs cycle, thus changes in their content during growth, development and ripening are expected. Most of the Krebs cycle acids are stored in the vacuole (Etienne et al., 2013).

During avocado ripening organic acids could be used as substrates in the glycolytic pathway, trimesic acid cycle, and gluconeogenesis (Mu et al., 2018); thus, the slight but significant decreases of total acids in avocado during ripening could be expected.

As stated by Mu et al. (2018), genetic regulation of organic acids not only is complex but involves several polygenes, and the fruit organic acid content is correlated with the activities of related enzymes. It has been reported for example that phosphoenolpyruvate carboxylase (PEPC), NAD-dependent malate dehydrogenase (NADMDH), and NADP-malic enzyme (NADP-ME) are important for the synthesis of malic acid and citric acid, but NADP-ME plays a major role in the degradation of malic acid (Mu et al., 2018). Related to *Persea americana*, Ibarra-Laclette et al. (2015) reported the transcriptome of the Mexican avocado and different organs (seeds, roots, stems, leaves, aerial buds, and flowers) and for three different fruit ripening stages defined as pre-climacteric, climacteric, and post-climacteric. A higher number of genes related to organic acid metabolic processes at the pre-climacteric stage followed by the climacteric and post-climacteric stages were reported by Ibarra-Laclette et al. (2015). Annotated reported polygenes corresponded to malate dehydrogenase, peroxisomal NAD malate dehydrogenase, malate synthase, aluminum activated malate transporter 9, aconitase, phosphoenolpyruvate carboxylase, and phosphoenol pyruvate carboxylase kinase, but their expression patterns at the three different stages previously mentioned are not available (Ibarra-Laclette et al., 2015). Kilaru et al. (2015) reported as supplementary information the pattern expression of key genes related to organic acid metabolism in Hass avocado at five different developmental stages (from 75 g fruit and 4% oil to 200 g fruit and 12% oil). The gene annotated as phosphoenolpyruvate carboxylase family protein increased in expression from stage I and II but then tended to decrease in expression reaching its lowest level at stage V corresponding to mature fruit. Genes annotated as malate dehydrogenase cytosolic and plastidial did not present clear differences in expression at the five different developmental stages. As previously mentioned, avocado is considered as non-acid fruit and previous studies concluded that there is no correlation between the expression of organic acid metabolism related genes and organic acid content (Mu et al., 2018).

Regarding the second metabolic group of organic acids represented by ascorbic and tartaric acid, information regarding the evolution of ascorbic acid in Hass avocado mesocarp, exocarp, and seed is available. Ascorbic acid is located in all compartments of the cell. Tesfay et al. (2010) reported ascorbic acid contents for Hass avocado fruit from 16 weeks after full bloom (~70 g fresh weight) to commercial maturity (~180 g fresh weight, 18–22% oil content FW) lowest in the mesocarp and highest in the exocarp. During these 6 months of growth and development, avocado fruit displayed average contents of ascorbic acid of 0.41 mg g DW^{-1} of mesocarp tissue, 2.77 mg ascorbic acid g DW^{-1} of exocarp tissue, and 1.84 mg ascorbic acid g DW^{-1} of seed. As fruit grew and developed, a decrease of ascorbic acid was observed for the mesocarp and seed but instead the exocarp displayed a mild increase of ascorbic acid during fruit growth and development. Defilippi et al. (2015) reported for Hass avocado subjected to ripening at 20°C during 15 days storage, that both tartaric acid and ascorbic acid remained relatively constant during this ripening period. The edible portion of Hass avocado has been reported to contain 8.8 mg ascorbic acid/100 g FW (Dreher and Davenport, 2013). It is important to highlight the cross talk between fruit firmness (cell wall related) and ascorbic acid content since their biosynthetic pathways share GDP-D-mannose epimerase (Mounet-Gilbert et al., 2016).

A very important group of organic acids present in avocados correspond to phenolic acids and fatty acids. Galacturonic acid, a very important component of pectins, will also be addressed in the section related to cell wall metabolism. However, Pedreschi et al. (2014) reported for Hass avocado at harvest, higher content of galacturonic acid in fast ripening fruit indicative of different physiological age. Phenolic acids do not participate in primary metabolism but mainly in secondary metabolism being precursors of other phenolic compounds and components of the cell wall. Thus, a very brief introduction to studies on the topic is incorporated in this review. Hurtado-Fernández et al. (2014, 2015a) evaluated 13 different avocado cultivars including Hass at two different ripeness stages (at physiological maturity and ready to eat ripeness stages) and reported that the concentration of organic acids and phenolic acids generally decreased as the fruit ripened, except for phenolic acids such as ferulic and *p*-coumaric which increased in concentration as the fruit ripened. Hass avocado at harvest presented a lower amount of benzoic acid compared to the ready to eat stage. But, the quinic acid concentration in Hass was higher at harvest and decreased at the ready to eat stage (Hurtado-Fernández et al., 2014). Lately, Hurtado-Fernández et al. (2016) reported the evolution of six important metabolites from the phenylpropanoid pathway including phenolic acids (pantothenic, p-coumaric, and ferulic acid) and epicatechin, chlorogenic acid, and abscisic in different avocado cultivars over different harvest seasons depending on the variety. Results showed that the phenolic acids previously mentioned were quite similar in the four avocado cultivars studied but the concentrations of chlorogenic acid, epicatechin, and abscisic acid differed.

METABOLISM OF AMINO ACIDS DURING AVOCADO FRUIT GROWTH, DEVELOPMENT, AND RIPENING

Amino acid metabolism provides precursors for protein synthesis, for respiration processes and for a range of specialized metabolites. Fruit imports amino acids from the phloem and less from the xylem *via* symplastic or apoplastic routes depending on the stage of development (Zhang et al., 2015). Amino acids and their derivatives have been reported to exert influence on taste and quality.

A thorough study on avocado fruit amino acid metabolism during growth, development, and ripening is not available up to our knowledge. The potential physiological role of polyamines involved in nucleic and protein synthesis in avocado was studied back in 1986 by Winer and Apelbaum. These authors studied the evolution of polyamines during avocado development and ripening. The levels of putrescine and spermidine decreased during fruit growth, being these decreases moderate during fruit development until maturation. However, the most striking decrease of these polyamines was observed during ripening (Winer and Apelbaum, 1986). Coincident with the climacteric peak of ethylene, putrescine, spermidine, and spermine decreased their contents to 0 and 20% of their initial values, respectively. Exogenous application of polyamines provoked a decrease in the level of endogenous ACC in avocado fruit. Interestingly, spermine in the mesocarp remained constant while the fruit was attached to the tree. From these findings, it was postulated that the high level of polyamines in avocado fruit during development provoke the repression of ethylene production and lack of ripening while the fruit remains attached to the tree (Winer and Apelbaum, 1986).

Avocado mesocarp contains a high content of 2% protein (Dreher and Davenport, 2013). During "Hass" avocado fruit ripening, variations in the content of soluble proteins have been reported (Blakey et al., 2010, 2012). Blakey et al. (2012) reported increases of soluble protein content during ripening of Hass avocado from 17.8 mg g^{-1} DW to 34.8 mg^{-1} g DW from day 2 to day 16. This increase in soluble protein content is associated with the ripening process which demands *de novo* synthesis of enzymes/proteins involved in the different pathways triggered by the ripening process. Previously, Blakey et al. (2010) reported similar increases in soluble protein content during ripening while C_7 sugars decreased while C_6 sugars tended to increase as ripening proceeded.

Recent studies related to the understanding of differences in ripening speed of avocado fruit have demonstrated the participation of amino acid metabolism, protein folding, transport, and translation (Pedreschi et al., 2014; Fuentealba et al., 2017; Uarrota et al., 2019). Glutamic acid has been reported to be highly correlated to a heat shock treatment (38°C for 1 h) that triggered ripening synchronization in early and middle season Hass avocado fruit. In the same study, differences 1 day after harvest, at the metabolome and proteome levels were observed between early and middle season fruit indicating differences in the physiological state at harvest and thus different response to the effectiveness of the heat treatment in terms of ripening (Uarrota et al., 2019). Previously, in a study of Pedreschi et al. (2014) fast ripening Hass avocado fruit at harvest displayed higher contents of glutamic and aspartic acids and alanine compared to the slow ripening phenotype. These differences were attributed to differences in physiological maturity at harvest not evident with classical commercial assessment of dry matter content and firmness. Fuentealba et al. (2017) recently reported higher expression of proteins involved in correct protein folding, translation and protein *de novo* synthesis in the fast ripening Hass avocado phenotype and higher amount of aminopeptidases and carboxypeptidases involved in post-translational modification in the slow ripening Hass avocado phenotype.

LIPID METABOLISM DURING AVOCADO FRUIT GROWTH, DEVELOPMENT AND RIPENING

Avocado is a very complex matrix formed by a wide variety of compounds and one of the main components is lipids mainly composed of triacylglycerols (TAGs). TAGs composed of monounsaturated fatty acids (MUFA; 9.80 g/100 g) are the predominant ones and also of polyunsaturated (PUFA) and saturated fatty acids (SFA; 2.13 g/100 g). The abundance of these substances, together with the fact that some of the main health benefits of avocado have been attributed to its high monounsaturated fatty acid content, makes lipids one of the most studied chemical families in avocado (Hurtado-Fernández et al., 2018).

Avocado lipids can be divided into (1) neutral lipids or also called triacylglycerols (TAGs), fats or oils (tri, di, and monoacylglycerols); (2) phospholipids; and (3) glycolipids. The neutral lipid fraction constitutes 96% of the total lipid content of avocado, mainly are TAGs. TAGs composed of 18:1, 18:2, 16:0, and 16:1 fatty acids are present, and the relative concentration (percentage of total lipid) of each is in the range 59–81% (18:1), 7–14% (18:2), 7–22% (16:0), and 3–11% (16:1). It is important to point out the differences in lipid content and profile in the mesocarp, exocarp, and seed in different cultivars of avocado. For instance, Galvao et al. (2014) reported average mesocarp fat contents (g/100 g) of 16.2, 13.6 and 11.9 for Fortuna, Collinson and Barker cultivars. Average seed fat contents (g/100 g) for the same cultivars corresponded to 1.8, 2.5, and 2.1, respectively and average fat content of the peel corresponded to 0.9, 1.7, and 1.1, respectively, for Fortuna, Collinson and Barker cultivars. In terms of fatty acid composition of the mesocarp except for Barker that presented a higher % of SFA, in the other cultivars Fortuna and Collinson, MUFA were the predominant fatty acids. The seed instead of Fortuna was characterized by a higher relative content of SFA followed by PUFA and MUFA, but the other two cultivars presented a higher proportion of PUFA followed by SFA and MUFA, respectively. The exocarp instead of the three avocado cultivars was characterized by a higher relative content of MUFA followed by SFA and PUFA, respectively.

Oil content increases in the mesocarp a few weeks after the fruit sets. As oil increases in the mesocarp, water content decreases by the same amount, so that the total percentage of oil and water remains constant during fruit life. Nevertheless, biosynthesis of triglycerides does not start at the beginning of the physiological life of the fruit and can change during fruit development and ripening (Ozdemir and Topuz, 2004). Avocado fruit is characterized by the presence of lipid-containing idioblasts, specialized cells, distributed uniformly in the mesocarp, which make up to 2% volume of the edible portion and are characterized by a thick (4 μm) wall consisting of three layers of cellulose, suberin, and lignified cellulose.

Idioblasts contain high acetogenin content. Acetogenin profiles are conserved in mesocarp, while seed profiles are dynamic during fruit growth (Rodríguez-López et al., 2017). During avocado fruit development, the mesocarp accumulates by dry weight 60–70% oil and 10% carbohydrates. The oil is stored in the form of triacylglycerol (TAG) and is predominantly composed of oleic acid (Kilaru et al., 2015). It has been observed that the increase in fruit weight is highly correlated with the accumulation of lipid content in the mesocarp tissue (Kilaru et al., 2015) and unlike mature oilseeds, mature avocados cv. Hass can accumulate up to 18% oil in the mesocarp. The avocado seed instead did not present much change in accumulation during growth and development of the fruit (Kilaru et al., 2015).

Fatty Acid Biosynthesis During Avocado Fruit Growth, Development and Ripening

At least six different metabolic pathways requiring the activation of over 200 genes are involved in oil metabolism in avocado fruit (Salas et al., 2000; Kilaru et al., 2015). Thus, sucrose to be converted into TAG requires the degradation of sucrose and generation of pyruvate in the plastid requiring the active participation of glycolysis, pentose phosphate pathway and plastid transporters and the synthesis of fatty acids in the plastid and TAG assembly in the endoplasmic reticulum. It consists of three events: (1) production of the glycerol backbone; (2) formation of fatty acids or fatty acyl moieties; and (3) esterification of glycerol with the fatty acid components to yield TAGs (Bewley et al., 2013). Carbohydrates are the carbon sources in plants for TAG biosynthesis. *De novo* fatty acids synthesis takes place in the plastid, acetyl-CoA is needed as precursor and it is derived from sucrose and by the action of two key enzymes acetyl-CoA carboxylase and fatty acid synthase, palmitate, stearate, and oleate are formed as main products. These fatty acids are then exported to the cytosol where they are activated to Acyl-CoAs and further modified. The desaturases introduce double bonds, and elongases elongate the fatty acid chain length. Kilaru et al. (2015) reported for Hass avocado at five different developmental stages high expression of transcripts levels for the orthologs of ribulose 1,5 biphosphate carboxylase (RBC), phosphoribulokinase, and phosphoenolpyruvate (PEPC) in avocado mesocarp, and it was consistent with their suggested role in carbon assimilation. In addition, same authors reported high expression of sucrose synthase (SuSy) in the cytosol during mesocarp development and highlighted the major role of SuSy in the generation of the hexoses needed for pyruvate synthesis. Starch is the main substrate for glycolysis in the plastids and in the same study of Kilaru et al. (2015), transcripts for starch synthesis and degradation gene orthologs were abundant through the different avocado mesocarp developmental stages. It has been previously reported that at early stages of fruit set in avocado, about 44% of the flesh weight is due to sugars which continue to increase during rapid fruit growth but then decline during oil accumulation (Liu et al., 1999b). One fourth of total oil content accumulation in avocado has been reported to occur during early stages of development and Vergara-Pulgar et al. (2019) have recently reported enrichment of transcripts related to lipid localization, fatty acid synthase activity and acyl carrier activity involved in fatty acid biosynthesis when comparing mesocarp of 150 DAFS (days after fruit set) with 240 DAFS, respectively. Acetyl-CoA carboxylase the regulatory enzyme that controls the rate of fatty acid synthesis and catalyzes the first reaction to generate malonyl-CoA was also detected in avocado and oil palm (Dussert et al., 2013; Kilaru et al., 2015). As previously mentioned, avocados are rich in C_7 sugars and it is postulated by Kilaru et al. (2015) that they might play in early stages of fruit growth, a regulatory role in the initiation of oil biosynthesis.

Kilaru et al. (2015) carried out a complete transcriptomic analysis targeting pathways related to oil metabolism in mesocarp and seeds from avocado cv. Hass at five different developmental stages (from 125 to 230 g FW ~ 4 to 12.5% oil content) and compared with that of oil rich monocot (palm oil) and dicots (rapeseed and castor) tissues in order to identify tissue and species specific regulation of TAG biosynthesis supposed to be considered a highly conserved process in plants. Avocado mesocarp presented about 45, 34, 9, 5, 4, and 3% (average of five developmental stages) of the transcripts to be genes involved in glycolysis and plastidial fatty acid biosynthesis, pentose phosphate pathway, sucrose degradation, phospholipid, and TAG assembly and plastid transporters, respectively (Kilaru et al., 2015). It was noted that across the five developmental stages of the mesocarp, the abundance of genes involved in glycolysis for the generation of pyruvate and further fatty acid synthesis was striking as the genes involved in the acyl group synthesis in the plastid in contrast to the abundance levels of genes involved in phospholipid and TAG assembly remained pretty constant among the different developmental stages of the mesocarp. Vergara-Pulgar et al. (2019) has recently reported a strong expression of transcripts related to fatty acid and TAG biosynthesis at the start of the developmental process of Hass avocado (150 DAFS and 240 DAFS) but downregulation for later stages of 300 DAFS and 390 DAFs, respectively, which support previous reports that most of the lipid content in avocado is produced during the first half of fruit development, then the processes are reduced but not culminated. Same authors have recently reported oleosin to be differentially expressed in the mesocarp between 240 DAFS and 390 DAFS possibly having a role as lipid storage stabilizer. Previously, Horn et al. (2013) and Gidda et al. (2013) reported two new lipid droplet associated proteins (LDAP1 and LDAP2) in avocado mesocarp. Kilaru et al. (2015) found higher expression of these proteins during the five evaluated developmental stages in avocado mesocarp. These proteins are supposed to bind and stabilize lipid particles in avocado mesocarp. Recently, Ge et al. (2019) reported a histological analysis of lipid droplets in avocado mesocarp during fruit development (from 65 days after pollination, DAP to 125 DAP for the avocado variety Guitenda N°2, *Persea americana* var. guatemalensis). Small lipid droplets of 2 μm of diameter were present throughout the periphery of the cells from 65 to 105 DAP. But at 125 DAP (harvest date corresponding to 11.12% total fatty acids

based on dry mass), larger lipid droplets of 10–25 μm occupying the volume of most of the cell were observed.

Kilaru et al. (2015) indicate based on this transcriptomic data that for oil rich tissues and diverse species, a conserved common stoichiometry and temporal regulation of transcripts with oil accumulation holds. However, only some orthologs of the fatty acid biosynthetic pathway in avocado were similar to that of monocots and dicots. The orthologs expressed in avocado for PDHC-E1β (pyruvate dehydrogenase complex E1β enzyme), HAD (HAD superfamily, subfamily IIIB acid phosphatase), and FATA (acyl ACP thioesterase A) were different than the one predominantly expressed in oil rich tissues of monocots and dicots. Thus, Kilaru et al. (2015) reported more than 60% of the transcripts encoding for fatty acid biosynthesis pathway belonged to stearoyl ACP desaturases (SAD/DES) and to ACP and their expression increased with the maturity of the mesocarp. ACP transcripts represented 24% of the total fatty acids synthesis gene expression, transcripts that mapped to ACP4 (acyl carrier protein 4, plastid) were by far the most abundant in avocado while the other four isoforms were barely detectable.

The expression pattern of stearoyl-ACP desaturase genes in avocado mesocarp reflects its lipid composition according to Kilaru et al. (2015). During mesocarp development, transcript levels coding for FAB2 were the most abundant than any other enzyme involved in lipid biosynthesis and represented 44% of the total plastidial fatty acid synthesis gene expression and increased during maturation and correlated with the increased oleic acid of avocado mesocarp. This was supported by Kilaru et al. (2015), who studied five different developmental stages of Hass avocado (I–V, from 125 to 230 g fresh weight and 4.5–12.5 lipid content fresh weight) and reported 18% of the total lipids to be polyunsaturated during stages I–III and then less than 10% were polyunsaturated in stages IV and V, respectively. The lipid composition agreed with the higher expression levels of LPCAT (lysophosphatidylcholine acyltransferase) and PDAT (phospholipid:diacylglycerol acyltransferase) in stages I–III compared to IV and V. Thus, a possible role for acyl editing during early stages of development is proposed and supported by higher levels of transcripts for an ortholog of oleate desaturase (FAD2) during early stages of development (I–III) compared to IV and V respectively. Recently, Ge et al. (2019) reported the fatty acid mesocarp composition during four stages of avocado fruit development (65–125 DAP; corresponding to 9.45–54.75 g of mesocarp mass) and the expression of key genes and regulators of glycolysis and fatty acid biosynthesis. The composition of fatty acids varied at the four stages of development. The concentration of palmitic and palmitoleic acids increased from 65 to 105 DAP followed by a rapid increase until 125 DAP. Oleic acid content only increased after 85 DAP and then increased dramatically by 70 at 125 DAP. Linoleic acid content fluctuated during fruit development and was higher than those of the other fatty acids, but linolenic acid content declined constantly during fruit development. These results showed that the content of palmitic, oleic, and linoleic acids all increased during the four stages of fruit development, reaching their maxima at the late stage. In addition, the expression of fatty acid related genes such as *PaWRI1, PaACP4-1, PaACP4-2,* and *PapPK-β1* significantly increased during fruit development with maximum expression at 125 DAP. From 65 to 125 DAP, *PaWRI1, PaACP4-2,* and *PapPK-β1* displayed a fourfold increase but kept showing significant differences among the four stages of fruit development but *PaACP4-1* only increased 0.5–1.0-fold expression at 65 DAP and 1.42 fold at 125 DAP. Thus, the observed total fatty acid changes during the four stages of avocado fruit development by Ge et al. (2019) were consistent with the changes of expression of *PaWRI1, PaACP4-2,* and *PapPK-β1* but inconsistent with *PaACP4-1* and *PaWRI2* expression patterns.

Up to our knowledge, the only complete study based on the avocado transcriptome of *P. americana* var. *drymifolia* related to patterns of genes involved in fatty acid metabolism and fruit ripening corresponds to Ibarra-Laclette et al. (2015). The transcriptome of avocado fruit from three different ripening stages corresponding to pre-climateric, climacteric and post-climateric was reported. From these data and based on the expression of transcripts related to "fatty acid synthesis" and "fatty acid elongation, desaturation and export" for these three postharvest ripening stages, Ibarra-Laclette et al. (2015) suggested that the initiation of fruit ripening determines the end of oil accumulation and fatty acid composition. Most of the genes expressed in the fruit homologous to fatty acid biosynthesis genes decreased as fruit ripened. These authors reported three homologs (UN066747, UN21149 and UN33083) of FAB2/SSI2 (stearoyl-acyl carrier protein desaturase) (AT2G43710) as differentially expressed unigenes during ripening. FAB2/SSI2 corresponds to the major enzyme that converts stearic acid ($C_{18:0}$) to monounsaturated acid ($C_{18:1}$) in chloroplasts. These results together with those of Kilaru et al. (2015) based on fruit growth and development of avocado support that lipid accumulation and changes in the fatty acid profile such as fatty acid desaturation occur during fruit development, and possibly, this process concludes a few days after harvest (pre-climacteric stage). The evident decrease of FAB2/SSI2 transcripts and genes involved in fatty acid biosynthesis during fruit ripening explains the non or minimal changes observed in the fatty acid composition during postharvest ripening of avocado. Ibarra-Laclette et al. (2015) hypothesize ethylene as the signaling molecule once perceived that halts lipid biosynthesis and programs changes in the fatty acid profile.

Minimal change in the fatty acid composition of the mesocarp during postharvest has been reported, and the increased oil concentration during storage and ripening has been related to postharvest dehydration and to increased lipid recovery due to partial cell wall breakdown (Mostert et al., 2007; Meyer and Terry, 2008). Pedreschi et al. (2016) reported no effect of postharvest ripening strategies such as temperature and ethylene on the fatty acid profile and content of Hass avocados. Instead the fatty acid profile is determined during growth and development and highly influenced by temperature, light, and photoperiod. Low temperatures during growth and development have been reported to favor the accumulation of polyunsaturated fatty acids having oxygen as the limiting factor for desaturases and membrane fluidity limit the activity

of desaturases. Several studies have reported differences in the profile of fatty acids of Hass avocado from different origin (Donetti and Terry, 2014; Pedreschi et al., 2016), which can have direct consequences on critical cold storage temperature. On this perspective, García-Rojas et al. (2012) reported potential candidate genes involved in loss of quality of late season Hass avocados stored under cold storage grown in Chile and identified stearoyl-ACP desaturase (*PamSAD*) and acyl-CoA synthase (PamACoAS1,2,3). Furthermore, Gudenschwager et al. (2013) on an attempt to understand darkening of the Hass avocado mesocarp under suboptimal cold storage, studied pattern expression of genes that encode the multi-subunit acetyl CoA carboxylase enzyme (MS-ACCase) as being key enzymes in fatty acid biosynthesis and potentially playing a role in membrane synthesis and maintenance. Two MS-ACCase subunits were identified *in silico*, a biotin carboxylase (BC) and a biotin carboxyl carrier protein (BCCP). Since these two genes (*PamACCase-BC* and *PamACCase-BCCP*) were increased in ripe avocados after cold storage, the authors suggested that these genes might be related to cold storage induced physiological disorders in Hass avocado. In addition, several studies have reported that biosynthesis of antifungal compounds in avocado fruit is connected with the activation of fatty acid biosynthetic pathways. Different elicitors (cold temperature, ethylene, and fungal infection) reported by Madi et al. (2003) and Wang et al. (2004) enhanced the expression/activity of Δ9 stearoyl-ACP desaturase (SAD) and Δ12 oleate desaturase (FAD2).

Fatty Acid Catabolism During Avocado Fruit Development and Ripening

Fatty acids to be further utilized by most lipid metabolic enzymes need to be thioesterified by long chain acyl CoA synthases (LACS). Nine different isoforms of LACS have been identified in *Arabidopsis* and in avocado mesocarp, transcripts for the ortholog of LACS4 have been reported as most abundant followed by LACS8, LACS1, and LACS9 (Kilaru et al., 2015). More than 80% of the transcripts of LACS orthologs were represented by the ER-associated isoforms (LACS1, LACS4, and LACS8) and only 16% by the ortholog of plastidial LACS9 in avocado mesocarp. Thus, it remains to be elucidated which of the LACS contributes to acyl activation and where it happens. In addition, orthologs of peroxisomal LACS6 and LACS7 were barely detectable in avocado mesocarp during fruit development. Thus, these results suggest that fatty acids undergo little β-oxidation during mesocarp development (Kilaru et al., 2015). Fatty acids can be degraded *via* peroxisomal β-oxidation, and it is supposed to operate during lipid synthesis or incorporated into triacylglycerols *via* the Kennedy pathway in the endoplasmic reticulum (Kessel-Vigelius et al., 2013).

Kilaru et al. (2015), reported up to 34 different lipase genes during five developmental stages of which four corresponded to monoacyl glycerol lipases (MAGL) and two to triacylglycerol lipases (TAGL). Two MAGL genes did not present changes in expression during the five developmental stages, while two other MAGL tended to decrease in expression as the developmental stage advanced. For the TAGL genes, no differences in expression were observed for the five developmental stages. These results confirm reduced expression of lipases during oil accumulation and furthermore during maturation, which confirms previous studies that reported no changes in oil content and fatty acid profile during avocado ripening.

Oil Regulation in Avocado Fruit

Previous studies of oil palm mesocarp revealed WRI1 to be correlated with oil accumulation. In avocado mesocarp, Kilaru et al. (2015) reported in addition to WRI1, transcripts for the isoforms WRI2 and WRI3 to be highly expressed but upstream regulators of WRI1 such as LEC1, LEC2, and FUS3 were not expressed or detected in avocado mesocarp. These results indicate that oil regulation in non-seed tissue is different than in seed tissues.

In avocado mesocarp, the overall expression pattern of WRI1 orthologs was similar to genes WRI1, which regulate ACP, BCCP (biotin carboxyl carrier protein of heteromeric ACCase), KASII (ketoacyl-ACP synthase II), and PDHC (pyruvate dehydrogenase) and the pattern of oil accumulation. In contrast to the role of WRI2 in *Arabidopsis* fatty acid biosynthesis, the high expression of this ortholog in avocado mesocarp during oil accumulation suggests a potential role in TAG accumulation in avocado. Ge et al. (2019) recently reported that while *PaWRI1, PaACP4-1,* and *PapPK-β1* seem to play key roles in the accumulation of oil in avocado mesocarp as also reported by Kilaru et al. (2015), the pattern of expression of *PaWRI2* differed indicating that this transcription factor contrary to what was reported by Kilaru et al. (2015) might not be influencing oil accumulation.

CELL WALL DYNAMICS METABOLISM DURING FRUIT DEVELOPMENT AND RIPENING IN AVOCADO

Avocados undergo a dramatically softening of the mesocarp during its climacteric period, and these changes in mesocarp firmness are accompanied by changes in skin color, influencing the fruit quality and storability (García-Rojas et al., 2016). Fruit softening is primarily associated to a cell wall disassembly attributed to modifications induced by ripening with an impact in the primary cell wall polysaccharides (Brummell, 2006). There is a consensus in the literature on the conformation of the primary cell walls of plants, including fruits, which corresponds to a pectin matrix and a cellulose-xyloglucan network (Brummell, 2006; Caffall and Mohnen, 2009). It is well known that cell wall structure can have an impact on fruit texture (Brady, 1987; Yakushiji et al., 2001; Vicente et al., 2007; Ng et al., 2014). During the maturation of avocados highly dynamic alterations have been described, involving mainly enzymatic cell wall degradation (Jeong et al., 2002, 2003; Defilippi et al., 2018). Despite the relevant implications of the remodeling of the cell wall and the physiology of avocado, there is very little information in early stages of fruit development.

Cell Wall Dynamics During Ripening of Avocado Fruit

Pectins are mainly composed of homogalacturonans (HG), rhamnogalacturonan type I and II (RG-I and RG-II, respectively; Ishii and Matsunaga, 2001; Coenen et al., 2007). The galacturonic acid (GalA) is the main constituent of the HG, which is secreted to the cell wall where they are de-esterified by the action of pectin methylesterase (PME) (Micheli, 2001; Baiano et al., 2011); leaving available sites for the action of polygalacturonase (endo-PG, exo-PG) and pectate lyases (PL, Brummell, 2006). During avocado softening, the cell wall content undergoes several modifications and pectins are the most studied component. The content of galacturonic acid decreases significantly throughout the softening. These changes in GalA content have been correlated with an increase in the PG activity (Jeong et al., 2002; Jeong and Huber, 2004). Moreover, during avocado softening, a polygalacturonase gene has been described showing an increased expression in postharvest storage (García-Rojas et al., 2012). Although PG participates in the softening of the fruit, this enzyme would not be so relevant in the phase when most significant loss of firmness of the fruit occurs (Jeong et al., 2003). Pectin modifications have been described as a polysaccharide solubilization, mainly in HG, and it is highly linked to PME and PG activity (Awad and Young, 1979; Wakabayashi, 2000). Consequently, these changes in the pectin matrix have been proposed as the main causes of avocado softening as a result of cell wall loosening (Jeong et al., 2002).

Additionally, several publications point out the importance of the modifications of the lateral chains of RG-I in the firmness of fruit (Gross, 1984; Orfila et al., 2001; Ng et al., 2014; Cornuault et al., 2018). RG-I consists of a backbone of rhamnose and galacturonic acid, with side chains of arabinan, galactan, and arabinogalactan, which can be linked to hemicelluloses/celluloses (Vidal et al., 2001; Guillon et al., 2017). Wang et al. (2018) indicated that RG-I content is three-fold the RG-II in the fruit cell wall, and RG-I represented the most important branched pectic component of the primary cell wall and middle lamella (Goulao et al., 2012). β-galactosidases and α-arabinosidases have been described as enzymes that remove galactan and arabinan side chains of RG-I in fruit ripening (Brummell, 2006). In avocados, the pectin distribution has not been described. However, RG-I has been recently associated with the softening in avocados, where galactose (the main component of the side chains of this polysaccharide) amount decreases as firmness decreases. Moreover, β-galactosidase activity increases during softening (Tateishi et al., 2001, 2007; Defilippi et al., 2018). Another component of the RG-I side chains, arabinose, also showed a decrease during softening and an α-L-arabinofuranosidase activity increase have been reported (Kamiyoshihara et al., 2018). Additionally, the rhamnose content remained constant suggesting that the changes observed in RG-I correspond to a disassembly of the RG-I side chains and not to its backbone structure (Sakurai and Nevins, 1997; Defilippi et al., 2018).

Hemicelluloses correspond to cell wall polymers that interact with cellulose (Hayashi and Kaida, 2011) and pectins (Fasoli et al., 2016). Hemicelluloses are conformed by linear chains of glucose, xylose, and/or mannose and possess different side chains involving these monosaccharides and fucose or arabinose. Xyloglucans are the most important polysaccharides in the fraction of hemicelluloses of the primary wall of dicots (Nishinari et al., 2007). In avocados, little is known about the hemicellulose dynamics during softening. Nevertheless, it has been reported that endoglucanase could play an essential role in xyloglucan remodeling (Hatfield and Nevins, 1986; O'Donoghue and Huber, 1992). Another cell wall enzyme, xyloglucan endotransglycosylase/hydrolase participates in the remodeling of hemicelluloses (Fasoli et al., 2016), presenting in tomato a high activity during the expansion of the fruit and either decreases or remains constant during maturation (Miedes and Lorences, 2009). However, in avocados no transcriptional or proteomic characterizations have been reported.

Expansins promote the elimination of hydrogen bonds between cellulose and hemicellulose fibers, and indirectly the degradation of pectins by increasing access to pectin remodeling enzymes (Cosgrove, 2000; Wang et al., 2018). In avocados, expansins are poorly understood, showing a little detection in ripen avocadoes (Rose et al., 2000). Likewise, cellulase activity has been widely studied in avocados. Similarly, to pectin-degrading enzymes, cellulase detection and activity increase during avocado softening, changing the elastic properties of cellulose matrix (Pesis et al., 1978; Sakurai and Nevins, 1997; Jeong et al., 2002; Jeong and Huber, 2004).

Agricultural Practices to Mitigate Cell Wall Disassembly in Avocado Fruit

Cold storage is extensively used by the avocado producers to improve the postharvest viability by delaying ripening-associated softening of avocados. In refrigerated storage, the cell wall of avocados continues to undergo modifications such as degradation of pectins by PG, PME, and β-Gal activities and cellulose degradation by endoglucanase activity at a slower rate (Chen et al., 2017; Defilippi et al., 2018). Defilippi et al. (2018) determined that the activity of PG and β-Gal increased in avocadoes after cold storage compared to harvest, which correlates with the depolymerization of HG and the solubility of pectin polysaccharides and reduction of flesh firmness. However, although this cold storage technology is critical in influencing avocado softening delay, there is insufficient information describing the cell wall changes in pectins, hemicellulose, and cellulose that contribute to the firmness loosening in cold-stored avocados.

The use of plant growth regulators (PGR) is a common practice in some tree fruit handling. One of the most studied PGR in fruit ripening and softening is ethylene because it plays a crucial role in the development of climacteric fruits by initiating and coordinating the ripening process (Jeong and Huber, 2004). Thus, 1-methylcyclopropene (1-MCP) binds to the ethylene receptor and prevents this gas from having a physiological effect (Sisler and Serek, 1997). 1-MCP has been widely used to delay the ripening process in avocados (Feng et al., 2000; Zhang et al., 2011). Defilippi et al. (2018) described that treatments with 1-MCP can extend the postharvest life of the avocado, delaying the enzymatic activity of PG, PME,

and β-Gal. Likewise, Tateishi et al. (2007) described that during treatments with 1-MCP, the β-Gal activity is delayed due to negative transcriptional regulation of these genes, impacting directly in the galactosidase proteins. Furthermore, the endoglucanase activity is also repressed during the treatments with 1-MCP, altering the composition and structure of the polysaccharides conforming the cell wall (Jeong et al., 2002; Jeong and Huber, 2004).

Post-genomic Tools for the Understanding of Cell Wall Metabolism in Avocado

The combination of "omics" techniques such as metabolomics, proteomics, and transcriptomics would help to better understand the processes that occur in avocado fruit as it has been used in other plant species (Shiratake and Suzuki, 2016; Fabres et al., 2017; Savoi et al., 2017). The study of the proteins involved in the development of the plants and the maturation of the fruit provides valuable information to understand processes, in particular, the metabolism of the remodeling of the cell wall (Vicente et al., 2007; Palma et al., 2011; Nilo et al., 2012; Shi et al., 2014; Tenhaken, 2015; Pedreschi, 2017; Martínez-Esteso and Bru-Martínez, 2018). In the case of avocado fruit, at the proteomic level, some analyses have been carried out to understand some physiological processes, such as ripening (Fuentealba et al., 2017), or for the identification of the protein profiling of avocados (Esteve et al., 2012; Righetti et al., 2015). About transcriptomic techniques, different efforts have been carried out to the study of fatty acid synthesis (Ibarra-Laclette et al., 2015; Kilaru et al., 2015), and the responses to both biotic and abiotic stresses (Chanderbali et al., 2009; Mahomed and van den Berg, 2011; Reeksting et al., 2014, 2016). A transcriptomic analysis revealed an increase in the expression of different gene clusters associated to cell wall macromolecule catabolic processes during avocado ripening, inducing changes in the composition of the cell wall. Moreover, genes involved in cell wall biogenesis showed a decreased expression during ripening (Ibarra-Laclette et al., 2015). Additionally, a recent transcriptomic study during avocado fruit development described that genes codifying cell wall structural proteins showed a constantly decreased expression through fruit development, suggesting a role of these genes in avocado fruit firmness. These genes are homologs to proline-rich proteins (PRPs), which constitute a large part of the cell wall structural proteins (Vergara-Pulgar et al., 2019). Furthermore, certain metabolomic studies have been developed in the understanding of the biology and evolution of this fruit (Contreras-Gutiérrez et al., 2013; Hurtado-Fernández et al., 2015b, 2016), the description of processes from the secondary metabolism (Rodríguez-López et al., 2015; Meléndez-González and Espinosa-García, 2018), and the ripening process of avocado (Pedreschi et al., 2014, 2016). However, there is a lack of information regarding the interactions between avocado cell wall remodeling, fruit development and postharvest modifications and changes regarding variety, agronomical cultural practices, and postharvest technology. Alternatively, these changes may impact on the cell wall metabolism that later translates into avocado firmness evolution. Therefore, by having physiological data and omics information (i.e., transcriptomics, proteomics, and metabolomics), we will be able to correlate these data with the firmness properties of the fruit, which will provide more precise ideas about the changes in the processes studied and their impact on the quality of avocados.

CONCLUSIONS AND PERSPECTIVES

A deep understanding of primary metabolism of avocado fruit during growth, development, and ripening and its relation to fruit quality is still to be achieved. Even though, the most recent studies have approached the understanding of this fruit species using new sequencing platforms and bioinformatic tools to critically study and deal with a non-model organism that lack a reference genome, still there is need for further studies that include a complete profiling of carbohydrates, lipids and hormones together with transcriptomic changes in mesocarp, peel, and seed. Further studies that incorporate stable isotope labeling approaches might be important to predict the flux of carbon in central metabolism in avocado. Thus, in depth understanding of avocado fruit development and carbon partitioning could be achieved. Amino acid and organic metabolism have been practically ignored in the studies reported. The role of C_7 sugars in early stages of mesocarp development and regulation of fruit ripening remains to be elucidated. In addition, the potential role of C_7 sugars and its coordination with the initiation of lipid biosynthesis remains to be studied. Regulation of oil biosynthesis in avocado mesocarp still deserves further studies, since fruit quality is determined in this fruit basically by the total oil content and composition of fatty acids. Cell wall metabolism has been poorly studied up to date in avocado and is crucial since it completely influences fruit firmness and other texture and sensory-related properties.

AUTHOR CONTRIBUTIONS

RP, CF, and JEA significantly contributed to the sections related to sugar, organic acid, and amino acid metabolism. In addition, they designed the outline of the content of the manuscript and leaded the editing of the manuscript. CM, VU, and RP significantly contributed to the section related to oil metabolism. RC-V, PO, and BD significantly contributed to the section related to cell wall metabolism.

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REVIEW
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From Central to Specialized Metabolism: An Overview of Some Secondary Compounds Derived From the Primary Metabolism for Their Role in Conferring Nutritional and Organoleptic Characteristics to Fruit

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Fruit flavor and nutritional characteristics are key quality traits and ones of the main factors influencing consumer preference. Central carbon metabolism, also known as primary metabolism, contributes to the synthesis of intermediate compounds that act as precursors for plant secondary metabolism. Specific and specialized metabolic pathways that evolved from primary metabolism play a key role in the plant's interaction with its environment. In particular, secondary metabolites present in the fruit serve to increase its attractiveness to seed dispersers and to protect it against biotic and abiotic stresses. As a consequence, several important organoleptic characteristics, such as aroma, color, and fruit nutritional value, rely upon secondary metabolite content. Phenolic and terpenoid compounds are large and diverse classes of secondary metabolites that contribute to fruit quality and have their origin in primary metabolic pathways, while the delicate aroma of ripe fruits is formed by a unique combination of hundreds of volatiles that are derived from primary metabolites. In this review, we show that the manipulation of primary metabolism is a powerful tool to engineer quality traits in fruits, such as the phenolic, terpenoid, and volatile content. The enzymatic reactions responsible for the accumulation of primary precursors are bottlenecks in the transfer of metabolic flux from central to specialized metabolism and should be taken into account to increase the yield of the final products of the biosynthetic pathways. In addition, understanding the connection and regulation of the carbon flow between primary and secondary metabolism is a key factor for the development of fruit cultivars with enhanced organoleptic and nutritional traits.

Keywords: quality traits, flavor, metabolic engineering, fruit, primary metabolism, secondary metabolism

INTRODUCTION

Plant metabolism can be sub-divided into primary (or central) metabolism, which encompasses reactions and pathways absolutely vital for survival, and secondary (or specialized) metabolism, which fulfills a multitude of important functions for growth and development, including the interaction of the plant with the environment. Primary metabolism products derived from glycolysis, the TCA cycle, or the shikimate pathway often serve as precursors for the synthesis of the tens of thousands of secondary metabolites that have already been described (Kroymann, 2011). Compared to the differences of primary metabolism reactions, which are highly conserved, a much greater diversity is observed in secondary metabolism pathways at the level of species, organs, tissues, and cell level and even at different developmental stages (Wink, 2010). Furthermore, another factor that is presumably necessary for the large diversity of secondary metabolism is its high level of catalytic promiscuity, which is most likely due to its recent divergence from primary metabolism and the weaker selection pressure applied to secondary metabolic enzymes than to primary metabolic enzymes (Tokuriki et al., 2012). A recent metabolomic and statistical study comparing fruits from wild and domesticated accessions of strawberry showed that domestication caused the general dysregulation of secondary metabolism, while the core primary metabolites were maintained, suggesting the looser regulation of specialized metabolism (Vallarino et al., 2018). In addition, a correlation was observed between the taxonomic distribution of secondary metabolites and the gradual development of specialized tissue types and lifestyles in land plants (Weng, 2013). One example of this is the burst in the chemical diversity of volatile compounds to attract co-evolving insects concomitant with the rise of the angiosperms (Pichersky et al., 2006).

Several studies supported the evolution of secondary metabolism by the recruitment of enzymes and pathways from primary metabolism (de Kraker and Gershenzon, 2011; Kroymann, 2011; Carrington et al., 2018). Indeed, secondary metabolic pathways originate from different nodes of core primary metabolic pathway, suggesting that emergent enzymatic activities against primary metabolites yielded new compounds that were able to increase plant adaptation to particular environments and were gradually converted into specialized metabolites (Weng, 2013). It is thought that gene duplication, which is a very common key process in the plant kingdom for gain of new gene functions, is the mechanism by which specialized metabolism expanded to reach its current high level of diversity (Carrington et al., 2018). However, it is still unclear whether neofunctionalization followed gene duplication or gene duplication occurred as a consequence of an adaptive conflict present in the ancestral gene (Kroymann, 2011). In addition, it is interesting to note that new protein folds were not necessary for the emergence of specialized metabolism; on the contrary, gene families involved in secondary metabolism evolved with the use of primary metabolism protein folds (Weng et al., 2012).

de Kraker and Gershenzon (2011) and Carrington et al. (2018) presented two examples of enzyme recruitment from primary to secondary metabolism following gene duplication and the gain of a new function. The primary metabolite shikimate and the secondary metabolite quinate (which are structurally similar) are synthesized by shikimate and quinate dehydrogenases, respectively. Interestingly, both enzymes are members of the same gene family; however, due to a gene duplication event prior to the angiosperm/gymnosperm split, the two genes diverged into two different clades, allowing the evolution of quinate metabolism from primary metabolism (Carrington et al., 2018). An even more recent phylogenetic study confirmed that quinate dehydrogenases emerged from shikimate dehydrogenase sequences, and then evolved through independent gene duplication events in eudicots (Gritsunov et al., 2018). In addition, the authors demonstrated that very few changes in the amino acid sequence were necessary to modify the enzyme activity toward quinate synthesis.

Another striking example of the emergence of secondary metabolism emerged from central metabolism is the evolution of methylthioalkylmalate synthase (MAM), which catalyzes the committed step in the biosynthesis of precursors to glucosinolate, a secondary metabolite class involved in defense mechanisms in plants of the *Brassicaceae* family (de Kraker and Gershenzon, 2011). The MAM sequence is very close to that of isopropylmalate synthase (IPMS), which is involved in leucine synthesis. Phylogenetic studies indicated that the MAM enzyme most likely evolved from IPMS through gene duplication and a change in enzyme function. Once again, a few changes in the MAM sequence, specifically a deletion at the C-terminus, removed leucine-mediated feedback inhibition, and two amino acid changes in the catalytic sites were able to explain the recruitment of the enzyme from primary to secondary metabolic pathways.

SECONDARY METABOLITES IN FRUIT

By modifying central metabolite precursors, secondary metabolism is able to fulfill key functions involved in the interaction of the plant with its environment, particularly in relation to its biotic entourage. Three main classes of secondary metabolites are produced by plants: (1) terpenoid/isoprenoid, (2) phenolic, and (3) nitrogen/sulfur-containing compounds (Aharoni and Galili, 2011), which are produced from primary metabolism like TCA cycle, glycolysis, amino acids, pentose phosphate, and shikimate pathways. The fruit, an organ dedicated to seed protection and dispersal, has evolved in a multitude of forms that favors both its attractiveness to dispersers and its repellence to pathogens. Secondary metabolism in fruit carries out most of these functions by producing compounds involved in defense, pigmentation, and aroma (Leitzmann, 2016). In particular, polyphenol and terpenoid compounds are the main families of secondary metabolites produced by fruit during its growth and development (Poiroux-Gonord et al., 2010). Together with the presence of volatiles, polyphenol and terpenoid compounds

are responsible for its unique aroma and its outstanding nutritional properties.

Phenolic Compounds

Phenolic compounds or polyphenols are mostly produced through the shikimate and phenylpropanoid pathways. They are key contributors to the responses of the plants toward biotic and abiotic stresses, such as the protection against solar radiation and the robustness toward mechanical damage and in mediating defense against pathogens and herbivores. In addition, they are involved in flower and fruit pigmentation, important aspects for reproduction, and seed dispersal (La Camera et al., 2004; Vogt, 2010; Fraser and Chapple, 2011; Hassan and Mathesius, 2012). Due to their antioxidant and antiproliferative properties, they are highly valuable in human nutrition, and epidemiological studies suggest that a high dietary intake of polyphenols is associated with a decreased risk of cardiovascular and cancer diseases (Rodriguez-Mateos et al., 2014; Giampieri et al., 2015). Although polyphenols are a large and heterogeneous group of secondary metabolites, they are surprisingly derived from a very restricted set of basic structures whose origin is set in a primary metabolic pathway, the shikimate pathway (Herrmann, 1995). Polyphenolic compounds consist of multiple phenol ring backbones with hydroxyl groups or other substitutes like sugar molecules and organic acids (Manach, 2004). Several thousands of compounds with a polyphenol structure have been characterized in higher plants (Vogt, 2010).

Tannins are a group of polyphenols which can be divided into two classes: (1) condensed tannins (*syn*. proanthocyanidins) composed by flavan-3-ols polymers subunits linked *via* 4-6 and 4-8 interflavan bonds and (2) hydrolysable tannins that can be described as esters of gallic acid with a central polyol, typically β-D-glucopyranose (Ossipov et al., 2003). Glycosylation reactions of gallic acid yield penta-*O*-galloyl-β-D-glucopyranose requires particular attention because this derivative is the common precursor of all hydrolysable tannins. Further galloylation of penta-*O*-galloyl-β-D-glucopyranose gives rise to gallotannins, one of the two subclasses of hydrolysable tannins. Alternatively, penta-*O*-galloyl-β-D-glucopyranose can suffer oxidation reactions between different galloyl residues, forming 3,4,5,3′,4′,5′-hexahydroxydiphenoyl (HHDP) moieties, the core structure of the second subclass (ellagitannins) (Niemetz and Gross, 2005). Contrary to gallotannins, ellagitannins are widely spread in plant kingdom and form the largest group of known tannins. They are particularly abundant in berries of the Rosaceae family (strawberry and raspberry) and pomegranate. Hydrolysis of ellagitannins releases HHDP which spontaneously forms ellagic acid. Nowadays, tannins are intensively investigated because of their antioxidant, antimicrobial, antiviral, and antitumor characteristics (Landete, 2011). Two of the final products of the shikimate pathway, the aromatic amino acids phenylalanine and tyrosine, are phenylpropanoid precursors directed toward the secondary metabolism by the action of aromatic amino acid lyases, leading to the synthesis of both volatiles and non-volatile phenylpropanoids. In particular, phenylalanine ammonia lyase (PAL) catalyzes the deamination of phenylalanine to cinnamic acid and is the gateway enzyme to the phenylpropanoid pathway, directing carbon flow from primary to secondary metabolism (Vogt, 2010). The activities of several enzyme superfamilies (oxygenases, ligases, oxidoreductases, and transferases) are then responsible for the huge diversity of phenylpropanoids, being the formation of 4-coumaroyl CoA a decisive branch point within the pathway (Vogt, 2010). Phenylpropanoids range from simple phenolic acids, including derivatives of benzoic and cinnamic acids, to more complex compounds, such as stilbenes, lignans (lignin precursors), or the ubiquitous and well-studied flavonoids, which are present in many fruits. The shared structure of all flavonoids is the flavan nucleus, formed by A, B, and C rings, which are two aromatic rings (A and B) connected by three carbon atoms forming an oxygenated heterocycle (C). The distinct groups of flavonoids differ in the composition of their C heterocycle (**Figure 1A**; Hannum, 2004; Manach, 2004; Leitzmann, 2016). The flavonoid pathway starts with the formation of flavanones, which originate from the condensation of coumaroyl CoA and malonyl CoA molecules and are the first compounds with a flavan nucleus. Dihydroflavonols are then synthesized from flavanones and can be converted to anthocyanidins, which are colorless and unstable pigments. Anthocyanidin oxidation and glycosylation forms anthocyanins; these stable and colored compounds accumulate in vacuoles and confer a red, blue, pink, or purple color to many fruits, including eggplants, cherries, or strawberries (Petrussa et al., 2013). The reduction of anthocyanidins

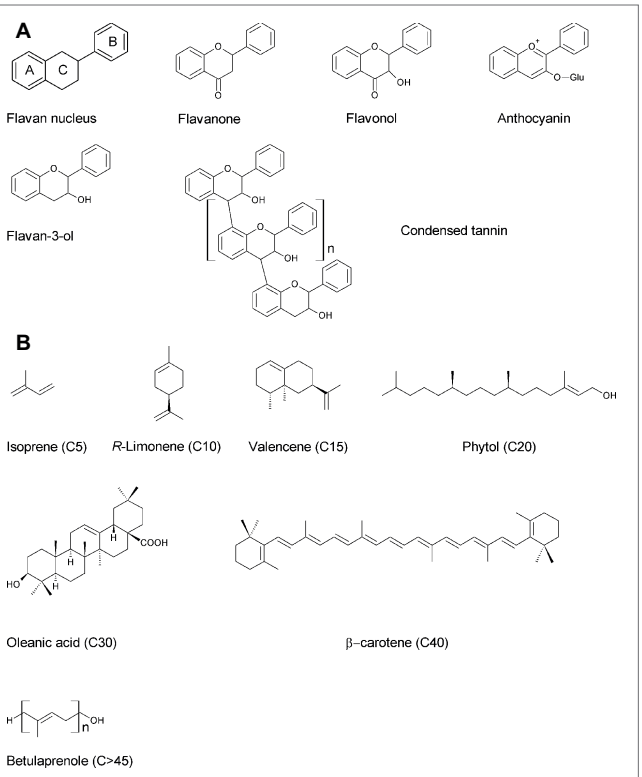

FIGURE 1 | (A) Flavonoid chemical structures, including the flavan nucleus and the main classes of flavonoids found in fruits. **(B)** Terpenoid chemical structures, including isoprene and an example of mono-(R-limonene), sesqui-(valencene), di-(phytol), tri-(oleanic acid), tetra-(β-carotene), and polyterpenoid (betulaprenole) compounds.

leads to the formation of flavan-3-ols, which are present in both monomeric (i.e., catechin) and oligomeric forms (condensed tannins). Indeed, the formation of condensed tannins occurs by the addition of anthocyanidin molecules to the terminal unit of flavan-3-ols (Bogs, 2005). Condensed tannins are present in many fruits, being responsible for their astringent flavor and preventing herbivore consumption when unripe (Manach, 2004).

A series of phenylalanine-derived volatiles with an aromatic ring include benzenoids, phenethyl compounds, phenylpropanes and phenylpropenes (Gonda et al., 2018). Phenylpropenes are present in many economically important fruits, such as tomato, strawberry, and grape, and originate from a side branch of the phenylpropanoid pathway. Indeed, their precursors are coniferyl and coumaryl alcohols, intermediates in lignin biosynthesis (Atkinson, 2018). The first committed step is the conversion of both alcohols to hydroxycinnamyl acetates, which are then reduced by phenylpropene reductases to produce volatiles such as eugenol, chavicol, and estragole, conferring aromatic spicy notes to the fruit (Araguez et al., 2013; Yauk et al., 2017). Other phenylalanine-derived volatiles, including methyl benzoate, benzyl acetate, and cinnamyl acetate, are synthesized from a lateral branch of the phenylpropanoid pathway, using PAL product cinnamic acid as precursor (Gonda et al., 2018). Finally, phenylalanine can produce phenethyl compounds in a PAL-independent manner, by the action of aromatic amino acid aminotransferase and decarboxylase (Tieman et al., 2006; Gonda et al., 2010; Atkinson, 2018).

Terpenoid Compounds

The largest family of secondary metabolites identified to date belongs to the isoprenoid class, also known as terpenoids. Many terpenoids have a commercial interest since they are applied as pesticides, antimicrobial agents, and dietary anticarcinogenics. In addition, they are also used as precursors to produce chemicals, such as vitamins. All terpenoids derive from the mevalonate (MVA) pathway which is active in cytosol and starts from acetyl CoA, or from the methylerythritol-4-phosphate pathway (MEP), which is active in the plastids and starts from pyruvate and glyceraldehyde-3-phosphate (Rodríguez-Concepción and Boronat, 2002). Both pathways lead to the formation of the two 5-carbon isopentenoid building blocks: isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP) (Laule et al., 2003). The MVA pathway produces IPP which is isomerized to DMAPP by isopentenyl diphosphate isomerase (IDI) (Hemmerlin et al., 2012). Isoprene, the smallest terpenoid with 5-carbon atoms, is produced from DMAPP (Behnke et al., 2007). Terpenoid larger than C5 are mono-(C10), sesqui-(C15), di-(C20), tri-(C30), tetra-(C40), and polyterpenes (>45) units (**Figure 1B**), that are formed by sequential head-to-tail condensation of IPP with DMAPP, geranyl diphosphate (GPP), farnesyl diphosphate (FPP), geranylgeranyl diphosphate (GGPP), and so on to increase the chain length (Jiang et al., 2016). These reactions are catalyzed by isoprenyl diphosphate synthases (IDS), also called prenyltransferases. In this review, we mainly focused on monoterpenes (C10), sesquiterpenes (C15), diterpenoid (C20), and carotenoids (C40) for their importance in the nutritional and aromatic quality of fruits.

Carotenoids

Carotenoids, a family of tetraterpenoid molecules, are widely distributed in plants, algae, fungi, and bacteria. In plants, carotenoids play a role as pigments, being responsible for the bright and appealing yellow, orange, and red tones of many fruits such as tomato, pumpkin, persimmon, and pepper (Sun et al., 2018). Furthermore, they play key roles in photosynthesis and photoprotection (Ruiz-Sola and Rodríguez-Concepción, 2012; Niyogi and Truong, 2013; Hashimoto et al., 2016), and they also provide precursors for the biosynthesis of the phytohormones, abscisic acids (ABA), and strigolactones (Nambara and Marion-Poll, 2005; Al-Babili and Bouwmeester, 2015). Moreover, they act as health-promoting phytonutrients and have been linked to the prevention of cardiovascular diseases, cancers, diabetes, Alzheimer's, and other age-related diseases (Fraser and Bramley, 2004; Rao and Rao, 2007; Fiedor and Burda, 2014; Nuutinen, 2018).

Because of their important functional roles, significant efforts have been made to understand carotenoid metabolism in plants (Hirschberg, 2001; Fraser and Bramley, 2004; Botella-Pavía and Rodríguez-Concepción, 2006; Ruiz-Sola and Rodríguez-Concepción, 2012; Nisar et al., 2015; Liu et al., 2015b; Ikoma et al., 2016). Carotenoid biosynthesis pathway is well established. Geranylgeranyl diphosphate (GGPP; C20) is the direct carotenoid precursor, being formed by the condensation of three IPP and one DMAPP molecules. The first step of carotenoid biosynthesis is the condensation of two GGPP to produce phytoene (C40) that is catalyzed by phytoene synthase (PSY), which is the major rate limiting step (Cazzonelli and Pogson, 2010). Next, lycopene is formed from phytoene by a series of desaturation and isomerization reactions catalyzed by phytoene desaturase (PDS), ζ-carotene desaturase (ZDS), ζ-carotene isomerase (Z-ISO), and carotenoid isomerase (CRTISO). Cyclization of lycopene in which lycopene ε-cyclase and lycopene β-cyclase are involved gives rise to α-carotene and β-carotene (orange pigments), while following hydroxylation by two non-heme carotene hydroxylases (BCH1 and BCH2) and two heme hydroxylases (CYP97A and CYP97C) produces yellow xanthophylls (Sun et al., 2018). Oxidative cleavage by carotenoid cleavage dioxygenases (CCDs) and non-enzymatic cleavage of carotenoid molecules between the C9 and C10 position, yield to apocarotenoid formation (also called norisoprenes), including phytohormones and volatile compounds such as α- and β-ionone, 6-methyl-5-hepten-2-one, or geranylacetone that play an important role in the aroma of fruits like tomato, melon, or apricot (Beltran and Stange, 2016; Hou et al., 2016; Tieman et al., 2017; Wang et al., 2019). Despite the intensive research in this field, little is known about the regulation of carotenoid metabolism.

Volatile Terpenoids

Volatile terpenoids constitute the largest class of plant volatiles. Monoterpenes and sesquiterpenes play a key role in the interaction of the plant with its environment, being the most studied because of their broad distribution among angiosperms (Dudareva et al., 2004; Dudareva and Pichersky, 2008). These volatiles greatly contribute to floral emissions and the aroma of several fruits, including citrus, mango, grape, and strawberry

(Hampel et al., 2006). As an example, the essential oil of *Citrus* fruits is mainly formed by the monoterpene *R*-limonene (Weiss, 1997; Feng et al., 2018), while *S*-linalool, which positively correlates with flavor intensity, is an important component of strawberry aroma (Aharoni et al., 2004; Yan et al., 2018). In addition, these volatiles play important roles in plant physiology such as signaling, attracting pollinators, and repelling or acting against predators and other leaf-damaging organisms (Loreto et al., 2014; Abbas et al., 2017). Recent studies demonstrated that emission of these volatiles by plants under biotic or abiotic stimuli, such as insect attacks or herbivore feeding, can lead to the transcriptional activation of defense genes in its neighbors (Tzin et al., 2015a; Richter et al., 2016; Markovic et al., 2019).

The pathway of volatile terpenoids biosynthesis can be summarized in three phases. As previously described, the first two phases are (1) the formation of IPP and DMAPP and (2) the sequential head-to-tail addition of IPP unit to DMAPP to form geranyl pyrophosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP). The third phase of terpene volatile biosynthesis involves the conversion of the various prenyl diphosphates DMAPP, GPP, FPP, and GGPP to hemiterpenes, monoterpenes, sesquiterpenes and diterpenes, respectively, by the action of a large family of enzymes called terpene synthases (Bohlmann et al., 1998). Moreover, the huge diversity of volatile terpenoids is achieved through the action of terpene synthases, since they are able to generate multiple products from a single prenyl diphosphate precursor and many of them can also accept more than one substrate (Degenhardt et al., 2009; Bleeker et al., 2011). Another layer in volatile terpenoid diversification is formed by the action of enzymes through the transformation of the initial products by oxidation, dehydrogenation, and other reactions to increase their volatility and modulate their aromatic characteristics (Dudareva et al., 2013).

Other Volatile Compounds

In addition to phenylpropanoid and terpenoid volatiles, primary metabolites, including carbohydrates, fatty acids, and amino acids, are also the direct precursors of many compounds that significantly contribute to the fruit aroma.

Substituted 4-hydroxy-3(2H)-furanones give caramel-like and sweet aromatic notes to some fruits, being particularly abundant in strawberry and pineapple, and are directly derived from carbohydrate metabolism. Indeed, D-fructose-1,6-diphosphate is the precursor of the 4-hydroxy-2,5-dimethyl-3(2)H-furanone, also known as furaneol. Methylation of furaneol produces 2,5-dimethyl-4-methoxy-3(2)H-furanone or mesifurane, which is another important component of the fruit aroma (Tokitomo et al., 2005; Raab et al., 2006).

Amino acid degradation is another important source of fruit volatiles. In particular, the catabolism of methionine, branched chain amino acids (leucine, isoleucine, and valine) and aromatic amino acids (phenylalanine, tyrosine, and tryptophan) yields a series of aldehydes, alcohols, and esters (Gonda et al., 2010).

The last steps of amino acid-derived volatile synthesis have been well studied in many fruits, being catalyzed by alcohol dehydrogenases (ADH, in which the aldehyde is reduced to the corresponding alcohol) and by alcohol aminotransferases (AATs), to form the corresponding esters (Pérez et al., 1996; Aharoni et al., 2000; Beekwilder et al., 2004; Manríquez et al., 2006; Yauk et al., 2017). However, the first steps of the pathway, upstream of ADH, have received less attention and are still not clearly defined (Tieman et al., 2006; Gonda et al., 2010; Kochevenko et al., 2012). Studies conducted in tomato and melon fruits using isotope labeling suggested that α-keto acids are key intermediates in the conversion of amino acids to volatiles and that, at least in the case of the branched-chain amino acids, they are probably more important precursors of the branched-chain volatiles than the amino acids themselves. The transamination of the amino acid to the corresponding α-keto acid occurs with the help of aminotransferase enzymes that reversibly catalyze the interconversion of amino acids and α-keto acids. In the second step, α-keto acids are decarboxylated to aldehydes, leading to the formation of volatiles (Gonda et al., 2010; Kochevenko et al., 2012).

Non-polar primary precursors, i.e., saturated and unsaturated fatty acids, also take part in generating plant volatiles, such as ketones, lactones, aldehydes, alcohols, and esters (Schwab et al., 2008). Indeed, free C18 unsaturated fatty acids, linoleic and linolenic acids, can be metabolized through the lipoxygenase (LOX) pathway, converting them into fatty acid hydroperoxides (HPOs). HPOs can be further converted by the action of fatty acid hydroperoxide lyases (HPLs), yielding aldehydes that can be reduced to their corresponding alcohols by the action of alcohol dehydrogenases. Alcohols generated through the LOX pathway can be further esterified by the action of AAT, forming straight chain esters (Rowan et al., 1999; Fellman et al., 2000; Li et al., 2014). These volatiles include C6 compounds, such as (Z)-3-hexenal, hexanal, hexanol, or hexyl acetate, known as "green leaf volatiles" due to their odor characteristics. Indeed, they confer the typical green, grassy and unripe notes of many fruits, and are released by plants under abiotic or biotic stress stimuli (Vogt et al., 2013; Mwenda and Matsui, 2014; Ul-Hassan et al., 2015; Vivaldo et al., 2017). In addition, the peroxidation of C18 polyunsaturated fatty acids by the LOX pathway is responsible for C5 volatile formation, in an HPL-independent manner (Shen et al., 2014). Together with "green leaf volatiles" and several C7, C8, and C10 volatiles, they are correlated with consumer preferences (Buttery et al., 1989; Hildebrand et al., 1989; Vogt et al., 2013; Mwenda and Matsui, 2014; Ul-Hassan et al., 2015).

The aroma of many ripe fruits is dominated by esters, which provide them with a fruity, sweet scent. Interestingly, the differences between aromatic and non-aromatic melon varieties lie in ester content. Indeed, both types produce amino acid-derived volatiles; however, in the aromatic varieties, these volatiles are normally esterified, while in non-volatile varieties, they are present as alcohols and aldehydes (Gonda et al., 2010). Fatty acids serve as precursors for straight chain esters, while branched chain esters originate from branched chain amino acids. In addition, aromatic esters (such as benzyl acetate) derive from phenylalanine (Wang et al., 2019).

METABOLIC ENGINEERING FOR FRUIT QUALITY TRAITS

For years of traditional breeding, farmers focused on yield, disease resistance, and fruit appearance. However, consumer preferences are currently forcing them to pay attention to fruit quality traits such as flavor and nutritional value (Tieman et al., 2017; Vallarino et al., 2019). For this reason, fruit metabolism has become an obvious target for the production of better-tasting and healthier fruits (Beauvoit et al., 2018). While primary metabolites, such as sugars and acids, directly influence fruit taste, secondary metabolites, such as polyphenols, terpenoids, and volatiles, are also responsible for their quality by being involved in their aroma, color, and health-promoting characteristics.

The modification of central metabolism in order to improve fruit aroma and nutrition by increasing the availability of precursor metabolites is an appealing idea; however, the regulation of primary metabolism is very tight, mainly because the plant needs to maintain a metabolic steady state. This is achieved by the multi-layered regulation of the involved enzymes and by the highly interconnected nature of the pathways, in which primary metabolic intermediates participate in several reactions (Sweetlove et al., 2017).

Secondary metabolic engineering for the (over)production of specialized metabolites is a much easier task, because the pathways are less interconnected than primary metabolism, and situated in a peripherical position of the network. Nevertheless, primary metabolism engineering for the accumulation of valuable secondary metabolites involved in fruit aroma and nutritional characteristics is a promising strategy, as it is reviewed here.

The Shikimate Pathway and the Engineering of Phenylalanine Synthesis

Phenylalanine, tyrosine, and tryptophan are synthesized through the primary metabolic shikimate pathway, being the first one the main aromatic amino acid produced (**Figure 2**); indeed, approximately 30% of the photosynthetically fixed carbon is directed through its synthesis to produce phenylpropanoids (Rippert and Matringe, 2002). For this reason, the shikimate pathway acts as a metabolic connection between central and specialized metabolism and a carbon flux checkpoint during the synthesis of secondary metabolites (Tzin et al., 2015b). In addition, bottlenecks in the conversion of primary metabolites into specialized metabolites must be identified to boost fruit aroma or the synthesis of health-promoting compounds. The transgenic expression of fundamental elements controlling a biosynthetic pathway can disturb the system and cause perturbations in metabolite accumulation, allowing the identification of bottlenecks in the process (Xie et al., 2016). Once identified, the genes (i.e., genes encoding enzymes or transcription factors) involved in the rate-limiting steps of the pathway can be converted into valuable tools for metabolic engineering. Such tools are available to study the synthesis of aromatic amino acids and their derivative secondary pathways are described below.

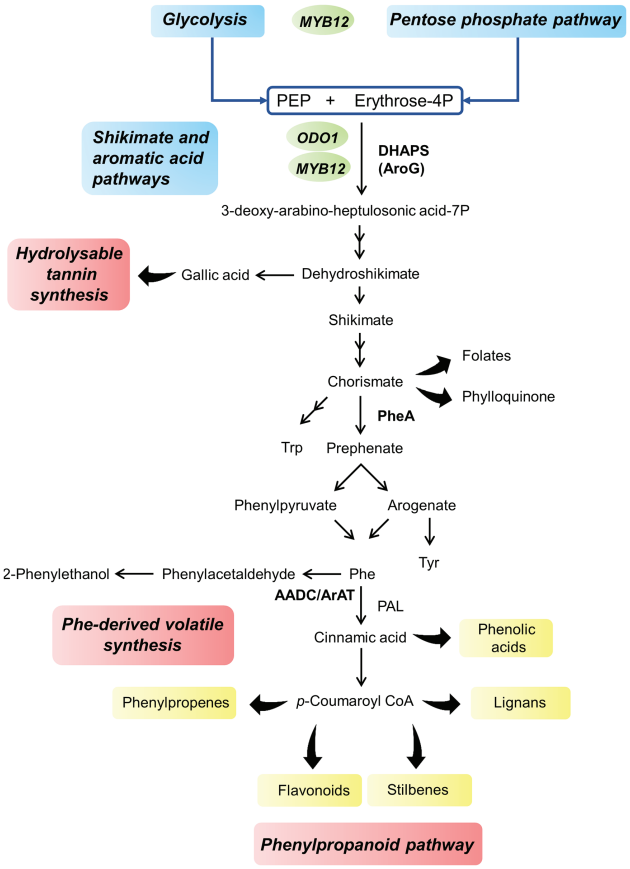

FIGURE 2 | Overview of the shikimate pathway leading to the synthesis of phenolic acid compounds, including soluble (or hydrolysable) tannins, phenylpropanoids and phenylalanine-derived volatiles. Main classes of phenylpropanoids are shown in yellow. Primary pathways are indicated in blue, while secondary pathways are in red. Enzymes involved in the accumulation of primary metabolite precursors and possible targets for metabolic engineering are indicated in bold. Transcription factors involved in the regulation of carbon flux toward phenylpropanoid synthesis are shown in green (*ODO1* from *Petunia* and *MYB12* from *Arabidopsis*), and are located next to the pathways they controlled. PEP, phosphoenolpyruvate; DHAPS, 3-deoxy-d-arabino-heptulosonate 7-phosphate synthase; AroG, bacterial feedback insensitive DAHPS; PheA, bacterial feedback insensitive chorismate mutase/prephenate dehydratase; AADC, aromatic amino acid decarboxylase; ArAT, aromatic amino acid aminotransferase; PAL, phenylalanine ammonia lyase.

The first key enzyme of the shikimate pathway, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS), controls the amount of carbon entering the pathway, and converts phosphoenolpyruvate and erythrose-4-phosphate into 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (Herrmann, 1995). Plants DAHPS enzymes are regulated by feedback inhibition loops (Graziana and Boudet, 1980; Reinink and Borstlap, 1982; Rubin and Jensen, 1985). Chimeric bacterial feedback-insensitive *DAHPS* genes have been suggested to inhibit the mechanism that promotes carbon flux toward the synthesis of aromatic amino acids and their specialized metabolite derivatives in *Arabidopsis* (Tzin et al., 2012), tomato (Tzin et al., 2013, 2015b; Xie et al., 2016), and red grape cv. Gamay cell suspensions (Manela et al., 2015).

Preliminary studies in the model plant *Arabidopsis* showed that the expression of bacterial feedback-insensitive DAHPS (*AroG* gene) increased the levels of shikimate, prephenate, and the aromatic amino acids phenylalanine and tryptophan compared to those observed in the absence of DAHPS expression. These results suggested that DAHPS enzymatically limits the flux of carbon from primary to secondary metabolism. Furthermore, the accumulation of the two aromatic acid precursor metabolites (shikimate and prephenate) indicated that the two downstream enzymatic steps are also possible bottlenecks in these pathways, at least in conditions in which DAHPS is not limiting (Tzin et al., 2012). *AroG* expression not only induced changes in central amino acid metabolism but also led to the accumulation of phenylalanine, which induced an increase of phenylalanine-derived secondary metabolites, such as lignin precursors, their derivatives, and flavonoids (Tzin et al., 2012). As many metabolites involved in fruit aroma and nutrition are phenylpropanoids and phenylalanine-derived volatiles (Tikunov, 2005), the regulatory effect of the DAHPS gene has been further studied in commercially important crops, (Tzin et al., 2013, 2015b). In addition, the phenylpropanoid pathway competes for the use of common precursors with terpenoids compounds (Negre-Zakharov et al., 2009). Alterations in primary metabolism were obvious in the fruit of tomatoes overexpressing the *AroG* gene under the *E8* promoter, a fruit-specific promoter that is induced by ethylene during fruit ripening (Tzin et al., 2013). As expected, the levels of shikimate and the three aromatic amino acids were increased, but alterations reached beyond the shikimate pathway, and the levels of several oligo- and monosaccharides, amino acids, and fumarate were altered compared to those in control tomato fruit. In addition, the modulation of secondary metabolism was also observed in fruits expressing the *AroG* transgene; on the one hand, the levels of several phenylpropanoids, both volatiles and non-volatiles, were increased in ripe fruits compared to those in control fruit, which was most likely due to the increase in the levels of their of primary precursors. On the other hand, the levels of carotenoids and volatile terpenoids decreased compared to those in control tomato fruit, confirming metabolic cross talk between the different primary and secondary pathways (Tzin et al., 2013). Indeed, the shift in metabolic flux toward oligosaccharides (*via* gluconeogenesis) and to the shikimate/phenylpropanoid pathways decreased precursor availability for terpenoid compounds.

Grape berries accumulate high levels of flavonoids, anthocyanins, benzenoids, and stilbenes. The ectopic overexpression of the *AroG* gene in a red grape cell suspension also led to the accumulation of phenylalanine and tyrosine (Manela et al., 2015). The levels of two specific polyphenols, the stilbene resveratrol and the flavonoid quercetin, increased by 20-fold and 150-fold, respectively, compared to those in control red grape cells as the consequence of phenylalanine accumulation. Interestingly, the authors confirmed that the increase in polyphenols was not the result of the enhanced expression of key genes of the stilbenes or phenylpropanoids pathways but was exclusively the result of increased phenylalanine content, suggesting that this amino acid is rate-limiting in the production of these metabolites. However, substrate availability probably does not control phenylpropanoid synthesis, as increased phenylalanine levels due to *AroG* expression did not lead to the accumulation of anthocyanins. Dihydroflavonol reductase, which is downstream of quercetin and upstream of anthocyanins in the flavonoid pathway, seemed to be unaffected by the increase in available substrate (Manela et al., 2015).

Another possible bottleneck in the synthesis of phenylalanine is the step catalyzed by the chorismate mutase/prephenate dehydratase, which converts chorismate into phenylpyruvate *via* prephenate. Following the same strategy, Tzin et al. (2009, 2015b) overexpressed a feedback-intensive bacterial form of the enzyme (*PheA* gene) in *Arabidopsis* and tomato fruit. In *Arabidopsis*, this led to an increase in phenylalanine levels compared to those in the control plants, which in turn modulated the secondary metabolite content; however, in tomato, *PheA* overexpression caused only insignificant changes (Tzin et al., 2015b). Interestingly, the ectopic co-expression of both the *AroG* and *PheA* genes produced a different metabolic profile than the one obtained with *AroG* expression alone. Levels of the phenylalanine-derived volatile phenylacetaldehyde were higher in the *AroG* and *PheA* co-expression lines than in the *AroG* lines, suggesting that additional regulatory mechanisms control the synthesis of metabolites derived from the shikimate and phenylalanine pathways. Phenylacetaldehyde contributes to the pleasant and fruity aroma of several fruits, such as tomato, grape, or plum, and is one of the most impactful compounds in persimmon aroma; as a consequence, its increase would be an interesting feature for quality trait breeding (Tieman et al., 2006; Pino and Quijano, 2012; Wang et al., 2012; Rambla et al., 2016). However, because of the cross talk between phenylpropanoid and terpenoid metabolism, the increase in phenylacetaldehyde in *AroG* and *PheA* co-expression lines was accompanied by a decrease in the levels of the terpenoid-derived volatiles β-ionone and geranylacetone, two compounds with a strong impact on overall fruit aroma appreciation (Zvi et al., 2012).

Due to the limitation in modulating the expression of enzymes controlling carbon flux from primary to secondary metabolism, the manipulation of transcription factors controlling a determined pathway appears to be a powerful tool for metabolic engineering.

Members of the R2R3-type MYB family act as regulators of aromatic amino acid biosynthesis and downstream secondary metabolites (Stracke et al., 2001; Liu et al., 2015a). The silencing of *ODO1*, a MYB factor in petunia required for the floral expression of *DAHPS* and other genes of the shikimate and phenylpropanoid pathways, caused a decrease in the levels of phenylalanine-derived volatiles compared to those in control plants (Verdonk, 2005).

The co-expression of *ODO1* and *AroG* in tomato fruit led to a higher phenylpropanoid content than that measured in either single transgene, probably for the combination of high substrate availability (higher phenylalanine content due to *AroG* expression) and the increased expression of key structural genes in the phenylpropanoid pathway (as a consequence of *ODO1* expression). *AroG*- and *ODO1*-expressing tomato fruits contained high levels of hydroxycinnamic acid derivatives,

which act as antioxidants and antimicrobials (Korkina, 2007). Interestingly, both the volatile profile and the aromatic acid content were improved when compared to those in the single transgene plants, producing healthier and more appealing fruits (Xie et al., 2016).

AtMYB12 is another MYB transcription factor that regulates flavonol synthesis in *Arabidopsis* (Luo et al., 2008). *AtMYB12* was overexpressed under the E8 promoter in tomato fruit, and resulted in 10% of fruit dry weight accumulation of flavonols and hydroxycinnamates (Zhang et al., 2015). Interestingly, this transcription factor was able to reprogram primary metabolism, driving carbon flux, ATP, and reducing power generated through central metabolism toward aromatic acid biosynthesis. This makes *AtMYB12* a great potential tool for engineering phenylpropanoid metabolism; indeed, Zhang et al. (2015) crossed *AtMYB12* with *Del/Ros1* tomato lines that accumulate high levels of anthocyanins in fruit (Butelli et al., 2008; Luo et al., 2008). Fruits resulting from the cross exhibited higher levels of chlorogenic acid, flavonols, and anthocyanins than either of the parental lines because of the activation of all the genes encoding primary metabolism enzymes related to flavonoid biosynthesis. Furthermore, *E8:AtMYB12* tomato lines can redirect metabolic flux toward the synthesis of the desired phenylpropanoids if the overexpression of the transcription factor is combined with a specific structural gene. As a proof of concept experiment, *E8: AtMYB12* co-expressing stilbene synthase from grape or isoflavone synthase from *Lotus japonicus* resulted in the highest yields reported to date of the stilbene resveratrol and the isoflavone genistein in tomato fruits (Zhang et al., 2015). As *AtMYB12* expression increases the aromatic amino acids content by the upstream activation of primary metabolism and by reprogramming carbon flux, this study opens the possibility of the manipulation of specialized metabolites derived from tyrosine and tryptophan in addition to the phenylpropanoids derived from phenylalanine.

In addition to serve as precursors for the synthesis of phenylpropanoids, the shikimate/aromatic amino acid pathway is also involved in the synthesis of essential micronutrients. In particular, phenylalanine-precursor chorismate is an important branch point within the pathway, leading to the synthesis of tetrahydrofolate (vitamin B9) (Wolak et al., 2017) and phylloquinone (vitamin K_1) (Basset et al., 2017).

Folates

Folates are a group of water-soluble B vitamins, derivatives of tetrahydrofolic acid, which are synthesized only by plants and microorganisms. Folates are important components of the human diet, as they are needed for a large set of physiological processes. In particular, they play important roles in the biosynthesis of DNA (purines and thymidylate), but also for the production of methionine and vitamin B5 (Basset et al., 2004). It is known that folate deficiency can not only cause megaloblastic anemia and birth defects but also a low folate intake is associated with a higher risk to suffer cardiovascular disorders and several cancers (Ramírez Rivera et al., 2016). Folates are particularly abundant in some vegetables, legumes, and fruits (Hossain et al., 2004). In plants, folates are synthetized from pterin, glutamate, and *p*-aminobenzoate (*p*ABA) which derived from chrorismate (Díaz de la Garza et al., 2004). Strategies for folate biofortification have favored the engineering of the pteridine branch by acting upon the committed step. Indeed, this reaction, catalyzed by the GTP cyclohydrolase I (GCHI), seems to be rate-limiting as *GCHI* overexpression has led to significant folate increase in different crops, including rice and tomato (Díaz de la Garza et al., 2004; Hossain et al., 2004; Storozhenko et al., 2007). An alternative approach is the overexpression of the *aminodeoxychorismate synthase* (*ADCS*), the key enzyme of the *p*ABA pathway, catalyzing the synthesis of aminodeoxychorismate from chorismate. In rice, overexpression of both *GCHI* and *ADCS* led to a 100-fold folate increase (Storozhenko et al., 2007). Interestingly, Watanabe et al. (2017) showed that fertilization of hydroponically cultivated spinach with phenylalanine conduced to an increase in folate content. As both *p*ABA and phenylalanine are synthesized from chorismate, a phenylalanine excess could induce a negative feedback on the chorismate mutase or arogenate dehydratase, and so favor carbon flux toward *p*ABA synthesis (Watanabe et al., 2017). Phenylalanine fertilization of hydroponically grown crops, including fruits such as strawberries that are among the richest natural source of folates, seems an appealing and easy strategy, which in addition, avoids the use of genetically modified organism.

Phylloquinone

Phylloquinone (vitamin K1), a terpenoid-quinone conjugated component of the photosystem I, is an essential component in the human diet for its role in blood coagulation and bone metabolism (Saxena et al., 2001). The main dietary source of phylloquinone is green leafy vegetables; however, small amounts of phylloquinone are also found in fruits (Jäpelt and Jakobsen, 2016). Phylloquinone is a prenylated naphthoquinone which synthesis derived from two metabolic branches: (1) *via* chorismate the precursor of the naphthoquinone ring and (2) *via* MEP pathway for the formation of the phytyl diphosphate precursor (Lichtenthaler, 2010). Isochorismate synthase drives carbon flux from shikimate and chorismate pathway toward phylloquinone synthase, outlining the central role of chorismate in the synthesis of both primary and secondary metabolites (Verberne et al., 2007).

Amino Acids Metabolism Engineering for Fruit Aroma

Aroma is generated by a complex mixture of volatiles emitted by the fruit; however, even if hundreds of volatiles are detected in most fruits, a small subset of them is thought to be actually responsible for their distinctive fragrance (Jetti et al., 2007; Rowan et al., 2009; Klee and Tieman, 2018). Interestingly, these key volatiles are derived from a small set of primary metabolites, including phenylalanine, valine, leucine, isoleucine, methionine, and fatty acids (**Figure 3**; Klee and Tieman, 2018).

The first steps in amino acid-derived volatile synthesis have not been clearly established yet. Gonda et al. (2010) identified two aminotransferases, CmArAT1 and CmBCAT1, with aromatic

amino acid (ArAT) and branched-chain amino acid aminotransferase (BCAT) activity, respectively, and able to convert amino acids into their respective α-keto acids. Their expression in melon fruit was similar to the pattern of accumulation of amino acid-derived volatiles and to the expression profiles of other known genes involved in volatile

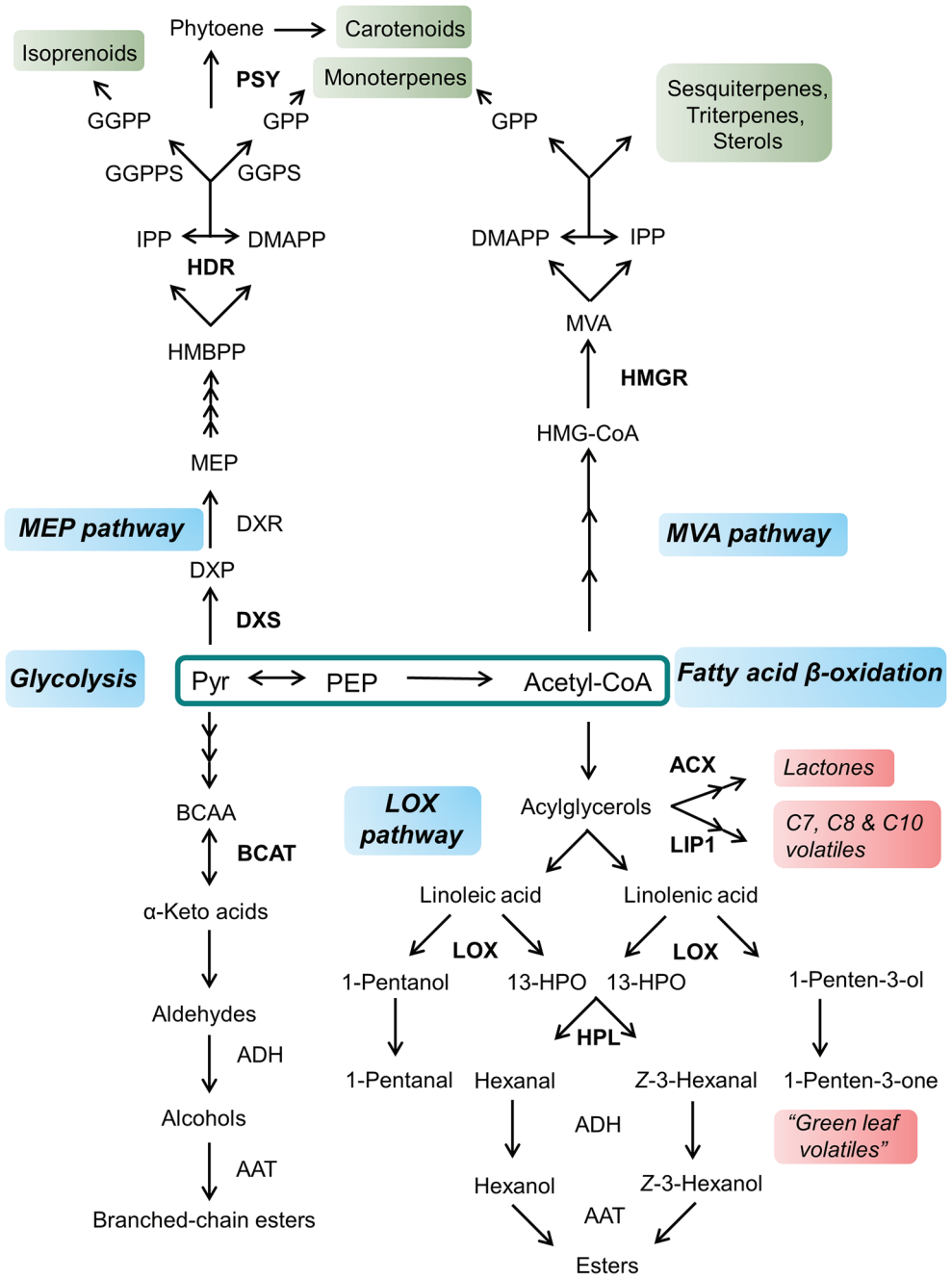

FIGURE 3 | General overview of volatile and terpenoid compounds synthesis from pyruvate and acetyl-CoA. Enzymes involved in the accumulation of primary metabolite precursors and possible targets for metabolic engineering are indicated in bold. Primary pathways are indicated in blue; main classes of terpenoid compounds are shown in green, while volatile classes are emphasized in red. Pyr, pyruvate; PEP, phosphoenolpyruvate; BCAA, branched-chain amino acid; BCAT, branched-chain aminotransferase; ADH, alcohol dehydrogenase; AAT, alcohol aminotransferase; ACX, acyl-CoA oxidase; LIP1, lipase1; LOX, lipoxygenase; HPL, hydroperoxide lyase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXP, 1-deoxy-D-xylulose-5-phosphate; DXR, DXP reductoisomerase; MEP, 2-C-methyl-D-erythritol-4-phosphate; HMBPP, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate; HDR, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate: GGPS, GPP synthase; GGPP, geranylgeranyl diphosphate; GGPPS, geranylgeranyl diphosphate synthase; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; MVA, mevalonate.

biosynthesis, such as AATs (Gonda et al., 2010; Li et al., 2016). In addition, climacteric melon cultivars exhibited higher *CmArAT1* and *CmBCAT1* expression levels than non-climacteric cultivars that accumulate fewer aroma-related compounds, supporting the role of both enzymes in volatile formation (Li et al., 2016). These results suggest that aminotransferases could be a suitable target in melon for metabolic engineering to favor carbon flux from primary precursors to volatile synthesis.

However, the overexpression of *BCAT1* (catabolic enzyme) and *BCAT3* (anabolic enzyme) in tomato plants showed minimal effects on the levels of branched-chain volatiles; even if an increase was observed compared to the levels of these volatiles in control plants, the effect was not large, indicating that further knowledge of the pathway is necessary to improve tomato fruit aroma (Kochevenko et al., 2012).

The lack of an effect on volatile synthesis observed in tomato overexpressing *BCAT1* and *BCAT3* is in accord with the absence of a correlation between the branched-chain amino acid pool and the expression levels of *BCAT1* and *BCAT3*, as described by Maloney et al. (2010). Additionally, there was no correlation between phenylalanine levels and the levels of its volatile derivatives (Tieman et al., 2006; Dal Cin et al., 2011). Rambla et al. (2016) did neither observe a significant correlation in different grape varieties between the abundance of branched-chain amino acids and the levels of their related volatiles, suggesting that this is a common characteristic of amino acids due to their involvement in a broad range of reactions and metabolic pathways. Taken together, these results indicate that amino acids, as central intermediates of cell metabolism, are most likely regulated by the multiple mechanisms, making it more difficult to use them as targets to improve aroma. Furthermore, it seems that the regulation of volatile synthesis occurs downstream of their precursor supply, at the level of α-keto acid precursors (Kochevenko et al., 2012). Indeed, Wang et al. (2019) showed that the decarboxylation of some α-keto acids by a pyruvate decarboxylase isoform (*PDC1*) highly expressed in melon fruit was a limiting step in the synthesis of ethyl and pentyl esters. In particular, the decarboxylation of 2-oxobutanoate by PDC1 leads to propanal synthesis, which is an intermediate in the formation of several straight chain esters (Wang et al., 2019).

Phenylalanine-derived volatiles, such as 2-phenylethanol, are also important for fruit flavor (Tieman et al., 2006). The formation of phenylalanine-derived volatiles is still not well understood and seems to differ between fruits from different species. In tomato, phenylalanine undergoes a two-step pathway, in which an initial decarboxylation reaction catalyzed by an aromatic amino acid decarboxylases (AADC) family member generates 2-phenethylamine, which is further deaminated to yield phenylacetaldehyde. The deamination reaction is probably carried out by an amine oxidase, dehydrogenase or transaminase, although the enzyme has not been identified. Finally, 2-phenylacetaldehyde reductase catalyzed the formation of 2-phenylethanol (Tieman et al., 2007). AADC activity seems to exercise major control over the metabolic flux from phenylalanine to multiple volatile compounds, which makes this pathway a good target for metabolic engineering (Tieman et al., 2006). Indeed, a tomato introgression line, IL-8-2-1, in which chromosome 8 was transferred from the wild tomato relative *Solanum pennellii* to the M82 cultivar, exhibited significantly higher AADC activity than the M82 tomato cultivar together with the increased emissions of 2-phenylacetaldehyde, 2-phenylethanol, 1-nitro-2-phenylethane, and 2-phenylacetonitrile and unchanged levels of free phenylalanine compared to those in the M82 tomato cultivar (Schauer et al., 2006). In addition, transgenic M82 plants overexpressing *AADC1A* or *AADC2* also showed increased levels of these volatiles compared to those in the M82 tomato cultivar (Tieman et al., 2006).

Using stable isotope feeding experiments in ripe melon fruit, Gonda et al. (2018) showed that phenylalanine is concomitantly metabolized by several biosynthetic pathways that operate in parallel, including both PAL-dependent and PAL-independent reactions. *CmArAT1* can convert phenylalanine to phenylpyruvate through a different pathway than that described in tomato (Gonda et al., 2010, 2018). In addition, both benzenoid and phenylpropanoid volatiles are generated through the action of PAL in the first committed step.

In addition to branched chain and aromatic aminotransferase activity, methionine aminotransferase activity was detected in melon fruit. Furthermore, exogenous methionine was able to increase the concentrations of sulfur-containing volatiles, which are an important aroma component of several fruits (Gonda et al., 2010, 2013). Using stable isotope-labeled precursors, Gonda et al. (2013) showed that two parallel pathways, involving a methionine aminotransferase and a methionine γ-lyase (MGL), respectively, were responsible for the degradation of methionine to sulfur volatiles. They confirmed that the second pathway was active in melon fruit by identifying *CmMGL*, a gene which expression increased along ripening and correlated with the presence of sulfur volatiles in different cultivars. More surprisingly, the degradation of methionine by MGL yielded isoleucine, suggesting a role of this amino acid in both sulfur and non-sulfur esters (Gonda et al., 2013).

While changes in the activity of the enzymes that transform amino acids to volatiles did not seem to have a strong effect on the precursor pools, another way of influencing it was suggested by Li et al. (2016). Indeed, they observed that melon treated with ethylene, which controls most ripening events in climacteric fruits, such as volatile biosynthesis (Giovannoni, 2004), showed increased levels of the vast majority of amino acids compared to those in melon without ethylene treatment. This result could indicate that not only ester formation but also the steps upstream of ester synthesis are under hormonal control. In contrast to the other cited studies, a positive correlation between phenylalanine content and most aromatic esters was observed, in addition to ethylene variation (Li et al., 2016).

Fatty Acid Metabolism Engineering for Fruit Aroma

Lipoxygenases gene (*LOX*) family including non-heme, iron-containing dioxygenases ubiquitously present in plant. In plants, lipoxygenases are involved in several processes like seed

germination, fruit ripening, and plant defense (Kessler et al., 2004; Yan et al., 2013). LOX catalyzes the oxidation of polyunsaturated fatty acids (linolenic acid, α-linolenic acid, and arachidonic acid) to form fatty acid hydroperoxides which serve as intermediates in the formation of physiologically active compounds such as oxylipins (Beaudoin and Rothstein, 1997). Depending on the positional specificity of fatty acid oxygenation, which can be C9 (9-LOX) or C13 (13-LOX) of the hydrocarbon backbone, two types of lipoxygenases are described (Feussner and Wasternack, 2002). Although many *LOX* genes have been identified in plants, only a reduced number of members have been described to be involved in aroma formation. As an example, 23 putative *LOX* genes were identified in apple; however only two, *MdLOX1a* and *MdLOX5e*, were associated with the production of green leaf volatiles by a QTL mapping (Vogt et al., 2013).

Tomato contains six *LOX*-encoding genes, named *TomloxA-F*, which can act on polyunsaturated fatty acids, at either the C9 or C13 position, yielding 9-hydroperoxides or 13-hydroperoxides (HPOs), respectively. Only 13-HPOs can be further metabolized into aromatic compounds *via* the action of hydroperoxide lyase (HPL), giving rise to C6 aldehydes (Chen et al., 2004). The *TomloxC* gene, which is highly expressed in ripening fruits, encodes a LOX enzyme essential for the generation of C5 and C6 aldehyde and alcohol volatiles, the most important contributors to consumer liking of fresh tomatoes (Shen et al., 2014). Silencing of the *TomloxC* gene led to a reduction in the level of both C6 and C5 fatty-acid-derived short-chain aldehydes and alcohols (Chen et al., 2004; Shen et al., 2014). However, the silencing of *HPL* resulted in a decrease in C6 volatiles and an increase in C5 volatiles compared to those in control plants, confirming that HPL is involved in the formation of C6 compounds, but not of C5 compounds (Shen et al., 2014). The higher emission of C5 volatiles in *HPL*-silenced plants than in control plants is most likely due to an increase in the content of 13-HPOs, which are driven toward C5 compound synthesis. This suggests that two separate LOX reactions first generate a hydroperoxide and then generate an alkoxyl radical that undergoes non-enzymatic cleavage to generate C5 alcohols (Shen et al., 2014). It would be interesting to see if the overexpression of *LOX* genes produced fruits with a better flavor, although there is no evidence that the activities of *LOX* isoforms are rate-limiting to volatile synthesis (Garbowicz et al., 2018). Zhang et al. (2017a) overexpressed a *13-LOX* gene from melon, *CmLOX18*, in tomato plants. Fruits from the transgenic lines showed enhanced emission of C6 volatiles together with an increased expression of *HPL* compared to the wild-type tomato plant. Interestingly, no changes in the expression of *Tomlox* genes or in the levels of C5 volatiles were observed, suggesting that *CmLOX18* is only involved in the synthesis of C6 volatiles in melon fruit (Zhang et al., 2017a). This result brought up the possibility of modulating *LOX* expression in the fruit to increase volatile emission and to determine whether this increase would correlate with better consumer acceptance.

The targeting of precursor content can be a valuable strategy to increase secondary metabolite and volatile levels, although, as previously described in this review, this approach remains limited, possibly because metabolic intermediates have a stronger influence on the final product of the pathway than the primary metabolite precursors (Gonda et al., 2010; Qin et al., 2014). The *in vitro* incubation of kiwi, melon, or tomato fruits with fatty acid precursor linoleic and linolenic acids increased the emission of C6 aldehydes, alcohols, and straight chain esters compared to that of the control fruit (Zhang et al., 2009; Ties and Barringer, 2012; Tang et al., 2015). Similar results were obtained in pear fruit; however, ester increase was more noticeable when the fruits were incubated with the direct C6 volatile precursors hexanol and hexanal (Qin et al., 2014). Contreras et al. (2016) monitored ripening-dependent changes in the pool of free fatty acids and volatiles in apple and observed an increase in the levels of hexanol, hexanal, and esters containing hexyl moieties derived from hexanol concomitant with the increase of linoleic acid over time. In addition, the absence of linolenic acid detection in the Jonagold apple cultivar was linked with a deficiency in the emission of *cis*-3-hexenal, *cis*-3-hexenol, and *cis*-3-hexenyl acetate, suggesting that the availability of the precursor fatty acid is a key element in the fruit aroma pattern (Contreras et al., 2016). Taken together, these results suggest that free fatty acid content is a good candidate for metabolic engineering. Furthermore, the regulation of fatty acid catabolism through the LOX pathway by phytohormones is another interesting factor to take into account for the accumulation of valuable aromatic compounds. Indeed, a study in grape cell culture suggested that ABA and methyl jasmonate have a positive effect on LOX activity, leading to a mayor accumulation of C6 and C9 volatiles (Ju et al., 2016).

To date, very little is known about the initial steps leading to fatty acid-derived volatile biosynthesis. The first step would likely include the release of polyunsaturated fatty acids from mono-, di-, and triglycerides by the action of a lipase. Very recently, a QTL analysis of introgression lines from the wild tomato *S. pennellii* into the cultivated species *S. lycopersicum* (Eshed and Zamir, 1995) allowed the identification of a class III lipase, LIP1, involved in the degradation of triacylglycerols into glycerol and free fatty acids in fruits (Garbowicz et al., 2018). The introgression line, IL 12-3, and backcrossed introgression lines (BILs) containing introgressions from *S. pennellii* that overlap those that of IL 12-3 contained a *S. pennellii* genome region that was correlated with significantly lower levels of diacylglycerols and triacylglycerols and higher levels of six fatty acid-derived volatiles, including several C5, C10 (*Z*-4-decenal), and C12 (*E,E*-2,4-decadienal) aldehydes, than those in control plants. Fine QTL mapping, using a sub-ILs population derived from a cross between *S. pennellii* IL 12-3 and M82 *S. lycopersicum* confirmed that the *S. pennellii* locus co-localized with the *LIP1* locus. In ripening fruits, *SpLIP1* transcripts accumulate to several thousand-fold higher levels than those in its *S. lycopersicum* ortholog, possibly due to an insertion into the *SlLIP1* promoter region. In addition, a positive correlation between *SpLIP1* transcript abundance and the emissions of C10 aldehydes was observed, suggesting that the content of these volatiles is controlled by *SpLIP1* expression.

The silencing of *SpLIP1* in IL 12-3 led to an increase in triacylglycerols and a reduction of C5-C10 volatiles, confirming the role of LIP1 in generating free fatty acid precursors for aroma compound synthesis. As expected, the overexpression of *SpLIP1* in the M82 cultivar decreased the levels of diacylglycerols and triacylglycerols and increased the glycerol content compared to those in the control M82 cultivar. However, no significant increase in the emissions of fatty acid-derived volatiles was observed, which was probably because of the initial position of *LIP1* in the metabolic pathway and its limited effect on the overall flux rate. Nevertheless, introducing the *SpLIP1* allele into elite tomato varieties would allow the specific increase in the content of multiple volatiles that are positively correlated with consumer preference and holds promise for the metabolic engineering of fruit aroma (Garbowicz et al., 2018).

Fatty acids are also precursors for the synthesis of lactones and furanones, which are important molecules that contribute to the aromas of fruits such as peach or mango (Deshpande et al., 2017; Zhang et al., 2017b). Several studies have demonstrated that the β-oxidation of fatty acids in the peroxisome is one of the main lactone biosynthesis pathways, as a positive correlation was found between lactone content and acyl-CoA oxidase (ACX) activity in peach (Zhang et al., 2017b). ACX catalyzes the first step in the fatty acid β-oxidation and is rate-limiting in the biosynthesis of lactone volatiles. Specifically, a high correlation was found between γ-decalactone accumulation, ACX activity against C_{16}-CoA substrate and *PpACX1* content in the mesocarps of ripe peaches (Zhang et al., 2017b).

In mango fruit, the lipoxygenase *Mi9LOX* utilizes linoleic and linolenic acids as substrates, and the overexpression of this gene induced a significant increase in the concentration of δ-valerolactone and δ-decalactone compared to that in control mango fruit. In addition, the overexpression of *MiEH2*, which encodes an epoxide hydrolase involved in lactone synthesis that catalyzes the hydrolysis of fatty acid epoxides (Haffner and Tressl, 1998), yielded higher levels of δ-valerolactone, δ-hexalactone, and γ-hexalactone than those observed in control fruit (Deshpande et al., 2017).

Mi9LOX and *MiEH2* are part of the lipoxygenase and monooxygenase pathways respectively. As previously described in this section, HPOs generated through the lipoxygenase pathway can be redirected to the formation of C6 aldehydes and ketones *via* the *HPL* pathway. In addition, Deshpande et al. (2017) suggested that HPOs can also serve as substrates for the peroxygenase pathway, producing epoxy and monohydroxy fatty acids and leading to the formation of lactones. Consequently, lactone synthesis in mango competes with HPL pathway for the use of common precursors.

The Engineering of Primary Terpenoid Precursors for Carotenoid and Monoterpene Content in Fruit

Generally, the MEP pathway supplies precursors to monoterpenes, diterpenes, and carotenoids, while the MVA pathway provides building blocks for sesquiterpene, triterpene, and sterol synthesis, although cross talk and exchange occurs between the metabolites of both pathways (**Figure 3**; Laule et al., 2003; Eisenreich et al., 2004; Gutensohn et al., 2013). Several studies have noted that the metabolic flux through the MEP pathway is often higher than through the MVA pathway, and the export of plastidial IPP plays an important role in cytosolic terpene synthesis in some plants (Dudareva et al., 2005; Wu et al., 2006; Orlova et al., 2009). It is thought that the metabolic flux in the MVA pathway is controlled by the 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), which catalyzes the formation of MVA from 3-hydroxy-3-methylglutaryl CoA. Indeed, overexpressing a *HMGR* gene from *Arabidopsis* in tomato plants conduced to an increase of phytosterol content in ripe fruits (Enfissi et al., 2005). In addition to phytosterols, the reaction synthesized by HMGR seems to be also the rate-limiting step in triterpene synthesis. Overexpression of the gene in *Platycodon grandiflorum*, a medicinal plant well known in Asia, led to enhanced levels of platycodins, a group of triterpene glycosides which are the main pharmacological components present in the roots (Kim et al., 2013).

Two primary metabolites, pyruvate and glyceraldehyde-3-phosphate, derived from glycolysis and the pentose phosphate pathway are the substrates of the first enzyme of the MEP pathway, 1-deoxy-D-xylulose 5-phosphate synthase (DXS). DXS catalyzes the condensation of pyruvate and glyceraldehyde-3-phosphate, producing 1-deoxy-D-xylulose-5-phosphate (DXP), which is then isomerized to MEP by DXP reductoisomerase (DXR). Five other steps lead to the formation of two terpene precursors, IPP and DMAPP, with 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (HMBPP reductase or HDR) as the last enzyme in the pathway (Saladié et al., 2014). The MEP pathway is tightly regulated at multiple levels, although studies in different plant species have noted that the control of the pathway is exerted mainly by DXS, both at the transcriptional level and in term of protein abundance and activity (Lois et al., 2000; Wright et al., 2014; Simpson et al., 2016).

The products of the MEP pathway, IPP and DMAPP, then condense to form GGPP in a reaction catalyzed by the GGPP synthase (GGPPS). As several genes encoding putative GGPPS have been identified in most plant genomes, it is likely that different isozymes could be involved in the synthesis of each specific group of isoprenoids (Lange and Ghassemian, 2003).

The MEP pathway and carotenoid biosynthesis are coordinated at the gene expression level, as the expression of the genes involved in the first pathway precedes or correlates with the expression of genes in the second one (Bouvier et al., 1998; Lois et al., 2000; Botella-Pavía et al., 2004). Regulation and cross talk between both pathways have been previously reviewed by Rodríguez-Concepción (2010) and will not be discussed here in detail. However, a few key steps seem to control metabolic flux from primary to secondary metabolism and will be summarized in the next section.

In tomato, *LeDXS1* is responsible for the accumulation of carotenoid pigments during fruit ripening (Lois et al., 2000). Indeed, an increase in *LeDXS1* expression was concomitant with

the largest accumulation of carotenoid at the orange fruit stage as observed in other fruits such as pepper (Bouvier et al., 1998). During ripening, a shift between lutein and β-carotene, which accumulate at the green stage, and lycopene, which is the main carotenoid at the red stage, occurs (Ronen et al., 1999). Significant cross talk occurs between the MEP and carotenoid pathways that may contribute to the regulation of pigment accumulation in the fruit, as the expression of *DXS* correlates with the transcript abundance of the committed enzyme of carotenoid biosynthesis, a fruit-specific phytoene synthase, *PSY1*. However, *PSY1* transcripts start to accumulate before *DXS* transcripts accumulation and the increase in carotenoid content, suggesting that the synthesis of the precursor, DXP, is the rate-limiting step in lycopene synthesis (Lois et al., 2000). In addition, the injection of 1-deoxy-D-xylose, which can enter the MEP pathway, into mature green tomatoes upregulated *PSY1* expression compared to that in control tomatoes, confirming the regulatory role of *DXS* in the carotenoid pathway (Lois et al., 2000). In orange (*Citrus sinensis*) juice sacs, carotenoid synthesis is also controlled by *DXS* and *PSY1* in a coordinated way during fruit ripening, leading to a general increase in the biosynthetic genes of the carotenoid pathway (Alós et al., 2006; Fanciullino et al., 2008).

In addition to DXS, the last enzyme of the MEP pathway, HDR, may play an important role in the regulation of plastidial isoprenoid precursors for carotenoid biosynthesis (Botella-Pavía et al., 2004; Vranová et al., 2012). Indeed, the increased expression of *HDR* was also observed simultaneously with carotenoid accumulation in ripening tomatoes. It is possible that the coordinated upregulation of *DXS*, *HDR*, and *PSY* transcript levels is necessary to drive metabolic flux toward carotenoid synthesis (Botella-Pavía et al., 2004).

Taking advantage of the regulatory function of *DXS*, Enfissi et al. (2005) showed that the overexpression of an *E. coli DXS* in tomato plants increased DXS enzyme activity and carotenoid content compared to those in control tomato plants, even though the accumulation of the final product, carotenoids, was disparate. Indeed, the phytoene content was strongly increased compared to that in control tomato plants, suggesting that phytoene desaturation is the limiting step in the absence of DXS bottleneck. This is yet another example of the challenge in precursor metabolite engineering; elevated *DXS* expression was only able to influence the pathway when adequate precursors from intermediary metabolism were accessible (Enfissi et al., 2005).

In tomato and *Citrus*, carotenoid formation during fruit ripening is dependent on type I DXS (Paetzold et al., 2010; Peng et al., 2013). In melon, however, two type II DXS enzymes (CmDXS2a and CmDXS2b) are the principal isoforms in the fruits of orange varieties and are probably responsible for plastidial isoprenoid accumulation. In varieties that lack carotenoid pigments, such as "Piel de Sapo," neither of these CmDXS2 enzymes were upregulated, implying that only varieties that contain high levels of carotenoids require elevated DXS activity (Saladié et al., 2014).

Another study in clementine pointed out the connection between carbohydrate metabolism and the accumulation of carotenoids. Indeed, they saw that sugar starvation at the early stages of fruit development led to an increase in carotenoid content. In this case, the relation between primary and specialized metabolism would be linked to plastid capacity in accumulating carotenoids, and not to precursor availability (Poiroux-Gonord et al., 2013).

The IPP and DMAPP produced through the MEP pathway can condense to form GPP with the help of GGP synthase (GGPS). GPP then serves as a substrate for monoterpene synthase, which catalyzes the branch point step in monoterpene biosynthesis (Gutensohn et al., 2013). GGPPS, which is involved in carotenoid synthesis, is influenced by heterodimeric GGPSs, which contain a small subunit able to interact with GGPPS and to change its product specificity from GGPP to GPP (Tholl et al., 2015). Based on this knowledge, tomato plants were produced that overexpress the small subunit of a heterodimeric *GGPS* (*GGPS-SSU*) from snapdragon under the control of a fruit-specific promoter (Gutensohn et al., 2013). The massive metabolic flux of the MEP pathway, which was induced during tomato ripening to support carotenoid accumulation, was driven toward monoterpene synthesis, and volatiles were almost not detected in non-transformed plants due to the low expression of monoterpene synthase in ripening tomato fruits (Buttery et al., 1989; Falara et al., 2011; Gutensohn et al., 2013). Even the monoterpene content was increased when geraniol (monoterpene) synthase was overexpressed together with *GGPS-SSU* (Gutensohn et al., 2013).

Other studies in grape correlate DXS activity with monoterpene content (Battilana et al., 2009; Duchêne et al., 2009; Emanuelli et al., 2010; Dalla Costa et al., 2018). Indeed, the well-appreciated floral flavor of Muscat varieties is associated with the presence of monoterpenoids, such as linalool, geraniol, nerol, citronellol, and α-terpineol (Ribéreau-Gayon et al., 1975). Interestingly, a major QTL for monoterpene production in Muscat varieties co-localized with *VvDXS1* (Battilana et al., 2009; Duchêne et al., 2009). Emanuelli et al. (2010) assessed the association between nucleotide variation in the *DXS* sequence and Muscat flavor, and found a non-synonymous amino acid difference in over 95% of the Muscat-flavored genotypes studied.

Dalla Costa et al. (2018) confirmed by analyzing a grapevine germplasm collection that the nucleotide changes in the sequence of *VSDXS1* were able to explain the differential accumulation of monoterpenes in Muscat and non-Muscat grape varieties. In addition, this genetic variation seemed to also modulate sesquiterpene content. Wild-type Muscat *VvDXS1* (with increased catalytic efficiency) alleles were overexpressed in the "microvine,", "Chardonnay," and "Brachetto" cultivars. At flowering, the transgenic plants showed the upregulation of the *HDR* gene and of three genes of the MVA pathway compared to those of the control plants, suggesting that both pathways could be more deeply integrated than previously thought (Chaurasiya et al., 2012; Dalla Costa et al., 2018). In addition, genes from the downstream carotenoid, monoterpene, and sesquiterpene pathways were slightly upregulated compared to those of the control plants. During veraison and at grape maturity, most of the MEP and MVA pathway genes were upregulated, together with the genes involved in the first steps of the carotenoid pathway compared to those of the control plants, while at maturity, few changes were observed between the transgenic plants and the control plants. When the fruits were ready to

harvest, the monoterpene content was significantly higher in the transgenic plants than in the control plants, and the increase was more pronounced in the plants overexpressing the Muscat allele, most likely due to its improved catalytic performance (Dalla Costa et al., 2018). This is another example of the ectopic expression of *DXS1* supporting metabolic flux through the MEP pathway to improve the volatile content of the fruit. Additionally, it was possible to increase final metabolites levels by searching for alleles with better catalytic efficiency.

Engineering for Tocopherol Content

Tocopherols are lipid-soluble antioxidants that are exclusively synthesized by photosynthetic organisms. They have a high nutritional value, as several of them show vitamin E activity (Pryor et al., 2000). These compounds contain a chromanol group derived from homogentisate and an isoprenoid-derived chain resulting from the MEP pathway. Homogentisate is derived from the shikimate pathway, where its formation is catalyzed by 4-hydroxyphenylpyruvate dioxygenase (HPPD) in the first step of the tocopherol-core pathway. *HPPD* expression increased along with the accumulation of tocopherol and carotenoid levels during mango fruit ripening (Singh et al., 2011). However, neither *HPPD* nor *DXS* overexpression alone in tomato was sufficient to redirect the flux toward tocopherol synthesis. The expression of another gene, *geranylgeranyl reductase (GGDR)*, which is responsible for the availability of the ultimate tocopherol precursor, phytyl-diphosphate, was correlated with an increase in vitamin E content in mature green tomato fruit and was a bottleneck for the isoprenoid precursor availability (Quadrana et al., 2013). In addition, an analysis of different tomato genotypes noted that the genotypes containing high-tocopherol levels presented attenuated carotenoid levels, suggesting that precursor competition is the main limiting factor for vitamin E synthesis (Quadrana et al., 2013). As a consequence, metabolic engineering for enhanced tocopherol content in fruit should include the manipulation of both structural genes from the core tocopherol-core pathway and the limiting reactions involved in precursor synthesis.

CONCLUSION

Metabolic flux through primary metabolism leads to the synthesis of several key intermediates necessary for the synthesis of the huge diversity of specialized compounds involved in fruit organoleptic and nutritional characteristics. Unfortunately, a limitation of primary metabolism engineering involves the lack of a correlation between primary metabolites pools and the levels of secondary metabolites that are derived from them. The emergence of new approaches like -omics (i.e., metabolomics and next-generation sequencing) technologies will allow us to combine multi-level transcriptional regulation and pathway rerouting to facilitate the metabolic engineering for fruit biofortification of these compounds. As described in this review, several approaches including crossing techniques and transformation could achieve the metabolic engineering to favor the accumulation of these specific beneficial metabolites derived from primary metabolism. In this sense, the use of genetically modified plants, overexpressing the genes responsible for the limiting steps in the interface between primary and secondary metabolism, is an essential tool, allowing a better understanding of the regulatory network that controls metabolic flux. Furthermore, the identification of natural allelic variation within germplasm is a powerful approach for fruit quality improvement in breeding programs, as it bypasses transgenic plant practice. Since obtaining high quality fruits has become one of the primary goals of breeding programs, application of these natural polymorphisms in marker-assisted selection can allow the rapid selection of varieties with enhanced organoleptic and nutritional characteristics and lead to greater economic returns for the industry.

AUTHOR CONTRIBUTIONS

All authors did the literature research, drafted the review, and helped write the final manuscript.

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REVIEW
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Fruit Salad in the Lab: Comparing Botanical Species to Help Deciphering Fruit Primary Metabolism

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Although fleshy fruit species are economically important worldwide and crucial for human nutrition, the regulation of their fruit metabolism remains to be described finely. Fruit species differ in the origin of the tissue constituting the flesh, duration of fruit development, coordination of ripening changes (climacteric vs. non-climacteric type) and biochemical composition at ripeness is linked to sweetness and acidity. The main constituents of mature fruit result from different strategies of carbon transport and metabolism. Thus, the timing and nature of phloem loading and unloading can largely differ from one species to another. Furthermore, accumulations and transformations of major soluble sugars, organic acids, amino acids, starch and cell walls are very variable among fruit species. Comparing fruit species therefore appears as a valuable way to get a better understanding of metabolism. On the one hand, the comparison of results of studies about species of different botanical families allows pointing the drivers of sugar or organic acid accumulation but this kind of comparison is often hampered by heterogeneous analysis approaches applied in each study and incomplete dataset. On the other hand, cross-species studies remain rare but have brought new insights into key aspects of primary metabolism regulation. In addition, new tools for multi-species comparisons are currently emerging, including meta-analyses or re-use of shared metabolic or genomic data, and comparative metabolic flux or process-based modeling. All these approaches contribute to the identification of the metabolic factors that influence fruit growth and quality, in order to adjust their levels with breeding or cultural practices, with respect to improving fruit traits.

Keywords: amino acids, cross-species, fleshy fruit, inter-species, metabolism regulation, organic acids, primary metabolism, sugars

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INTRODUCTION

Fresh fruits (866 Mt worldwide in 2016[1]) and their derived products are economically important. Besides their energetic role in human diet linked notably with their carbohydrate contents, they are also crucial for human nutrition and health, especially in relation with their contents in vitamins, anti-oxidants and fibers (Baldet et al., 2014; Rodriguez-Casado, 2016; Wang et al., 2016;

[1] www.fao.org/faostat

Aune et al., 2017; Padayachee et al., 2017). In fruit tissues, primary metabolism can be defined as the biochemical processes that are necessary for growth and development and shared by a large number of taxonomic groups (Verpoorte, 2000), and produces metabolites that are generally essential for organism survival (Aharoni and Galili, 2011). It contributes to flesh growth and ripening, and final fruit quality, particularly sweetness and acidity. Its operation varies according to botanical species and developmental stages. Different botanical species may differ in the composition of the phloem sap originating from leaves and unloaded into the fruit, the occurrence of transient starch accumulation during development, the hormonal orchestration of ripening changes, and the major metabolites accumulated at ripening (**Figure 1**). All these aspects are linked with primary metabolic pathways involving carbohydrates, organic acids, and amino acids. These pathways are regulated along fruit development that can last from a few dozens to more than 200 days-post-anthesis (DPA), from fruit set to ripe fruit, depending on the species (**Table 1**). Early development stages after fruit set are usually characterized by a high concentration of organic acids whereas ripening is associated with soluble sugar accumulation (Famiani et al., 2015; Beauvoit et al., 2018). However, the regulation of metabolic pathways is not that simple.

Studies dealing with fruit metabolism include biochemical analyses of metabolites from targeted analyses to metabolomics in tissues or sap, measurement of enzyme activity and regulation, transcriptomics, map-based cloning or genome (re)sequencing. Several of these approaches can be combined for one cultivar across fruit development or use a large collection of genetic resources. This review focuses on comparing species for the programming and integration of primary metabolic pathways with growth and fruit quality, mostly for temperate fruits. Such comparisons should help identifying key regulation points, for instance regarding the trade-offs between fruit yield and quality, and possibly propose hypotheses for breeding or agricultural practices.

SIMILARITIES AND DISSIMILARITIES ARE NOTICED IN THE COMPOSITION OF PRIMARY METABOLITES IN FRUITS

Fruit taste is strongly influenced by sweetness and acidity, which are associated to sugars and organic acids, respectively. Sugars are abundant in most fleshy fruits; so much that fructose has been named after them (i.e., after the Latin *fructus*). Besides, several amino acids influence the so-called umami taste. The composition and concentrations of these major constituents of the ripe fruit vary according to species (**Table 1**).

Soluble Sugars and Organic Acids

Concerning soluble sugars, hexoses are usually more abundant than sucrose. This is the case for most berries, e.g., raspberries, blackberry (Mikulic-Petkovsek et al., 2012) and grape berry (Dai et al., 2013), as well as kiwifruit (Richardson et al., 2011), pepper (Osorio et al., 2012), eggplant (Makrogianni et al., 2017), and cherry (Wills et al., 1983; Usenik et al., 2008). In some species, fructose is more abundant than glucose and sucrose, such as in apple and pear (Drake and Eisele, 1999) or blackcurrant (Mikulic-Petkovsek et al., 2012). However, there are also species in which sucrose is the most abundant sugar, such as mandarin (Legua et al., 2014), peach (Wills et al., 1983), watermelon (Gao et al., 2018), melon (Wang et al., 1996), and hardy kiwi (Klages et al., 1998; Mikulic-Petkovsek et al., 2012). Finally, some species contain sucrose in almost the same proportion as hexoses, for instance several cultivars of litchi (*Litchi chinensis*) (Wang et al., 2006) and cultivated as well as wild strawberry (Moing et al., 2001; Mikulic-Petkovsek et al., 2012).

Sugar alcohols are a major component for some fruit species. Sorbitol, which is common in Rosaceae trees, is present in notable quantities in the developing peach and apricot fruit (Bae et al., 2014). It is also one of the main sugars in ripe chokeberry, rowanberry and eastern shadbush (Mikulic-Petkovsek et al., 2012). Another sugar-alcohol, *myo*-inositol, is present in the early stages of kiwifruit and hardy kiwi development (Klages et al., 1998).

Concerning organic acids, a recent review (Famiani et al., 2015) and research study (Mikulic-Petkovsek et al., 2012) listed the main organic acids found in fruits of more than 50 species. Citrate and malate are the major organic acids in many fruit species. Typically, young fruits are likely to accumulate malate, which will tend to be replaced by citrate at ripening (McFeeters et al., 1982; Flores et al., 2012). Thus, species such as lime, orange, raspberry, strawberry, blueberry, and melon (Wang et al., 1996) accumulate high levels of citrate, while other species such as apple, cherry, chokeberry, rowanberry, eastern shadbush, watermelon (Gao et al., 2018), and eggplant (Kozukue et al., 1978) build up in malate. Other species, e.g., pear, apricot, goji berry, and blackcurrant accumulate both organic acids. In some cases, other organic acids are also overrepresented, as for example isocitrate in blackberry, tartrate in grape berry and lychee, or quinate in kiwifruit and hardy kiwi (Kim et al., 2012).

Large compositional differences for major compounds have been reported between domesticated species and wild relatives. For instance, the tomato domesticated species (*Solanum lycopersicum* L.) accumulates hexoses whereas several wild species (*Solanum neorickii*, *Solanum chmielewskii*, *Solanum habrochaites*) accumulate sucrose as the major sugar (Yelle et al., 1988; Schauer et al., 2004). Furthermore, wild tomato species (*Solanum pennellii*, *S. neorickii*, *S. chmielewskii*, *S. habrochaites*) were found to accumulate higher levels of malate and citrate (Schauer et al., 2004; Steinhauser et al., 2010) than cultivated tomatoes. Similarly, the domestication of mandarin led to a strong decrease in citrate (Wang et al., 2018). Large compositional differences may even be found between cultivars of a given species. An example is given for acidic lemon and lime, where glucose and sucrose are the major sugars, and citrate the main organic acid, whilst in acidless lemon and lime, fructose is the major sugar and citrate, malate and quinate are equally present (Albertini et al., 2006).

Amino Acids

Regarding amino acids, there are also differences according to botanical species. For berries such as strawberry, glutamine,

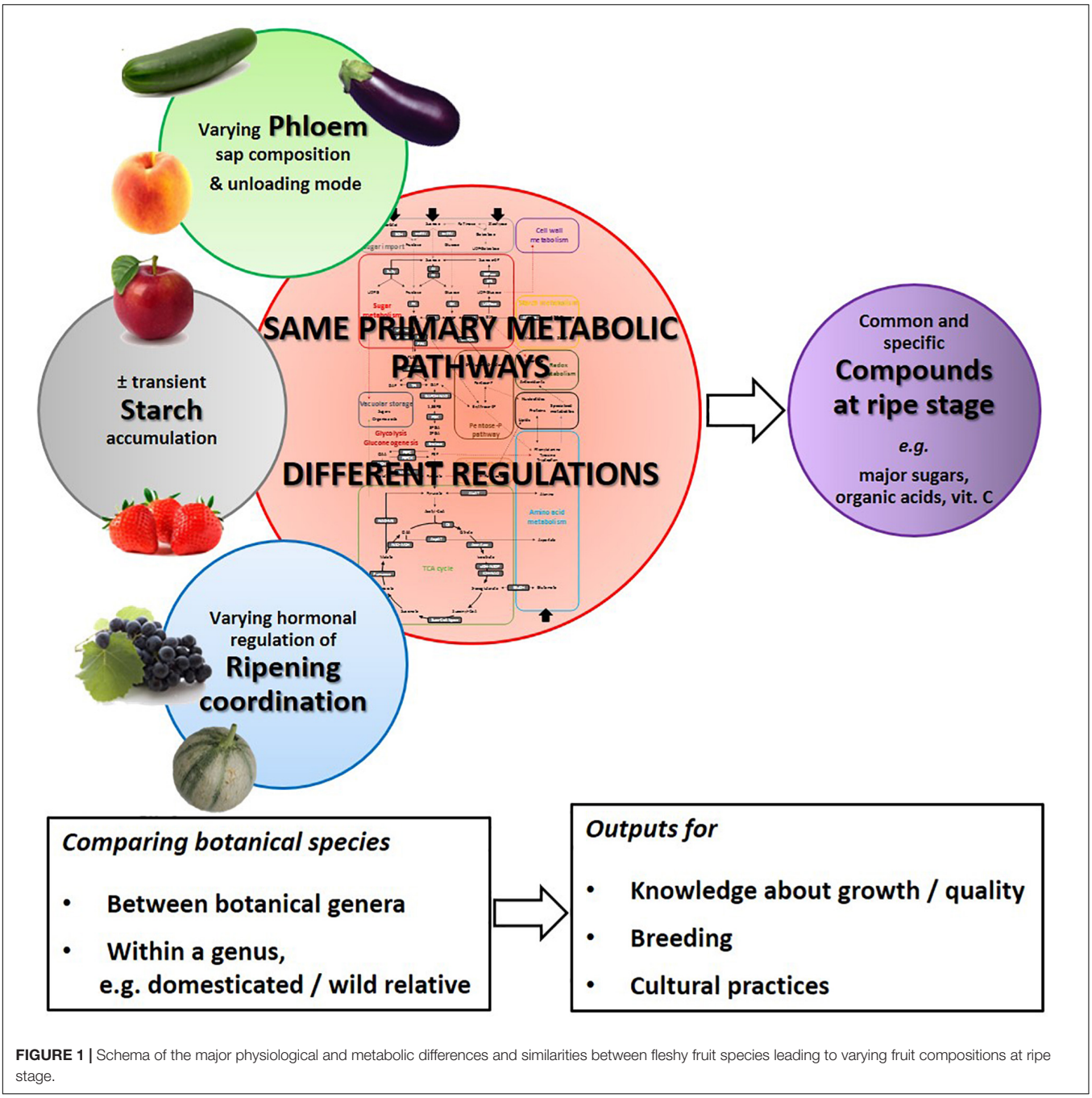

FIGURE 1 | Schema of the major physiological and metabolic differences and similarities between fleshy fruit species leading to varying fruit compositions at ripe stage.

and asparagine are the most abundant ones whereas blackberry accumulate asparagine and glutamate. For blackcurrant, orange, and lemon glutamine is the major amino acid besides alanine (Burroughs, 1960; Brückner and Westhauser, 1994). The latter one is also abundant in raspberry with serine (Burroughs, 1960). In grape berry, proline, arginine, glutamine and alanine dominate in berry skin, while proline, alanine and γ-aminobutyric acid (GABA) dominate in berry pulp (Guan et al., 2017). For several Rosaceae trees, asparagine dominates with glutamate and aspartate in apple (Zhang et al., 2010), with serine in pear (Chen et al., 2007), and with glutamate and proline in peach (Moing et al., 1998). Concerning Solanaceae, asparagine, GABA, and proline seem to be the predominant amino acids in pepper (Osorio et al., 2012). Glutamine and glutamate are the major ones in domesticated tomatoes (*S. lycopersicum*) together with GABA which is also present in high quantities (Schauer et al., 2004). However, two wild tomato species largely differ according to the latter authors: *S. habrochaites* harbors a high tryptophan content, and *S. pennellii* a high GABA pool, five times higher than in the domesticated species. In kiwifruit, aspartate is the main amino acid (Nardozza et al., 2013).

TABLE 1 | Major characters linked with primary metabolism and differing between temperate fruit species.

Botanical family	Species	Fruit development duration (DPA)	Climacteric ripening [a]	Major phloem transported sugar[a]	Transient starch storage	Major soluble sugars in ripe fruit	Major organic acids in ripe fruit	Major amino acids in ripe fruit	References
Actinidiaceae	Kiwifruit *Actinidia deliciosa Actinidia chinensis*	237	Yes[2]	Sucrose	Yes	Glucose/fructose	Quinate/citrate	Aspartate	Chen et al., 2017; Klages et al., 1998; Nardozza et al., 2013; Richardson et al., 2011
	Hardy kiwifruit (kiwai) *Actinidia arguta*	140	Yes		Yes	Sucrose	Quinate/citrate	–	Kim et al., 2012; Klages et al., 1998; Mikulic-Petkovsek et al., 2012
Cucurbitaceae	Cucumber *Cucumis sativus*	20	No[2]	Raffinose/ stachyose/ sucrose[4]		Fructose/glucose	Malate (commercial)/ citrate (physiological)	–	Hu et al., 2009; McFeeters et al., 1982
	Melon *Cucumis melo*	48	Yes for cantaloupe[2] No for honeydew[1]	Raffinose/ stachyose/ sucrose		Sucrose	Citrate	Glutamate/ glutamine/ GABA	Mitchell et al., 1992; Wang et al., 1996
	Watermelon *Citrullus lanatus*	55	No[2]	Raffinose/ stachyose/ sucrose[3]		Sucrose	Malate		Gao et al., 2018; Zhang and Ge, 2016
Ericaceae	Blueberry *Vaccinium corymbosum*		Yes[2]			Glucose/fructose	Citrate		Mikulic-Petkovsek et al., 2012
Grossulariaceae	Blackcurrant *Ribes nigrum*					Glucose/fructose	Citrate	Glutamine/ α-alanine	Burroughs, 1960; Mikulic-Petkovsek et al., 2012
	Redcurrant *Ribes rubrum*					Glucose/fructose	Citrate	Glutamine/ α-alanine	Burroughs, 1960; Mikulic-Petkovsek et al., 2012
Rosaceae	Apple *Malus domestica*	160	Yes[2]	Sucrose/sorbitol[4]	Yes	Fructose	Malate	Asparagine/ aspartate/ glutamate	Brookfield et al., 1997; Drake and Eisele, 1999; Zhang et al., 2010
	Apricot *Prunus armeniaca*	65	Yes[2]	Sucrose/sorbitol[4]		Glucose/fructose (cv. Harcot) sucrose (cvs Bavinity and Trevatt)	Citrate/malate		Bae et al., 2014; Wills et al., 1983
	Blackberry *Rubus fruticosus*		Yes/no			Glucose/fructose	Isocitrate	Asparagine/ glutamate	Burdon and Sexton, 1993; Burroughs, 1960; Mikulic-Petkovsek et al., 2012; Whiting, 1958
	Sweet cherry *Prunus avium*		No[2]	Sucrose/ sorbitol[4]		Glucose/ fructose	Malate		Usenik et al., 2008; Wills et al., 1983
	Chokeberry *Aronia melanocarpa*					Sorbitol/glucose	Malate		Mikulic-Petkovsek et al., 2012
	Eastern shadbush *Amelanchier canadensis*					Glucose/fructose/ sorbitol	Malate		Mikulic-Petkovsek et al., 2012

(Continued)

TABLE 1 | Continued

Botanical family	Species	Fruit development duration (DPA)	Climacteric ripening [a]	Major phloem transported sugar[a]	Transient starch storage	Major soluble sugars in ripe fruit	Major organic acids in ripe fruit	Major amino acids in ripe fruit	References
	Peach *Prunus persica*	125	Yes[2]	Sucrose/sorbitol[4]	Yes	Sucrose	Malate/citrate	Asparagine/ glutamate/ proline	Moing et al., 1998; Wills et al., 1983
	Pear *Pyrus communis Pyrus pyrifolia*		Yes[2]	Sucrose/sorbitol[4]	Yes	Fructose	Malate/citrate	Asparagine/serine	Chen et al., 2007; Drake and Eisele, 1999; Mesa et al., 2016
	Prune *Prunus domestica*			Sucrose/sorbitol[4]		Glucose/ fructose/ sucrose (cultivar dependent)	Malate		Wills et al., 1983
	Plum *Prunus salicina*	91	Yes/no			Glucose/fructose	Quinate		Bae et al., 2014
	Raspberry *Rubus idaeus*		No[2]			Glucose/fructose	Citrate	Serine/α-alanine	Burroughs, 1960; Mikulic-Petkovsek et al., 2012
	Rowanberry *Sorbus aucuparia*					Sorbitol	Malate		Mikulic-Petkovsek et al., 2012
	Strawberry *Fragaria x ananassa*	39	No[2]	Sucrose[3]	Yes	Glucose/fructose or glucose/fructose/ sucrose cultivar dependent	Citrate	Asparagine/ glutamine	Burroughs, 1960; Mikulic-Petkovsek et al., 2012; Moing et al., 2001; Souleyre et al., 2004
	Wild strawberry *Fragaria vesca*					Glucose/ fructose/ sucrose	Citrate		Mikulic-Petkovsek et al., 2012
Rutaceae	Clementine/mandarine *Citrus clementina Citrus unshiu*		No[2]		No	Sucrose	Citrate		Legua et al., 2014; Mehouachi et al., 1995
	Acidic lemon *Citrus limon*	150	No[2]			Glucose/sucrose	Citrate		Albertini et al., 2006
	Acidless lemon *Citrus limon*	150	No[2]			Fructose	Citrate/ malate/ quinate	Glutamate/alanine	Albertini et al., 2006; Brückner and Westhauser, 1994
	Acidic lime *Citrus latifolia*	150	No[2]			Glucose/sucrose	Citrate		Albertini et al., 2006
	Acidless lime *Citrus limettioides*	150	No[2]			Fructose	Citrate/malate/ quinate		Albertini et al., 2006
	Acidic orange *Citrus sinensis*	150	No[2]			Glucose/fructose	Citrate		Albertini et al., 2006
	Acidless orange *Citrus sinensis*	150	No[2]	Sucrose		Fructose	Quinate	Glutamate/alanine	Albertini et al., 2006; Brückner and Westhauser, 1994; Hijaz and Killiny, 2014
Solanaceae	Eggplant *Solanum melongena*		Yes/No[2]			Glucose/fructose	Malate		Kozukue et al., 1978; Makrogianni et al., 2017
	Pepper *Capsicum chilense*	70	Yes/no[1]			Glucose/fructose	Citrate (red) Malate (green)	GABA/proline/ asparagine	Flores et al., 2012; Osorio et al., 2012

(Continued)

TABLE 1 | Continued

Botanical family	Species	Fruit development duration (DPA)	Climacteric ripening [a]	Major phloem transported sugar[a]	Transient starch storage	Major soluble sugars in ripe fruit	Major organic acids in ripe fruit	Major amino acids in ripe fruit	References
	Goji berry *Lycium barbarum*					Glucose/fructose	Citrate/malate		Mikulic-Petkovsek et al., 2012
	cultivated tomato, *Solanum lycopersicum*	40–60	Yes[1,2]	Sucrose[4]	Yes	Glucose/fructose	Citrate/malate	Glutamate/ glutamine	Schaffer and Petreikov, 1997; Schauer et al., 2004
	Wild tomato, *S. neorickii*, *S. chmielewskii*, *S. habrochaites S. pennellii*	40–60				Sucrose	Citrate/malate	Tryptophan (*S. habrochaites*) Aspartate (*S. chmielewskii*) Pyoglutamate/ aspartate (*S. neorickii*) GABA (*S. pennellii*)	Schauer et al., 2004
Vitaceae	Grape berry *Vitis vinifera*	100–110	No[2]	Sucrose	Little or no	Glucose/fructose	Malate/tartrate	Proline/alanine/ GABA	Dai et al., 2013; Guan et al., 2017; Ollat et al., 2002; Swanson and El-Shishiny, 1958

Fruit development duration in days post-anthesis (DPA), ripening type (climacteric or non-climacteric) and compositional characteristics of phloem sap and ripe fruit. [a] *Defined according to Barry and Giovannoni (2007)*[1], *Paul et al. (2012)*[2], *Rennie and Turgeon (2009)*[3], *Fu et al. (2011)*[4].

PHLOEM LOADING AND UNLOADING STRATEGIES DIFFER AMONG FRUITS

Fruits are strong sinks attracting plenty of photoassimilates transported from leaves via phloem. From photosynthetic site to sink, photoassimilates need at least three transporting steps, including phloem loading, phloem long-distance transport, and phloem unloading. The strategies of phloem loading, unloading, and the transported forms of carbon are diverse (Rennie and Turgeon, 2009; Braun et al., 2013). Sucrose is the main photoassimilate transported in phloem in most fruit species such as cultivated tomato, grape, sweet orange and cultivated strawberry (Swanson and El-Shishiny, 1958; Rennie and Turgeon, 2009; Fu et al., 2011; Hijaz and Killiny, 2014). However, several fruit species of the Cucurbitaceae family, such as cucumber, watermelon, and melon also transport oligosaccharides including raffinose and stachyose, in higher or equal concentration than sucrose (Mitchell et al., 1992; Rennie and Turgeon, 2009; Fu et al., 2011). Tree species from the Rosaceae family, such as apple, peach, plum or prune, apricot and sweet cherry also transport sugar alcohols (e.g., sorbitol) (Rennie and Turgeon, 2009; Fu et al., 2011). For example, sorbitol can account for 60–90% of the carbon transported in phloem in peach tree (Moing et al., 1997).

Phloem Loading

Sucrose can be loaded into phloem by three loading strategies, including active apoplasmic, active symplasmic (also called polymer trapping), and passive symplasmic routes (Rennie and Turgeon, 2009; Fu et al., 2011). Active apoplasmic loaders normally have low-abundant plasmodesmata in leaf and require the presence of sucrose transporters (SUTs) and hexose and sucrose transporters (SWEETs), such as in tomato leaf (Fu et al., 2011; Jensen et al., 2016; Liesche and Patrick, 2017). In active symplasmic loaders, sucrose diffuses into the companion cells through abundant plasmodesmata, and is enzymatically converted into sugar oligomers (e.g., raffinose and stachyose), which are molecularly larger and cannot diffuse back to phloem parenchyma, forming a polymer trapping mechanism. Fruit species of Cucurbitaceae family, such as cucumber, watermelon and melon, are active symplasmic loaders (Rennie and Turgeon, 2009; Fu et al., 2011). Passive symplasmic loading requires abundant plasmodesmata to allow sucrose diffusion or convection from mesophyll cells to sieve elements following the sugar concentration gradient. Strawberry is a passive loader, and grape is a candidate passive loader (Rennie and Turgeon, 2009). The phloem loading strategies for sugar-alcohols (e.g., sorbitol, mannitol) can be active apoplasmic or passive symplasmic (Reidel et al., 2009). Most fruit trees of Rosaceae family, including apple, apricot, sweet cherry, peach, and pear are passive symplasmic loaders (Reidel et al., 2009; Fu et al., 2011). Multi-species comparison analysis showed that active loading is associated with efficient water conduction and maximized carbon efficiency and growth, while the reverse is true for passive loading (Fu et al., 2011). A meta-analysis of 41 species with a modeling approach further showed that phloem sugar concentrations are in average at 21.1% for active loaders and 15.4% for passive

loaders (Jensen et al., 2013). The theoretical optimum sugar concentration in phloem sap proposed was 23.5%. Organic acids are also transported in phloem (Fiehn, 2003), although references are rare. Amino acids, for instance arginine and glycine in grapevine (Gourieroux et al., 2016), also use phloem as the main transport route from source to sink, and are also transported in xylem sap (Tegeder and Hammes, 2018).

Phloem Unloading

In fruit sinks, photoassimilates (sucrose, sugar-alcohols, or oligosaccharides) need to be unloaded following symplasmic or apoplasmic pathways (Braun et al., 2013). In apple and cucumber fruits, phloem unloading is apoplasmic throughout fruit development (Zhang et al., 2004; Hu et al., 2011). In several fruits, shifts between the two phloem unloading strategies can occur during development. Tomato and grape fruits operate symplasmic unloading during early development stage when soluble sugar is low, and switches to apoplasmic unloading during fruit ripening when soluble sugars accumulate (Ruan and Patrick, 1995; Patrick, 1997; Zhang et al., 2006). For kiwifruit, Chen et al. (2017) showed that sucrose phloem unloading occurs mainly through the apoplasmic route along fruit development (44–135 days after blooming). However, Gould et al. (2013), working from 22 to 200 days after anthesis, found that phloem unloading dominantly appeared via symplasmic route in early fruit development, while an apoplasmic route becomes important during the later developmental stages. The dominant symplastic import of sugar at the initial stages of fruit development allows a high inflow of carbon input via mass flow. Shifting from symplasmic to apoplasmic unloading during fruit ripening limits back-flow of assimilates from fruit sink to sieve elements and likely facilitates sugar accumulation to high concentrations in fruit tissues (Ruan and Patrick, 1995; Patrick, 1997; Zhang et al., 2006). For amino acid unloading, most plant species follow a symplasmic process driven by a downhill concentration gradient (Tegeder and Hammes, 2018). The question whether and how loading strategies, carbon loading forms, and unloading strategies influence fruit growth and quality are still under debate.

PRIMARY METABOLISM PATHWAYS ARE DIFFERENTIALLY REGULATED

Sugar and Sugar-Alcohol Metabolism

In most fruits, the main source of carbon is imported via phloem in form of sucrose, which can be degraded via the reactions catalyzed by cell wall invertase in the apoplast, neutral invertase or sucrose synthase following symplastic import into the cytosol, or acid invertase following subsequent import into the vacuole. Carbon import patterns are highly variable from one species to another. For example, in sweet pepper both vacuolar and neutral invertases have been proposed as determining carbon import at young stages (Nielsen et al., 1991). This contrasts with kiwifruit, in which sucrose synthase has been proposed as controlling most of the carbon import in growing fruits (Chen et al., 2017). In the same species, the previous measurement of neutral invertase and sucrose synthase cytosolic enzymes seemed in agreement with symplastic phloem unloading throughout fruit development before ripening (Nardozza et al., 2013). Parietal invertase has been found to impact significantly tomato sugar content at maturity (Fridman et al., 2004). A low level of acid invertase activity and the absence of sucrose synthase activity in *S. chmielewskii*, a wild tomato species, were associated with the high content of sucrose (Yelle et al., 1988). In contrast to *S. lycopersicum*, the capacity of most enzymes of glycolysis and the tricarboxylic acid (TCA) cycle of *S. pennellii*, which also accumulates hexoses, is maintained and increases even during the ripening of the fruit, probably reflecting the fact that the fruit continues to grow until maturity (Steinhauser et al., 2010). For sorbitol-transporting fruit species, imported sorbitol is converted into fructose by sorbitol dehydrogenases (Park et al., 2002). In apple, for instance, fructose is stored in the vacuole or metabolized (Berüter et al., 1997). Sorbitol oxidase (Lo Bianco and Rieger, 2002) and sorbitol-6-phosphate dehydrogenase (S6PDH) (Ohkawa et al., 2008) may also play a role in sorbitol metabolism in Rosaceae fruit trees. For Cucurbitaceae, imported raffinose and stachyose are rapidly metabolized via a pathway that includes enzymes of sugar hydrolysis, phosphorylation, transglycosylation, nucleotide sugar metabolism, sucrose cleavage and synthesis, with an initial implication of α-galactosidases (Dai et al., 2011).

In the cytosol, hexoses resulting from import, degradation or export from the vacuole are phosphorylated via the reactions catalyzed by hexokinases (both hexoses) or fructokinases (fructose only). It has been proposed that the high capacities found for these enzymes in young growing fruits promote high fluxes through glycolysis (Biais et al., 2014). Hexoses phosphates are partitioned between cytosol and plastids, although, unlike leaf plastids, fruit plastids are capable of importing hexoses phosphates (Batz et al., 1995; Butowt et al., 2003). Fructose-6-phosphate is phosphorylated via the reaction catalyzed by ATP- or pyrophosphate-dependent phosphofructokinases (only the former is found in both cytosol and plastid), which enables its breakdown via glycolysis in both compartments. Results obtained in banana suggest that these enzymes are inhibited by phospho*enol*pyruvate (PEP) via allosteric feedback, indicating that there is a crossed glycolytic flux control between PEP and fructose-6-phosphate, which activates PEP carboxylase (Turner and Plaxton, 2003). In both the cytosol and plastid, glucose-6-phosphate tends to equilibrate with fructose-6-phosphate and glucose-1-phosphate via the reaction catalyzed by phosphoglucose isomerase and phosphoglucomutase, respectively, which are present in both compartments. In the cytosol, glucose-1-phosphate is the precursor of uridine diphosphate glucose (UDP-glucose, via the reaction catalyzed by UDP-glucose pyrophosphorylase), precursor of cell wall (Reiter, 2002, 2008; Mohnen, 2008), ascorbate and sucrose (Reiter and Vanzin, 2001). In the chloroplast, glucose-1-phosphate is converted into adenosine diphosphate glucose (ADP-glucose), the precursor of starch. In most fruits, the acquisition of sweetness at maturation is the result of important metabolic changes leading to sugar accumulation (Bonghi and Manganaris, 2012). Of these, starch degradation is often a major source of sugars and

energy as detailed below. Finally, sugar vacuolar storage is probably one of the most important, although overlooked, features regarding fruit sweetness. In particular, modeling sugar metabolism in tomato fruit suggested that tonoplastic sucrose and hexose transporters are major control points that condition fruit sugar content (Beauvoit et al., 2014), in line with dramatic alterations in fruit sugar accumulation provoked by the overexpression of a tonoplast transporters in melon (Cheng et al., 2018).

Organic Acid Metabolism

Malate, citrate, quinate, and tartrate constitute the four main organic acids accumulated to high levels in the vacuoles of fleshy fruits, during their development (DeBolt et al., 2006; Richardson et al., 2011; Tril et al., 2014; Hussain et al., 2017). In fruit, malate is mostly synthetized by the pyruvate kinase bypass, which involves the irreversible carboxylation of phosph*enol*pyruvate into oxaloacetate (OAA) by phosph*enol*pyruvate carboxylase, and OAA is subsequently reduced to malate by cytosolic NAD-dependent malate dehydrogenase (Sweetman et al., 2009; Yao et al., 2011). Citrate is produced from OAA by the TCA pathway, operating in a non-cyclic mode, which is known to take place in plants (Sweetlove et al., 2010) and evidenced in citrus fruits (Katz et al., 2011) with the involvement of mitochondrial citrate synthase (Sadka et al., 2001). Quinate is produced at a branch point of the shikimate biosynthesis pathway by the enzyme quinate dehydrogenase (Marsh et al., 2009; Gritsunov et al., 2018). It is a precursor of chlorogenic acids that are major specialized metabolites in a range of fruit species. Tartrate synthesis results from L-ascorbic acid catabolism through the Smirnoff-Weelher pathway (Melino et al., 2009). L-idonate dehydrogenase, which catalyzes a step in this pathway, is present in grape during the green stage of berry development, concomitantly with the tartrate synthesis peak (DeBolt et al., 2006). Once produced, organic acids are stored into flesh cell vacuoles thanks to an acid trap mechanism, which relies on (i) the existence of a strong pH difference between the cytosol (neutral or slightly alkaline) and the vacuole (highly acidic, pH down to 2.5 in citrus) and (ii) the existence of passive di- and tri-anions transporters on the tonoplast (De Angeli et al., 2013; Etienne et al., 2013). For citrate, the existence of a proton coupled active symporter, CsCit1, has also been reported (Shimada et al., 2006). The regulation of vacuolar malate storage has recently begun to be deciphered (Jia et al., 2018). Once the ripening phase starts, organic acids exit the vacuole and are metabolized to (i) fuel the respiration increase linked to climacteric crisis in climacteric fruits (Colombié et al., 2015) or to meet higher energy demand in non-climacteric fruits such as grapes (Sweetman et al., 2009), or (ii) produce hexoses by neoglucogenesis (Walker et al., 2015; Famiani et al., 2016).

Amino Acid Metabolism

Amino acid accumulation in developing fruits is the result of both import from phloem and xylem translocation, and *in situ* synthesis (Beshir and Mbong, 2017; Wang L. et al., 2017; Mechthild and Hammes, 2018). Several enzymes of amino acid biosynthesis, including, among others, glutamine synthetase, asparagine synthetase, alanine aminotransferase, and methionine synthase have been detected in global proteomic studies in developing grape berries (Wang G. et al., 2017) or by ^{13}C-based flux variance analysis in apple (Beshir and Mbong, 2017). Beside the classical 20 amino acids, fruits can also produce other, non-proteogenic amino acids, such as GABA, which is synthesized through the GABA shunt (Bouché and Fromm, 2004), and possibly β-aminobutyric acid (Thevenet et al., 2017), or citrulline for instance in cucurbits (Fish and Bruton, 2010) including melon (Bernillon et al., 2013) that is produced from arginine (Joshi and Fernie, 2017). Amino acids are not just bricks to build protein in fruits, but also contribute to the global organoleptic qualities of fruits. For example, levels of glutamate contribute to the so-called "umami" taste of tomato (Kurihara, 2015). Amino acid catabolism has been particularly studied in fruits, as it produces numerous quality-related compounds. Phenylalanine leads to the production of polyphenols through the phenylpropanoid pathway, which have antioxidant properties and are health-promoting compounds (Butelli et al., 2008; Cirillo et al., 2014). It is also the starting point of volatile aromas (3-phenylpropanol, 2-phenethylacetate) in melon fruit (Gonda et al., 2018). Isoleucine was shown to be the precursor for 2-methylbutyl ester aromas in strawberry (Pérez et al., 2002) and methoxypyrazines in grape berries (Guillaumie et al., 2013). Thus, amino acid metabolism is a key determinant of fruit quality and palatability.

Cell Walls and Specialized Metabolites

Fruit primary metabolism also provides building blocks for the synthesis of cell-walls, and non-volatile specialized metabolites (Verpoorte, 2000) besides those mentioned above (e.g., flavonoids, alkaloids, anthocyanins, isoprenoids). Primary cell-wall precursors are mainly supplied as nucleoside diphosphate (NDP) derivatives to produce cellulose, hemicelluloses and pectins (Reiter, 2002, 2008; Mohnen, 2008). Secondary cell-wall lignin precursors, monolignols, are produced by the phenylpropanoid pathway (Zhong and Ye, 2015). Flavonoid and anthocyanin precursors are 4-coumaroyl-coenzyme A (4-coumaroyl-CoA) and malonyl-CoA molecules condensed by chalcone synthase (Jaakola, 2013). Alkaloids are a diverse family of specialized metabolites and are synthesized from various precursors. For instance, steroidal alkaloids of tomato fruit derive from cholesterol (Itkin et al., 2013), whereas tropane alkaloids of deadly nightshade come from arginine and ornithine (Sato et al., 2001). Carotenoids come from both the mevalonic (MVA) and the MVA-independent pathway. Their precursor isopentenyl-diphosphate is either produced from acetyl-CoA or pyruvate and glyceraldehyde-3-phosphate (Fraser and Bramley, 2004). Furthermore, most of these specialized metabolites are decorated with sugars and organic acids. Specialized metabolites have a role in plant defense, but their biosynthesis has a metabolic cost. Thus, allocation theory has been developed to explain resource-based trade-off between plant physiological functions (Bazzaz et al., 1987)

and was confirmed experimentally at the plant level (Caretto et al., 2015).

STARCH DOES NOT ALWAYS ACCUMULATE TRANSIENTLY DURING FRUIT DEVELOPMENT

Starch transient accumulation occurs during fruit development in several fleshy fruits such as strawberry, tomato, banana, kiwifruit, apple, and pear. In strawberry, starch accumulates extremely early in the fruit formation process to 3–5% dry weight, and starch degradation predominates thereafter (Moing et al., 2001; Souleyre et al., 2004). In tomato fruit, starch amount peaks at immature green stage, contributing around 20% dry weight (Schaffer and Petreikov, 1997). In apple, starch accumulation occurs continuously from 4 weeks after anthesis until maximal concentration at about 15–17 weeks, then follows a continuous net degradation (Brookfield et al., 1997). In pear, starch degradation starts several weeks before fruit harvest (Mesa et al., 2016). Though kiwifruit and bananas can accumulate more starch than the abovementioned fruit species during fruit growth, nearly 40 and 70% dry weight, respectively, a similar temporal accumulation/degradation pattern is observed (Zhang et al., 2005; Hall et al., 2013; Li and Zhu, 2017). Because of their conserved temporal profiles, starch levels are used to define a ripening index for fruit harvest in several species including apple (Doerflinger et al., 2015). In all these fruits, in addition to a temporal accumulation, starch also shows spatial distribution patterns. In tomato fruit, starch accumulates more in parenchyma (inner pericarp) than in columella (Schaffer and Petreikov, 1997). For different apple cultivars, along with fruit ripening, different spatial starch accumulation/degradation patterns, such as ring or star-shaped pattern, were observed (Szalay et al., 2013). In bananas, starch is lost from the fruit center to the banana outward (Blankenship et al., 1993). Both the temporal and spatial variations of starch in fruits are linked with sucrose-to-starch metabolic enzyme activities (Schaffer and Petreikov, 1997). For example, Shinozaki et al. (2018) showed that the genes encoding enzymes involved in starch biosynthesis, including ADP-glucose pyrophosphorylase (AGPase) and starch-branching enzyme, showed higher expression in parenchyma, which is coherent with the AGPase enzyme activity and starch amount abundance observed in tomato pericarp. Moreover, the AGPase large subunit allele from *S. habrochaites* is characterized by increased AGPase activity in line with higher immature fruit starch content, compared to *S. lycopersicum*. Near-isogenic lines resulting from the interspecific cross of *S. habrochaites* and *S. lycopersicum* allowed showing that the high-starch phenotype was related to a temporal extension of transcription of an AGPase large subunit gene that also conferred higher AGPase activity to the high-starch tomato line (Petreikov et al., 2006, 2009).

Starch plays multiple roles during fruit development. At early fruit set, it is suggested to be a carbon reserve, particularly under mild stress conditions (Ruan et al., 2012). A study on kiwifruit suggested that starch turnover occurs at early developmental stage during cell division (Nardozza et al., 2013). When tomato plants were grown under control, shading or water shortage conditions, fruit hexose and sucrose amounts were similar, but fruit starch contents showed large fluctuations during fruit growth, which suggested that starch may play a buffering role for carbon supply under different abiotic stresses (Biais et al., 2014). Fruit species that do not store carbohydrate reserves such as starch, for instance muskmelon, must remain attached to the plant for the accumulation of soluble sugars to occur during ripening (Hubbard et al., 1990). In fruit species that store starch as a reserve of carbohydrates when fruit is ripening, net starch degradation, attributed to the complex actions of a range of enzymes related to starch breakdown at transcriptional and translational levels in banana (Xiao et al., 2018), also contributes to sugar content in banana (Prabha and Bhagyalakshmi, 1998) or kiwifruit (Nardozza et al., 2013). Petreikov et al. (2009) proposed an increase in transient starch accumulation in tomatoes as a valuable strategy for increasing the sink strength of the developing fruit and its final size and sugar levels. However, starch is not always degraded at fruit maturity. A striking example is the *Musa* genus, where we find dessert bananas characterized by a record degradation of starch (sometimes more than 10% of the dry matter) but also the cooking banana that remains rich in starch at maturity (Hill and Ap Rees, 1994; Jourda et al., 2016).

SEVERAL CROSS-SPECIES STUDIES HIGHLIGHT DOMESTICATION EFFECTS AS WELL AS MECHANISMS SHARED ACROSS PLANT FAMILIES

Studies comparing two or more fruit species are usually conducted with species belonging to the same genus or family. They rely on approaches ranging from simple biochemical analysis of metabolites to a combination of omics approaches. The use of introgression lines between a cultivated and a wild fruit species will not be considered in this paragraph, although a range of interesting works contributed to decipher the complexity of sugar or carboxylic acid metabolism, especially in tomato (see Ofner et al., 2016 for a summary of *S. pennellii* introgression lines for instance).

For comparisons within a genus, the parallel study of a cultivated species and one of its close wild relatives may provide insights into the effect of domestication on a primary metabolism pathway and its regulation. For instance, large-scale resequencing of 10 wild and 74 cultivated peach cultivars allowed comparative population genomics that showed an enrichment of gene families related to the carbohydrate metabolic process and TCA cycle within the edible group of peach genotypes (Cao et al., 2014). This work also identified a set of domestication genes, including one encoding a sorbitol-6-phosphate dehydrogenase. The draft genome of peach and whole-genome resequencing of 14 *Prunus* accessions paved the way to comparative and

phylogenetic analyses on manually annotated gene families among peach and other sequenced species, and enabled the identification of members with specific roles in peach metabolic processes for instance for sorbitol metabolism, and stressed common features with other Rosaceae species (The International Peach Genome Initiative, Verde et al., 2013).

Regarding another Rosaceae species, apple, a large-scale biochemical study on several hundreds of accessions, revealed that fruits of wild species showed significantly higher level of ascorbic acid than fruits of cultivated species (Fang et al., 2017). Ascorbic acid content was highly positively correlated with malic acid content, but negatively correlated with fruit weight and soluble solid content. As the expression levels of three genes involved in ascorbic acid accumulation were significantly negatively correlated with ascorbic acid contents in fruits, the latter authors suggested a feedback regulation mechanism in ascorbic acid related gene expression. They attributed the differences observed for fruit ascorbic acid content between the wild and cultivated species to an indirect consequence of human selection for increased fruit size and sweetness and decreased acidity.

For tomato, a combination of genome, transcriptome, and metabolome data from several hundreds of genotypes (wild tomato, *S. pimpinellifolium*, *S. lycopersicum* var *cerasiforme*, and *S. lycopersicum* accessions) showed how breeding altered fruit metabolite contents (Zhu et al., 2018). During fruit-size targeted selection, the contents of hundreds of metabolites, including primary metabolites, were changed. The authors propose that the increased primary metabolite content between their big-fruit and their small-fruit accession-pools might be the consequence of a larger metabolic sink in domesticated fruits, and that a range of the related metabolic changes may not be caused by the fruit weight genes themselves but rather be the consequence of linked genes. A study involving *S. pimpinellifolium*, *S. lycopersicum* var *cerasiforme*, and *S. lycopersicum* (Ye et al., 2017) that used a metabolite-based genome-wide association study with linkage mapping and gene functional studies identified a malate transporter (Sl-ALMT9) as being required for malate accumulation during ripening. It also showed that tomato domestication was associated with fixation and extension of favored alleles or mutations that increased malate accumulation.

A comparison of two citrus species, mandarin and orange with a difference in ascorbate content in the pulp (Yang et al., 2011) showed that higher expression of four genes along with lower activity of oxidation enzymes contributes to higher ascorbate in orange. A comparative study of two species of two different genera (Osorio et al., 2012), tomato (climacteric) and pepper (nonclimacteric), based on transcript and metabolite data, unraveled the similarities and differences of the regulatory processes underlying ethylene-mediated signaling in these two fruit types: differences in signaling sensitivity or regulators and activation of a common set of ripening genes influencing metabolic traits.

Finally, an elegant study combining species of three different genera concerns flesh acidity (Cohen et al., 2014). After map-based cloning of *Cucumis melo PH* gene (encoding a membrane protein) from melon, metabolites that changed in a common and consistent manner between high- and low-acid fruits of three species from three different genera, melon, tomato and cucumber, were searched using metabolic profiling. Functional silencing of orthologous *PH* genes in the latter two distantly related botanical families led to fruits with low acidity, revealing that the function of *PH* genes is conserved across plant families.

NEW TOOLS ARE EMERGING FOR MULTI-SPECIES COMPARISONS

Inter-species comparisons should not be comparing apples and oranges. In this perspective, an early study highlighted the challenge of aligning the different developmental stages (Klie et al., 2014). It could be partially solved by a more systematic use of development ontologies (Jaiswal et al., 2005) for omics approaches, or by the use of metabolic modeling along development and the cross-species comparison of model topologies and model parameters.

Metabolomics profiling has been used to study fruit metabolism within and between species. Thus, comparison by metabolic profiling of 15 peach cultivars pointed to cultivar-dependent and -independent metabolic changes associated with ripening and to the identification of ripening markers (Monti et al., 2016). The latter authors propose that metabolomics, revealing compositional diversity, will help improve fruit quality. Similarly, the profiling of volatile compounds in nine fruit species revealed that differences were mostly qualitative, with only seven common compounds (Porto-Figueira et al., 2015). Classical multivariate analyses such as principal component analysis (PCA), or more elaborated ones such as STATIS, which handles multiple data tables, are being used to mine metabolite data for comparisons between species. This latter statistical analysis was used at the fruit level to compare five species based on the pattern of 16 primary metabolites, and showed that climacteric species most significantly differed from non-climacteric ones with respect to the metabolism of some sugars and amino acids (Klie et al., 2014). However, tools are still required to take full advantage of the metabolomics datasets describing fruit composition that have been or will be, collected in repositories such as MetaboLights (Haug et al., 2013[2]) or the Metabolomics Workbench.[3] Although absolute quantitative data are easily reusable and comparable, this is not the gold standard for metabolomics data collected in these repositories, which are generally relative quantification datasets. Normalization methods for appropriate comparison of those data still need to be developed.

The comparative analysis of transcriptomic profiles in varieties of climacteric and non-climacteric melon has highlighted differences, in particular for genes related to ethylene biosynthesis and signaling, but also in gene expression related to sugar metabolism. Indeed, the upward regulation of

[2]https://www.ebi.ac.uk/metabolights/

[3]http://www.metabolomicsworkbench.org/

a soluble (vacuolar) acid invertase could influence the sucrose content of ripe fruit and post-harvest sucrose losses in climacteric fruit, while the upward regulation of invertase inhibitors would explain the high and stable sucrose levels in the non-climacteric variety and could be an important factor in their prolonged shelf-life (Saladié et al., 2015). A comparative study about tomato (climacteric) and pepper (non-climacteric) fruit combined analyses of transcriptomic and metabolic profiles (Osorio et al., 2012). As expected, it showed that genes involved in ethylene biosynthesis were not induced in pepper. However, genes downstream of ethylene perception, such as those implicated in fruit cell wall metabolism or carotenoid biosynthesis, were clearly induced in both Solanaceae species.

For genomics, a computational pipeline has been proposed to identify metabolic enzymes, pathways and gene clusters for about 20 plant species from their sequenced genome including tomato, grapevine and papaya fruit species (Schläpfer et al., 2017). Metabolic pathway databases were generated for 22 species and metabolic gene clusters were identified from 18 species. These vast resources can be used to conduct comparative studies of metabolism regulation between species, with the challenge to decipher organ specificities. Recently, an ambitious study about the evolution of fruit ripening involving transcriptomics, accessible chromatin study and histone and DNA methylation profiling of 11 fruit species revealed three types of transcriptional feedback circuits controlling ethylene-dependent fruit ripening (Lü et al., 2018). Similar approaches could highlight the circuits controlling primary metabolism during fruit growth.

While data on fruit metabolism of different species have been accumulated through years, their use to produce knowledge is now ranging from established statistical approaches to emerging modeling ones (Beauvoit et al., 2018). Modeling approaches involve several tools such as kinetic, stoichiometric or process-based modeling. For tomato, a kinetic metabolic model pointed to the importance of vacuolar storage for sugars (Beauvoit et al., 2014). A stoichiometric model highlighted a climacteric behavior as an emergent property of the metabolic system (Colombié et al., 2015). However, these properties are to be confirmed or infirmed for other fruit species. Recently, process-based models allowed the comparison of sugar concentration in fruits of four species or varieties and showed three species-related modes of sugar concentration control (Dai et al., 2016).

CONCLUSION

Although different botanical species share the same primary metabolism pathways, the regulation of these pathways is finely tuned along fruit development in particular ways in different species and results in compositional differences of the ripe fruit (**Figure 1** and **Table 1**). These differences result from genetic and epigenetic modifications linked with evolution, adaptation of species to their environment, domestication or breeding. It seems interesting although challenging, to search whether differences between fruit species for fruit development duration are directly or indirectly related to fruit metabolic characteristics as shown for metabolic profiles and lifespan of yeast mutants (Yoshida et al., 2010), or if differences in maturation duration may be related to mitochondrial metabolism as shown for yeast mitochondrial respiration and redox state and lifespan (Barros et al., 2010). Fruit quality improvement remains one of the major objectives of recent years for breeding. Many tools have been developed to achieve this objective, for instance the use of wild genetic material, omics technology, high-throughput phenotyping or biotechnology (Gascuel et al., 2017). Possible targets to improve sugar levels for instance include adjusting the time of shifting from symplasmic to apoplasmic phloem unloading, modifying sugar vacuolar storage, increasing transient starch storage, or increasing early organic acid accumulation and late neoglucogenesis. Most of the latter targets are linked directly with primary metabolism, but fine regulation networks need further attention. In a comparative study of orange varieties (*Citrus sinensis*), a gene coexpression analysis showed that the sugar/acid ratio-related genes not only encoded enzymes involved in metabolism and transport but also were predicted to be involved in regulatory functions like signaling and transcription (Qiao et al., 2017).

Comparing species helps to identify metabolic factors that influence fruit growth and quality, with a view to manipulating these levels to improve fruit traits. New strategies in species comparison, for instance omics, statistics and modeling, are promising and should continue to be developed in response to the large amount of metabolic data generated by increasingly efficient quantification and identification technologies.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Get the Balance Right: ROS Homeostasis and Redox Signalling in Fruit

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Plant central metabolism generates reactive oxygen species (ROS), which are key regulators that mediate signalling pathways involved in developmental processes and plant responses to environmental fluctuations. These highly reactive metabolites can lead to cellular damage when the reduction-oxidation (redox) homeostasis becomes unbalanced. Whilst decades of research have studied redox homeostasis in leaves, fundamental knowledge in fruit biology is still fragmentary. This is even more surprising when considering the natural profusion of fruit antioxidants that can process ROS and benefit human health. In this review, we explore redox biology in fruit and provide an overview of fruit antioxidants with recent examples. We further examine the central role of the redox hub in signalling during development and stress, with particular emphasis on ascorbate, also referred to as vitamin C. Progress in understanding the molecular mechanisms involved in the redox regulations that are linked to central metabolism and stress pathways will help to define novel strategies for optimising fruit nutritional quality, fruit production and storage.

Keywords: redox, fruit, ROS, metabolism, NAD, glutathione, tomato, ascorbate

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INTRODUCTION

Reduction-oxidation (redox) processes are a major consequence of the presence of ground-state oxygen gas (O_2, constituting c.a. 20.8% of the atmosphere) as a natural oxidant on Earth. Photosynthetic organisms (e.g. cyanobacteria, green algae, plants) produced O_2 by the light-driven splitting of water (H_2O) during oxygenic photosynthesis (Foyer, 2018). In other words, photosynthesis functionally houses redox reactions in plants that are underpinned by the transfer of electrons between a donor

Abbreviations: ABA, Abscisic acid; AGPase, ADP-glucose pyrophosphorylase; AltDH, Alternative dehydrogenases; AO, Ascorbate oxidase; AOX, Alternative oxidase; APX, Ascorbate peroxidase; ASC, Ascorbate; BABA, β-Aminobutyrate; CAT, Catalase; Chl, Chlorophyll; DHA, Dehydroascorbate; DHAR, Dehydroascorbate reductase; GABA, γ-Aminobutyrate; GP, Guaiacol peroxidase; GPX, Glutathione peroxidase; GR, Glutathione reductase; GRX, Glutaredoxins; GSH, Glutathione (reduced form); GSSG, Disulphide glutathione (oxidised form); GST, Glutathione S-transferase; JA, Jasmonic acid; LCMS, Liquid chromatography–mass spectrometry; MDHA, Monodehydroascorbate; MDHAR: Monodehydroascorbate reductase; Met, Methionine; MSR, Met sulphoxide reductase; NAD, Nicotinamide adenine dinucleotide; NADP, Nicotinamide adenine dinucleotide phosphate; NMR, Nuclear magnetic resonance; PS, Photosystem; PX, Peroxidase; RBOH, Respiratory burst oxidase homolog; ROS, Reactive oxygen species; $^1O_2$, Singlet oxygen; H_2O_2, Hydrogen peroxide; $O_2^{\bullet -}$, Superoxide anion; OH•, Hydroxyl radical; SA, Salicylic acid; SOD, Superoxide dismutase; TCA, Tricarboxylic acid; TRXs, Thioredoxins; XDH, Xanthine dehydrogenases.

and an acceptor. Consequently, this redox biochemistry generates the so-called reactive oxygen species (ROS). In tissues with low or no photosynthesis, such as roots and fruits, mitochondria can also drive the flow of electrons, thereby generating energy and ROS (Schertl and Braun, 2014).

Reactive oxygen species encompass highly reactive molecules that are partially reduced or excited forms of O_2 including singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), the superoxide anion ($O_2^{\bullet -}$) and the hydroxyl radical (OH•) (Apel and Hirt, 2004) (**Figure 1A**). Decades of research on redox biology pointed to a dual role for ROS both as toxic by-products of aerobic metabolism and as powerful signals that modulate plant functions (Mittler et al., 2011; Mittler, 2017; Foyer, 2018). With respect to this ambivalent concept, several ROS (e.g. H_2O_2) are produced during plant metabolism and development and in response to a fluctuating environment.

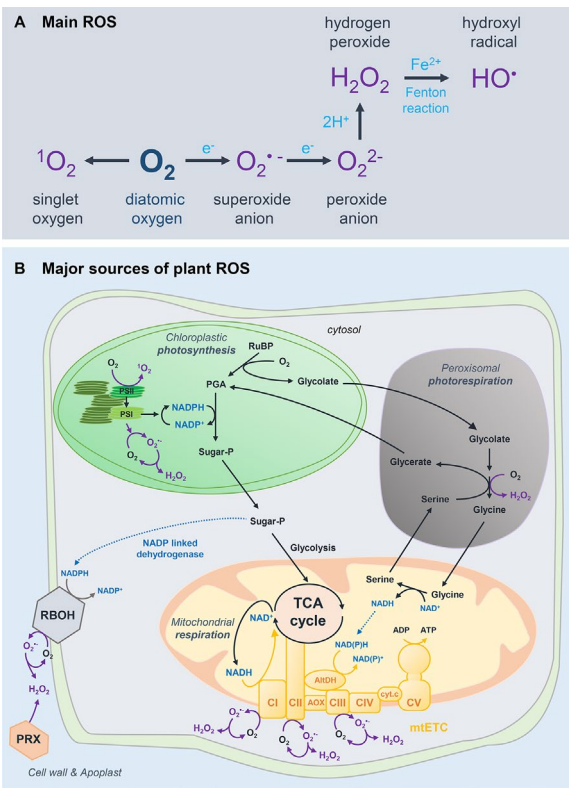

FIGURE 1 | Basics of ROS biology in plants. **(A)** Main reactive oxygen species (ROS) formed from atmospheric oxygen (O_2). **(B)** Major sources of ROS in plant cells. Excited triplet chlorophylls (Chl) can exacerbate the formation of singlet oxygen, mostly by the photosystem (PS) II reaction centre by photodynamic activation of ground-state oxygen (Fischer et al., 2013). In addition to singlet oxygen, superoxide and hydrogen peroxide can be formed in the chloroplast through the reduction of molecular oxygen, especially by the PSI. The four main complexes (CI–CIV) of the mitochondrial electron transport chain (mtETC) and the ATP synthase (CV) are represented in yellow. Among these complexes, CI (EC 1.6.5.3, NADH: ubiquinone oxidoreductase), CII (EC 1.3.5.1, succinate dehydrogenase) and CIII (EC 1.10.2.2, ubiquinol: cytochrome C oxidoreductase) are ROS-generating systems (Quinlan et al., 2012). mtETC, mitochondrial electron transfer chain; PRX, peroxidase; TCA, tricarboxylic acid; CI-V, complex I-V; AOX, alternative oxidase; AltDH, alternative dehydrogenase; cyt. c, cytochrome c; RuBP, ribulose 1,5-bisphosphate; PGA, phosphoglycerate; Sugar-P, sugar-phosphate; PS, photosystem.

Fruits, including fleshy fruits, are peculiar plant organs of great economic importance (e.g. 866 Mt worldwide in 2016, www.fao.org/faostat). They constitute a remarkable source of food worldwide and contain a plethora of natural compounds with various benefits for human health and nutrition, including vitamins, nutrients, fibres, proteins and minerals (Baldet et al., 2014; Rodriguez-Casado, 2016; Padayachee et al., 2017). Despite having high concentrations in carbohydrates, fruits usually exhibit reduced photosynthetic activity, but sometimes high respiration rates, in particular for climacteric fruits, such as tomato (Roch et al., 2019). As for other vegetative plant tissues, fruit biology involves redox reactions and generates ROS. Some fruits are major sources of antioxidants, such as ascorbate, which scavenge ROS (Gest et al., 2013b; Smirnoff, 2018).

To date, there is no global overview of the involvement of oxidative metabolism in fruit biology, despite some fairly recent reviews on ripening and photo-oxidative stress (Tian et al., 2013; Osorio et al., 2013a; Cocaliadis et al., 2014; Muñoz and Munné-Bosch, 2018). This present review aims at updating our current knowledge on redox biology of fleshy fruits. We provide an overview of the profusion of natural compounds having antioxidant properties and examine the importance of redox regulation in plant metabolism for development and stress responses. We also discuss the relevance of metabolic modelling for the study of redox fluxes in plants, which should help to improve knowledge on the link between metabolism and cell redox status and therefore to evaluate strategies for optimal fruit production and storage.

THE BASICS OF REDOX BIOLOGY IN PLANT CELLS

For decades, redox signalling has been perceived as a balance between low levels of ROS acting as signals to trigger signalling cascades that adjust plant functions and high levels of ROS causing oxidative cellular damage (Apel and Hirt, 2004). Currently, the paradigm of redox biology tends to display a bigger and clearer picture of the redox network, especially in plants where multiple sources of ROS are possible and associated with many 'ROS-processing systems' (Noctor et al., 2018). Spatial, temporal, metabolic and antioxidant specificities are multiple factors that can influence redox signalling. Whilst redox biology in fruit is clearly fragmentary, the concepts that originate from foliar tissues are useful whilst waiting for comprehensive studies that bring more substantial levels of knowledge. This section briefly describes the major sources of ROS that are found in plant cells and the systems that process them.

ROS Formation in Plants

The three main sources of plant ROS are the chloroplastic photosynthesis, the mitochondrial respiration and the peroxisomal photorespiration cycle (**Figure 1B**). The photosynthetic transport chain is assumed to be the major

source of plant ROS in photosynthetic tissues. Superoxide can directly exert its signalling function or be chemically reduced or dismutated to H_2O_2. Dismutation of H_2O_2 can be accelerated by superoxide dismutases (SODs; EC 1.15.1.1), which are pivotal in regulating the redox status of the plant cell (Smirnoff and Arnaud, 2019). Importantly, H_2O_2 is more likely to trigger transduction signals over longer cellular distances (e.g. into the nucleus) as it has a longer lifespan, a greater diffusion distance and stability as compared to $^1O_2$ (Exposito-Rodriguez et al., 2017; Mittler, 2017).

The photorespiratory cycle makes photosynthesis possible by scavenging 2-phosphoglycolate, which is toxic for the cell (Hodges et al., 2016). This highly compartmentalised pathway involving the chloroplast, peroxisome and mitochondrion is critical in generating H_2O_2 through the activity of peroxisomal glycolate oxidase (EC 1.1.3.15). Of course, the contribution of peroxisomal volume to total cell volume is small: 1% for peroxisomes compared to 12% for chloroplasts in leaves (Queval et al., 2011). Nonetheless, peroxisomes are predicted to be a major source of hydrogen peroxide in active photorespiratory cells. Furthermore, photorespiration-driven H_2O_2 is solely dismutated by peroxisomal catalase, which is commonly used as a redox marker of the peroxisome (Smirnoff and Arnaud, 2019, 202). In fruit, a high activity of the ascorbate recycling enzyme monodehydroascorbate reductase was observed in tomato fruit peroxisomes (Gest et al., 2013a), which supports the idea of an important role for peroxisomes in fruit redox homeostasis.

In nonphotosynthetic tissues, energy mostly originates from mitochondrial activity, which also contributes to generate ROS (Quinlan et al., 2012) (**Figure 1B**). The tricarboxylic acid cycle reduces NAD^+ into NADH in the mitochondrion, which is fundamental to ensure that cellular respiration produces ATP *via* oxidative phosphorylation (Millar et al., 2011) (**Figure 1B**). Thus, mitochondria are tightly linked to NAD(H) turnover (Gakière et al., 2018a). As for the chloroplast, specific SODs dismutate rapidly $O_2^{\bullet-}$ into H_2O_2 (Smirnoff and Arnaud, 2019). Besides ROS-generating systems, plant mitochondria specifically harbour alternative NADP(H) dehydrogenases that face both the matrix and the intermembrane space, as well as alternative oxidase (AOX) (**Figure 1B**). These enzymes are alternative respiratory routes, which do not produce energy, but allow viability when the enzymes of the main pathway are affected (Rasmusson et al., 2008, Rasmusson et al., 2009; Schertl and Braun, 2014). Alternative NADP(H) dehydrogenases can remove excess of reducing power in the mitochondria, which will balance the redox poise.

In addition, plant ROS can originate from other ROS-generating systems, including NADPH and xanthine oxidases. The NADPH oxidases (EC 1.6.3.1) are well-studied key players in ROS production (**Figure 1B**), most particularly with respect to biotic and abiotic environmental stresses (Torres and Dangl, 2005; Suzuki et al., 2011; Mittler, 2017). Xanthine dehydrogenases (EC 1.17.1.4, XDH) are important enzymes involved in the hydroxylation of hypoxanthine to xanthine, but can also form $O_2^{\bullet-}$ when molecular oxygen is used as the electron acceptor. Whilst XDHs in mammals can be converted into xanthine oxidases that produce both $O_2^{\bullet-}$ and H_2O_2, plant XDHs only form $O_2^{\bullet-}$, which can be swiftly dismutated into H_2O_2 (Yesbergenova et al., 2005; Ma et al., 2016). In complement, class III peroxidases (PXs; EC 1.11.1.7) are heme-containing enzymes that produce $O_2^{\bullet-}$ and H_2O_2 at the apoplast (Bindschedler et al., 2006; Cosio and Dunand, 2009; Daudi et al., 2012), although H_2O_2 formation is favoured at high pH in the presence of reductants (O'Brien et al., 2012). Peroxidases are also able to oxidise a donor and thereby process H_2O_2 (Lüthje and Martinez-Cortes, 2018).

For fruit tissues, however, knowledge is still lacking on the exact contribution of each source of ROS. Of course, due to low photosynthetic metabolism in fruit, one could predict different contributions than for leaves, which further depends on the plant species that exhibit diverse biochemical pathways able to scavenge and process cellular ROS. Even though mitochondria, peroxisomes and the apoplast are assumed to be leaders in ROS production in flowers and fruits (Qin et al., 2009a, Qin et al., 2009b; Rogers and Munné-Bosch, 2016), further research on fruit ROS is necessary to unveil the actual ROS-generating compartments and processes that mostly contribute to ROS production in fruit tissues.

Systems for ROS Scavenging and Processing in Plants

Reactive oxygen species produced in the plant cell can be scavenged, or processed, by highly efficient antioxidant systems. If this were not the case, ROS levels exceeding the requirement of metabolic processes would damage cellular structures and functions involving nucleic acids, proteins and lipids (Apel and Hirt, 2004; Muñoz and Munné-Bosch, 2018). Antioxidants include metabolites with antioxidant properties, which in fruit are profuse in their diversity and quantity and are found in all organelles. Besides metabolites, the antioxidant machinery is composed of a few major enzymes that rapidly process ROS, i.e. catalase (CAT; EC 1.11.1.21), SOD (EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1), glutathione S-transferase (GST; EC 2.5.1.18), glutathione peroxidase (**GPX**; EC 1.11.1.9), glutathione reductase (GR; EC 1.8.1.7) and guaiacol peroxidase (**GX**; EC 1.11.1.7). Hence, redox biology presents another level of ambiguity as enzymes such as peroxidase or dismutase can be considered as both ROS-generating and ROS-processing components (**Figure 2**). These enzymes tightly link to the pool of the redox buffers ascorbate, glutathione and pyridine nucleotides, which serve as reductants to recycle repeatedly glutathione and ascorbate *via* the so-called Foyer-Halliwell (or ascorbate-glutathione) cycle (Foyer and Noctor, 2011) (**Figure 2**). In addition, thioredoxins (TRXs) are widely distributed small proteins, which modulate the redox state of target proteins *via* transfer reactions of thiol-disulphide using NADP(H) as a cofactor (Geigenberger et al., 2017). These ROS-processing systems are also important for fruit metabolism, and they could link to developmental processes or responses to environmental changes, as we detail further below.

Fruit Antioxidants

Fruits, especially citrus and berry fruits, are well-known sources of antioxidants conferring plenty of beneficial effects for human

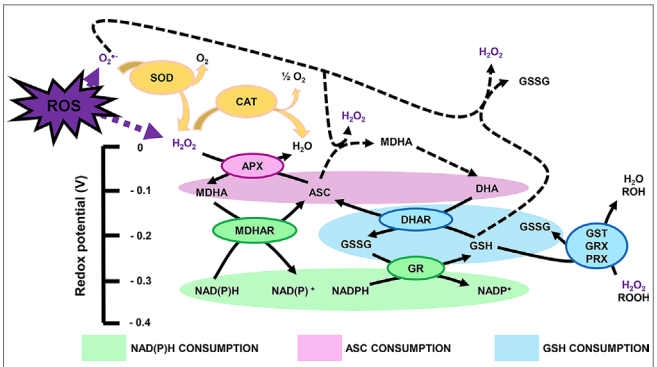

FIGURE 2 | Major cellular redox buffers: a *ménage-à-trois* to process ROS. Plain and dashed arrows represent enzymatic and nonenzymatic reactions, respectively. ASC, reduced ascorbate; APX, ascorbate peroxidase; CAT, catalase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GSH, reduced glutathione; GSSG, glutathione disulphide; GR, glutathione reductase; GRX, glutaredoxin; GST, glutathione S-transferase; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; PRX, GRX-dependent peroxiredoxin; ROH, organic compound with alcohol group; ROOH, organic compound with peroxide group; SOD, superoxide dismutase.

health (Gomes-Rochette et al., 2016). Because of their intricate oxidative metabolism (ROS production, described above), plants have developed a wide range of antioxidant metabolites as well as pathways to synthetize, catabolise and regenerate them. Basically, antioxidants refer to all biomolecules, including metabolites, which can process ROS and/or reactive nitrogen species to delay or avoid cell damage and for signalling processes (Nimse and Pal, 2015). Antioxidants can be distributed into several biochemical classes (**Figure 3**), including phenolics, terpenoids, thiol derivatives and vitamins, for which common metabolites and their antioxidant mechanisms are listed in

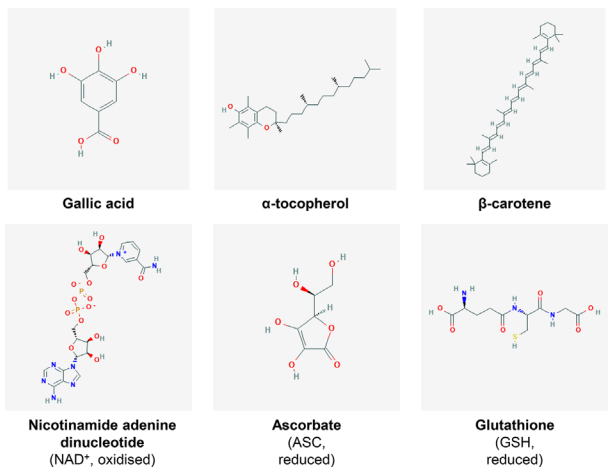

FIGURE 3 | Examples of fruit antioxidants. Chemical structures of several metabolites presenting antioxidant properties. The major redox buffers NAD^+, ASC and GSH are also presented. For further detail, refer to **Table 1**. Individual structures were obtained from PubChem (pubchem.ncbi.nlm.nih.gov/).

Table 1. Terpenoids, also known as isoprenoids for their core structure, can be divided into several classes based on their carbon skeleton, and among them, carotenoids are the main group with more than 600 having been identified and characterised (Graßmann, 2005). They are pigments used for light harvesting, preventing photo-oxidation and increasing fruit attractiveness for seed dispersion (Young and Lowe, 2018). Carotenoids among other terpenoids are widely studied with respect to their antioxidant properties and biological effects in plants and mammals. Whilst antioxidants are often shared by plant species, most plant families have developed their own range of specific antioxidant metabolites within their botanical taxa. Quite importantly, some major redox buffers shared between species, such as ferredoxins, pyridine nucleotides, TRXs, glutathione and ascorbate, can be distinguished as they play a fundamental role in the development of plants and their responses to the environment and thus in plant performance (Balmer et al., 2004; Geigenberger and Fernie, 2014; Geigenberger et al., 2017; Noctor et al., 2018; Gakière et al., 2018a).

Due to the wide diversity of fruit metabolites harbouring antioxidant activity, fruit antioxidants can process ROS in many ways. Most antioxidants spontaneously react with ROS, although enzymes such as APXs and glutaredoxins (GRX) catalyse several reactions. As previously mentioned, antioxidants remarkably participate in recycling pathways, such as the glutathione-ascorbate cycle, to maintain the redox state of the main redox buffers through the activity of GR, DHAR and MDHAR (**Figure 2**). The importance of such systems for fruit biology is detailed in Section 4.

Three Major Cellular Redox Buffers: A Ménage-À-Trois to Manage ROS

Ascorbate (ASC) and glutathione (GSH) sit at the top of plant soluble antioxidants because they process ROS rapidly using specific enzymes such as peroxidases belonging to the ascorbate-glutathione pathway (Foyer and Noctor, 2011) (**Figure 2**). In brief, ROS react preferentially with GSH and ASC: the latter can reduce H_2O_2 *via* APX to produce water and MDHA that will be reduced by MDHAR using NAD(P)H, or be transformed spontaneously in DHA that will be reduced by DHAR using GSH (**Figure 2**). These repetitive redox cycles allow for the regeneration of the pools and the maintenance of the cellular redox buffers in a highly reduced state in most cellular compartments under unstressed conditions. In addition, pyridine nucleotides (i.e. NAD(P)H and $NAD(P)^+$; **Figure 3**) are crucial for the regeneration of GSH and ASC through GR and MDHAR enzymes as well as being involved in other metabolic pathways, thereby linking redox homeostasis to central metabolism (Gakière et al., 2018a). Strikingly, fruit-specific concentrations and redox states of the pools are difficult to find in the literature (**Table 2**). In unstressed conditions, ASC and GSH are in a highly reduced state (> 90%), NAD(H) is 60% to 65% reduced, and NADP(H) is at 90% reduced in red ripe tomato fruits (Araújo et al., 2012; Centeno et al., 2011; Jimenez et al., 2002). However, NAD(H) is 12% to 20% reduced, and NADP(H) is 50% to 55% reduced in orange, apple, pear and

TABLE 1 | Examples of major antioxidant metabolites present in fruits.

Biochemical class	Compound class	Antioxidative metabolite	Antioxidant activity	Effect on human health	Source example (per 100 g FW)	Key references
Polyphenols	Hydroxycinnamic acids	Caffeic acid	Scavenge ROS and peroxyl radicals Inhibit lipid peroxidation	Anti-inflammatory Preventive effects for diabetes Cardiovascular protective effects	0.1–1.3 mg in tomato 0.4–35 µg in blueberries	Fu et al., 2011 Wolfe et al., 2008 Wang et al., 2017 Olas, 2018
		Ferulic acid			0.2–0.5 mg in tomato 26–185 µg in blueberries	Martí et al., 2016 Wang et al., 2017
		p-coumaric acid			0–0.6 mg in tomato 89–225 µg in blueberries 15–42 mg in strawberries	Skupien and Oszmianski, 2004 Martí et al., 2016 Wang et al., 2017
	Hydroxybenzoic acid	**Gallic acid**	Scavenge peroxyl radicals and ROS		2–9 mg in different cultivars of blackberries	Wada and Ou, 2002 Wang et al., 2017
	Flavonoids	Anthocyanins	Scavenge free radicals Acylation of anthocyanins with phenolic acid increase the antioxidant activity Prevent lipid peroxidation	Neuroprotective effects Anti-cancer involved in treatment of cardiovascular diseases	154–1001 µg in blueberries of Cyanidin 25–40 mg in strawberries of total anthocyanins	Skupien and Oszmianski, 2004 Khoo et al., 2017 Wang et al., 2017 Olas, 2018
		Catechin	Prevent lipid peroxydation Scavenge NO and ROS	Regulate superoxide production Regulation of transcription factors involved in oxidative stress responses	180–338 µg in blueberies 6–19 mg in different cultivars of strawberries	Fraga et al., 2018 Wang et al., 2017 Skupien and Oszmianski, 2004
		Quercitin		Neuroprotective and cardioprotective effects Anti-cancer	0.7–4.4 mg in tomato 202–266 µg in blueberries	Chaudhary et al., 2018 Martí et al., 2016 Wang et al., 2017
	Stilbenes	Resveratrol	Scavenge ROS and peroxyl radicals Inhibit lipid peroxidation	Neuroprotective and cardioprotective effects	51–97 µg in blueberries	Wang et al., 2017 Cory et al., 2018
Carotenoids		Lycopene	Process singlet oxygen Trap peroxyl radicals Inhibit radical-induced lipid peroxidation Reduce ROS production by nonphotochemical quenching of chlorophyll fluorescence	Anti-inflammatory Pro-vitamin A activity, converted to retinoids after breaking (oculo protective effects) Enhance immune system Anti-proliferative and anti-carcinogenic	7.8–18.1 mg in tomato 1.82–3.6 g in different buffaloberry cultivars	Eldahshan and Singab, 2013 Martí et al., 2016 Chaudhary et al., 2018 Murillo et al., 2010 Graßmann, 2005 Riedl et al., 2013
		Zeaxanthin			200 µg in mandarins 7.92 mg in South American sapote 6 mg in orange pepper 340 µg in tomato	Murillo et al., 2010
		β-Carotene			0.1–1.2 mg in tomato 1.5–3.8 mg in apricot	Martí et al., 2016 Sass-Kiss et al., 2005
Thiols		**Glutathione**	Process ROS *via* enzymatic and non-enzymatic reactions ROS scavenging Maintain thiol equilibrium S-glutathionylation of Cys residues allowing regulation of central metabolism during oxidative stresses	Neuroprotective effects Involve in asthma prevention and treatment	1,3 mg in mango 210–298 µg in strawberries 16–19.5 mg in tomato	Ding et al., 2007 Fitzpatrick et al., 2012 Smeyne and Smeyne, 2013 Erkan et al., 2008 Martins et al., 2018 Noctor et al., 2018 Keutgen and Pawelzik, 2007

(*Continued*)

TABLE 1 | Continued

Biochemical class	Compound class	Antioxidative metabolite	Antioxidant activity	Effect on human health	Source example (per 100 g FW)	Key references
Vitamins	Tocochromanols	**α-Tocopherol (VE)**	Prevent lipid peroxidation by scavenging free radicals (donating hydrogens) using ascorbate to be regenerated Prevent the oxidation of carotenoids Essential macronutrient for human maintaining cell membrane integrity	Anti-anemia Neuroprotective effects	0.5–1,1 mg in tomato; 0.6–0.8 µg in MoneyMaker cultivar 1,6–3,2 mg in red sweet pepper 3.8 mg in green olives of total tocopherol + tocotrienols	Gugliandolo et al., 2017 Giovinazzo et al., 2004 Chaudhary et al., 2018 Dasgupta and Klein, 2014 Raiola et al., 2015 Chun et al., 2006 Knecht et al., 2015
		Ascorbate (VC)	Process ROS *via* enzymatic and non-enzymatic reactions Allow the regeneration of tocopherols and carotenoids	Anti-scurvy Anti-inflammatory Anti-cancer	10–15 mg in commercial cultivars of tomato and until 70 mg in ancestral cultivars 54–87 mg in different cultivars of strawberries 2.4–3g in camu-camu	Chaudhary et al., 2018 Martins et al., 2018 Stevens et al., 2007 Skupien and Oszmianski, 2004 Justi et al., 2000

TABLE 2 | Examples of ASC, GSH and NAD/P(H) sources in fruits.

	Source example (per 100 g FW)	References
ASC	10 to 15 mg in tomato 54–87 mg in strawberries 2.4-3 g in camu-camu	Stevens et al., 2007 Skupien and Oszmianski, 2004 Justi et al., 2000
GSH	1.3 mg in mango 16–19.5 mg in tomato 210–298 µg in strawberries	Ding et al., 2007 Giovinazzo et al., 2004 Cervilla et al., 2007 Keutgen and Pawelzik, 2007
NAD^+	3.21 mg in red fruits and 2.22 mg at breaker stage in tomato 780 µg in orange 400 µg in grapefruit	Osorio et al., 2013b Centeno et al., 2011 Bruemmer, 1969
NADH	5.82 mg in red fruits and 4.94 mg at breaker stage in tomato 170 µg in orange 50 µg in grapefruit	Osorio et al., 2013b Centeno et al., 2011 Bruemmer, 1969
$NADP^+$	0.46 mg in red fruits and 0.77 mg at breaker stage in tomato 89 µg in orange 69 µg in grapefruit	Osorio et al., 2013b Centeno et al., 2011 Bruemmer, 1969
NADPH	3.88 mg in red fruits and 3.23 mg at breaker stage in tomato 119 µg in orange 89 µg in grapefruit	Osorio et al., 2013b Centeno et al., 2011 Bruemmer, 1969

grapefruits (Bruemmer, 1969), which is congruent with the redox status of photosynthetic tissues (Gakière et al., 2018b). This clearly suggests a diversity in fruit redox homeostasis as fruit growth influences the redox state of pyridine nucleotides. Furthermore, these three major cellular redox buffers display distinct redox potential: −0.1, −0.23 and −0.32 mV for the ASC/DHA, GSH/GSSG and $NAD(P)^+$/NAD(P)H couples, respectively (**Figure 2**). In this case, as pyridine nucleotides have a lower redox potential, they will be detrimental for electron transfer to GSH and ASC during redox mechanisms.

THE IMPORTANCE OF THE REDOX HUB FOR FRUIT SIGNALLING

The redox hub consists of all the molecular partners able to generate, process or trigger oxidative signals, whilst the resulting redox signalling can modulate the physiology of plant organs including fruits (Mittler, 2017; Noctor et al., 2018). Fruits are a major source of central metabolites (Osorio et al., 2013a; Roch et al., 2019), such as carbohydrates, lipids, amino and organic acids, but also vitamins and other antioxidant metabolites that play important roles in fruit biology (**Figure 3**). Besides, redox status is also at the heart of the control of metabolic processes (Geigenberger and Fernie, 2014). One among many reasons is the prominence of pyridine nucleotides (NAD/P(H)) as master regulators of hundreds of biochemical reactions (Gakière et al., 2018a), together with ascorbate/dehydroascorbate (ASC/DHA) and glutathione (GSH/GSSG) couples (Noctor et al., 2018). In this context, we will present recent advances in our understanding of key spatiotemporal redox signals that occur during developmental processes and in response to environmental changes, including redox buffers that balance the redox poise (**Figure 4**).

Redox and Central Metabolism During Fruit Development

Fruit development comprises three main phases: cell division, cell expansion and ripening. As green organs, young fruits and leaves share some similarities due to the presence of photosynthetically active chloroplasts driving central metabolism, hence developmental processes (Cocaliadis et al., 2014). Fruit photosynthesis can contribute to the production of starch, which can then be turned into soluble carbohydrates during ripening. In tomato, a number of studies points towards the importance of the mitochondrial malate valve in transmitting redox status to the plastids, which will influence plastidial metabolism (Centeno et al., 2011; Osorio et al., 2013b). In fact,

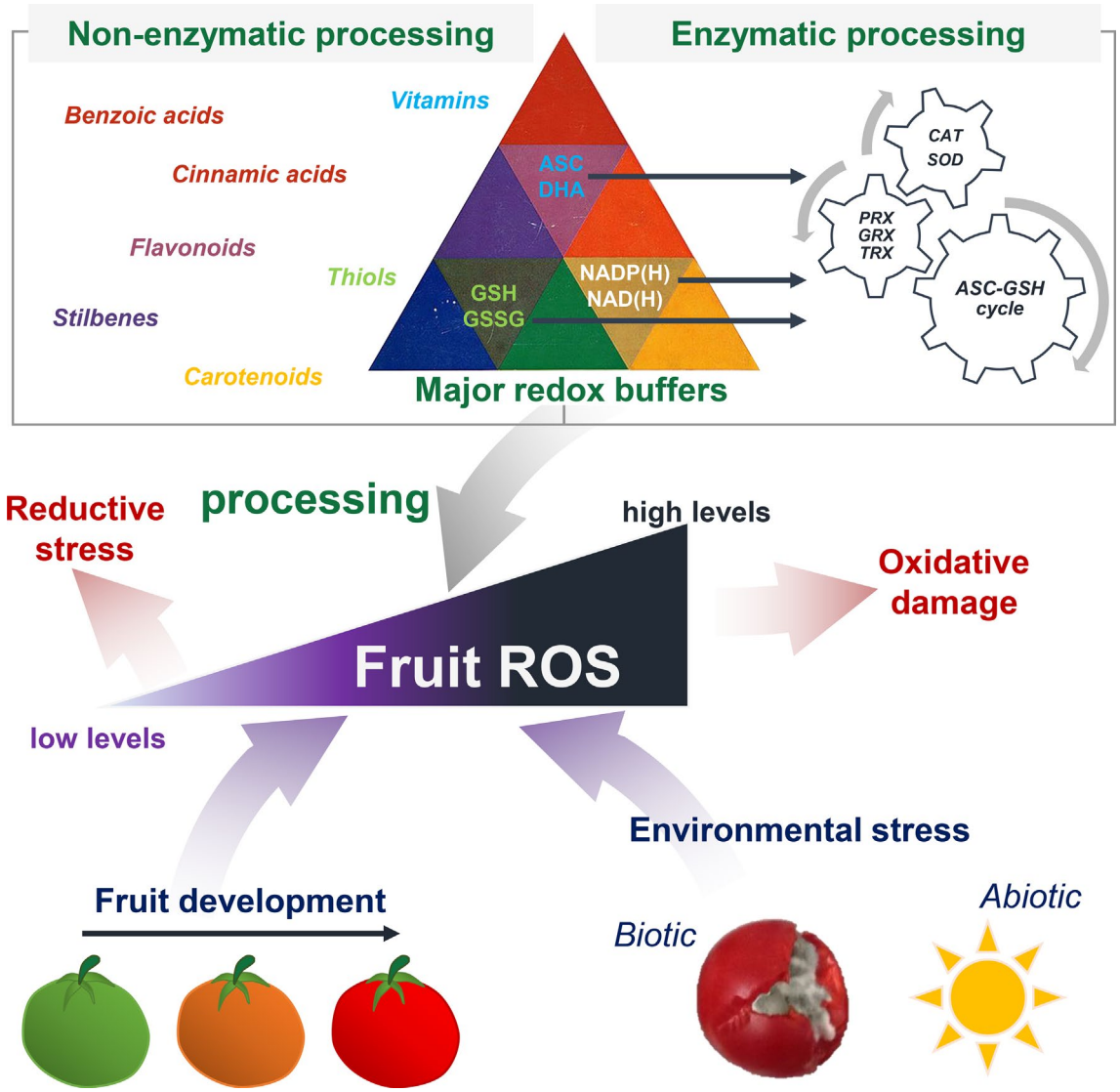

FIGURE 4 | Pivotal role of redox signalling for fruit growth and stress responses. Fruits produce ROS and other redox signals during development and in response to environmental stress. Reactive oxygen species homeostasis is finely tuned between ROS production and processing. This involves several enzymatic and non-enzymatic mechanisms, including antioxidant metabolites and major redox buffers (NAD/P(H), ASC and GSH) that allow antioxidant cycles to process excess of ROS. When ROS levels are too high, the resulting oxidative stress would damage cellular structures.

decreasing malate content in the growing fruit could stimulate the activation state of AGPase (EC 2.7.7.27), leading to increased starch and soluble sugar pools in ripening tomato. Intriguingly, such metabolic repercussions tend to provide tolerance of tomato to water loss, wrinkling and pathogenic infections. This supports the paradigm of a versatile role of redox signals in metabolic regulation throughout development and in response to stress (Tian et al., 2013). Another hallmark of this growth phase is when chloroplasts become chromoplasts by losing green chlorophylls at the expense of coloured antioxidants like carotenoids (Lado et al., 2015; Martí et al., 2009). Concomitantly, the expression of nuclear- and plastid-encoded photosynthetic genes drops as the fruit ripens. It is noteworthy that ROS synthesis influences the accumulation of carotenoids (Pan et al., 2009), which are major scavengers of singlet oxygen, specifically β-carotene, tocopherol and plastoquinone (Miret and Munné-Bosch, 2015). Besides carotenoids, anthocyanins can accumulate in the growing fruits and contribute to both red/purple/blue colours and antioxidant properties [**Table 1**; (Muñoz and Munné-Bosch, 2018)]. Whilst carotenoids accumulate primarily within lipophilic membranes, anthocyanins are stored in the vacuole where their colour depends on their chemical structure, which is influenced by vacuolar pH (Jaakola, 2013). In this context, recent studies have reported a critical role for epigenetic processes in growing tomato fruit by linking DNA demethylation levels with transcriptomic changes of genes involved in fruit antioxidant biosynthesis (e.g. flavonoids, carotenoids) (Lang et al., 2017). Conversely, however, development and ripening of orange,

a nonclimacteric fruit, were correlated with an increase in DNA methylation levels, together with repression of photosynthetic genes (Huang et al., 2019). Thus, fruit development is likely to present remarkable discrepancies in terms of redox signals, their source (e.g. chloroplastic, mitochondrial, peroxisomal, apoplastic) and the duration and extent of oxidative stress, even at early stages of fruit growth in comparison to leaves (Muñoz and Munné-Bosch, 2018).

Ripening is an important end process of fruit development that involves multiple molecular regulations (Osorio et al., 2013a). It is mediated by redox signalling, more specifically during the chloroplast-to-chromoplast transition and in the mitochondrial compartment, where protein carbonylation occurs and respiration rates increase, thus affecting the redox state when sugar supply becomes limiting (Qin et al., 2009a; Tian et al., 2013). As the fruit ripens, oxidative stress progressively augments, like in peach, tomato, pepper and grape berries (*Vitis vinifera*), where H_2O_2 pools accumulate upon changes in skin colour (Jimenez et al., 2002; Martí et al., 2009; Qin et al., 2009a; Pilati et al., 2014; Kumar et al., 2016). In fact, it is assumed that ROS accumulation produces two distinct peaks during fruit growth: first at the onset of ripening and second at overripening either preharvest or postharvest (Muñoz and Munné-Bosch, 2018). It is possible that increased oxidative stress might favour fruit softening, which is beneficial for seed release (Jimenez et al., 2002). This would explain why short life tomato cultivars are redox-stressed and present lower antioxidant activities (Cocaliadis et al., 2014).

Because of ROS imbalance, oxidative signals need processing *via* cellular redox buffering and the antioxidant machinery to avoid cellular damage (**Figure 4**). In ripening grape berries, accumulated levels of H_2O_2 are accompanied with a concomitant stimulation of CAT activity (Pilati et al., 2014). Moreover, under oxidative stress, increases in activities of APX, MDHAR and GR are seen in peach (Camejo et al., 2010). Contrasting observations are reported for peroxidases: ripening phase is associated with increased PX activities in mango, apples and banana fruit, whilst tomato, strawberry and capsicum show a decline in these activities (Pandey et al., 2012, and references therein). The importance of antioxidant systems for ripening is also exemplified in grapevines where there is a strong developmental modulation of ASC metabolism at the biosynthetic, recycling and catabolic levels (Melino et al., 2009). Fruit growth of wine grapes can witness a gradual induction of ASC biosynthesis and subsequent changes in the accumulation of ASC and two derivatives: tartaric and oxalic acids. Whilst immature berries showed a swift accumulation of ASC together with a low ASC/DHA redox ratio, ripe fruits instead showed an increased accumulation of ASC and higher ASC/DHA ratio. Additionally, acerola, an exotic fruit cultivated mostly for its ascorbic acid content, shows differential regulation of MDHAR and DHAR genes during fruit ripening (Eltelib et al., 2011). A comparison of ASC metabolism in mandarin and orange, two citrus species harbouring different ASC contents in pulp, deepens our understanding of the differences in ASC concentrations in fruit (Yang et al., 2011b). This study revealed that higher ASC in ripening orange was associated with an augmented expression of four genes involved in ASC biosynthesis, encoding GDP-D-mannose-3′,5′-epimerase (EC 5.1.3.18), GDP-L-galactose-phosphorylase (EC 2.7.7.69), L-galactose dehydrogenase (EC 1.1.1.316) and L-galactono-1,4-lactone dehydrogenase (EC 1.3.2.3), together with attenuated activities of ASC oxidase and ASC peroxidase, which are involved in ASC degradation. Another elegant work on isolated mitochondria from ripening tomato fruits has reported a global stimulation of the ASC-GSH cycle at the enzyme level (López-Vidal et al., 2016). Recently, a system biology approach in tomato has been conducted based on large-scale transcriptomic, proteomic, metabolic and phenotypic data for orange fruit of RNAi lines for three enzymes involved in ASC metabolism (AO, GLD and MDHAR) (Stevens et al., 2018). The ASC redox state has been reported to influence the expression of genes involved in cellular protein synthesis and stability and ribosomal function. Besides redox functions, synthesis of ASC is also crucial for tomato fruit growth (Garcia et al., 2009; Mounet-Gilbert et al., 2016), as exemplified by profound growth stunting of tomato fruits silenced in mitochondrial ASC synthesis (Alhagdow et al., 2007). Another recent study of protein turnover at global scale in the developing tomato fruit revealed a stage-specific response of protein profiles that were associated to various redox functions (Belouah et al., 2019). Changes in redox-related proteins were represented in the young fruit (e.g. SOD, APX) and at ripening (e.g. MDHAR, GR). Hence, ASC metabolism appears to be central to redox homeostasis during fruit development.

Upon stress and senescence (i.e. ageing), oxidative alterations can drastically target proteins, resulting in conformational changes and thus impairing their catalytic functions. Methionine (Met) and cysteine, which contain sulphur, are probably the most susceptible to ROS oxidation (Davies, 2005). In the case of Met, oxidation can be reversed by Met sulphoxide reductase (MSR, EC 1.8.4.11/12) (Emes, 2009; Rey and Tarrago, 2018), which has been reported to play a role in senescing litchi fruit through down-regulation of *MSR* genes (Jiang et al., 2018). In leaves, previous works have suggested a link between MSR and the homeostasis and redox balance of NAD/P(H) (Pétriacq et al., 2012, Pétriacq et al., 2013). Besides MSR, a stimulation of the antioxidant systems in tomato fruit mitochondria has been reported to be associated with a differential carbonylation of mitochondrial proteins in breaker and light red tomato fruits, which might participate in protein degradation and cellular signalling (López-Vidal et al., 2016). Besides targeting proteins, aging of fruit encompasses other redox-related changes. In the pulp of Kyoho grape, postharvest senescence and rotting are accompanied by an accumulation of oxidative signals (e.g. malondialdehyde, hydrogen peroxide, superoxide anion) and a concomitant depletion of several antioxidant systems (e.g. ascorbate, flavonoids, total phenolics, reducing sugars) (Ni et al., 2016). Interestingly, exogenous treatment with hydrogen sulphide could alleviate those redox perturbations by enhancing the activity of antioxidant enzymes, such as CAT and APX, and by attenuating those of lipoxygenase in the pulp and peel of Kyoho grape.

Additionally, not only central metabolism links to redox signalling in fruit but also more specialised pathways involving

phytohormones (Symons et al., 2012; Leng et al., 2013). In red raspberry, a nonclimacteric crop fruit, the stage of ripeness at the time of harvest determines the antioxidant contents (e.g. anthocyanins, ellagitannins, vitamins C and E, carotenoids) (Beekwilder et al., 2005; Miret and Munné-Bosch, 2016). Application of the carotenoid-derivative hormone ABA after fruit set modulates the ASC/DHA ratio in young berries and more than doubles ASC pools in ripe fruit. Such an effect was partially explained by alterations of ASC oxidation and recycling through the activities of AO, APX, DHAR and MDHAR (Miret and Munné-Bosch, 2016). In postharvest conditions, fruit decay is a major issue caused by perturbation of the redox balance, including ROS production (Pétriacq et al., 2018). Thus, antioxidant mechanisms (e.g. ASC total pool and redox state, ASC-GSH cycle) are important actors throughout fruit growth, which is further evidence for the idea that ROS act as metabolic by-products requiring a finely tuned homeostasis (**Figure 4**). In an agri-food context, further research is required to disentangle the implication of each redox event occurring during fruit development, so that efficient strategies can be adopted to improve fruit production and storage.

Nevertheless, the active depiction of redox fluxes by deciphering redox signatures in plant biology is extremely tedious, if not impossible, probably due to the extreme reactivity of ROS and related redox signals and to the intricacy of the redox hub. However, a very interesting and promising alternative to measurements of redox pools and antioxidant systems is the use of mathematical modelling of metabolism, in particular for redox branches. In the context of central metabolism, previous studies elegantly shed a different light on climacteric respiration in tomato fruit using stoichiometric models (Colombié et al., 2015, Colombié et al., 2017). Using a medium-scale stoichiometric model, energy and the redox cofactors NAD(H) and NADP(H) were defined as internal metabolites and balanced so that constraining of the metabolic network was possible not only through C and N homeostasis, but also through the redox and energy status (Colombié et al., 2015). This model suggested a consistent requirement of NADPH for biomass synthesis and demonstrated that higher ATP hydrolysis was required for growth starting at the end of cell expansion and that a peak of CO_2 was released at the end of tomato ripening. This coincided with climacteric respiration of tomato fruit and involved energy dissipation by the AOX (**Figure 1B**), a redox marker of the mitochondrial compartment (Polidoros et al., 2009; Pétriacq et al., 2016). This was further confirmed by a more detailed stoichiometric model of the respiratory pathway, including AOX and uncoupling proteins (Colombié et al., 2017). Moreover, the recent flux analysis performed with grape cells under nitrogen limitation showed differently regulated fluxes were involved in the flavonoid (phenylpropanoid) pathway and in major carbon fluxes supporting a strong link between central metabolism and cell redox status by energy (ATP) and reducing power equivalents (NADPH and NADH) (Soubeyrand et al., 2018). Thus, mitochondrial function plays a notable role along fruit development in mitigating the redox poise upon an imbalance between energy supply and demand. In complement, when omics strategies failed to measure oxidative fluxes accurately, kinetic modelling of metabolism has proven to be a complementary and promising approach as it offers, with enzymatic and metabolic parameters, the possibility to describe quantitatively fluxes of cycling pathways such as redox metabolism. For instance, this was achieved previously for sucrose metabolism in the developing tomato fruit *via* a model of 13 differential equations describing the variations of hexoses, hexoses-phosphates and sucrose as a function of 24 enzyme reactions (Beauvoit et al., 2014). Similar approaches to redox cycles are necessary to obtain novel insights into the active redox dynamics involved in fruit biology.

The Key Role of ROS and Cognate Redox Signals in Fruit Responses to Environmental Constraints

The generation of ROS is a crucial process in response of plants to a changing environment and contributes to establish adaptive signalling pathways (Noctor et al., 2014). Oxidative stress typically comes as a secondary stress after primary stresses, whether they are abiotic constraints (**Figure 4**), such as drought or flooding, wounding, high light, cold or heat stress or biotic stresses including pest attacks or bacterial and fungal infections. Fruits are no exception to this rule: ROS can originate from NADPH oxidases (**Figure 1B**), specifically with respect to biotic and abiotic environmental challenges (Torres and Dangl, 2005; Suzuki et al., 2011; Mittler, 2017). Upon cold stress in apple fruits, NADPH oxidases might function *via* a regulatory node that integrates ethylene and ROS signalling pathways (Zermiani et al., 2015). In strawberry fruits, recent identification of NAPDH oxidase genes indicated that *FvRbohA* and *FvRbohD* might be involved in cold stress and defence responses (Zhang et al., 2018).

At present, it is assumed that major redox couples (NAD/P(H), ASC, GSH) are integral regulators of stress responses in plants (**Figure 4**), including both abiotic and biotic stresses (Pétriacq et al., 2013; Noctor and Mhamdi, 2014; Smirnoff, 2018; Gakière et al., 2018a). For instance, exogenous application of NAD^+ confers resistance to citrus canker disease in citrus (Alferez et al., 2018). In coherence with a modulation of these redox buffers, the antioxidant system further contributes in processing excess of ROS within stressed tissues (Foyer and Noctor, 2011; Smirnoff and Arnaud, 2019) (**Figure 4**). Additionally, redox processes dominate hormonal signalling *via* the stress hormones salicylic (SA), jasmonic (JA) and abscisic acids, which play a critical role in metabolic adjustments under stress conditions (Leng et al., 2013; Geigenberger and Fernie, 2014; Gakière et al., 2018a). Thus, a complex signalling network is devoted to shaping the fruit responses to stress. However, the interrelation between these multiple signalling partners is poorly understood, and its study will necessitate further research.

As for developmental processes (**Figure 4**), a hallmark of plant responses to stress is the activation of the ASC-GSH cycle (**Figure 2**). Upon arsenic and silicon exposure, fruits of two tomato cultivars exhibited different but profound redox

perturbations of H_2O_2 and antioxidant contents (e.g. lycopene, carotenoids and phenolics), ASC and GSH redox states and lipid peroxidation (Marmiroli et al., 2017a). Alternatively, a detailed proteomic study on tomato fruit confirmed the implication of ASC- and GSH-related proteins in response to this abiotic stress (Marmiroli et al., 2017b). Some of these redox alterations (H_2O_2, ASC and GSH redox states, total carotenoids and phenolics) were proposed as reliable arsenic exposition biomarkers for further studies that could broaden our knowledge on arsenic-induced abiotic stress in fruit (Marmiroli et al., 2017a). Besides arsenic, hot air treatment of strawberry fruits directly triggered the induction of antioxidant enzymes (e.g. CAT, APX and SOD), which further leads to a reduction of necrotrophic lesions caused by the fungal pathogen *Botrytis cinerea* (Jin et al., 2016). Additionally, a study of cold and light stress in tomato fruit unveils an interaction between temperature and light to modulate synthesis, recycling and oxidation of ASC in fruit (Massot et al., 2013). Light promoted the accumulation of ASC and GSH in tomato fruit, thus supporting the hypothesis of a stimulation in ASC synthesis by light (Gautier et al., 2009; Massot et al., 2012; Baldet et al., 2013; Smirnoff, 2018).

Redox signalling is associated with physiological disorders in fruits stored under multiple environmental stresses, such as for pome fruit, where redox-related metabolites are likely to accumulate (e.g. γ-aminobutyrate [GABA]) or rapidly decline (e.g. ASC, GSH) after exposure to low O_2 and/or elevated CO_2 environments (Lum et al., 2016). This in turn results in disturbances of the energetic and oxidative balance. In this context, both GABA and antioxidant metabolism are regulated by NAD/P(H) ratios, which confirms the tight link between cellular redox buffers and the regulation of oxidative metabolism (Trobacher et al., 2013; Lum et al., 2016) (**Figure 4**). A characterisation of TRX genes in harvested banana fruit suggests that the protein MaTrx12 regulates redox homeostasis, which impacts chilling tolerance (Wu et al., 2016). In tomato fruit, a combination of deep sequencing and bioinformatics revealed 163 circular RNAs that exhibited chilling responsive expression, among them several ones predicted to be involved in redox reactions and various stress signalling pathways (e.g. heat/cold shock protein, energy metabolism, hormonal responses, salt stress, cold-responsive transcription factors) (Zuo et al., 2016).

Infection of fruits with pathogenic microbes is a pressing issue due to dramatic postharvest diseases that can claim up to 50% of the total production worldwide (Romanazzi et al., 2016; Pétriacq et al., 2018). Resistance inducers have been used as promising strategies to elicit fruit defences against phytopathogens (Pétriacq et al., 2018). A global transcriptional analysis of strawberry fruit has demonstrated that the fungal elicitor chitosan and the salicylate-mimicking compound benzothiadiazole modulate chloroplastic signals to trigger various defence responses through redox alterations (e.g. *PX*, *GST*, *GRX*) (Landi et al., 2017). Accordingly, induction of sweet orange with chitosan or salicylic acid also alters the redox status of the cell (e.g. TRX, SOD, PX), as exemplified through RNAseq data (Coqueiro et al., 2015). Another example comes from *Peronophythora litchii*–infected litchi fruits that exhibit lower infection symptoms after treatment with a novel chitosan formulation (Jiang et al., 2018). Disease tolerance was correlated in litchi pericarp with higher activities of defensive (e.g. chitinase, phenylalanine lyase, glucanase) and antioxidant enzymes (e.g. SOD, CAT, APX), a lower O_2^- generation rate and lower malondialdehyde levels and higher contents of redox buffers including ascorbic acid and glutathione and reducing power. Moreover, priming of tomato seedlings with β-aminobutyrate (BABA), a novel phytohormone (Thevenet et al., 2017), confers resistance of tomato fruits to the fungal pathogen *B. cinerea* through metabolic rearrangements including antioxidant (e.g. flavonoids, polyphenols) and ABA contents (Wilkinson et al., 2018). This resistance was also associated with a delay in fruit ripening, which suggests a metabolic trade-off for defence metabolism versus fruit growth. Together, phytopathologic studies confirm the trigger of an oxidative burst in infected fruit tissues, for which excess ROS are mitigated both by a stimulation of enzymatic antioxidant systems and nonenzymatic protective, scavenging molecules (Tian et al., 2013). Hence, unsurprisingly, induction of antioxidant functions has proven to be effective in controlling postharvest diseases in fruits (Romanazzi et al., 2016; Pétriacq et al., 2018).

Practical Applications Towards Modifying Redox Metabolism in Fruits

Although the precise functions of redox regulators remain to be evidenced, a few practical applications are currently explored towards modifying redox biology in fruits. From a human health perspective, fruit redox metabolism received much attention since fruits and vegetables are major sources of essential antioxidative metabolites and thus recommended in human diet (e.g. five a day, http://www.fao.org/). Due to the intensively studied health effects of antioxidants for their numerous benefits for aging, cancer and chronic disorders, research focused on strategies to increase the antioxidant contents in consumable product. Moderate success has been obtained in engineering plants to increase antioxidants content such as ASC, GSH and vitamin E (Wargovich et al., 2012; Gallie, 2013). However, the *Golden Rice*, enriched in β-carotene (provitamin A), remains a successful story for redox application in crops combining plant biotechnologies, antioxidant synthesis pathway and human health (Botella-Pavía and Rodríguez-Concepción, 2006). Nevertheless, due to the importance of ROS signalling in developmental processes, the modulation of oxidative mechanisms can alter fruit growth. For instance, engineering tomato fruits to increase levels of antioxidants by enhancing chloroplast functions results in longer-lasting and firmer fruits (Mehta et al., 2002; Zhang et al., 2013). Thus, future applications need to consider the spatial and temporal regulations of redox homeostasis during plant development to improve significantly plant productivity.

Fruit physiological disorders during storage under multiple environmental stresses are also associated with redox perturbations (Lum et al., 2016). Fruit decay is a major issue caused by changes of the redox balance, including ROS production, in postharvest conditions (Pétriacq et al., 2018). From an agri-food perspective, chilling stress is oxidative but also particularly critical as low temperatures are often used

to delay senescence of many fruits (Lallu, 1997; Bustamante et al., 2016; Valenzuela et al., 2017; Alhassan et al., 2019). Reactive oxygen species accumulate during fruit overripening, which thus puts the improvement of fruit storage conditions in the forefront of redox signalling applications (Muñoz and Munné-Bosch, 2018). Furthermore, diverse chemical treatments have been identified to limit ROS accumulation by affecting either their production or processing. For instance, nitric oxide postharvest treatment in cucumber was associated with a decrease in ROS content and an increase of APX, CAT and SOD activities (Yang et al., 2011a; Liu et al., 2016). Other examples come from the use of chlorine dioxide fumigation in longan fruit that displays a reduction in enzymatic fruit browning (Saengnil et al., 2014) and ozone applications in citrus industry that allow to improve fruit shelf-life (Karaca, 2010). In addition, the plant defence hormones methyl-jasmonate (MeJA) and methyl-salicylate (MeSA) promote AOX gene expression in green pepper (Purvis, 1997). More recently, it was reported that MeJA also improved chilling tolerance of cucumber by increasing both CAT gene expression and enzyme activity (Liu et al., 2016). Biotechnological approaches have been further used to reduce oxidative stress in fruits mostly by overexpressing main ROS-processing enzymes (**Figure 2**) but also by increasing the total antioxidant content. In this context, anthocyanin- and flavonoids-enriched mango fruits have shown a better tolerance to cold during storage (Sudheeran et al., 2018).

Importantly, practical applications to modulate redox metabolism trigger plant resistance to biotic stresses. Fruits can suffer substantial yield losses from diseases as fruit decay at a postharvest level can claim up to 50% of the total production worldwide (Pétriacq et al., 2018). Given that ROS signalling is central to plant-pathogen interactions (Mittler, 2017), and main redox buffers are linked to defence hormonal signalling (Pétriacq et al., 2013; Pétriacq et al., 2016; Pétriacq et al., 2018), diverse treatment building on hormonal and redox signalling has shown a lower disease incidence and symptoms. For instance, nitric oxide treatment inhibits anthracnose (*Colletotrichum gloeosporioides*) in ripening mango (Hu et al., 2014) and further improves chilling tolerance in banana fruit *via* an induction of the antioxidative defence system (Wu et al., 2014). Additionally, MeSA and MeJA treatments can be used to stimulate pathogen resistance and increase the antioxidant content without affecting fruit quality in kiwi, tomato and peach (Tzortzakis and Economakis, 2007; Zhang et al., 2008; Fatemi et al., 2013).

CONCLUDING REMARKS AND FUTURE OUTLOOKS

Not before time, the simple Manichean belief of 'good' reductants and 'bad' oxidants, such as ROS, has become erroneous. There is so much to learn from future molecular studies of redox metabolism, particularly in fruit, for which an obvious lack of fundamental knowledge needs to be addressed. Reactive oxygen species production and cognate redox signals are key to harmonious metabolism and contribute to establishing adaptive signalling pathways throughout development and in response of fruits to environmental events. Whilst redox buffers, specifically ascorbate, clearly appear at the forefront of oxidative regulation, these redox mechanisms also seem to depend on the fruit species. Recent years have witnessed a growing interest in developing both analytical technologies (e.g. LCMS, NMR, ROS detection, redox proteomics) and mathematical modelling to provide quantitative description of the central metabolism and specialised pathways including antioxidant processes (Qin et al., 2009a; Beauvoit et al., 2014; Colombié et al., 2015; Colombié et al., 2017; Deborde et al., 2017). In tomato fruit, for instance, spatially resolved distribution of metabolites including antioxidants will help to decipher the involvement of such redox compounds in physiological responses (Nakamura et al., 2017).

Studying key spatiotemporal redox processes involved in fruit is of paramount importance. Numerous fruits, such as the ones from the Solanaceae family (e.g. tomato, pepper, eggplant), not only contain a cocktail of antioxidants (vitamins A and C, flavonoids), but also domestication of these plants has reduced the content in prohealth molecules such as vitamin C. Indeed, ascorbate was higher in ancestral cultivars of tomato (Gest et al., 2013b; Palma et al., 2015). These are among the many reasons for ascorbate to be at the heart of research on the plant redox hub, where plant scientists endeavour to increase fruit ASC content, which should improve human nutrition and plant tolerance to stress (Macknight et al., 2017). Progress in understanding the molecular signatures involved in the redox regulations that link central metabolism and stress pathways will help to define novel strategies for optimal fruit production and storage (Beauvoit et al., 2018).

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Primary Metabolism in Citrus Fruit as Affected by Its Unique Structure

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Citrus is one of the world's most important fruit crops, contributing essential nutrients, such as vitamin C and minerals, to the human diet. It is characterized by two important traits: first, its major edible part is composed of juice sacs, a unique structure among fruit, and second, relatively high levels of citric acid are accumulated in the vacuole of the juice sac cell. Although the major routes of primary metabolism are generally the same in citrus fruit and other plant systems, the fruit's unique structural features challenge our understanding of carbon flow into the fruit and its movement through all of its parts. In fact, acid metabolism and accumulation have only been summarized in a few reviews. Here we present a comprehensive view of sugar, acid and amino acid metabolism and their connections within the fruit, all in relation to the fruit's unique structure.

Keywords: acidity, amino acids, citrate, citrus fruit, sugars, primary metabolism, quality

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CITRUS FRUIT MORPHOLOGY — A UNIQUE STRUCTURE THAT DETERMINES CRITICAL ASPECTS OF PRIMARY METABOLISM

The citrus fruit, termed hesperidium, is a fleshy fruit which, like all berry-type fruit, is characterized by a thick and fleshy pericarp (Esau, 1966; Fahn, 1990). The pericarp is usually divided into three tissues: the exocarp, which is the outer skin, the mesocarp, which usually refers to the major fleshy, edible interior, and the endocarp, an internal tissue composed of one (as in tomato) or several cell layers. In true fruit, which develop from the ovary, these three tissues are part of the ovary wall.

The exocarp of citrus fruit is the outer colored peel, often referred to as the flavedo (Schneider, 1968) (**Figure 1A**). Proceeding inward is the albedo, the spongy white part of the peel. Most cell layers of the albedo are considered to be mesocarpal tissue, and the two or three innermost cell layers are referred to as endocarp (**Figure 1**). In mandarins, the albedo disintegrates during fruit maturation, leaving only the vascular system (reticula), which gives this group its name, *Citrus reticulata*. The pulp, the edible part of the fruit, is composed of juice sacs/vesicles that develop from the endocarp at an early stage of fruit development (**Figure 1**). Some authors refer to the juice sacs as endocarp, while others consider them to be a separate tissue. The juice sacs develop into the ovary locule, defined as the section in which the ovary wall that develops into fruit. The carpel and the juice sacs are covered by the same epidermal layer of segment epidermis (**Figure 1B**). The juice sac is connected to the wall by a stalk, which joins the segment epidermis, so the latter provides one continuous layer covering both the segment and the juice sac. Three major vascular bundles, a dorsal and two side (septal) bundles, are found in each section. Most juice sacs initiate from the dorsal wall, but some develop from the side wall, adjacent to the side vascular bundle (Koch and Avigne, 1990). When present, seeds develop in the inner side of the fruit, where the carpels merge or along the ovary wall. Nutrition is supplied by a specific bundle, termed seed (or central) bundle, reaching from the fruit pedicle to the center of the fruit.

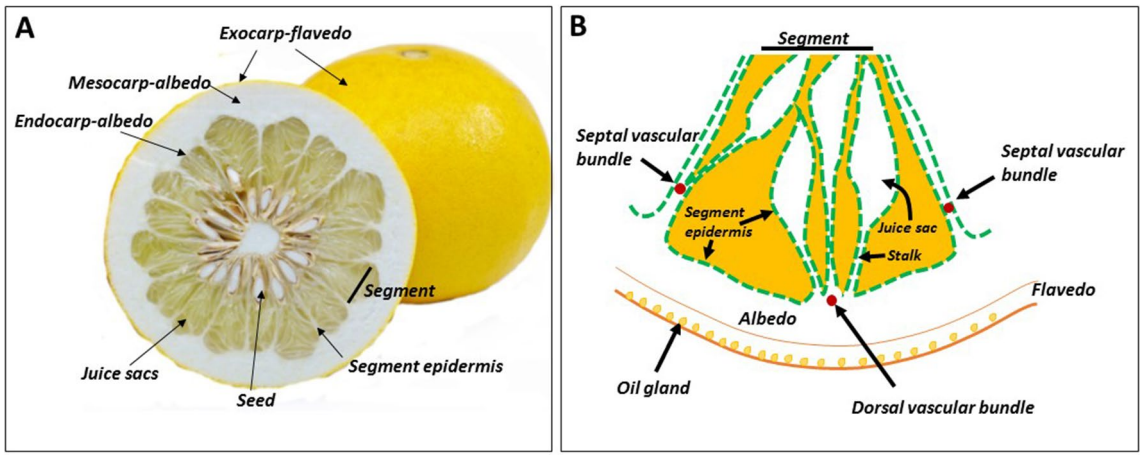

FIGURE 1 | Citrus fruit morphology. Cross section of the fruit **(A)**, and schematic representation of a segment, including the peel tissues **(B)**. The segment epidermis also covers the juice sacs, and the three segment vascular bundles — dorsal and two septal — do not reach the pulp tissues.

The juice sac is a unique structure, found only in fruit of the genus *Citrus* and its close relatives. It is often referred to as a "sac of juice," but this is misleading; the juice sac is composed of various layers of cells, each with distinct morphology (Shomer, 1975; Burns et al., 1992; Burns et al., 1994). The vesicle primordia emerge from the endocarp soon after fertilization and fruit set. In a few cases, juice sac primordia are visible even before fertilization and fruit set, mainly when fertilization does not occur and parthenocarpic fruit develop (this is the case in many commercial citrus cultivars) (Burns et al., 1992). During fruit development, the vacuole of the juice sac cell becomes greatly enlarged, occupying over 90% of the total cell volume, and releases its content as juice. At fruit maturity, the vacuole contains about 100, 75, and 90% of the total cellular sucrose, hexose and citrate, respectively (Echeverria and Valich, 1988).

The juice sac is considered the major fruit sink; however, it is disconnected from the vascular system, which ends in the albedo (**Figure 1B**). This unique characteristic determines photoassimilate translocation rate into the sink cells and therefore, rate of fruit development, and the time required to reach maturity.

CITRUS FRUIT DEVELOPMENT IN RELATION TO CHANGES IN SUGAR AND ACID CONTENTS AND CLIMATIC EFFECTS

In many citrus cultivars, the major external change that marks the conversion of the citrus ovary into a fruitlet is usually petal fall (Spiegel-Roy and Goldschmidt, 1996). Fruit development is divided into three overlapping stages: cell division (stage I), cell expansion (stage II), and fruit maturation (stage III) (Bain, 1958). During stage I, fruit growth is relatively moderate, and the peel, especially the albedo, thickens by cell division. During this stage, juice sacs grow out *via* cell division into the locule. Stage II is characterized by rapid fruit growth, mostly due to juice cell expansion. During stage III, the rate of fruit volume increase is greatly reduced. Externally, the major change is color break, and internally, sugar and acid levels reach the desired levels for harvesting and consumption, as discussed further by Spiegel-Roy and Goldschmidt (1996). Changes in secondary metabolites give the fruit its unique aroma and flavor (Tadeo et al., 2008). As there is no respiration burst or autocatalytic ethylene production, the citrus fruit does not undergo the classical ripening process, typical of climacteric fruits. For a given citrus cultivar, the final flavor quality of the fruit has to be determined empirically and depends, largely, on consumer preference (Goldenberg et al., 2018). The completion of fruit development is cultivar-dependent, with some cultivars, such as Satsuma mandarin (*Citrus unshiu*), being ready for harvest 5–6 months after flowering, whereas others, such as Valencia orange (*Citrus sinensis*), are harvested 12–14 months after flowering (Ladaniya, 2008). In hot climates, fruit development is accelerated, potentially reducing the time needed for fruit maturation by ca. 50% (Reuther, 1973). Sugar and acid level in the pulp are the two major fruit quality determinants. The major organic acid associated with pulp total acidity is citrate, which begins to accumulate during stage II of fruit development, when the fruit and its juice vesicle cells enlarge rapidly (Hussain et al., 2017). The accumulation continues for a few weeks, reaching a peak when the fruit volume is about 50% of its final value, then the acid declines gradually as the fruit matures. In most varieties, there is a slight increase in sugar content early in fruit development, but the major increase occurs during stage III, when the acid content declines (Sinclair, 1984). In citrus, the major translocated sugar is sucrose and in many varieties, it accumulates to double the level of glucose or fructose (Goldschmidt and Koch, 1996). Maturation index, which determines the fruit's internal quality, is the ratio between total soluble solids (TSS, BRIX) and total

acidity. As the acid content declines toward harvest, sugars account for most of the TSS.

As already noted, climate plays a major role in fruit development and maturation. Most of the commercial citrus cultivars were selected or bred in the subtropical regions of the world, and they are therefore adapted to regions where maturation occurs during the cool season (Wu et al., 2018). In hot climates, such as in the tropics, fruit maturation is accelerated and the major factor affected by temperature is the fruit acid level, with a linear relationship between the accumulation of heat hours and acid decline (Reuther, 1973). Therefore, in hot climates, the fruit reaches its maturation index faster than in colder climates, and tends to be too sweet. However, this is only part of the problem. Citrate catabolism is associated with an increase in alcohols, aldehydes and other secondary metabolites associated with reduced flavor and fruit decay (Porat et al., 2002). Therefore, in hot climates, the time during which the fruit is harvestable and marketable is considerably shortened, and fruit decay occurs faster than in the colder regions (Reuther, 1973). Hot climate has the opposite effect on color break, which requires the correct number of cold night-time to develop (Goldschmidt, 1988; Iglesias et al., 2007; Tadeo et al., 2008). Therefore, not only the fruit decay faster in hot climates, but their color does not fully develop, and in extreme cases may even remain green. One of the expected outcomes of climate change is warmer winter temperatures with shorter cold-night times (Cleland et al., 2007). As most citrus cultivars are harvested during this season, the effect of global warming is expected to be negative on both internal and external citrus fruit quality.

PHOTOASSIMILATE TRANSLOCATION INTO FRUIT AND SUGAR METABOLISM

Sink Strength and Its Control by Sucrose Hydrolysis in the Sink

Sink strength is determined by the sink's size and activity (reviewed in Sonnewald et al., 1994; Chang and Zhu, 2017; Smith et al., 2018). In crop plants, it is defined in practice by yield parameters (fruit quantity, fruit size, etc.), and quality parameters, such as carbohydrate (BRIX) and protein levels. Fruit size is genetically controlled, but physiological parameters, such as sink position in relation to other sinks and source tissues, and the time it takes to develop, also affect sink size and therefore, its strength (Bangerth and Ho, 1984; Ross-Ibarra, 2005). In tomato, there are over 30 loci that define fruit size, with many genes acting to control cell division at various developmental stages (Barrero et al., 2006). Practically, sink activity is defined as the rate of photoassimilate translocation and their contribution to growth and developmental processes relative to their accumulation. To simplify the discussion, we will refer here only to sugars, as the major photoassimilates in fruit in general, and in citrus fruit in particular. As with many other plant species, in citrus, sucrose is the major sugar translocated from the leaves to the fruit (Goldschmidt and Koch, 1996). During fruit maturation, it is the major accumulated sugar, with a sucrose:glucose:fructose ratio of 2:1:1 in many cultivars (Komatsu et al., 2002) and references therein). In many cases, sucrose accumulation is detected early in fruit development, indicating a higher translocation than utilization rate (Hiratsuka et al., 2017). As discussed further on, sucrose catabolism into hexoses within the fruit provides the central mechanism controlling sink activity, and therefore sink strength. Sucrose is hydrolyzed either to fructose and UDP-glucose by sucrose synthase (SuSy), a bidirectional enzyme, or to glucose and fructose by invertase, a unidirectional enzyme (**Figure 2**) (Roitsch and Gonzalez, 2004). Following hydrolysis, glucose and fructose are phosphorylated to glucose-6-phosphate and fructose-6-phosphate by hexose kinase and fructokinase, respectively, while UDP-glucose is phosphorylated to glucose-1-phosphate by UDP-glucose phosphorylase. While SuSy is cytosolic, sucrose hydrolysis by invertase is performed in the apoplasm by cell-wall invertases, in the cytosol by neutral/alkaline invertases, and in the vacuole by acidic invertases. The enzyme is modulated post-translationally by invertase inhibitor, which might act *in vivo*, but not necessarily *in vitro* (Roitsch and Gonzalez, 2004; Katz et al., 2007; Palmer et al., 2015). In a few plant systems, it has been shown that alteration of the activities of SuSy and various forms of invertase results in altered yield and/or carbohydrate levels, and thus altered sink strength. For example, one amino acid change in the tomato cell-wall invertase LIN5 enhanced specific activity of the enzyme, the rate of sucrose uptake and, overall, BRIX (Fridman et al., 2004; Baxter et al., 2005). Apoplasmic expression of a yeast invertase gene in potato enhanced tuber size (Sonnewald et al., 1997). Increased expression of cucumber SuSy induced sucrose and starch accumulation and increased fruit size (Fan et al., 2019). Transgenic downregulation of tomato SuSy resulted in reduced sucrose uptake early in fruit development, reduced fruit set, reduced fruit number and reduced fruit size (D'Aoust et al., 1999). Similarly, reduced expression of acid invertase and SuSy in muskmelon and cucumber, respectively, reduced fruit size and sucrose level (Yu et al., 2008; Fan et al., 2019). Phenotypes associated with reduced sink strength were also demonstrated in carrot roots by downregulating vacuolar and cell-wall invertases as well as SuSy (Tang et al., 1999; Tang and Sturm, 1999). Taken together, these studies demonstrated the importance of invertases and SuSy for sink strength and supported the notion that sink strength is controlled, at least in part, within the sink cells and/or at translocation points, i.e., zones of phloem unloading.

Mechanisms of Phloem Unloading

Sugar transport from the leaf to the collecting phloem is defined as sugar or phloem loading, and its release from the transport system, the releasing phloem, into the sink cell is defined as sugar or phloem unloading (reviewed in Rennie and Turgeon, 2009; Turgeon and Wolf, 2009; Zhang and Turgeon, 2018). Movement of photoassimilates from the leaves to the sink through the stem *via* the transport phloem is a complex process. Although a major driving force is the concentration gradient between source and sink according to the pressure flow hypothesis (Münch, 1930), long-distance movement requires in and out movement of solutes from the transport system to the surrounding tissue, temporal accumulation, and energy investment (Thompson, 2006). The mechanism(s) of sugar unloading has been investigated in a number of fruit and other sink organs, such as tomato, grape

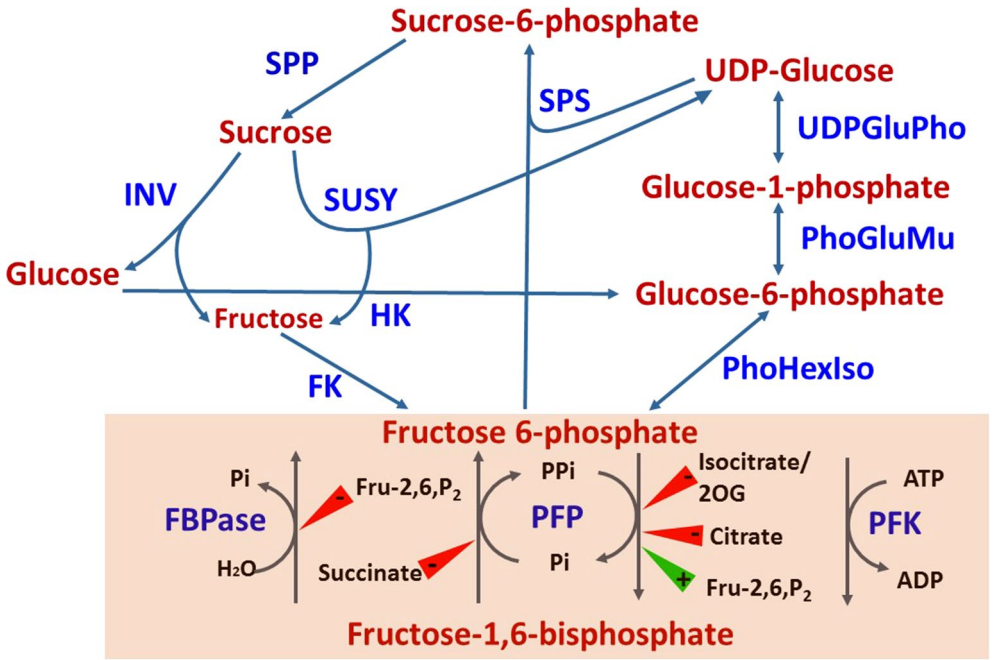

FIGURE 2 | Sugar metabolism and the interconversion of fructose-6-phosphate to fructose-1,6-bisphosphate (shaded). Compound inhibiting (red triangle) or inducing (green triangle) the activities of FBPase, PFP and PFK are shown. INV, invertase; SUSY, sucrose synthase; SPS, sucrose phosphate synthase; SPP, sucrose phosphate phosphatase; HK, hexose kinase; FK, fructokinase; UDPGluPho, UDP glucose phosphatase; PhoGluMu, phosphoglucose mutase; PhHexIso, phosphohexose isomerase; FBPase, fructose bisphosphate phosphatase; PFP, pyrophosphate-dependent fructose 6-phosphate kinase; PFK, ATP-dependent fructose 6-phosphate kinase; Fru-2,6-P_2, fructose-2,6, bisphosphate; 2OG, 2-oxoglutarate.

berry, cucumber, apple, walnut and potato tuber, by microscopy, fluorescent dyes, immunolocalization of sugar transporters, and use of transporter inhibitors (Ruan and Patrick, 1995; Wu et al., 2004; Zhang et al., 2004; Zhang et al., 2006; Nie et al., 2010; Hu et al., 2011; Palmer et al., 2015; Chen et al., 2017). Unloading is operated by two major mechanisms, symplasmic, in which sugar transport occurs through the plasmodesmata connecting the transport cells and the sink cells, and apoplasmic, where sink cells are not connected symplasmically to the transport cells, and transport must therefore cross membranes through the apoplasm, usually using facilitated transport mechanisms (reviewed in Rennie and Turgeon, 2009; Turgeon and Wolf, 2009; Zhang and Turgeon, 2018). In some fruit, such as cucumber, apple, and kiwifruit, unloading is apoplasmic throughout fruit development (Zhang et al., 2004; Hu et al., 2011; Chen et al., 2017). In other cases, such as tomato fruit and grape berry, there is a shift between symplasmic and apoplasmic unloading (Ruan and Patrick, 1995; Zhang et al., 2006), whereas in the potato tuber, the shift is from apoplasmic to symplasmic (Viola et al., 2001). In jujube, two shifts occur during fruit development; apoplasmic unloading early in fruit development shifts to symplasmic unloading, which then shifts back to apoplasmic unloading close to ripening (Nie et al., 2010). The two types of unloading occur simultaneously during walnut fruit and seed development, symplasmic in the seed and apoplasmic in the fruit (Wu et al., 2004). Overall, symplasmic unloading is considered faster than apoplasmic unloading (Zhang and Turgeon, 2018). Therefore, the mechanism is dependent on the rates of sink development and photoassimilate utilization versus the rate and form of their accumulation, in order to avoid an increase in osmoactive molecules in the cytosol. For instance, in tomato fruit and grape berry, fruit development and sugar utilization are rapid during the first half of their development, requiring symplasmic unloading. Later, when the fruit shifts from a utilizing to accumulating sink, the unloading rate is reduced by shifting it to apoplasmic. During the first half of potato tuber development, it accumulates soluble, osmoactive sugars, and therefore apoplasmic unloading is required, but during later stages, starch (a non-osmoactive compound) is accumulated, allowing a faster unloading. Jujube fruit is characterized by rapid growth during the middle stages of its development, and therefore apoplasmic unloading is interrupted by symplasmic unloading during this stage.

Sugar Metabolism in Citrus Fruit and Possible Mechanisms of Its Unloading and Movement

As phloem unloading has never been investigated directly in citrus fruit, the mechanisms in the various fruit tissues are unclear (Goldschmidt and Koch, 1996). Nevertheless, potential mechanisms can be discussed based on the following: (i) the kinetics of sugar transport into the various fruit tissues, vascular bundles, segment epidermis, stalk of the juice sac, and juice sac, as determined using photoassimilate distribution by $^{14}CO_2$-feeding of leaves, through either continuous labeling or

pulse-chase experiments (Koch, 1984; Koch and Avigne, 1984, Koch and Avigne, 1990; Hiratsuka et al., 2017), (ii) the steady-state distribution of sucrose and hexoses, as well as the activities of sugar-metabolizing enzymes and their protein levels in the various fruit tissues, especially during intensive sugar uptake (Echeverria and Valich, 1988; Lowell et al., 1989; Tomlinson et al., 1991; Echeverria, 1992; Echeverria et al., 1992; Nolte and Koch, 1993a; Nolte and Koch, 1993b; Kubo et al., 2001; Hiratsuka et al., 2017). While the activities of sugar-metabolizing enzymes have been well-studied and characterized, understanding their physiological role during the various stages of fruit development is more challenging. In tomato, for instance, about 20 days post-anthesis, SuSy activity decreased and the activity of an apoplasmic invertase, eventually identified as LIN5, was induced (Yelle et al., 1991; Fridman et al., 2004). This shift was associated with the well-studied shift from symplasmic to apoplasmic unloading and with the conversion of the fruit from utilizing to accumulating sink (Ruan and Patrick, 1995). It might be concluded, therefore, that in tomato fruit, SuSy activity is required to maintain a high rate of sucrose utilization, whereas invertase activity is associated with hexose accumulation. Clearly, the equivalent information is still missing in citrus fruit. In the following, photoassimilate movement and distribution, as well as the activities of sugar-metabolizing enzymes and their protein levels are described for the various fruit tissues (**Figure 3**). In most of the studies, the activities of the various forms of invertases are defined by their pH optima and solubility. Herein, alkaline/neutral-soluble invertase is referred to as cytosolic invertase, acid-soluble invertase as vacuolar invertase, and acid-insoluble invertase as cell-wall invertase (Roitsch and Gonzalez, 2004).

Vascular Bundle

^{14}C-photoassimilates, mostly as sucrose, were first detected in the dorsal vascular bundle, which seems to be the major transporting bundle, and to a lesser extent in the septal and central vascular bundles as well (**Figure 1**) (Koch, 1984; Koch and Avigne, 1990; Hiratsuka et al., 2017). Using pulse-chase experiments, maximal radiolabel was recovered after 6 h of labeling, but it was remarkably reduced after 24 h. Companion cells, phloem parenchyma and sieve elements are usually connected through plasmodesmata, thus allowing relatively rapid sugar movements. As this movement is slowed down in the albedo and pulp tissues, it can be assumed that temporal storage of sugars occurs in the companion cells/phloem parenchyma. The activity of vacuolar and cytosolic invertases, as well as of SuSy, might well be indicative of such storage. Indeed, SuSy activity was relatively high in the vascular bundle, especially during high sugar translocation, and the protein was strongly immunolabeled in the companion cells (**Figure 3**) (Lowell et al., 1989; Tomlinson et al., 1991; Nolte and Koch, 1993b; Hiratsuka et al., 2017). Acid and soluble invertase activities were also relatively high in the vascular bundle (Lowell et al., 1989; Tomlinson et al., 1991), supporting this notion. Cell-wall invertase was also present in the vascular bundle (Lowell et al., 1989), but this might represent apoplasmic movement from the vascular bundle into the surrounding albedo cells.

Albedo Cells

The vascular bundle terminates near the segment epidermis. However, albedo cells are present between the bundle and the segment epidermis, and therefore phloem unloading is expected to occur primarily into albedo cells before sugar reaches the pulp

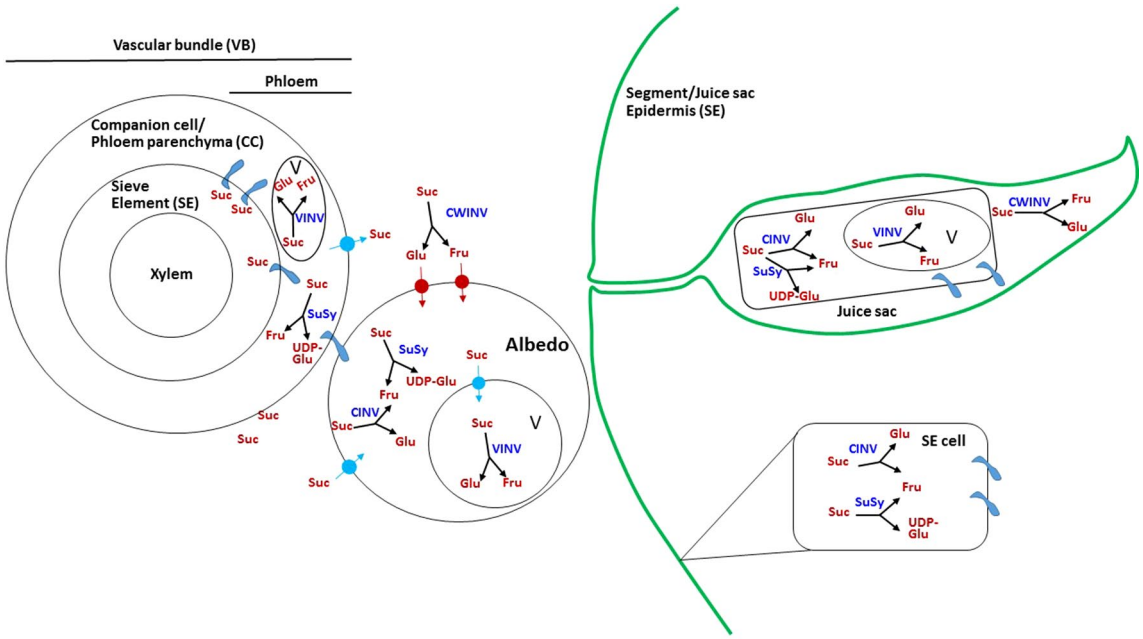

FIGURE 3 | Possible mechanism of sugar transport and metabolism in citrus fruit. Schematic presentation showing the possible paths of sucrose movement and hydrolysis in the various fruit tissues. Suc, sucrose; Glu, glucose; Fru, fructose; UDP-Glu, UDP glucose; V, vacuole; SUSY, sucrose synthase; VINV, vacuolar invertase; CWINV, cell-wall invertase; CINV, cytosolic invertase; plasmodesmata.

tissue (**Figure 3**) (Tomlinson et al., 1991). In young fruitlets and fruit, the albedo is the major tissue; cell division terminates within 4–5 weeks post-anthesis, and fruit growth during stage II is achieved by pulp expansion (Bain, 1958). Therefore, it might be assumed that most of the sugar in the albedo is transported, while only a minor part of it is required for albedo cell metabolism and development. A considerable amount of sucrose and hexoses are found in albedo cells (Lowell et al., 1989; Hiratsuka et al., 2017). Pulse-chase experiments demonstrated that most of the radiolabel remains in the post-phloem compartment, i.e., albedo cells, for about 24 h before reaching the pulp tissues, juice sac and segment epidermis (Koch and Avigne, 1990). This slowing of sugar movement could indicate that most of the transport is *via* the apoplasmic path. The presence of insoluble acid invertase activity could provide an indication for this type of unloading; such activity has been detected in albedo cells, although at a lower level than in other tissues—the vascular bundle, segment epidermis, and juice sacs (Tomlinson et al., 1991). In addition, the albedo contained considerable activities of SuSy, vacuolar invertase and cytosolic invertase (Lowell et al., 1989; Kubo et al., 2001; Hiratsuka et al., 2017); in fact, the activity of vacuolar invertase was strongest in the albedo compared to other fruit tissues, indicating active storage of sucrose/hexoses in the albedo after unloading. Obviously, the presence of plasmodesmata and a symplasmic pathway between the vascular bundle and albedo cells cannot be ruled out at this stage.

Segment Epidermis

As already noted, the segment epidermis provides a continuous layer with the juice sac epidermis (**Figure 1**). It is considered part of the transport tissues, and therefore enzymatic activities are sometimes reported for the vascular bundle and segment epidermis together (Lowell et al., 1989), although in other cases they are separated (Tomlinson et al., 1991; Kubo et al., 2001; Hiratsuka et al., 2017). A considerable percentage, about 30%, of the total radiolabel could be recovered in the epidermis, with maximal accumulation between 24 and 48 h after feeding in a pulse-chase experiment using grapefruit (*Citrus* × *paradisi*) (Koch and Avigne, 1990). With continuous labeling, about 50% of the total radiolabel was recovered in the segment epidermis within 24 h. However, when radiolabeled sugars were quantified in Satsuma mandarin after 48 h of feeding with $^{14}CO_2$, the segment epidermis displayed the lowest amount per fresh weight or per fruit (Hiratsuka et al., 2017). These discrepancies could be due to different experimental designs or reflect cultivar differences. Regardless, the segment epidermis provides a strong sink, and movement of photoassimilates from this sink to the juice sac cells cannot be ruled out. Sucrose hydrolysis in the segment epidermis was mediated by relatively high activities of SuSy and soluble invertase (**Figure 3**) (Tomlinson et al., 1991; Kubo et al., 2001; Hiratsuka et al., 2017). The activities of vacuolar and cell-wall invertases were not reported, and it might therefore be assumed that most of the cell-to-cell movement is through the symplastic pathway.

Juice Sac Stalk

Photoassimilates were detectable in the stalk of the juice sacs as early as 6 h after $^{14}CO_2$ feeding, as found by pulse-chase experiment. However, with continuous exposure, the kinetics of radioactivity accumulation were higher between 24 and 48 h of exposure (Koch and Avigne, 1990). Sugar-metabolizing enzymes were not monitored in the stalk separately from the juice sac, but the same mechanisms are likely to be operating in both parts of the juice sacs.

Juice Sacs

As the edible part of the fruit, sugar metabolism and transport in the juice sac have received more attention than in other fruit parts. Photoassimilate transport proceeds to the inner part of the juice sac (**Figure 3**). Following 1 h of $^{14}CO_2$ feeding to a source leaf next to grapefruit fruit, and 1 week of translocation, about 60% of the label was found in the juice sacs, with similar results in Satsuma mandarin (Koch and Avigne, 1984; Hiratsuka et al., 2017). A maximal rate of radiolabel accumulation in pulse-chase experiments was reached between 24 and 48 h of labeling (Koch and Avigne, 1990). Movement from the stalk to the distal part of the juice vesicle is relatively slow, and may take up to 96 h in the case of pomelo juice vesicles, which can reach 3 cm in length (Goldschmidt and Koch, 1996). Interestingly, whereas in grapefruit juice sacs, most of the labeled assimilates were recovered as sucrose, in Satsuma mandarin, fructose was predominant (Lowell et al., 1989; Hiratsuka et al., 2017). The accumulation of sucrose per fresh weight peaked in the juice sacs during stage II of fruit development (Lowell et al., 1989). Sucrose hydrolysis seemed to be mediated by all enzymes, as the activity of SuSy and that of the three forms of invertase were detected in the juice sacs (Echeverria and Valich, 1988; Lowell et al., 1989; Echeverria, 1992; Kubo et al., 2001; Hiratsuka et al., 2017). However, most studies showed that the activity of vacuolar invertase was relatively high, followed by SuSy activity. The activity of cell-wall invertase was also detected, but at a lower level, and soluble invertase activity was lowest. The relatively slow sugar transport in the juice sacs suggests diffusion. The presence of plasmodesmata has so far not been demonstrated, and cell-to cell movement might also follow a symplasmic pathway. Considering the relatively high activity of the vacuolar invertase, temporal storage and compartmentalization of sugars should occur during transport. Moreover, as the activity of cell-wall invertase was also demonstrated, apoplasmic movement cannot be ruled out, and it might also play a role in temporal storage. Lowell et al. (1989) indicated that young fruit might behave differently than mature ones, as the former displayed uphill transport in terms of sugar concentration whereas fully grown fruit displayed downhill transport (Lowell et al., 1989). Interestingly, out of the six SuSy genes in the citrus genome, two were induced in juice sacs during development, with one of them induced in the segment epidermis as well, suggesting that SuSy acts in sucrose mobilization within the juice sacs (Komatsu et al., 1999; Islam et al., 2014). As expected, invertase activity in all cellular compartments was reduced toward fruit maturation, in good correlation with the reduction in the invertase transcripts (Lowell et al., 1989; Katz et al., 2011). The activity and transcript levels of sucrose phosphate synthase genes were induced in Satsuma fruit juice sacs toward maturation, in accordance with an increase in sucrose level; however, in grapefruit, enzyme activity was induced from stage I to stage II of fruit development, and decreased toward

maturation (Lowell et al., 1989; Komatsu et al., 1996; Komatsu et al., 1999). This might explain the difference in sucrose levels between the two cultivars, as grapefruit accumulates less sucrose than Satsuma mandarin. Sucrose phosphate phosphatase was also induced during later stages of fruit development, suggesting that sucrose accumulation did not result only from translocation from the leaves but also from active synthesis within the juice sac cells (Komatsu et al., 1999; Katz et al., 2011). Nonutilized sucrose is stored in the vacuole and therefore, sucrose transport across the tonoplast might well play a role in regulating its levels within the cell and even its unloading rate. Sucrose and hexose uptake into tonoplast vesicles of sweet lime (*Citrus limetta*) was not induced by ATP, suggesting facilitated diffusion (Echeverria et al., 1992; Echeverria et al., 1997). Inclusion of acid invertase protein in the vesicles induced sucrose uptake, suggesting that sucrose hydrolysis by invertase or chemical acid hydrolysis within the vacuole provided the driving force for its uptake (Echeverria et al., 1992; Echeverria et al., 1997). An endocytic mechanism for sucrose transport across the tonoplast was also suggested (Etxeberria et al., 2005).

THE INTERCONVERSION OF FRUCTOSE-1-PHOSPHATE AND FRUCTOSE-1,6-BIPHOSPHATE, A CENTRAL STEP CONNECTING SUGAR AND ORGANIC ACID METABOLISM

While being transported into the fruit, sucrose can undergo metabolism in a few directions. Hexose phosphate synthesis is an important metabolic step, with the reversible conversion of fructose-1-phosphate (Fru-1-P) and fructose-1,6-biphosphate (Fru-1,6-P_2) providing a link between sugar and organic metabolism *via* glycolysis/gluconeogenesis pathways (**Figure 2**) (Plaxton, 1996; Fernie et al., 2004). The reaction is catalyzed by two independent mechanisms (Uyeda, 1979; Hofer, 1987; Yang et al., 2014). One involves two enzymes, an ATP-dependent phosphofructokinase (PFK) catalyzing the glycolytic conversion of Fru-6-P to Fru-1,6-P_2, and fructose-1,6-bisphosphatase (FBPase), catalyzing the reverse, gluconeogenic reaction. The other mechanism is composed of one bidirectional enzyme, pyrophosphate-dependent PFK (PFP) composed of two subunits, PFPα and PFPβ (Mertens, 1991; Muchut et al., 2019). Whereas PFK is generally considered ubiquitous, PFP has been described in prokaryotes and lower eukaryotes, including some bacteria, and some protozoan parasites (Bapteste et al., 2003). In addition, it is found in higher plants, where it is expressed in various tissues (Muchut et al., 2019 and references therein). While plants contain both PFP and PFK, bacteria and protozoa appear to have either one or the other, and yeast and animals contain only the latter (Bapteste et al., 2003). PFK is considered the more abundant enzyme, but its activity in plants is less characterized than that of PFP, due to its instability upon purification. PFK is found in both the cytosol and the plastids, whereas PFP is a cytosolic enzyme. Several hypotheses have been raised to explain the role of PFP in plants, including activation during stress (Krook et al., 2000; Fernie et al., 2001; Mutuku and Nose, 2012; Panozzo et al., 2019). Transgenic up/downregulation of PFP in tobacco, potato, and sugarcane resulted in only minor alternations in plant growth and metabolism (Hajirezaei et al., 1994; Paul et al., 1995; Nielsen and Stitt, 2001; Wood et al., 2002a; Wood et al., 2002b; Groenewald and Botha, 2008; Besir and Cuce, 2018). However, reduced expression of PFP in *Arabidopsis* resulted in delayed development, while higher expression resulted in induced development (Lim et al., 2009). Moreover, knockout mutants suggested that PFP is required for adaptation to salt and osmotic stress during germination and seedling growth (Lim et al., 2014). While Fru-2,6-P_2 is the major PFK activator in microorganisms and animals, in plants it does not activate PFK but rather PFP (Stitt, 1990). Citrate was found to be an inhibitor of PFP activity, especially in the glycolytic direction (Carnal and Black, 1983), and was suggested to affect the affinity of Fru-2,6-P_2 binding (Van Praag, 1997a; Van Praag et al., 1998).

PFP was detected in the juice sac cells of Valencia orange and grapefruit along with PFK and FBPase (Echeverria and Valich, 1988; Van Praag et al., 1999). While grapefruit PFP was strongly induced by Fru-2,6-P_2 in the forward reaction, it was barely affected by the activator in the reverse reaction (**Figure 2**) (Van Praag, 1997a; Van Praag, 1997b; Van Praag et al., 1998), as also demonstrated for potato, pineapple and tomato fruit (Van Schaftingen et al., 1982; Kobayashi et al., 1992; Tripodi and Podesta, 1997). It was also shown that citrate, and to some extent other intermediates of the tricarboxylic acid cycle, inhibit the glycolytic reaction of PFP in grapefruit, whereas the gluconeogenic reaction was barely affected (Van Praag, 1997a). Reduction in PFP activity in the ovaries of open versus closed flowers paralleled the reduction in protein levels of the two subunits, suggesting that the enzyme activity was regulated by its protein levels in the ovary (Kapri, 2003). However, more complex relationships were detected in the fruit, demonstrating the involvement of other mechanisms in regulating PFP activity. Recently, the two subunits of citrus PFP were coexpressed and expressed separately in bacteria (Muchut et al., 2019). Monomeric forms of both subunits were able to catalyze phosphorylation of Fru-1-P, but when coexpressed, the heteromeric form generated activity that was two orders of magnitude larger. While the activity of the heteromeric form was induced by Fru-2,6-P_2, that of the β-monomer was repressed and the activity of the α-monomer was barely affected.

CITRATE METABOLISM AND VACUOLAR PH HOMEOSTASIS IN CITRUS FRUIT

Citrate metabolism, transport and accumulation in citrus fruit have been recently reviewed (Hussain et al., 2017). Here they are described only briefly, with an emphasis on the biochemistry and control of transport mechanisms associated with proton and citrate translocation which have been characterized in citrus fruit.

Pulp Acidity and Citrate Level

Pulp acidity in citrus fruit is determined by two separate processes, citrate content in the vacuole of the juice sac cell

and vacuolar acidification, which can reach 0.3 M and pH 2.0, respectively in lemon and other acidic cultivars (Hussain et al., 2017). Although separate, these two processes are bioenergetically coregulated (Sadka et al., 2000a; Sadka et al., 2000b). During the first half of fruit development, citrate accumulation is accompanied by proton influx which reduces the vacuolar pH. Citrate has three dissociation constants (pKa) — 6.39, 4.77 and 3.14 — and in the vacuole it acts as a buffer by binding protons as they accumulate and reducing the pH, thus providing a driving force for additional proton influx (Müller and Taiz, 2002; Shimada et al., 2006). On the other hand, proton influx provides a driving force for citrate uptake, and probably also for its synthesis. When the vacuolar pH of Navel orange juice sacs was below 3.5, two forms of citrate were detected, citrateH_3 and citrateH_2^- (**Figure 4**) (Shimada et al., 2006). CitrateH^{2-} and citrateH^{3-} could be detected in pH ≥ 3.5 and pH ≥ 5.0, respectively. During the second half of fruit development, when the acid level declines, citrate removal is accompanied by proton efflux and increasing pH. There is a good correlation among different citrus cultivars between the level of juice pH (representing mostly vacuolar pH) and citric acid concentration (Etienne et al., 2013), and there are no reported cases in which pulp pH and citrate level are both low; therefore, altering citrate concentration will change pH homeostasis, and vice versa. However, early in fruit development, the two processes can be distinguished (Sadka et al., 2000a). Citrate accumulation in Minneola tangelo (*Citrus* × *tangelo*) starts in early June and continues for approximately 3 weeks; during this time, pH is slightly increased, probably due to the dilution effect associated with cell division. Significant pH reduction is only detected after 4 weeks, suggesting that the buildup of some citrate accumulation is required to induce proton influx into the vacuole. This also suggests that citrate accumulation precedes proton accumulation. In other fruit of low and moderate acidity levels, such as melons, i.e., pH 4.5–6.5, some inbred lines with higher pH and higher citrate + malate content than their parents were reported (Burger et al., 2003).

Although citrate is the major organic acid accumulated in citrus fruit, accounting for 90% of the total acids, the synthesis and accumulation of other organic acids have also been reported (Albertini et al., 2006). For instance, in orange, there is a transient increase in quinic and oxalic acids early in fruit development. Malic acid also accumulates to some extent during the maturation of lemon, lime and orange fruit.

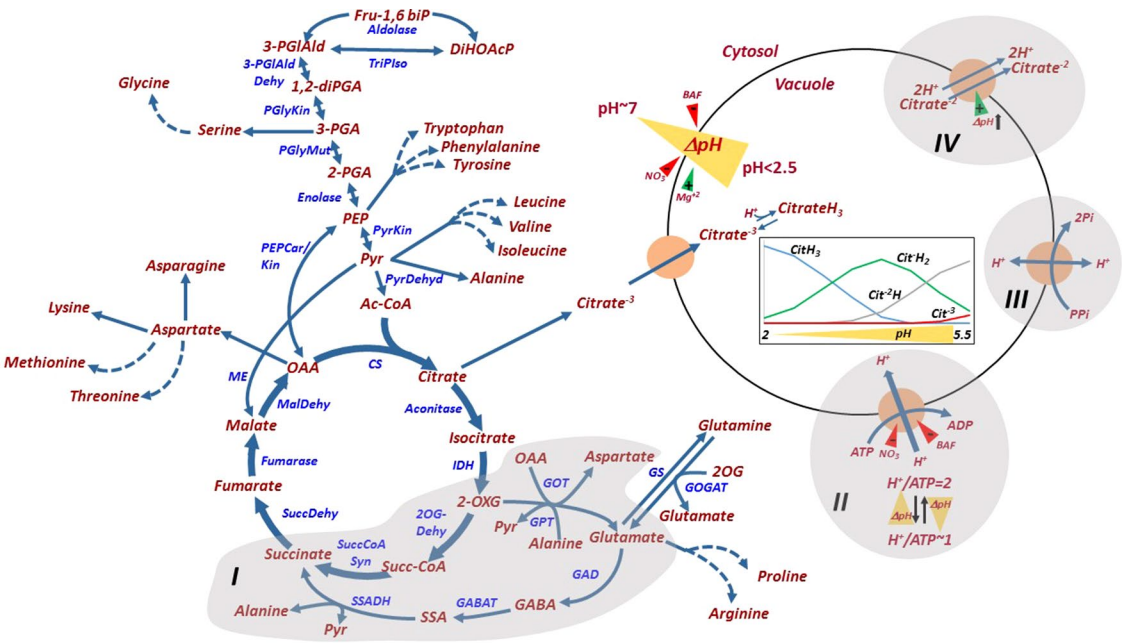

FIGURE 4 | Glycolysis, tricarboxylic acid metabolism, their metabolic connections to amino acids, and citrate and pH homeosthasis in the juice sac cell. The GABA shunt connecting glutamate and 2-oxoglutarate (I), the V-type H^+-ATPAse (II), the H^+-pyrophosphatase (III) and the Citrate/H^+ symporter (IV) are shaded. H^+/ATP coupling ratio and its pH dependence are indicated. ΔpH (yellow triangle) across the tonoplast is generated by the activities of the H^+-ATPase and the H^+-PPiase, and is induced by Mg^{+2} and reduced by BAF and nitrate. V-ATPase is inhibited by bafilomycin (BAF) and nitrate. The Citrate/H^+ symporter is driven by the acidification of the vacuole (pH decrease). The relative distribution of the various forms of citrate under different vacuolar pH values are shown in the inset figure, as explained in the text. Fru-1,6biP, fructose 1,6-bisphosphatase; DiHOAcP, dihydroxyacetone phosphate; 3-PGlAld, 3-phosphoglyceraldehyde; 1,2-diPGA, 1,2-diphosphoglycerate; 3-PGA, 3-phosphoglyceric acid; 2-PGA, 2-phosphoglyceric acid; PEP, phosphoenolpyruvate; Pyr, pyruvate; Ac-CoA, acetyl-Coenzyme A; 2-OXG, 2-oxoglutarate; Succ-CoA, succinyl-coenzyme A; OAA, oxaloacetate; 2OX, 2-oxoglutarate; GABA, γ-aminobutyric acid; SSA, succinic semialdehyde; PGlyMut, phosphoglycerate mutase; PyrKin, pyruvate kinase; PyrDehyd, pyruvate Dehydrogenase; CS, citrate synthase; IDH, isocitrate dehydrogenase; 2-OGDehyd, 2-oxoglutarate dehydrogenase; Succ-CoA Syn, succinyl-CoA synthase; SuccDehyd, succinate dehydrogenase; MalDehyd, malate dehydrogenase; PEPCar/Kin, phosphoenolpyruvate carboxykinase; GOT, glucooxaloacetate transaminase; GPT, glucopyruvate transaminase; GOGAT, glutamine oxoglutarate aminotransferase; GS, glutamine Synthetase; GAD, glutamate decarboxylase; GABAT,4-aminobutyrate-2-ketoglutarate transaminase; SSADH, succinic semialdehyde dehydrogenase.

Transport of Citrate and Protons Across the Tonoplast

So far, three mechanisms associated with proton movement across the tonoplast have been identified and characterized in citrus juice sac cells (Müller et al., 1996; Echeverria et al., 1997; Marsh et al., 2000; Shimada et al., 2006): V-type H^+-ATPase, the major enzyme driving proton influx; H^+-pyrophosphatase; and citrate/H^+ symporter, most likely acting to remove $citrate^{-2}$ out of the vacuole along with $2H^+$ (**Figure 4**). Other transport mechanisms, associated with citrate transport across the mitochondrial membrane and citrate movement into the vacuole, have been predicted for other plant species, but not for citrus fruit (Etienne et al., 2013). A P-type ATPase, homologous to the petunia PH5 and PH8, was suggested to play a role in vacuolar hyperacidification (Aprile et al., 2011; Shi et al., 2015). *PH5* and *PH5* were recently shown to be highly expressed in acid cultivars and downregulated in acidless cultivars, due to mutations in the MYB, HLH and/or WRKY transcription factors (Strazzer et al., 2019). While PH5 and PH8 were shown to localize to the vacuole in petunia, their membrane localization and biochemical properties in citrus require further research (Faraco et al., 2014; Verweij et al., 2008).

The identification and characterization of vacuolar transport mechanisms require isolating purified tonoplast vesicles or intact vacuoles (Lin et al., 1977). An array of experimental tools can then be used to study transport across the membranes, such as radiolabeled molecules (citrate), pH-dependent fluorescent dyes such as acridine orange or quinacrine (Stadelmann and Kinzel, 1972). An acidic-inside can be generated in isolated tonoplast vesicles or intact vacuoles through the activation of the V-type H^+-ATPase or the H^+-pyrophosphate with Mg–ATP or Mg–PPi and the use of inhibitors (bafilomycin A) or protonophores (gramicidin) to alter the pH gradient. For example, the addition of bafilomycin A inhibits the V-ATPase activity while gramicidin permeabilize the membrane to protons, thus abolishing the DpH across the membrane without affecting the pump hydrolytic activity. Tonoplast vesicles of juice sacs were isolated and purified from acidic cultivars and their acidless counterparts. ^{14}C-citrate uptake of acidless pomelo vesicles was about 20% higher than that of acid pomelo, eliminating the possibility that the difference in fruit acidity between these two cultivars was due to citrate transport into the vacuole (Canel et al., 1995). The uptake was enhanced by ATP (**Figure 4**) (Echeverria et al., 1997). Generation of a pH gradient was investigated in tonoplast vesicles of acid (*Citrus aurantifolia*) and acidless lime. As expected, it was induced by Mg–ATP, while bafilomycin and nitrate inhibited ATP hydrolysis and abolished the pH-gradient formation (**Figure 4**) (Brune et al., 2002). Sweet lime tonoplast vesicles appeared to generate a DpH four times faster than those of acid lime, but they had higher H^+ leakage following H^+-ATPase inhibition by EDTA than the acid lime, possibly representing their limited *in-vivo* capacity for H^+ retention. The lemon vacuolar H^+-ATPase was purified and characterized by Taiz's group (Müller et al., 1996; Müller et al., 1997; Müller et al., 1999; Müller and Taiz, 2002). They revealed that, in fact, two tonoplast-bound ATPase activities exist, a nitrate-sensitive V-type ATPase that is partially inhibited by vanadate, and a vanadate-sensitive ATPase that is partially inhibited by nitrate (Müller and Taiz, 2002). These results should be taken with caution because of the possible cross-contamination of the tonoplast vesicles with other membrane vesicles. Nitrate inhibition seemed to be dependent on the time of tonoplast vesicle preparation; for the same phenological stage, inhibition peaked during the spring and was minimal during the autumn–winter, suggesting an environmental effect resulting in seasonal changes in membrane lipid composition (Müller et al., 1999). Moreover, the H^+/ATP coupling ratio varied between 1 to 2 as the DpH increased, displaying a pH-dependent slippage, where the hydrolytic activity and the H^+ transport are partially uncoupled. Further, the fruit V-ATPase reconstituted into artificial proteoliposomes showed a steeper pH gradient than the corresponding reconstituted epicotyl enzyme (Müller et al., 1997). Overall, the following characteristics seem to allow lemon fruit V-ATPase to generate a steep pH gradient: (i) variable coupling, (ii) low pH-dependent slip rate, (iii) low proton permeability of the membrane, (iv) lower H^+/ATP stoichiometry, and (v) improved coupling by citrate, the major accumulated organic acid, which also enhance the enzyme's ability to generate a pH gradient. The pyrophosphatase activity in acid lime fruit was much lower than that of H^+-ATPase, suggesting the latter as the major mechanism for proton influx (Echeverria et al., 1997). Tonoplast vesicles isolated from juice cells of 'Valencia' oranges (*Citrus sinensis* L.) displayed similar V-type ATPase and V-PPiase activities, although a steady-state was reached faster with ATP as substrate. At a DpH of 3 units, V-PPiase synthesized PPi in the presence of Pi, indicating that mature orange juice cells acted as a source of PPi, providing a mechanism for recovery of stored energy in the form of the pH gradient across the vacuole during later stages of development and postharvest storage (Marsh et al., 2000). In summary, in light of the possible presence of an additional tonoplastic H^+ transport mechanism, P-ATPase, vacuolar proton homeostasis and transport across the tonoplast require further biochemical research.

A vacuolar citrate/H^+ symporter, CsCit1 (**Figure 4**), homologous to the *Arabidopsis* decarboxylate transporter, was characterized in orange fruit; its mRNA and protein levels coincided with the acid-decline stage, suggesting its role in citrate efflux (Shimada et al., 2006). Yeast cells expressing the *CsCit1* displayed electroneutral coupled citrate–H^+ cotransport with a stoichiometry of 1citrate/$2H^+$.

AMINO ACID HOMEOSTASIS IN CITRUS FRUIT

Amino acids have been studied in citrus fruit in relation to the nutritional value of the juice provided the motivation, mostly for early workers, to analyze the levels of free amino acids and their patterns of accumulation during fruit development and storage (reviewed in Sinclair, 1984). The exposure of fruit to stress on-the-tree and cold or heat treatments during storage was associated with the accumulation of several amino acids. Glycolysis and the tricarboxylic acid cycle are metabolically associated to amino acid metabolism (**Figure 4**), its relation to citrate decline

and the induction of a γ-aminobutyric acid (GABA) shunt during the second half of fruit development. Moreover, the possible relationships between amino acid accumulation and Huanglongbing (HLB) resistance/tolerance mechanisms have been recently investigated (Killiny and Hijaz, 2016; Killiny et al., 2018; Setamou et al., 2017; Yao et al., 2019).

Changes in Amino Acid Metabolism During Fruit Development and Upon External Stimuli

In general, all of the amino acids are detected in the juice of mature fruit, with aspartic acid, asparagine, serine, glutamic acid, proline and GABA being the more abundant (reviewed in Sinclair, 1984). A gradual increase in most of the free amino acids was detected during fruit development and toward maturation of Valencia orange (Sinclair, 1984). This increase is associated with citrate decline and it is common to all citrus cultivars (Kimura et al., 2017). However, different trends were detected in Navel oranges (*Citrus* × *sinensis*), with most amino acids and their metabolites decreasing from stage II to III of fruit development (Katz et al., 2011). A comparative analysis of total amino acid contents among various citrus cultivars showed lemon and mandarin with overall higher contents of essential amino acids than pomelo, grapefruit or sweet orange (Wang et al., 2016). Moreover, lemon displayed higher levels of amino acids with bitter taste, such as histidine, phenylalanine and valine, as well as acidic amino acids, aspartic acid and glutamic acid.

Following harvest, citrus fruit are usually subjected to relatively long storage periods at low temperatures. However, heat treatments, which vary from 37°C for 24 h to ~50°C for a few minutes, prior to storage, are common to reduce pathogenic agents, as well as to induce resistance to chilling and pathogens. The effects of such treatments on amino acid contents and metabolism were investigated, with conflicting results. In Satsuma mandarins, the contents of most amino acids were reduced or remained unchanged following heat treatment and only ornithine showed a consistent increase following the treatment (Yun et al., 2013). On the other hand, Matsumoto and Ikoma (2012) found that most Satsuma mandarin amino acids were heat-responsive, showing a remarkable contents increase during postharvest storage at 20°C or 30°C, but not at 5°C or 10°C. However, two amino acids, ornithine and glutamine, were cold-responsive, suggesting active metabolism during postharvest cold storage.

Changes in amino acid metabolism during fruit development of various cultivars and in the presence of external stimuli have been studied mostly by transcriptomic and metabolomic analyses. The activation of the GABA shunt, a major route for citrate catabolism (**Figure 4**), was identified in a transcriptomic analysis (Cercos et al., 2006) and confirmed by proteomics (Katz et al., 2007); these analyses identified an increase in the transcript of glutamate dehydrogenase, aspartate/alanine aminotransferase, glutamate dehydrogenase, glutamine synthase, GABA amino transferase and succinate semialdehyde dehydrogenase during fruit development, and the presence of their corresponding proteins during the declining-citrate stage of fruit development (Cercos et al., 2006; Katz et al., 2007; Katz et al., 2010; Katz et al., 2011; Lin et al., 2015). Moreover, use of an aconitase inhibitor, which induces citrate accumulation, resulted in induced activities of some of the enzymes of the GABA shunt (Degu et al., 2011). In addition, proteins of most amino acid-synthesis enzymes were induced either from early stage II to stage II or from stage II to stage III of fruit development, including pathways leading to the synthesis of cysteine, glycine, serine, leucine, valine, asparagine, aspartate, alanine, ornithine and glutamine (Katz et al., 2011). Induction of amino acid metabolism was suggested to play a role in the accumulation flavor-associated volatiles (Yu et al., 2015). Comparative transcriptomic analysis of high- and low-citrate oranges showed elevated transcript levels of phenylalanine-, arginine-, proline-, cysteine- and methionine-metabolism genes in the high-citrate orange (Lu et al., 2016). Cold storage of mandarins resulted in major alterations in amino acid metabolism, including the biosynthesis of proline and arginine, and significant enhancement of the catabolism of branched-chain amino acids (Yun et al., 2010; Tietel et al., 2011; Yun et al., 2012). Catabolism of the branched-chain amino acids leucine, isoleucine, and valine releases acetyl-CoA, providing a precursor for amino acid-derived volatiles that are associated with off-flavor development during fruit storage (Tietel et al., 2011). Water stress also induced alterations in the amino acid metabolism suggested to be involved in defense mechanisms against stress (Oliveira et al., 2015).

Amino Acids and Defense Against HLB

Citrus HLB, caused by the phloem sap-restricted bacterium *Candidatus* Liberibacter, is a serious production threat to the citrus industry in various regions of the world. The bacteria are transmitted by phloem sap-piercing citrus psyllids while they feed, mostly on young expanding vegetative shoots. Different citrus cultivars show varied susceptibility/tolerance to HLB. The differential response seems to be associated with psyllid feeding preferences and with plant tolerance to the bacteria. Based on controlled graft-inoculation experiments, cultivars were classified into three major groups, sensitive, moderately tolerant and tolerant, each showing different symptoms, from severe leaf chlorosis, depressed growth and death in the sensitive cultivars, to fewer and lesser severe symptoms in the tolerant cultivars. The bacteria appeared to be auxotrophic for a few amino acids, supplied by their host. The bacteria were suggested to affect free amino acid availability by altering the expression of amino acid storage proteins, at least in the insect host. To assess whether amino acid metabolism plays a role in the variable citrus tolerance to HLB, metabolomics analyses were performed in various cultivars on healthy and infected trees. Although most of the analyses were performed with phloem sap, and not the fruit, we include their brief description, as some fruit symptoms might also be associated with changes in amino acid metabolism. In a metabolic survey of phloem sap and leaves of citrus cultivars showing varied sensitivity/tolerance to HLB, the levels of all amino acids were elevated in the tolerant cultivars (Killiny and Hijaz, 2016; Killiny et al., 2018). Comparative analyses of amino acid contents in the phloem sap of bacterium-permissive (*Citrus* and psyllid) and non-permissive (non-*Citrus*) hosts showed

that seven amino acids, mostly of the glutamate family, were associated with susceptibility, whereas five amino acids, mostly of the serine family, were associated with tolerance/resistance (Setamou et al., 2017). Moreover, high proline-to-glycine ratios were associated with bacterium-permissive hosts. Overall, the level of consistency in these studies in relation to amino acid composition in sensitive/tolerant plant species was not high. HLB-symptomatic Valencia orange fruits showed an overall increase in the level of most detected amino acids as compared to no symptomatic fruit, possibly due to protein degradation (Yao et al., 2019).

CONCLUDING REMARKS

Along with secondary metabolites, products of primary metabolism — carbohydrates, organic acids, amino acids, fatty acids, and their polymeric forms — provide important components to fruit taste, aroma and nutritional value. Fruit vary in their structure, and this variation affects developmental, as well as primary and secondary metabolic processes. The juice sacs — the major pulp component in citrus — are unique among fruit. In this review, we summarize how this unique structure affects photoassimilate translocation, movement, metabolism, and accumulation. Surprisingly, despite intensive research on many aspects of citrus fruit development and metabolism, the mechanisms of photoassimilate unloading have so far not been investigated as in other fruit and sinks, although the research tools are quite well-developed. Here, sugars, organic acids, and amino acids are metabolically connected, and special attention is given to the connecting steps, i.e., the interconversion of Fru-6-P and Fru-1,6-P_2, and the GABA shunt. In summary, this review attempts to summarize research of primary metabolism in citrus fruit, emphasizing open questions deserving further research.

AUTHOR CONTRIBUTIONS

AS and EB wrote the text; LS and IK made the figures and helped with literature search.

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REVIEW
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Chemical Composition of Mango (*Mangifera indica* L.) Fruit: Nutritional and Phytochemical Compounds

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Mango fruit has a high nutritional value and health benefits due to important components. The present manuscript is a comprehensive update on the composition of mango fruit, including nutritional and phytochemical compounds, and the changes of these during development and postharvest. Mango components can be grouped into macronutrients (carbohydrates, proteins, amino acids, lipids, fatty, and organic acids), micronutrients (vitamins and minerals), and phytochemicals (phenolic, polyphenol, pigments, and volatile constituents). Mango fruit also contains structural carbohydrates such as pectins and cellulose. The major amino acids include lysine, leucine, cysteine, valine, arginine, phenylalanine, and methionine. The lipid composition increases during ripening, particularly the omega-3 and omega-6 fatty acids. The most important pigments of mango fruit include chlorophylls (*a* and *b*) and carotenoids. The most important organic acids include malic and citric acids, and they confer the fruit acidity. The volatile constituents are a heterogeneous group with different chemical functions that contribute to the aromatic profile of the fruit. During development and maturity stages occur important biochemical, physiological, and structural changes affecting mainly the nutritional and phytochemical composition, producing softening, and modifying aroma, flavor, and antioxidant capacity. In addition, postharvest handling practices influence total content of carotenoids, phenolic compounds, vitamin C, antioxidant capacity, and organoleptic properties.

Keywords: *Mangifera indica*, mango, maturation, postharvest, nutrition, antioxidants, polyphenols, carotenoids

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INTRODUCTION

This manuscript presents a compilation of updated information on the nutritional composition of different mango varieties (**Figure 1**), as well as their main phytochemical components, useful for human nutrition, health, and other applications for agricultural, pharmaceutical, and food industries and the changes of these components during development and postharvest. This knowledge should contribute to control fruit deterioration, greater use, and valorization of the fruit.

The review consists of four parts for better understanding of the reader. The first part corresponds to the description on the nutritional content of mango fruit based on its macronutrients

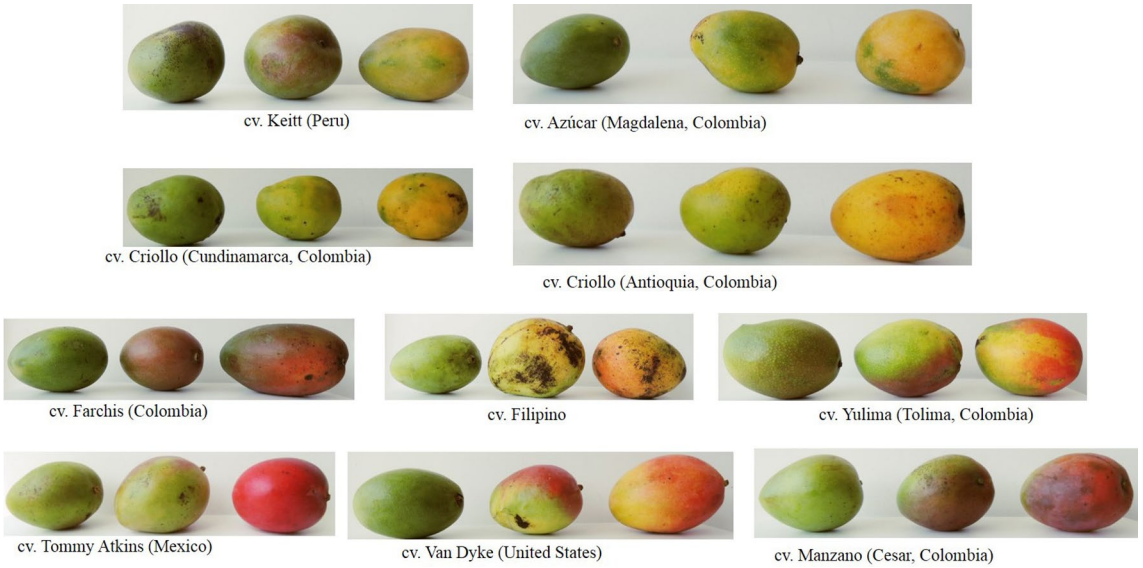

FIGURE 1 | Photographs of mango cultivars in different ripening stages.

(carbohydrates, lipids and fatty acids, proteins and amino acids, and organic acids) and micronutrients (vitamins and minerals). The second part continues with the analysis of the most relevant phytochemical compounds identified in mango fruit (phenolic acids, flavonoids, and pigments such as chlorophyll and carotenoids) not only in the edible portion of the fruit but also in the seed and skin, in order to show that by-products of the mango fruit can be used with potential benefit for health and industry.

In the third part, the changes on nutritional and phytochemical composition of mango fruit during the development and ripening are explained. It is well known that chemical components of mango vary according to the region of planting, cultivar, cultural practices, and nutritional conditions of the plant. However, the mango fruit development and ripening involve a series of biochemical, physiological, and structural controlled changes that affect the content of nutrients, phytochemical compounds, and the organoleptic characteristics leading to a soft, ripe, and edible mango fruit with desirable attributes for consumer acceptance.

Finally, taking into account that mango is a climacteric fruit that continues the ripening processes after detachment from the parent plant, attributed to the increases in the rate of respiration and ethylene production (Tharanathan et al., 2006), several postharvest handling processes can reduce their postharvest life and also the fruit composition, leading to losses in terms of quality and quantity, which can be considerably reduced by applying adequate and improved strategies and technologies to prolong the shelf life of mango fruit.

NUTRITIONAL COMPOSITION

Mango fruit is an important source of macronutrients such as carbohydrates, lipid and fatty acids, protein and amino acids, and organic acids. Also, mango has micronutrients such as vitamins and minerals and, finally, non-nutrients compound such as phenolic compounds, flavonoids and other polyphenols, chlorophyll, carotenoids, and volatile compounds. The energy value for 100 g of the pulp ranges from 60 to 190 kcal (250–795 kJ), being an important fruit for the human diet (**Table 1**). The nutritional, non-nutritional, and water contents of mango fruit vary depending of the cultivar and several preharvest and postharvest factors. For example, according to the United States Department of Agriculture (USDA) data of nutrient report, the mature mango pulp of Haden, Kent, Keitt, and/or Tommy Atkins varieties present 83.4 g of water per 100 g of fresh fruit, while the cultivar Azúcar from Colombia contain 79.3 g (Corrales-Bernal et al., 2014).

Macronutrients

Carbohydrates

Ripened mango fruit is a major source of sugars (glucose, fructose, and sucrose) and other carbohydrates such as starch and pectins (Bello-Pérez et al., 2007). All these are significant compounds from a nutritional and flavor aspect. The fruit flesh

TABLE 1 | Proximal composition analysis of mature mango fruit (*Mangifera indica* L.) (taken in part from Tharanathan et al., 2006).

Parameter	Content (g per 100 g of fruit dry weight basis)
Water	78.9–82.8
Ashes	0.34–0.52
Total lipid	0.30–0.53
Total protein	0.36–0.40
Total carbohydrate	16.20–17.18
Total dietary fiber	0.85–1.06
Energy (kcal)	62.1–190

of ripe mango contains about 15% of total sugars. Fructose is the major monosaccharide during the preclimateric phase (Bernardes et al., 2008), while sucrose is the principal sugar in ripe mango fruit (Instituto Colombiano de Bienestar Familiar (ICBF), 2015; Saleem-Dar et al., 2016; United States Department of Agriculture, Agricultural Research Service, 2018).

The USDA Nutrient Database (United States Department of Agriculture, Agricultural Research Service, 2018) reports that the total carbohydrate and sugar contents of Tommy Atkins, Haden, Kent, and Keitt cultivars per 100 g fruit is 14.98 and 13.66 g, respectively (sucrose, 6.97 g; glucose, 2.01; and fructose, 4.68 g), and 1.6 g of dietary fiber/100 g of fruit. The table of food composition from Colombia (Instituto Colombiano de Bienestar Familiar (ICBF), 2015) presented the content of glucose, fructose, sucrose, starch, and pectin in mango pulp (3.9, 1.0, 8.8, 1.8, and 8.2 g/100 g of pulp, respectively) and 2.6 g of dietary fiber. In African mango cultivars, the total sugar content varied between 10.5% and 32.4% (Othman and Mbogo, 2009), while the interval diminishes to 10–12% in Sudanese varieties (Nour et al., 2011). In general, many cultivars of mango contain sucrose, fructose, and glucose in order of highest to lowest (Bello-Pérez et al., 2007).

Starch is the most important from a quantitative point of view in the unripe mango fruit. During ripening, starch is hydrolyzed to glucose (Derese et al., 2017). After phosphorylation, glucose phosphate enters the hexose phosphate pool and fuels a "futile cycle" of sucrose synthesis and degradation (Geigenberger and Stitt, 1991) that controls the content of glucose, fructose, and sucrose, as it was found in kiwifruit (Moscatello et al., 2011). Thus, during ripening, glucose, fructose, and sucrose generally increase (Bernardes et al., 2008). The increase of these monosaccharides and disaccharides during maturation has been observed in cultivars Baladi (Sharaf et al., 1989), Haden (Castrillo et al., 1992), Alphonso (Yashoda et al., 2005), Dashehari (Kalra and Tandon, 1983), Keitt (Medlicott et al., 1986), and Tommy Atkins (Tasneem, 2004).

Pectin is a structural carbohydrate abundant in mango pulp and is considered an important component as a gelling sugar. When fruit is unripe, pectin is accumulated, but during ripening, its molecular weight decreases (Bello-Pérez et al., 2007; Saleem-Dar et al., 2016); this is attributed to the activity of hydrolysis of pectin enzymes in this stage (Prasanna et al., 2004).

Proteins and Amino Acids

Mango, like many fruits, has a low protein content with respect to the other macronutrients; by example, mango pulp from Colombia contributes to 0–0.6% of protein (Instituto Colombiano de Bienestar Familiar (ICBF), 2015; Corrales-Bernal et al., 2014), while in Peru, mango contains 1.5 to 5.5% total protein; in other cultivars like Java, mango has 1–2%, and in India, cultivars present low contents of total protein (0.5–1%) (Saleem-Dar et al., 2016).

The amino acid composition also varies among cultivars and maturation levels (Augustin et al., 1978). The amino acids alanine, arginine, glycine, serine, leucine, and isoleucine have been detected in considerable amounts in the ripe state, while all other amino acids are present in trace amounts (Tharanathan et al., 2006). **Table 2** presents the content of amino acids described by the United States Department of Agriculture, Agricultural Research Service, (2018).

TABLE 2 | Amino acid composition in edible portion of mango fruit (United States Department of Agriculture, Agricultural Research Service, 2018).

Amino acid	Content of g/100 g
Isoleucine	0–0.029
Leucine	0–0.050
Lysine	0–0.066
Methionine	0–0.008
Phenylalanine	0–0.027
Tyrosine	0–0.016
Tryptophan	0–0.013
Threonine	0–0.031
Valine	0–0.042
Histidine	0–0.019
Arginine	0–0.031
Alanine	0–0.082
Aspartic acid	0–0.068
Glutamic acid	0–0.096
Glycine	0–0.034
Proline	0–0.029
Serine	0–0.035

Lipids and Fatty Acids

Lipids are nutrients present in small quantities in mango pulp; however, the seed and the peel have been considered a source of fatty acids and are compared with cocoa butter (**Table 3**). These fatty acids are useful mango by-products and can be used in the pharmaceutical and food industries.

The prominent fatty acids found in the mango kernel are palmitic, stearic, oleic, and linoleic. Lignoceric, arachidic, linolenic, and behenic acids are present in lower concentrations (**Table 3**) (Jahurul et al., 2015). The content of triglycerides determined in blend of mango seed was 11% to 38.8% of 1,3-dipalmitoyl-2-oleoyl-glycerol (POP), 22.1% to 36.9% of 1,3-distearoyl-2-oleoyl-glycerol (SOS), and 15.4% to 16.2% of 1-palmitoyl-3-stearoyl-2 oleoyl-glycerol (POS) (Jahurul et al., 2014a). It has been reported that the incorporation of SOS-rich fats in chocolate products increases the solid fat content, leading to the inhibition of fat bloom and reducing the tempering time (Reddy and Prabhakar, 1994; Maheshwari and Reddy, 2005). Therefore, SOS-rich fractions could be used as an ingredient for the production of temperature-resistant hard butter, which is particularly useful in countries with high temperatures (Jahurul et al., 2014c); thus, mango seed fat is a potential cocoa butter alternative (Solís-Fuentes and Durán-de-Bazúa, 2004; Sonwai et al., 2012; Jahurul et al., 2014b; Jahurul et al., 2014c).

The fatty acid content in the peel and pulp has been also studied in different varieties of mango such as Malgoa, Totapuri, Benishan, Sundari, and Neelam and was found to range from 0.75% to 1.7% in the skin and 0.8% to 1.36 in the flesh (Pathak and Sarada, 1974), with the triglycerides being the major components of the pulp, while monoglycerides and diglycerides are minor components (Selvaraj et al., 1989). In the cultivars Alphonso, Pairi, and Kent, 17 different fatty acids were identified and quantified during development and ripening of mango fruit with an increase of unsaturated fatty acids and an omega-6/omega-3 ≤ 1 at the ripe stage, which suggests that mango fruit is a good source of essential fatty acids (**Table 3**) (Desphande et al.,

TABLE 3 | Content of fatty acids in mango fruit.

Carbon skeleton	Common name	Variety	Content	Part of the fruit
16:0[a]	Palmitic acid	Malaysia	6.95–10.93	Seed
		Mixed Egypt	5.8	
		Manila Mexico	9.29	
		Kaew Thailand	5.4	
		4 varieties Kenya	4.87–10.57	
18:0[a]	Stearic acid	Malaysia	32.8–47.62	Seed
		Mixed Egypt	38.3	
		Manila Mexico	39.07	
		Kaew Thailand	46.6	
		4 varieties Kenya	24.22–32.80	
20:0[a]	Arachidic acid	Malaysia	1.77–2.43	Seed
		Mixed Egypt	—	
		Manila Mexico	2.48	
		Kaew Thailand	1.7	
		4 varieties Kenya	0.67–1.64	
24:0[a]	Lignoceric acid	—	—	Seed
18:1 (Δ^{9})[a]	Oleic acid	Malaysia	37.01-47.28	Seed
		Mixed Egypt	46.1	
		Manila Mexico	40.81	
		Kaew Thailand	41.1	
		4 varieties Kenya	46.37–58.59	
18:2 ($\Delta^{9,12}$)[a]	Linoleic acid	Malaysia	3.66–6.87	Seed
		Mixed Egypt	8.2	
		Manila Mexico	6.06	
		Kaew Thailand	3.8	
		4 varieties Kenya	6.73–10.4	
18:3 ($\Delta^{9,12,15}$)[a]	α-Linoleic acid	—	—	Seed
14:0[b]	Myristic acid	Alphonso	174.29, 231.21	Pulp, peel
		Pairi	74.03, 295.16	
		Kent	40.57, 323.9	
16:0[b]	Palmitic acid	Alphonso	1,933.43, 2,682.16	Pulp, peel
		Pairi	896, 3,460.13	
		Kent	560.88, 2,883.29	
18:0[b]	Stearic acid	Alphonso	75.63, 123.57	Pulp, peel
		Pairi	33.36, 238.57	
		Kent	29.76,116.39	
20:0[b]	Arachidic acid	Alphonso	19.01, 29.21	Pulp, peel
		Pairi	7.2, 55.24	
		Kent	3.2, 32.56	
22:0[b]	Behenic acid	Alphonso	24.90, 43.83	Pulp, peel
		Pairi	8.88, 55.38	
		Kent	3.67, 43.73	
24:0[b]	Lignoceric acid	Alphonso	35.85, 86.16	Pulp, peel
		Pairi	27.04, 1,17.24	
		Kent	24.88, 71.15	
16:1, *n*–7[b]	Palmitoleic acid	Alphonso	2,881.90, 1,986.59	Pulp, peel
		Pairi	599.84, 533.59	
		Kent	314.28, 1,527.72	
16:1, *n*–5[b]	11-Hexadecenoic acid	Alphonso	146.22, 119.07	Pulp, peel
		Pairi	51.49, 58.01	
		Kent	22.42, 147.05	
17:1, *n*–7[b]	10-Heptadecenoic acid	Alphonso	11.82, n.d.	Pulp, peel
		Pairi	8.76, n.d.	
		Kent	3.76, n.d.	
18:1, *n*–9[b]	Oleic acid	Alphonso	856.59, 2,376.3	Pulp, peel
		Pairi	761.79, 2,847.25	
		Kent	261.3, 778.48	
18:1, *n*–7[b]	11-Octadecenoic acid	Alphonso	646.48, 480.59	Pulp, peel
		Pairi	248.78, 321.16	
		Kent	176.61, 282.14	
20:1, *n*–9[b]	11-Eicosenoic acid	Alphonso	6.57, 10.01	Pulp, peel
		Pairi	2.39, 10.49	
		Kent	n.d., n.d.	

(*Continued*)

TABLE 3 | Continued

Carbon skeleton	Common name	Variety	Content	Part of the fruit
16:2, *n*–4	9,12-Hexadecadienoic acid	Alphonso	33.86, n.d.	Pulp, peel
		Pairi	17.71, n.d.	
		Kent	16.09, n.d.	
18:2, *n*–6	Linoleic acid	Alphonso	83.58, 422.83	Pulp, peel
		Pairi	139.44, 1,956.03	
		Kent	80.05, 1,277.41	
18:2, *n*–3	9,15-Octadecadienoic acid	Alphonso	61.58, n.d	Pulp, peel
		Pairi	20.24, n.d.	
		Kent	20.93, n.d.	
7:2, *n*–3	Hepta-2,4(*E*,*E*)-dienoic acid	Alphonso	698.01, 265.93	Pulp, peel
		Pairi	662.32, 1,152.72	
		Kent	835.33, 352.98	
18:3, *n*–3	Linolenic acid	Alphonso	840.37, 1,149.88	Pulp, peel
		Pairi	522.23, 1,991.68	
		Kent	408.42, 1,201.18	

n.d., not detected.
[a]*(Jahurul et al., 2015).*
[b]*µg/g tissue (Desphande et al., 2016).*

2016). This increase in fatty acid content during maturation has also been observed in Harumanis, Kalabau, Stam Panjang, African Bush, Fazil, and Kanchamithia varieties (Saleem-Dar et al., 2016).

Bandyopadhyay and Gholap (1973) showed the association between the intensity of aroma and flavor and determined the ratio of palmitic–palmitoleic acid in ripening mango pulp to apply as an index of aroma and flavor of mangoes. When this ratio is greater or less than 1, the fruit has a mild or strong aroma and flavor, respectively. It has been proposed that fatty acids are probably precursors for the biosynthesis of lactones. These compounds are important in the research on flavor biochemistry of foods (Desphande et al., 2016).

Organic Acids

Organic acids are characterized by weak acidic properties. These compounds may have low molecular weight such as oxalic and citric acids or too high molecular weight like humic acids with aromatic nuclei composed of carboxylic and phenolic functional substituents (Richter et al., 2007). Organic acids are necessary for aerobic metabolism and as flavor constituents that contribute to fruit quality, organoleptic properties, and fruit acidity (Vallarino and Osorio, 2019).

Organic acid compounds have been identified in different varieties of mango, and the content depends on acid synthesis, degradation, utilization, compartmentation, and external factors such as temperature, light, fertilization, water supply, and other plant management practices (Vallarino and Osorio, 2019).

Fruit acidity of mango is attributed mainly to the content citric and malic acids (Matheyambath et al., 2016), although other common organic acids from the tricarboxylic acid cycle have been reported in mango fruit including citric, oxalic, succinic, malic, and pyruvic as well as tartaric, muconic, galipic, glucuronic, and galacturonic acids; of these, citric is the major organic acid [0.13% to 0.71% fresh weight (FW)] (Shashirekha and Patwardhan, 1976; Sarker and Muhsi, 1981; Medlicott and Thompson, 1985; Tharanathan et al., 2006). For example, citric and malic acids are the major organic acids in Keitt mango, whereas α-ketoglutaric, ascorbic, oxalic, and tartaric acids are found in lower concentrations (Medlicott and Thompson, 1985; Medlicott et al., 1986). In mangoes from Badami, citric acid presents the highest concentration, but succinic and malic acids were also detected (Shashirekha and Patwardhan, 1976). A similar profile was described in Fazli variety (pyruvic, citric, succinic, oxalic, and malic acids); in addition to those, tartaric acid was determined in Zardalu mangoes (Kumar et al., 1993). On the contrary, mango cultivars from Africa present a moderate content of citric acid (0.2% to 1.3%) (Othman and Mbogo, 2009).

Micronutrients

Vitamins

The nutrient database of USDA (National Nutrient Database for Standard Reference) reported the values of water-soluble and fat-soluble vitamins analyzed from Tommy Atkins, Keitt, Kent, and Haden cultivars (**Table 4**). Vitamin C and vitamin A are dominant, suggesting that regular consumption of mango fruit

TABLE 4 | Vitamin composition in 100 g of edible portion of mango fruit (United States Department of Agriculture, Agricultural Research Service, 2018).

Vitamin	Value per 100 g
Ascorbic acid (Vit C)	13.2–92.8 mg
Thiamine (Vit B1)	0.01–0.04 mg
Riboflavin (Vit B2)	0.02–0.07 mg
Niacin (Vit B3)	0.2–1.31 mg
Pantothenic acid (Vit B5)	0.16–0.24 mg
Pyridoxine (Vit B6)	0.05–0.16 mg
Folate total	20–69 µg
Folic acid	0 µg
Folate food	20–69 µg
B12	0.00 mg
Vitamin A	54 µg
Vitamin E (α-tocopherol)	0.79–1.02 mg
Vitamin K	4.2 µg

can provide the necessary dietary requirements of these vitamins (WHO/FAO, 2003).

Great variations exist in vitamin C content, fluctuating from 9.79 to 186 mg/100 g of mango pulp (Vazquez-Salinas and Lakshminarayana, 1985; Manthey and Perkins-Veazie, 2009; Wongmetha and Ke, 2012; ICBF, 2015; Matheyambath et al., 2016; USDA, 2018). The cultivars Kent, Tommy Atkins, and Keitt from Mexico, Peru, Brazil, and Ecuador; Haden from Peru and Mexico; and Ataulfo from Mexico were analyzed by Manthey and Perkins-Veazie (2009) who reported that the average vitamin C level over all harvest locations were 19.3, 24.7, 25.6, 31.0, and 125.4 mg/100 g of pulp in the Tommy Atkins, Keitt, Kent, Haden, and Ataulfo cultivars, respectively (Manthey and Perkins-Veazie, 2009). The higher vitamin C concentration in Ataulfo was comparable with the concentrations in Ubá cultivar from Brazil (77.7 mg/100 g FW) (Ribeiro et al., 2007). Sellamuthu et al., (2013) analyzed six African varieties (Tommy Atkins, Zill, Peach, Sabre, Rosa, and Phiva) and found that vitamin C content oscillated from 50.71 to 17.01 mg/100 g FW, being significantly higher ($P < 0.05$) in cv. Sabre and lower ($P < 0.05$) in cv. Tommy Atkins; and for the Palmer cultivar from Brazil, it was 40.9 mg/100 g FW (Valente et al., 2011). These variations are attributed to several different preharvest and postharvest factors, which all can influence the synthesis.

Vitamin C content changes during ripening; its content is higher in less ripe mango fruit compared with fully ripe mango (Matheyambath et al., 2016). Vitamin C decreases quickly 5 to 7 weeks after fruit setting and when ripe fruit is stored at room temperature (Yahia, 2011; Ibarraz-Garza et al., 2015). The vitamin C decrease may be due to the involvement of different metabolic pathways such as ethylene, oxalate, and tartrate biosynthesis because vitamin C is a coenzyme of their respective enzymes (Singh et al., 2011). In the analysis of vitamin C concentration in the pulp of Keitt, Sensation, and Xiangya mango cultivars from China at different stages (green and ripe mangoes), it was observed that the fruit pulp showed an important decrease during ripening (Keitt, 163.94 to 46.87 mg ascorbic acid equivalent (AAE)/100 g; Sensation, 176.03 to 29.34 mg AAE/100 g; and Xiangya, 160.35 to 30.84 mg AAE/100 g) (Hu et al., 2018).

The content of vitamin A in the fruit varies from 1,000 to 6,000 IU (Matheyambath et al., 2016). Thus, mango consumption is very important, especially for those regions where there is a deficiency of vitamin A (Muoki et al., 2009). Consumption of a single fruit (around 300 g) would supply 15–69 retinol equivalents (REs)/day depending on the cultivar. This would correspond to 11.5% of REs requirement per day for both teenagers and adults. For example, the consumption of three times per day of Tommy Atkins would provide more than 50% of the daily requirements of women and children aged 3–6 years (Muoki et al., 2009).

Similar conclusions were reported by Nana et al. (2005, 2006) who indicated that intervention strategies with mango improve the vitamin A intake by 50% and serum retinol concentrations by 26% of children (2 to 3 years) in Western Africa, over a 15-week period where mangoes are consumed little or nothing due to low seasonal availability.

The E and K vitamins are found in minor quantities (**Table 4**), while vitamin D has not been detected in any cultivars until now (ICBF, 2015; Saleem-Dar et al., 2016; USDA, 2018). The content of vitamin E is commonly low or moderate in fruit and can occur in tocopherols α-, β-, λ-, and γ-T; its corresponding tocotrienols are α-, β-, λ-, and γ-T3. The most biologically active form is α-tocopherol. In Ataulfo cultivar from Mexico, it was determined that fresh-cut mangoes contain 1.33 mg/100 g FW (Robles-Sanchez et al., 2009), which is greater than that informed by the USDA Nutrient Database (USDA, 2018) for Tommy Atkins, Kent, Keitt, and Haden.

Vitamin E increases from green mature stage to mature stage in Tommy-Kent mangoes, while in Tommy Atkins and Dasherai cultivars, the content of vitamin E is high at the unripe stage but later decreased (Barbosa Gámez et al., 2017; Singh et al., 2011). These changes in vitamin E content may in part be explained by the fact that vitamin C contributes to the biosynthesis of the oxidized form of vitamin E, tocopheroxyl radical, leading to the production of α-tocopherol (Méne-Saffrané, 2018). The opposite also occurs; that is, when vitamin C decreases, α-tocopherol content also decreases (Joas et al., 2009).

The vitamin B complex of mango fruit refers to water-soluble enzyme cofactors and their derivatives, which participate in different metabolic processes in plants and in humans. The vitamins included are thiamin (B1); riboflavin (B2); niacin (B3); pantothenic acid (B5); pyridoxine, pyridoxal, and pyridoxamine (B6); biotin (B8); and folate or folic acid (B9), except for biotin all the others vitamins, have been found in mango fruit (Saleem-Dar et al., 2016). All these vitamins B are important for proper human nutrition because humans are not able to synthesize these micronutrients. These vitamins can be also affected by several preharvest and postharvest factors, as well as the maturity stage because vitamin B synthesis is associated with the state of differentiation of cells (Aslam et al., 2010).

Minerals

According to the Recommended Daily Allowance (RDA) recommended levels by the National Research Council of USA (1989), mango may provide enough amount of essential minerals for human health, such as calcium, iron, magnesium, phosphorus, potassium, sodium, zinc, copper, manganese, and selenium. **Table 5** shows the essential mineral contents of Tommy

TABLE 5 | Mineral composition in edible portion of mango fruit.

Mineral	Value (mg) per 100 g[a]	Value (mg) per 100 g[b]
Calcium	7–16	9–21
Iron	0.09–0.41	0.1–0.9
Magnesium	8–19	10–38
Phosphorus	10–18	19–23
Potassium	120–211	147–617
Sodium	0–3	0–4
Zinc	0.06–0.15	0–0.1
Copper	0.04–0.32	n.d.
Manganese	0.03–0.12	1.6–18.2
Selenium	0–0.6	n.d.

n.d., not determined.

[a]*United States Department of Agriculture, Agricultural Research Service, (2018).*

[b]*Instituto Colombiano de Bienestar Familiar (ICBF), (2015).*

Atkins, Keitt, Kent, and/or Haden (USDA, 2018) and Colombian cultivars (ICBF 2015). The major essential minerals that mango pulp contributes are K, P and Ca, while the levels of Na, Zn, and Fe were the lowest, and the seeds and peels contain significantly higher levels than does the pulp in the following order: Ca > K > Mg > Na > Fe > Mn > Zn > Cu (Njiru et al., 2014).

PHYTOCHEMICAL COMPONENTS

Phenolic Acids

Phenolic acids are plant secondary metabolites that form part of human diet and are of significant importance because of their biological abilities and health benefits (Brglez et al., 2016; Yahia et al., 2017). Mango pulp includes the two major categories of phenolic acids in plants, hydroxybenzoic and hydroxycinnamic acid derivatives. These phenolic acids may be present free or conjugated forms with glucose or quinic acid (Mattila and Kumpulainen, 2002, Burton-Freeman et al., 2017). The hydroxybenzoic acids that have been detected in the mango pulp are gallic, vanillic, syringic, protocatechuic, and *p*-hydroxybenzoic acids, while the hydroxycinnamic acid derivatives are *p*-coumaric, chlorogenic, ferulic, and caffeic acids (Masibo and Qian, 2008; Ediriweera et al., 2017). The content and characteristics of phenolic acids depend on the cultivar, crop, and ripening stage (Corrales-Bernal et al., 2014, Burton-Freeman et al., 2017).

Different phenolic acids were identified in the flesh and skin of nine mango varieties cultivated in China (Abbasi et al., 2015). The highest phenolic acid in 100 g FW of pulp was ferulic acid (33.75 mg), followed by protocatechuic (0.77 mg), chlorogenic (0.96–6.20 mg), gallic (0.93–2.98 mg), vanillic (0.57–1.63 mg), and caffeic acids (0.25–0.10 mg) (Abbasi et al., 2015). Similarly, the major phenolic acids in Ataulfo mango from Mexico were protocatechuic acid (0.48–1.1 mg/100 g dry weight (DW)), vanillic acid (16.9–24.4 mg/100 g DW), gallic acid (94.6–98.7 mg/100 g DW), and chlorogenic acid (28–301 mg/100 g DW) (Palafox-Carlos et al., 2012a; Palafox-Carlos et al., 2012b). Contrary to these studies, Corrales-Bernal et al. (2016) and Kim et al. (2009) found that the major phenolic acid in mango pulp of Azúcar and Tommy Atkins varieties was gallic acid; however, these authors were unable to quantify the other well-known phenolic acids (*p*-hydroxybenzoic, *p*-coumaric, and ferulic acids) because of low concentration. The peel extracts of the mango cultivars Ataulfo, Keitt, Osteen, and Sensation have been found to have high concentrations of phenolic acids and derivatives such as gallic, syringic, methyl digallate ester, methyl gallate, gallotannins, galloyl glucose, theogallin, protocatechuic, and ferulic acid (Gómez-Caravaca et al., 2015; López-Cobo et al., 2017; Pacheco-Ordaz et al., 2018).

Hu et al. (2018) recently identified tentatively 34 compounds as derivatives of phenolic acids including gallotannins and quercetin derivatives, reporting for first time the detection of rosmarinic acid in mango fruit in different stages of ripeness, both in the peel and in the pulp. All the determinations were done by ultra-performance liquid chromatography in combination with electrospray ionization and quadrupole time-of-flight mass spectrometry (UPLC–ESI–QTOFMS).

Several authors, using different methods such as high-performance liquid chromatography (HPLC)/ESI–MS (Berardini et al., 2003) and HPLC–photodiode array (PDA)–MS (Ramirez et al., 2013) to detect and identify other derivatives of phenolic compounds. Berardini et al. (2003) have detected in the peel of mango Tommy Atkins 18 gallotannins (1.4 mg/g dry matter (DM) expressed as gallic acid) and five benzophenone derivatives identified tentatively as galloylated maclurin and iriflophenone glucosides, and 21 (15.5 mg/g DM) and eight gallotannins (0.2 mg/g DM) found in the seed and pulp, respectively. Among the gallotannins, some identified derivative compounds were assigned provisionally as iso-penta-*O*-galloyl-glucose, iso-hexa-*O*-galloy-glucose, penta-*O*-galloyl-glucose, tetra-*O*-galloyl-glucose, and hexa-*O*-galloy glucose (Berardini et al., 2003). Some identified derivatives of quercetin were consigned as quercetin-3-*O*-galactoside and quercetin-3-*O*-glucoside (Ramirez et al., 2013), quercetin-3-*O*-xyloside, quercetin-3-*O*-arabinopyranoside, and quercetin-3-*O*-arabinofuranoside (Schieber et al., 2003).

Ramirez et al. (2013) reported that the peel of Pica cultivar from Chile presented the highest content of total phenolic compounds (66.02 mg/100 g FW) analyzed by HPLC–PDA, and these authors detected 18 compounds present in Pica pulp and 13 in Pica peel more than what was detected in the peel and pulp of Tommy Atkins from Chile. The phenolic compounds identified in these fruits were three procyanidin dimers, seven phenolic acid derivatives, and four xanthones including homomangiferina, mangiferin, and mangiferin gallate in both peel and pulp of Pica and Tommy Atkins cultivars; only dimethyl mangiferin was identified in Tommy pulp (Ramírez et al., 2013).

Flavonoids and Other Polyphenolic Compounds

Polyphenols are a class of phytochemicals abundant throughout the plant kingdom. These molecules are generally involved in protecting plants from the ultraviolet radiation, aggression by pathogens, and reactive oxygen species (ROS) (Manach et al., 2004; Manach et al., 2005; Matheyambath et al., 2016). The most abundantly occurring polyphenols in plants are flavonoids, stilbenes, and lignans, of which flavonoids account for 60% of dietary polyphenols (Ramos, 2007; Van Breda et al., 2008).

Current interests are the antioxidant, anti-inflammatory, and anticarcinogenic activities of polyphenolic phytochemicals. The relevant polyphenols in the mango fruit related with the antioxidant capacity and/or quantity are the class of flavonoids (catechins, quercetin, kaempferol, rhamnetin, anthocyanins, and tannic acid) and the class of xanthones: mangiferin (Manach et al., 2004; Masibo and Qian, 2008). In the pulp of mango, the major flavonols are glycosides of quercetin (glucose, galactose, rhamnose, xylose, and arabinose), whereas kaempferol, isorhamnetin, fisetin, and myricetin are present in minor levels (Berardini et al., 2003; Ribeiro et al., 2008; Ramirez et al., 2013; USDA, 2018).

The USDA Nutrient Data Laboratory Flavonoid Database (https://www.ars.usda.gov/northeast-area/beltsville-md-bhnrc/beltsville-human-nutrition-research-center/nutrient-data-laboratory/) includes the data for 500 food items and 28 relevant

monomeric dietary flavonoids (flavonols, flavones, flavanones, flavan-3-ols, and anthocyanidins). The USDA, through this flavonoid database, has reported that 100 g of edible portion of mango fruit contains anthocyanidins (cyanidin, 0.10 mg; delphinidin 0.02 mg; and pelargonidin, 0.02 mg), the flavan-3-ol (+)-catechin (1.72 mg), traces of the flavones apigenin (0.01 mg) and luteolin (0.02), the flavonols kaempferol (0.05 mg) and myricetin (0.06 mg) (Haytowitz et al., 2018). In addition, the Nutrient Database of USDA has reported that mango fruit (Tommy Atkins, Kent, Keitt, and Haden) contains isoflavones (0.01 mg), proanthocyanidins dimers (1.8 mg), trimers (1.4), and four to six dimers (7.2 mg). Thus, the main flavonoids that have detected in mango flesh are quercetin and glycosides derivatives; the most relevant is the flavonol glycoside quercetin 3-galactoside (22.1 mg/kg), followed by quercetin 3-glucoside (16.0 mg/kg), quercetin 3-arabinoside (5.0 mg/kg), and quercetin aglycone (3.5 mg/kg) (Ediriweera et al., 2017; Matheyambath et al., 2016). Some mango cultivars were grown in Thailand (Tommy Atkins, Mani, Ngowe, R2E2, Kent, Jose, Mini-mango, Haden, Heidi, and Kaew Mon Duen Gao) has been found to contain glycosides of quercetin between 3.5 and 1,309.1 mg/100 g fruit (diglycoside, 3-*O*-gal, 3-*O*-glc, 3-*O*-xyl, 3-*O*-arap, 3-*O*-araf, and 3-*O*-rha), kaempferol 3-glc (6.7–77.3 mg), rhamnetin-3-*O*-gal/glc (5.4–734.4 mg), and quercetin (1.7–19.3 mg) (Berardini et al., 2003).

The seed and peel of mango fruit are also considered promising sources of polyphenols (Ribeiro et al., 2007; Ribeiro et al., 2008), with a total phenolic content for these residues of 6–8% of DM in Uba cultivar from Brazil, which is 4.6 and 7.3 times higher, respectively, than the content of the pulp, and a similar profile was reported for the flavonoids and xanthones of this variety (Ribeiro et al., 2008).

The xanthones are molecules formed by a C6–C3–C6 backbone structure with hydroxyl, methoxyl, and isoprene units linked to the A and B rings, which mostly occur as ethers or glycosides (Negi et al., 2013). Six xanthone derivatives have been identified (mangiferin, dimethyl mangiferin, homomangiferin, mangiferin gallate, isomangiferin, and isomangiferin gallate); among this group mangiferin (C2-*b*-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone), a C-glucosyl xanthone, is broadly distributed in higher plants, with demonstrated pharmacological and antioxidant activities. Mangiferin can be obtained from the bark, fruits, roots, and leaves of *Mangifera indica* Linn (Matheyambath et al., 2016). It has also described that mangiferin is able to activate anticancer, antimicrobial, antiatherosclerotic, antiallergenic, anti-inflammatory, analgesic, and immunomodulatory activities (Berardini et al., 2003; Ribeiro et al., 2008; Saleem-Dar et al., 2016; Ediriweera et al., 2017; Imran et al., 2017).

The content of mangiferin and derivatives is higher in the peel from Pica and Tommy Atkins mango fruit (22.15 and 9.68 mg/100 g FW, respectively) than in the pulp, 4.24 and 3.25 mg/100 g FW, respectively (Ramirez et al., 2013). In Uba and Tommy Atkins cultivars from Brazil, mangiferin was detected as 12.4 and 2.9 mg/kg DM, respectively, but it was not detected in Palmer pulp (Ribeiro et al., 2008).

In another analysis of mangiferin of the pulp of 11 cultivars grown in China, only in five of them was mangiferin reported (0.032–3.20 mg/100 g FW) (Luo et al., 2012), but interestingly, in the pulp of Azúcar cultivar grown in Colombia, it was 11.5 mg/100 g FW (data not shown).

For the derivative compounds of mangiferin, some characteristic peaks were identified as corresponding to compounds identified provisionally as maclurin-mono-*O*-galloyl-glucose, maclurin-di-*O*-galloyl-glucose, iriflophenone di-*O*-galloyl-glucose (Berardini et al., 2003), and mangiferin gallate (Schieber et al., 2003).

Pigments: Chlorophylls, Carotenoids, and Flavonoids

Chlorophylls

The color of the fruit peel is an important factor of mango maturation indices and quality, which changes from green to orange, yellow, or red flush, depending on the type of cultivar. In mango fruit, the green pigmentation is attributed to the presence of chlorophylls (Sudhakar et al., 2016; Nelson and Cox, 2017). Two types of chlorophyll have been detected in mango fruit, chlorophylls *a* (blue-green) and *b* (yellow-green), in a ratio of 3:1 (Medlicott et al., 1986; Saengnil and Kaewlublae, 1997; Lee and Schwartz, 2005).

In Tommy Atkins mango fruit, chloroplasts contain between 7 and 10 thylakoids per stack, connected through single intergranular thylakoids (Medlicott et al., 1985; Medlicott et al., 1986). Thylakoid system collapse inside the chloroplast at the beginning of ripening is associated with the loss of chlorophyll (Medlicott et al., 1986). Moreover, the reduction of chlorophyll levels in the peel is correlated with the increase of β-carotene content as ripening advances (Ketsa et al., 1999).

Chlorophyll and carotenoids are responsible for the color in some fruits (Sánchez-González et al., 2016). Several studies describe that chlorophyll breakdown is associated with the maturity of some fruits; (Du et al., 2014; Charoenchongsuk et al., 2015; Sánchez-González et al., 2016; Wei et al., 2019). When fruit appears green, an abundance of chlorophyll masks the carotenoids. The yellow color of carotenoids is unmasked by chlorophyll degradation during ripening (Charoenchongsuk et al., 2015). Taking this into account, the content of chlorophyll could be used as an indicator for the harvest in some fruits but not in others; for example, Charoenchongsuk et al. (2015) in their study show that 'Le Lectier' pears turned yellow during ripening with concomitant loss of chlorophylls *a* and *b* and carotenoids. In contrast, 'La France' pears stayed green even when fully ripe, because chlorophyll content did not change significantly.

Lechaudel et al. (2010) demonstrated that the greener color of the fruit is affected according to the position in the tree. The high values of chlorophyll correspond to green color of the peel and the flesh for fruits inside the canopy, whereas low values of chlorophyll are present in the fruits located in the top of the canopy.

The reduction in the chlorophyll content in the fruit is attributed to ethylene, which up-regulates the *de novo* synthesis of the enzyme chlorophyllase in the peel during ripening (Mir et al., 2001; Choo, 2018). Besides, chlorophyll can be also degraded by the peroxidase activity able to open the porphyrin ring, producing the loss of color (Kato and Shimizu, 1985). The peroxidase activity in the

peel of unripe and ripe Tongdum Thai mango was lesser than that in Nam Dokmai mango, which can explain the increased content of chlorophyll in Tongdum (Ketsa et al., 1999).

Carotenoids

Mango fruit is rich in carotenoid compounds. These molecules are lipid-soluble stains contributing to yellow-orange colors of mango fruit and red colors when mango is ripe, although the reddish color of peel in several varieties is due to anthocyanins (Masibo and Qian, 2008; Sivankalyani et al., 2016). Carotenoids are located in the chromoplasts, often masked by chlorophyll and non-photosynthetic plant tissues (Tanaka et al., 2008; Choo, 2018). In the chloroplasts, carotenoids act as an accessory pigment in light harvesting and as antioxidants converting the triplet chlorophyll to the singlet ground state (Arafat, 2005; Alcaíno et al., 2016). These compounds are classified in carotenes (α-carotene, β-carotene and γ-carotene), and xanthophylls (auroxanthin, antheraxanthin, neoxanthin, lutein, violaxanthin, and zeaxanthin) (Cano and Ancos, 1994; Varakumar et al., 2011; Eskin and Hoehn, 2013).

Sixteen carotenoids have been identified in fully ripe mango fruit, of which β-carotene (all-trans) account for 60% of the total carotenoids in the fruit (Saleem-Dar et al., 2016), while at the green stage, lutein (9- or 9′-*cis*-lutein) is the most representative, followed by many xanthophylls during the early ripening stage and during late ripening phases (Bramley, 2013; Ediriweera et al., 2017).

The carotenoid levels in fruit are directly affected by the development and the environmental conditions during fruit growth (Bramley, 2013). The variation in total carotenoid content in four different varieties of mango ripe and unripe was analyzed by Ellong et al. (2015). They reported that total carotenoids of mango cultivars Bassignac, Green, Julie, and Moussache were between 276.17 (Green) and 2,183 μg (Bassignac) per 100 g FW in the unripe stage. These values increased at the ripe stage, between 603.35 (Moussache) and 4,138.50 μg (Bassignac) of total carotenoids per 100 g FW (Ellong et al., 2015). A similar profile was observed in the total carotenoid contents of 12 mango varieties from Bangladesh at three stages of development: green (0.003 mg/100 g of pulp), semi-ripe (0.07 mg/100 g of pulp), and ripe (0.25 mg/100 g of pulp), indicating that the carotenoid content increased from green into ripe stage (Haque et al., 2015). Another study also compared the total carotenoid levels in the edible portion of the mango, variety Azúcar, at green, semi-ripe, and ripe stages (11.1, 11.8, and 10.7 mg of total carotenoids/100 g FW of edible portion, respectively). A significant difference only was found between the ripe and semi-ripe stages (Corrales-Bernal et al., 2014). The total carotenoid contents of the variety Azúcar were similar to those reported for the cultivars Keitt and Kent (10.4 and 12.9 mg/100 g pulp, respectively); higher than those of Tommy Atkins, Tainong No. 1, Irwin, and JinHwang cultivars (4.9, 5.2, 3.7, and 2.6, respectively) (Liu et al., 2013); but lower than those of Ataulfo mango (26.1 mg) (Manthey and Perkins-Veazie, 2009) at the ripe stage.

The total carotenoid content was also analyzed for the cultivars Haden, Tommy Atkins, Ubá, and Palmer from Brazil; this content ranged from 1.91 mg/100 g (Haden) to 2.63 mg/100 g (Palmer). The total carotenoid, β-carotene, accounted for 661.27 (Palmer) to 2.220 μg/100 g (Ubá) (Ribeiro et al., 2007). These values are higher than those in the Indian cultivars (Saleem-Dar et al., 2016). Comparable results were reported by the Nutrient Database of USDA (2018) for β-carotene (640 μg/100 g), α-carotene (9 μg/100 g), β-cryptoxanthin (10 μg/100 g), lutein and zeaxanthin (23 μg/100 g), and lycopene (3 μg/100 g) in Tommy Atkins, Kent, Keitt, and Haden mangoes.

Although the differences in the composition of carotenoids between different varieties may be due to environmental and genetic factors, state of maturation, production, and postharvest handling techniques, they can also be attributed to the analytical methods employed and to the unstable nature of carotenoids (Burton-Freeman et al., 2017). More than 25 carotenoids (free form, butyrates, and esterified), have been identified, but the major carotenoids in mango flesh seem to be all-*trans*-β-carotene, and all-*trans*- and 9-*cis*-violaxanthin (Mercadante et al., 1997; Ornelas-Paz et al., 2007; Petry and Mercadante, 2016).

The analysis of seven ripe mango cultivars (Ataulfo, Manila, Criollo, Paraíso, Haden, Kent, and Tommy Atkins) by HPLC coupled to a C30 stationary phase and diode array, fluorescence, and mass detectors showed that the highest content of carotenoid was contributed by all-*trans*-β-carotene (0.4 and 2.8 mg/100 g FW), with mangoes Haden and Ataulfo having the highest concentration, followed by all-*trans*-violaxanthin (0.5–2.8 mg/100 g FW) and 9-*cis*-violaxanthin (0.4–2.0 mg/100 g FW) (Ornelas-Paz et al., 2007). In a mango cultivar from Brazil, it was identified by HPLC and MS that all-*trans*-violaxanthin (2.1 mg/100 g FW) was the major carotenoid, followed by all-*trans*-β-carotene (1.5 mg/100 g FW) and 9-*cis*-violaxanthin (1.0 mg/100 g FW) (Mercadante et al., 1997).

A study of five mango varieties harvested at different times [Tommy Atkins (México, Brazil, and Ecuador), Kent and Keitt (Mexico), and Haden (Peru)] was performed to compare the β-carotene content over 1 year of harvest. This study showed that the concentration of β-carotene ranged from 5 (Tommy Atkins) to 30 mg/kg FW (Ataulfo) among the five varieties (Manthey and Perkins-Veazie, 2009), indicating that the type of cultivar had a greater influence on the levels of β-carotene but not the country of origin or harvest date (Manthey and Perkins-Veazie, 2009).

Volatile Compounds

The volatile compounds of mango are characterized by having low molecular weight (<400 Da), and in a wide range of functional groups, they can be in free or glycoside form. Also, they have a high vapor pressure that allows them to disperse easily and quickly in air, water, and soil (Sharifi and Ryu, 2018). This group of compounds that determine the characteristic aroma of the fruit is commonly present in small quantities, approximately 50 ppm or less, which comprises mixtures of monoterpenes, sesquiterpenes, and volatile oxygenates (monoterpenes, sesquiterpenes, esters, lactones, alcohols, aldehydes, ketones, volatile fatty acids, some degradation product of phenols, and some carotenoids) (Bender et al., 2000; Pino et al., 2005; Lebrun et al., 2008; Pandit et al., 2010; Li et al., 2017). They have a heterogeneous distribution quantitatively

and qualitatively among cultivars, maturity stage, and tissues of the fruit (MacLeod and Pieris, 1984; Adedeji et al., 1992; Andrade et al., 2000; Lalel et al., 2003a; Lalel et al., 2003b; Lalel et al., 2003c; Estarrón and Martindel Campo, 2016). Singh et al., (2004) analyzed more than 285 different volatile compounds in mango fruit including 7 acids, 55 alcohols, 31 aldehydes, 26 ketones, 14 lactones, 74 esters, 69 terpene hydrocarbons, and 9 other compounds (Singh et al., 2004). The most abundant volatile components in mango germplasms from China, the Americas, Thailand, India, Cuba, Indonesia, and the Philippines are monoterpenes (Li et al., 2017).

The content of these molecules can vary according to the method used for their extraction and identification and quantification, such as solid-phase microextraction (SPME) (Lalel et al., 2003a; Lalel et al., 2003b; Lalel et al., 2003c; Rivera et al., 2017), liquid–liquid extraction (Ollé et al., 1997), simultaneous distillation–extraction (MacLeod and Pieris, 1984; Pino et al., 1989; Andrade et al., 2000; Pino et al., 2005), static or dynamic headspace (Malundo et al., 1997), and solid-phase extraction (Adedeji et al., 1992).

Pino et al., (2005) analyzed the volatile compounds of 20 mango cultivars from the National Botanic Garden in Havana, Cuba, using simultaneously distillation–extraction, gas chromatography (GC), and GC coupled to MS (GC–MS). They reported 180 volatile compounds from 372 identified with a concentration ranging from 18 to 123 mg/kg of fresh fruit (Pino et al., 2005). In these cultivars, the major volatile compounds were terpene hydrocarbons such as delta-3-carene, a dominant compound present in other mango cultivar grown in Venezuela extracted by similar methods (MacLeod and Gonzalez de Troconis, 1982); followed by limonene also present in cultivars Baladi (Ollé et al., 1998), Carlota, and Bacuri (Andrade et al., 2000); terpinolene characteristics of Sri Lanka (MacLeod and Pieris, 1984); and cultivars grown in Australia (Bartley, 1988). The following quantitative important class of volatile compounds in mango grown in Cuba was 90 aliphatic, 16 aromatics, and 8 terpene esters found, with ethyl acetate and ethyl butanoate as the major esters; lactones were also detected (Pino et al., 2005; Desphande et al., 2016).

Some other examples of volatile constituents detected in the pulp of Tommy Atkins mangoes are pinene, limonene, α-terpinolene, D-carvone, β-elemene, α-bourbonene, β-cubebene, α-cubebene, aromadendrene, α-humulene, germacrene D, and *cis*-caryophyllene (Rivera et al., 2017). Some of the compounds found in the variety Bowen grown in Australia include ethyl butanoate, thujene, ethyl decanoate, β-caryophyllene, 3-carene, myrcene, ethyl butenaonate, and β-phellandrene (Correa, 2012). Some of the compounds found in the sap of seven mango varieties grown in India include β-myrcene, *trans*-/*cis*-ocimene, and limonene (Johna et al., 1999).

Some of the compounds detected in the mango sap from nine cultivars grown in Pakistan included (+)-3-carene, sabinene, α-phellandrene, α-humulene, γ-terpinene, α-pinene, and (−)-*trans*-caryophyllene (Musharraf et al., 2016). The 3-carene is reported as the most abundant for the varieties Bizcochuelo, S. Hayden, Tommy Atkins, Kent, Keitt, M. Bingué, Tête de chat, Palmer, and Irwin (Torres et al., 2012; Liu et al., 2013). In the varieties Bizcochuelo, S. Hayden, and Amelie, the caryophyllene is abundant (Pino et al., 1989), while α-terpinolene, a representative volatile compound, is in the cultivars Palmer, Kensington Pride, Tainong No. 1, JinHwang, and Keitt (Liu et al., 2013).

Volatiles in Tommy Atkins mangoes grown in Mexico were identified using SPME and GC–MS, which included pinene, limonene, α-terpinolene, D-carvone, β-elemene, α-bourbonene, β-cubebene, α-cubebene, aromadendrene, α-humulene, germacrene D, and *cis*-caryophyllene (Rivera et al., 2017). It has also been observed that mango fruit grown in the American continent commonly contains terpinene, sabinene, α-copaene, ethyl dodecanoate, and benzaldehyde, whereas the following terpenes were found in all the varieties studied: limonene, α-pinene, β-pinene, 3-carene, α-terpinolene, and α-humulene (Pino et al., 2005; Correa 2012; Estarrón and Martindel Campo, 2016).

CHANGES IN NUTRITIONAL AND PHYTOCHEMICAL COMPONENTS DURING FRUIT DEVELOPMENT, RIPENING, AND SENESCENCE

The beneficial effects of the mango components in human health due to their dietary, nutritional, and biological properties are affected by fruit development, ripening, and senescence. The maturity stage is a significant aspect that affects the compositional quality of fruit including nutritional factors, since during fruit ripening occur important biochemical, physiological, and structural changes.

The development of mango fruit occurs in four phases: 1) the juvenile until 21 days from fruit setting, when a rapid cellular growth occurs; 2) phase of maximum growth between 21 and 49 days from fruit setting, involving cell enlargement and initiation of maturation; 3) maturation and ripening stage between 49 and 77 days from fruit set, when the respiration climacteric and ripening process occur; and 4) senescence stage from day 77 from fruit set onwards, considered as the post-ripening stage, which is susceptible to microbial attack followed by decay and death (Tharanathan et al., 2006).

During the growth and ripening processes of mango fruit, changes in chemical composition occur (**Figure 2**), including decrease in ash level with some rise when nearing maturity, fiber remaining more or less constant, increase in the content of alcohol-insoluble solids due to starch accumulation, change of structural polysaccharides, and hydrolysis of starch into sugars, followed by fruit softening, biosynthesis of volatile compounds, chloroplast degradation, and chromoplast and carotenoid biosynthesis (Joas et al., 2012; Wongmetha et al., 2015; Matheyambath et al., 2016; Saleem-Dar et al., 2016). All these changes are the consequence of physiological and biochemical events controlled during ripening of fruit involving the fruit softening that affects the eating quality (Gill et al., 2017).

The increased activity of some enzymes through ripening may favor internal physiological disorders referred to as the internal flesh breakdowns such as a soft nose, jelly seed, and spongy

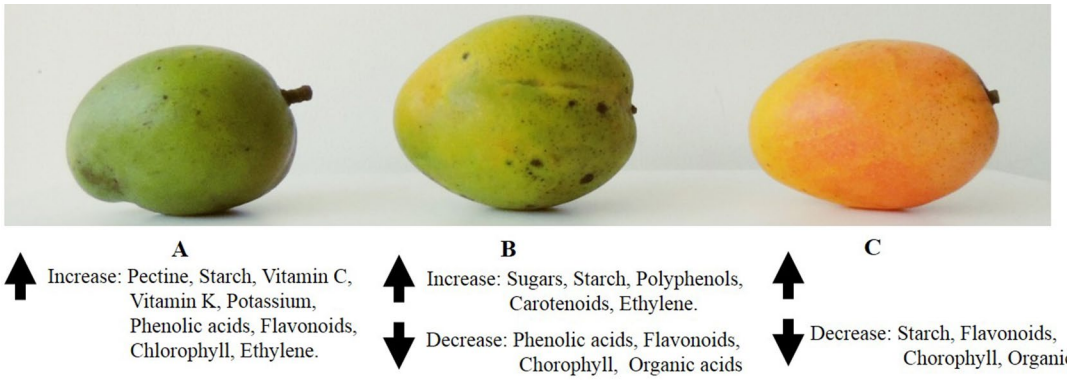

FIGURE 2 | Pictorial representation of ripening stages of "Criollo" mango fruit where changes of most representative phytochemicals are indicated.

tissue (Wainwright and Burbage, 1989). The occurrence and intensity of the alterations mentioned above depend on climate, geographical localization, and varieties; and the symptoms exhibit at the final step of fruit growth and maturation (Thomas et al., 1993; de Oliveira Lima et al., 1999). Biochemical studies showed increasing activity of the enzyme amylase through fruit growth, and this activity decreases towards the maturity stage (Sen et al., 1985).

It is important to note that depending on the variety of mango, the maturity process occurs starting in the skin towards the seed, as in the case of the Alphonso variety, while other varieties mature in the opposite direction such as in Haden, Kent, and Tommy Atkins.

The development of the fruit and the maturation process entail changes at the transcriptional level as demonstrated in the Alphonso variety, where it was observed that 4,611 transcripts correspond to different enzymes (oxidoreductase, transferase, hydrolase, ligase, lyase, and isomerase) and in which transferase enzymes were abundant followed by hydrolases in the tissues analyzed: flower, whole fruit 30 and 60 days after pollination, mature raw fruit, and pulp (green, mid-ripe, and ripe fruit) (Deshpande et al., 2017). This study identified genes encoding enzymes involved in 142 metabolic pathways of primary and secondary metabolites (Deshpande et al., 2017). Novel transcripts involved in the biosynthesis of monoterpenes, sesquiterpenes, diterpenes, lactones, and furanones related with flavor have been also identified in Ataulfo mangoes, which are differentially regulated during fruit development; in addition, 79 novel transcripts of inhibitors of cell wall-modifying enzymes were also identified, which suggests that the activity of enzymes can be controlled (Deshpande et al., 2017).

Starch exhibits a rapid rate of accumulation at the beginning of fruit development and decreases later, but it continues increasing until maturity (Tharanathan et al., 2006). For example, Alphonso mango shows a rise from 1% to 14% in starch content throughout development (Quintana et al., 1984). A similar profile was observed in JinHwang mango grown in Taiwan (Wongmetha et al., 2015). At the end of the maturity stage, reducing and non-reducing sugars are found to be increasing (Mann et al., 1974). During Tommy Atkins mango ripening, starch is hydrolyzed during the first week of harvest, but as the fruit becomes over-ripe, the content of starch and amylase activity is substantially reduced (de Oliveira Lima et al., 2001).

Several studies showed many compositional and metabolic differences between the healthy and damaged tissues. de Oliveira Lima et al. showed that sugars in the spongy pulp tissue (disease of fruit) are a consequence of the presence of starch that has not been hydrolyzed because of low activity of amylase enzyme during ripening in this disorder (de Oliveira Lima et al., 2001).

The last ones are responsible for the hydrolysis of sucrose. The accumulation of sucrose in Irwin mango was associated with the decrease of sucrose phosphate synthase activity, whereas the activities of acid invertase, neutral invertase, and sucrose synthase decreased at five developmental stages (50, 70, 90, 110, and 130 days after anthesis). A similar result was observed in JinHwang mango 110 days after anthesis (Wongmetha et al., 2015); moreover, the acid invertase activity was the dominant enzyme in sugar accumulation and quality of mango fruit, which propose that mango fruit of Irwin and JinHwang cultivars should be harvested after physiological maturity at 110 days after anthesis. During the initial stages of fruit development, sucrose phosphate synthase activity was higher than sucrose synthase activity and then decreased during the advances of the development of the fruit; in contrast, sucrose synthase activity increased from 0.27 U sucrose/min/g FW at 50 days after anthesis to 2.32 U sucrose/min/g FW at 130 days after anthesis (Wongmetha et al., 2015). During ripening, sucrose increased from 5.8% to 14.2% FW, while the pH changed from 3.0 to 5.2, whereas in the post-climacteric stage, the total acidity ranged from 0.13% to 0.71%, and the level of non-reducing sugars diminishes to 0.6% (Tharanathan et al., 2006).

Pectins are responsible for fruit texture. Pectin rises in the fifth week of mango fruit setting until the stone is formed, and then the pectin content decreases, leading to the fruit softening because of enzymatic degradation and solubilization of protopectin (Jain, 1961). Mango fruit pulp is composed of parenchymatous tissues that consist of calcium salts of pectin located in the cell wall during the early stages of cell growth (Voragen et al., 1995). The deesterification of pectins and losses of calcium ions are characteristic of ripening fruit because of cell

wall breakdown and dissolution of middle lamella (Tharanathan et al., 2006). When mango cell walls are degraded during ripening, monosaccharides of the pectin complex are released, and the resulting water-soluble pectic materials in the cell walls lose arabinose and galactose accounting for the galacturonan-rich polysaccharides in the mesocarp (Lizada, 1993).

Moisture content was observed to range from 780.78 to 790.78 g/kg FW in green Tommy-Kent and Tommy Atkins mangoes and increased significantly in the mature stage (835.55 to 849.04 g/kg FW), which can be attributed to the catabolism or modifications of cell wall fibers and, therefore, to water liberation (Barbosa Gámez et al., 2017).

A reduction in the soluble protein content happens up to 44 days after fruit setting and rises until 96 days (Lakshminarayana et al., 1970). At maturity, the amino acids alanine–arginine, glycine–serine, and leucine–isoleucine are predominant but decrease during ripening, with the exception of alanine (Lakshminarayana et al., 1970). Barbosa Gámez et al. (2017) reported that protein content in immature Tommy-Kent and Tommy Atkins mangoes was 40.45 and 37.02 g/kg FW, respectively, higher than in mature stage (12.10 and 13.87 g/kg FW, respectively); this is because there is an increase of metabolic enzymes required in the maturation and ripening process, while at the senescence stage, enzymes decrease (Andrade et al., 2012; Barbosa Gámez et al., 2017).

With respect to fatty acid content during mango ripening, it has been reported that there is an increase in triglyceride content in Alphonso mango fruit pulp, followed by modifications in the composition of fatty acid of the pulp (Bandyopadhyay and Gholap, 1973). Similar patterns have been also observed in mango cultivars from India (Selvaraj et al., 1989). During the ripening, an equal distribution of palmitic acid and palmitoleic acid it has observed, as well as a reduction in linoleic acid content and an increase in linolenic acid content (Desphande et al., 2016). The palmitic acid is incorporated to hydroxy fatty acids that are precursors of lactones; thus, a correlation between the aroma and flavor was observed using the ratio of palmitic acid to palmitoleic acid (Bandyopadhyay and Gholap, 1973; Lizada, 1993). During the preclimateric and climateric stages, the mitochondria increase its capacity to oxidize fatty acids such as stearic and oleic acids, producing precursors for the synthesis of carotenoids and terpenoid volatiles (Lizada, 1993).

Organic acid content decreases as the mango fruit ripens, and consequently, the titratable acidity declines (Shashirekha and Patwardhan, 1976). It has been reported that the enzyme activities involved in the Krebs cycle of mango fruit change during ripening (Baqui et al., 1974; Lizada, 1993). For example, the citrate synthase activity is drastically decreased, the activities of isocitrate and succinate dehydrogenases increase (Lizada, 1993), and malic enzyme in mango pulp presents the highest activity just before the climacteric peak (Dubery et al., 1984). Citrate synthase activity decreases during ripening, while isocitrate dehydrogenase and succinate dehydrogenase activities increase (Baqui et al., 1974).

Vitamin C contents in the pulp decrease during mango fruit ripening and are maximum in the early stages of growth (Robles-Sanchez et al., 2009); in spite of that, the ripe mango is an important source of vitamin C (Matheyambath et al., 2016). In addition, the amount of loss varied by species (Hu et al., 2018). One of the first studies on vitamin C developmental patterns in different mango cultivars (Amini, Mullgoa, Pico, and Turpentine) reported a reduction in the levels of the flesh 5 weeks after fruit setting until maturity, and smaller fruits with average age of about 5 weeks had an average vitamin C value of 88 mg/100 g of flesh mango fresh, but at maturity (16 weeks), these same changes were around only 22 mg/100 g (Spencer et al., 1956). A similar pattern has been reported for Tommy Atkins, Keitt, and Kent mangoes (Manthey and Perkins-Veazie, 2009). Moreover, Keitt mango showed significantly higher vitamin C content than did the other two varieties in the ripe phases, because of its direct inhibition of polyphenol oxidase (PPO) that probably confers better color and flavor retention during handling and processing (Robinson et al., 1993; Palma-Orozco et al., 2014). This reduction in vitamin C levels has been also explained based on the coenzyme function for the ACC-oxidase involved for ethylene synthesis, or as a substrate for the oxalate and tartrate biosynthesis (Mazid et al., 2011). In spite of this, the consumption of 300 g of mature Kent and Tommy Atkins mangoes contains 645 and 1,410 mg vitamin C/kg FW, respectively, which exceeds the dietary recommendations of 75–90 mg/day (WHO, 2003; Ibarra-Garza et al., 2015; Barbosa Gámez et al., 2017).

The content of vitamin E also changes through the ripening of mango fruit. Barbosa Gámez et al. (2017) reported that the content of this vitamin is highest at the immature stage (cv. Tommy-Atkins 91 mg/kg FW); however, when the fruit is ripened, the values were lower (65.81 mg/kg FW). On the other hand, Tommy-Kent mango presented an increase in vitamin E from the immature to mature stage (51.96 to 77.50 mg/kg).

The content of the group of B vitamins changed significantly at the mature stage compared with the immature stage (Barbosa Gámez et al., 2017). Niacin increased by 3.0- and 1.75-folds, pyridone increased by 2.8- and 3.4-folds, riboflavin increased by 3.1- and 2.9-folds, and thiamine increased by 2.2- and 2.5-folds in mature Tommy-Kent and Tommy Atkins mangoes, respectively (Barbosa Gámez et al., 2017).

The content of phenolic compounds (polyphenols and phenolic acids) changes during development until maturity due to their capacity for neutralizing free radicals, which are naturally produced during ripening processes or senescence (Palafox-Carlos et al., 2012a; Palafox-Carlos et al., 2012b). Total polyphenol contents in the immature stage in Kent and Tommy Atkins mango fruit were 4.81 and 4.42 g/kg, respectively, while in the mature stage, they were 5.24 and 4.18 g/kg, respectively (Barbosa Gámez et al., 2017). Flavonoids were low during mango ripening because of low expression of flavonol synthase as observed in Ataulfo mango Palafox-Carlos et al., 2012a; Palafox-Carlos et al., 2012b). A similar profile was observed in Keitt and Xiangya mangoes grown in China for the content of total polyphenols in the pulp and peel, where a decrease was observed during ripening [in Keitt, 97.59 to 59.43 and 1,207.02 to 641.90 mg gallic acid equivalent (GAE)/100 g FW; in Xiangya, 96.15 to 45.15 and 723.67 to 358.62 mg GAE/100 g FW], and flavonoids in green mango were 45% more than those in mature mango (Hu et al., 2018).

Several phenolic compounds have been identified in four ripening stages selected according to the percentage of yellow color in the peel of Ataulfo mango, including gallic, chlorogenic, protocatechuic, and vanillic acids (Palafox-Carlos et al., 2012a; Palafox-Carlos et al., 2012b). Chlorogenic acid in Ataulfo mango showed a content of 28 mg/100 g DW in the ripening stage 1 (0–10% yellow surface) and increased to 301 mg/100 g DW in the ripening stage 4 (71–100% yellow surface), whereas gallic acid content in the first ripening stage (0–10% yellow surface) was 94.6 mg/100 g DW, without significant differences with respect to ripening stages 2 and 3 (11–40% and 41–70%, yellow surface, respectively), but in ripening stage 4, it reached 98.7 mg/100 g DW. A similar profile was reported for vanillic acid (16.9 mg/100 g DW) in the first ripening stage to 24.4 mg/100 g DW in ripening stage 4; and the protocatechuic acid had a concentration of 0.48 mg/100 g DW in the first ripening stage and increased to 1.1 mg/100 g DW in the last ripening stage (Palafox-Carlos et al., 2012a; Palafox-Carlos et al., 2012b).

The pigmentation of the pulp in mango fruit occurs from the seed outwards (Tharanathan et al., 2006). The chlorophyll present in the unripe stage is degraded during mango ripening, and earlier present pigments and biosynthesis of anthocyanins and carotenoids are uncovered (Medlicott et al., 1986; Lizada, 1993; Tharanathan et al., 2006). Mature green mango contains three times more chlorophyll and marginally more β-carotene in the peel, where the enzyme activities of chlorophyllase and peroxidase are about half those of the ripe yellow fruit (Medlicott et al., 1986; Selvaraj and Kumar, 1994; Ketsa et al., 1999). The activity of these enzymes leads to a complete reduction of peel chlorophyll *a* during ripening from 2.2 in unripe fruit to 0.8 μg/cm^2 in ripe fruit (Medlicott et al., 1985; Medlicott et al., 1986).

On the other hand, the total carotenoid content of the peel increases approximately fivefold during ripening, probably due to carotenoid synthesis, in addition to underlying pulp carotenoids (Medlicott et al., 1986). The increase in total carotenoids during maturity has been considered as ripening index and harvest indicator; that is, the cultivars Sensation and Xiangya grown in China showed significantly higher total carotenoid content values in peels than in pulp; on the contrary, the variety Keitt also grown in China exhibited an adverse result (Hu et al., 2018). Ajila et al. (2007) reported that total carotenoid content was fourfold to eightfold greater in ripe mango peel than in unripe mango peel, whereas the research of Hu et al. (2018) showed around three to eight times higher content in ripe mango peel (Hu et al., 2018).

The accumulation of anthocyanins, which contribute to the red coloration of the mango peel, is dependent on the level of sun exposure (Medlicott and Thompson, 1985). For example, Saengnil and co-workers showed that mangoes (cv. Kent) that were covered with brown paper bags had lower levels of anthocyanins and less redness in the peel than were mangoes not covered (Saengnil and Kaewlublae, 1997). The accumulation of anthocyanins and flavonoids in the peel of mango fruit protects against the chilling injury and pathogen infection, probably because of the antioxidant capacity of these polyphenols inhibiting lipid peroxidation and reduction of decay incidence (Sivankalyani et al., 2016). These findings may lead to new strategies as selection of the resistant red fruit or technical methodologies that would increase the content of anthocyanins and flavonoids in mango fruit peel for improving postharvest traits and for improving diminishing losses (Sivankalyani et al., 2016).

The synthesis of a mixture of volatile compounds is associated with the flavor and aroma during fruit ripening as mentioned in a previous section. This is a consequence of ethylene production as volatile components accumulate from skin and pulp, improving fruit aroma and flavor (Lizada, 1993; Sergent et al., 1993; Singh and Janes, 2001; Tharanathan et al., 2006). The skin of Alphonso mango has an important terpene content, while the pulp is rich in lactone (Chidley et al., 2013). A similar profile of the volatile composition of skin and pulp of green Khieosawoei mango grown in Thailand (Tamura et al., 2001) and Kensington Pride mangoes (Lalel et al., 2003a; Lalel et al., 2003b; Lalel et al., 2003c) was observed. The major volatile components present in ripe mango fruit are terpenes, while some other hydrocarbons, esters, and alcohols have also been detected in this stage (Hunter et al., 1974, Pino et al., 1989).

CHANGES IN NUTRITIONAL AND PHYTOCHEMICAL COMPONENTS DURING POSTHARVEST HANDLING PRACTICES

After harvest of mango fruit, losses in quantity and quality occur, affecting the content of nutritional and phytochemical components at different points in the handling chain (Esguerra and Rolle, 2018). This is very important because consumers are interested not only in visual quality but also in health components and fruit safety (Esguerra and Rolle, 2018). There is limited information about how postharvest handling practices influence nutrient and phytochemical levels in mango fruit.

Postharvest activities include all those that are carried out with the fresh product, which can be done in the field after harvest, in collection centers, packing plant, during transport, during storage, or during marketing (Esguerra and Rolle, 2018). Some of the postharvest practices used for mango include i) trimming, ii) delatexing/desapping, iii) sorting/grading, iv) disease control, and v) procedures for extending shelf life of mangoes, such as packaging and transport (Tharanathan et al., 2006; Yahia, 2011).

Trimming is the cutting of stem resulting in latex or sap stains deposited on the fruit surface, since the sap stored in the fruit ducts is under significant pressure, and after pedicel abscission, the sap falls on the peel of mango fruit (Loveys et al., 1992; Esguerra and Rolle, 2018). The main cause of mango sap burn is attributed to a deposit of volatile compounds as terpinolene and car-3-ene through the lenticels, producing a tissue damage and the enzymatic browning (Loveys et al., 1992). The development of these pigmented lesions is a response associated with stress indicators and the successive release of PPO (Dixon and Paiva, 1995) that results in membrane damage with liberation of phenolic compounds deposited into the cell wall (Beckman, 2000; Bezuidenhout et al., 2005; Du Plooy et al., 2009).

The contact of sap or latex with the skin of mango induces lenticel discoloration, a red pigmentation described as red spots on the fruit surface caused by the synthesis of anthocyanins (Kangatharalingam et al., 2002), flavonoids (Dixon and Paiva, 1995) and phenylpropanoid derivatives (Du Plooy et al., 2009). However, this lenticel spotting can be induced at storage temperatures below 10–12°C, accelerating the occurrence of red spots related with chilling injury (Pesis et al., 2000).

Prasad and Sharma (2018) compared the effect of manual and mechanical harvesting practices on total soluble solids (TSS), total carotenoids, and antioxidant activity in four Indian commercial mango cultivars grown in India. Amrapali, Chausa, Dushehari, and Langra were stored at room temperature [25 ± 4°C and 65 ± 5% relative humidity (RH)]. Prasad and Sharma (2018) reported that independent of storage days and cultivar, no significant difference was observed in TSS between the manually harvested [20.4°B (Brix)] and mechanically harvested (20.6°B) fruit. Amrapali mangoes had the highest TSS when harvested manually and mechanically (22.1°B and 22.6°B, respectively). These differing results might be due to the fact that mechanically harvested fruit continues to ripen after harvesting, allowing for high accumulation of TSS, while manually harvested fruit does not ripen post-harvesting (Pacheco et al., 2017). In relation to total carotenoid content, Prasad and Sharma (2018) reported that independent of storage days and varieties analyzed, an overall slight increase was observed in total carotenoid content when fruits were mechanically harvested (5.4 mg/100 g FW) compared with the mangoes harvested manually (5.3 mg/100 g FW). This slight increase could be due to the effect of mechanical harvesting technique on proper attainment of ripening throughout the shelf life of mango fruit (Pacheco et al., 2017), but also subjected to varietal morphology of mango fruit (Abu-Gaukh and Mohamed, 2004). However, significant differences were found for total antioxidant activity in both manually and mechanically harvested mangoes. A slight increase of antioxidant activity was observed in fruit harvested mechanically (3.00 μmol Trolox eq/g FW) than that of manually harvested fruit (2.98 μmol Trolox eq/g FW). This difference was even more obvious at the ninth day of storage, as fruit harvested mechanically had an antioxidant activity of 4.29 μmol Trolox eq/g FW compared with 4.23 μmol Trolox eq/g FW in manually harvested fruit. These results were attributed to the extent of physical abrasion, a physical damage that directly affects antioxidants such as vitamin C present inside the fruit, because this component is used up by the fruit for combating external stresses (Pacheco et al., 2017; Prasad and Sharma, 2018).

Postharvest disease control measures such as for Anthracnose are an important aspect of postharvest practices that affect the content of mango phytochemical components (Rodríguez et al., 2009; Kamle et al., 2013). At the green stage, anthracnose cannot be perceived, and the symptoms of infection are obvious when the mango ripens; this disease is caused by the *Colletotrichum gloeosporioides* (Penz) fungi that produce the enzymes polygalacturonase and pectolyase, able to degrade the cell wall (Rodríguez et al., 2009; Kamle et al., 2013), favoring the oxidation of phenol compounds catalyzed by the enzyme PPO-producing quinones that are polymerized and form the characteristic brown spots. On the contrary, when the tissues of the mango fruit are healthy and intact, the PPO enzyme is located in chloroplasts and the phenolic compounds in vacuoles, both separated, and thus, the reaction is avoided (Ploetz et al., 1994; Pérez-Márquez et al., 2016).

Pérez-Márquez et al. (2016) analyzed the content of total phenols in 'Super Haden' mangos grown in Cuba that are damaged and treated after harvest, to establish their relationship with the defense mechanisms against the infection. In the skin of Super Haden mangoes affected by anthracnose, a reduction in the total phenol content levels was observed (59.25, 58.63, and 56.52 mg GAE/g FW) at different degrees of infection (mild, moderate, and severe damage, respectively), whereas the total phenol content in healthy fruit was 60.5 mg GAE/g FW (Pérez-Márquez et al., 2016).

Postharvest disease control is carried out by hydrothermal and/or chemical methods (López and Castaño, 2010). Pérez-Márquez et al. (2016) compared a treatment using hot water (53°C for 5 min) with polyethylene wax [10% Total solids (ST)] and imazalil (800 mg/L), and other with polyethylene wax (10% ST) plus imazalil (800 mg/L) on Super Haden mango fruit infected with anthracnose (mild, moderate, and severe levels); they observed that the total phenol compound content of mango peel increased after treatments. On the other hand, by using hot water with polyethylene wax plus imazalil and two bags of Conserver 21 (an ethylene absorber product), the total phenol content was 37.58 and 37.11 mg GAE/g FW, respectively, compared with the control value (33.94 mg GAE/g FW). These results also indicated a correspondence with a lower disease occurrence (Pérez-Márquez et al., 2016), because this treatment disinfects the fruit by eliminating or destroying the spores or fungal mycelium (Escribano and Mitcham, 2014). Treatment with hot water also induces the expression of the protein of polygalacturonase that can inhibit fungal endopolygalacturonase, considered as an important factor for the resistance of plants to phytopathogenic fungi (Li et al., 2013).

A similar result was reported by Kim et al. (2007) in fresh, mature, green mangoes to inhibit anthracnose. Hot water treatment (46.1°C, 75 min) combined with controlled atmosphere (3 kPa of O_2 + 97 kPa of N_2, or 3 kPa of O_2 + 10 kPa of CO_2 + 87 kPa of N_2) after 2 weeks of storage at 10°C and ripening in air at 25°C did not affect the content of gallic acid and hydrolysable tannins, while total polyphenols decreased naturally during ripening, regardless of hot water treatment or controlled atmosphere (Kim et al., 2007).

On the other hand, imazalil is a fungicide that inhibits the biosynthesis of ergosterol, an inhibitor of cytochrome P-450 enzyme that demethylates ergosterol (Pérez-Márquez et al., 2016). Imazalil acts directly on the fungus, affecting its cellular permeability and lipid biosynthesis reduces spore germination and the inflammation of the germ tube distortion and cytoplasm loss in germinated cells (Pérez-Márquez et al., 2016). In addition, the application of essential oil (a mix of eugenol, menthol, and carvacrol), decreases cellular respiration in the fruit and the production of ethylene (Guillén et al., 2012).

Although thermal-processing techniques inactivate microorganisms and spoil enzymes, inadequate handling of

heat-processing methods may induce several chemical changes in the fruit and reduces not only the content or bioavailability of some phytochemical compounds but also the organoleptic properties (Patras et al., 2010).

For example, Beyers and Thomas (1979) showed that blanching at 80°C for 5 min of mango fruit produces loss of carotenoids. Kim et al. (2009) also evidenced that total soluble phenolics, gallic acid, and gallotannins of mango fruit diminished as a result of prolonged hot water treatment (46°C for 70 to 110 min).

On the contrary, Keitt mango fruit treated with hot water dipping at 50°C for 30 min had slightly lower content of TSS until 6 days of storage, but after 9 days of storage, the final value of TSS was higher than at the beginning of the experiment, and the acidity was not significantly affected by the heat treatment (Djioua et al., 2009). Hot water dipping at 50°C for 30 min and 46°C for 75 min maintained the contents of vitamin C until 3 days of storage, whereas at 46°C for 30 min and 50°C for 75 min, the content of vitamin C quickly decreased (Djioua et al., 2009). These results suggest that with a proper time–temperature combination, these losses can be diminished, and fresh-cut products can still contain important levels of vitamin C during storage (Djioua et al., 2009). Finally, the carotenoid content of Keitt mango treated with hot water (46°C/75 min, 50°C/30 min, and 50°C/75 min) increased because of disruption of cell membrane induced by heat treatments, and extraction enhanced and chemically enhanced extraction of carotenoids; moreover, during storage, total carotenoid contents remained stable compared with those in non-treated fruit, which could increase the antioxidant capacity (Djioua et al., 2009).

Currently, the methods employed to extend the shelf life of mangoes include physical and chemical treatments to reduce respiration and ethylene production, but the storage techniques are expensive and not fully satisfactory and may lead to the development of off-flavor if temperatures used are lower than the optimum.

Treatment with plant hormones, such as methyl jasmonate, and their synthetic derivatives are used to improve mango fruit quality, to enhance color uniformity, to increase the activity of phenylalanine ammonia lyase, and to increase the content of total phenolic compounds including anthocyanins (Lalel et al., 2003a; Lalel et al., 2003b; Lalel et al., 2003c). They also improve β-carotene, vitamin C, glucose, fructose, and sucrose contents and the ratio of soluble solids/titratable acidity, increase firmness, and reduce weight loss (Muengkaew et al., 2016). Other treatments such as 1-methylcyclopropene (1-MCP) are used commercially to retard ripening, while ethrel is used to accelerate. 1-MCP treatment decreases lipid peroxidation and increases activities and isozymes of catalase and superoxide dismutase in Dashehari mango, while ethrel treatment has opposite effects (Singh and Dwivedi, 2008). It was observed that the use of 1-MCP in Kensington Pride cultivar at higher concentrations (10 and 25 μl/L) affect the production of monoterpenes, esters, aldehydes, and aroma volatile compounds, whereas application of low concentrations of 1-MCP (1 μl/L) has less effect on the aroma profiles of this mango cultivar (Lalel et al., 2003a; Lalel et al., 2003b; Lalel et al., 2003c). In relation to chemical treatments to slow fruit softening, the addition of divalent calcium ions solutions promotes the formation of calcium bridges between the pectic polysaccharide chains (Tharanathan et al., 2006).

The non-thermal-processing technologies such as irradiation has been used as treatments for preservation, causing minimal modifications to the quality attributes of food, but might affect levels of nutrients and phytochemical compounds conditioned to the dose and radiation source used (i.e., gamma, X-ray, UV, and electron beam) (Bhat and Sridhar, 2008). Reyes and Cisneros-Zevallos (2007) evaluated the effect on the total phenolic, carotenoids, and vitamin C content of Tommy Atkins mango after electron-beam ionizing radiation in a dose range of 1–3.1 Gy and storage. They observed that flavonols after 18 days in storage (3.1 Gy) increased, total phenolics were not affected, but vitamin C decreased around 50–54% during storage (≥1.5 kGy), but no important alterations in carotenoid content were reported, indicating a retardation in ripening of treated mangoes (1–3.1 kGy) compared with non-irradiated fruits. However, at the lowest dose (≥1.5 kGy), flesh pitting was observed, indicating presence of death tissue due to the induced oxidative stress (Reyes and Cisneros-Zevallos, 2007). Cruz et al. (2012) compared the effect of irradiation at 0.4 and 1.0 kGy with the hot water dipping treatment (46°C for 90 min) on organic acid content, among other attributes of Tommy Atkins mangoes from Brazil for export stored for 14 days at 11°C and then 23°C until the end of the study. They reported that the higher levels of citric and succinic acids were present in the control group (untreated fruit) on the last day of the study. In addition, no significant differences in the total sugar content were observed between groups (control vs. treated); finally, gamma radiation does not seem to negatively affect the quality attributes of mangoes.

At present, molecular biology techniques are good options for the control of ripening, like the antisense RNA technology, which signifies a single type of DNA transcript of 19–23 nucleotides and is complementary to mRNA (Xu et al., 2018). This RNA technology has been used to regulate gene expressions during the replication, transcription, and translation. For example, antisense RNA of 1-aminocyclopropane-1-carboxylate oxidase inhibits the expression of rate-limiting enzymes involved in the biosynthesis of ethylene in tomatoes, delaying its maturation (Oeller et al., 1991). At present, ongoing studies have been applied to mangoes, among other fruits (Xu et al., 2018).

CONCLUSIONS AND PERSPECTIVES

Mango is a valuable fruit from a nutritional point of view, providing fiber, micronutrients as carbohydrates (10–32% in ripe pulp), proteins (0–5%), amino acids (alanine, arginine, glycine, serine, leucine, and isoleucine), lipids (0.75% to 1.7%), and organic acids (citric is the major organic acid, 0.13% to 0.71% FW).

Mango fruit also provides macronutrients such as vitamins (vitamin C, from 9.79 to 186 mg/100 g of mango pulp; vitamin A, from 1,000 to 6,000 IU; E and K vitamins are found in minor quantities; D vitamin has not been detected in any cultivars until now). Except for biotin, all the other B vitamins have been found in mango fruit. In addition, mango fruit is an important source

of polyphenols (catechins, quercetin, kaempferol, rhamnetin, anthocyanins, tannic acid, and mangiferin; carotenoids, organic acids, and volatile compounds), useful for medicinal applications and also as indicators of fruit quality. All these concentrations depend on ripe state of the mango pulp and peel.

This review shows changes in nutritional and phytochemical components during postharvest handling practices, such as trimming, delatexing/desapping, sorting/grading, and disease control. Knowing and understanding the changes in the chemical composition in mango fruit during its development will allow producers to better characterize their cultivars and select those that have phytochemical characteristics that give added value to the fruit, for example, enhancing fruit color, delaying the maturation process, selecting fruit with a greater contribution of certain nutrients, increasing antioxidant components, and improving fruit characteristics for export purposes or greater use for agro-industry and processing.

AUTHOR CONTRIBUTIONS

MEM-C and EY collected the literature; wrote the sections related to nutritional composition, phenolic and pigments compounds, and the changes of nutritional and phytochemical components during the postharvest process of the fruit; made critical edits; and reviewed the whole manuscript before submission. RB collected the literature, wrote the section on carbohydrates, and prepared the figures presented. PL and JCGO collected the literature and wrote the sections related to volatile compounds and organic acids. BR collected the literature and contributed to the section on phenolic compounds. NL and JA collected the literature and wrote the sections related to changes of the nutritional and phytochemical compositions during growth and ripening.

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REVIEW
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Regulation of the Central Carbon Metabolism in Apple Fruit Exposed to Postharvest Low-Oxygen Stress

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After harvest, fruit remain metabolically active and continue to ripen. The main goal of postharvest storage is to slow down the metabolic activity of the detached fruit. In many cases, this is accomplished by storing fruit at low temperature in combination with low oxygen (O_2) and high carbon dioxide (CO_2) partial pressures. However, altering the normal atmospheric conditions is not without any risk and can induce low-O_2 stress. This review focuses on the central carbon metabolism of apple fruit during postharvest storage, both under normal O_2 conditions and under low-O_2 stress conditions. While the current review is focused on apple fruit, most research on the central carbon metabolism, low-O_2 stress, and O_2 sensing has been done on a range of different model plants (e.g., *Arabidopsis*, potato, rice, and maize) using various plant organs (e.g., seedlings, tubers, roots, and leaves). This review pulls together this information from the various sources into a coherent overview to facilitate the research on the central carbon metabolism in apple fruit exposed to postharvest low-O_2 stress.

Keywords: low-oxygen stress, postharvest storage, apple fruit, central carbon metabolism, oxygen sensing

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POSTHARVEST STORAGE OF APPLE FRUIT

Fruit Ripening

Fruit ripening is the process through which a fully grown mature but inedible plant organ is transformed into an attractive edible fruit with an optimum blend of colour, aroma, and texture. During this complex, highly coordinated and genetically programmed transformation process a sequence of changes occurs (Giovannoni, 2001), which is reflected by the metabolic activity of the fruit. Fruit can be divided into two groups based on their respiratory behavior during ripening: climacteric and nonclimacteric fruit (Saltveit, 1999; Wills and Golding, 2016). For climacteric fruit like pears and apples, the ripening process is characterized by a rise in respiratory activity and ethylene production. This respiratory climacteric rise marks the end of a period of active synthesis and maintenance and the start of fruit senescence. For nonclimacteric fruit, such as orange, lemon, pineapple, grapes, and strawberry, there is no peak in respiration and ethylene production rates remain low (Alexander and Grierson, 2002; Paul et al., 2012; Wills and Golding, 2016). To maximize their storage potential, apples are harvested 1 or 2 weeks before their climacteric rise in respiration. Subsequently, the mature but unripe fruit is typically stored under low O_2 conditions retarding the metabolic activity of the fruit to delay fruit ripening and senescence.

Factors Controlling Fruit Respiration Rate

Postharvest respiration rate can be controlled through temperature, O_2 and CO_2 concentration (Lammertyn et al., 2001; Cameron et al., 1994; Hertog et al., 1998). Ho et al. (2018) showed that temperature is the most influential factor controlling the respiration rate of apple fruit. The inhibitory effect of low temperature on the respiration rate is due to the decrease in catalytic activity of respiratory enzymes (Lyons, 1973; Graham and Patterson, 1982; Atkin et al., 2005; Bron et al., 2005; Falagán and Terry, 2018). Therefore, to minimize respiration, fruit is stored at the lowest possible temperature (Jackman et al., 1988; Wang, 2010).

Next to temperature, storage atmosphere composition has an influence on fruit respiration rate. O_2 is the final electron acceptor in the electron transport chain. By lowering the O_2 concentration in the storage atmosphere, the respiration of many fruit and vegetables slows down (Biale, 1946; Ke et al., 1991; Yearsley et al., 1996; Teixeira and Durigan, 2010; Thompson, 2010). The metabolic respiration chain consist of different pathways in which CO_2 is produced (glycolysis and TCA cycle) and O_2 is consumed (mitochondrial electron transport chain). As indicated in **Figure 1**, the respiration rate, represented by the O_2 consumption rate (blue line) and the total CO_2 production rate (green line) until the fermentation threshold (FT), decreases as the O_2 level decreases. However, when the O_2 level moves toward anoxia, the fruit metabolism shifts from aerobic respiration to fermentation (Geigenberger, 2003; Prange et al., 2005) (see also section on the regulation of central carbon metabolism under low-O_2 stress conditions), resulting in flavor and storage disorders (Kader et al., 1989; Franck et al., 2007; Thompson, 2010). Due to this metabolic shift, the CO_2 produced by the glycolysis will increase again, as well as the CO_2 produced by fermentation (red line), leading to an increase in the total CO_2 production (green line). The O_2 concentration at which the total respiratory CO_2 production rate is minimal is called the anaerobic compensation point (ACP) (Prange et al., 2005).

Finally, also the CO_2 concentration can influence the respiration rate of the fruit, but its mode of action is still not well understood. In addition, the effect of CO_2 depends on the fruit (Kubo et al., 1989; Peppelenbos and van't Leven, 1996; Hertog et al., 1998).

Low-O_2 Storage of Fruit

Apple fruit is commonly stored under low O_2 conditions at low temperature with the optimal conditions varying by geographic location, harvest date, storage duration, cultivar, and season (Saltveit, 2003; Dilley, 2010; Watkins and Nock, 2012).

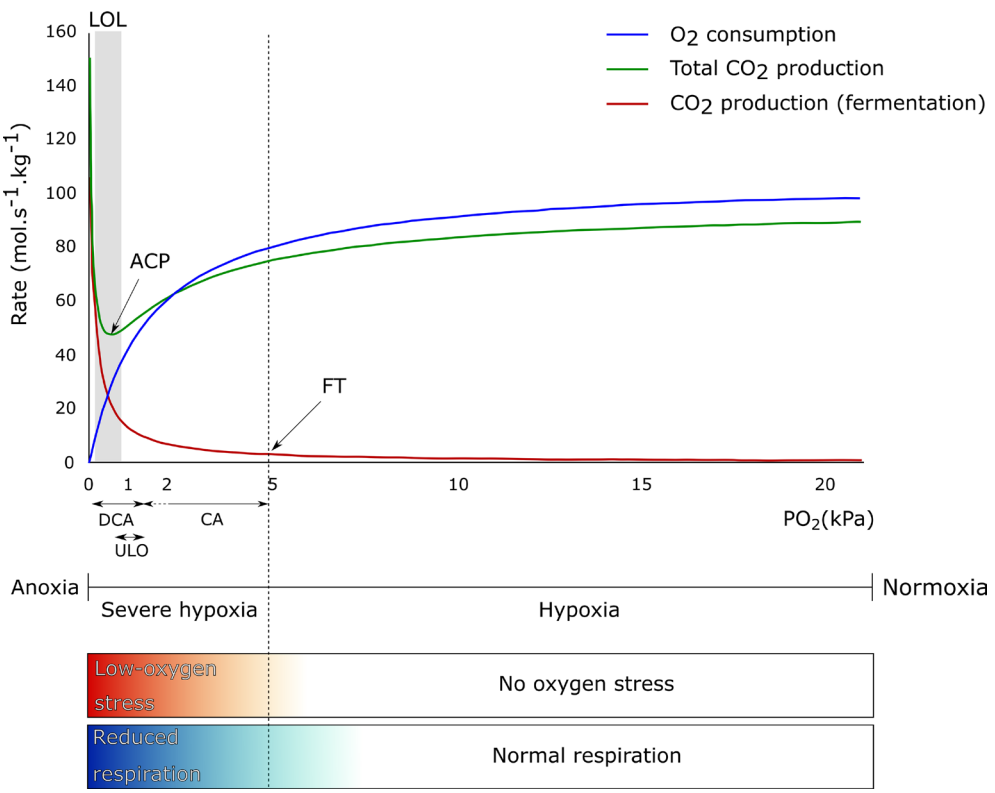

FIGURE 1 | Theoretical effect of O_2 concentration on O_2 consumption (blue line), total CO_2 production (green line), and CO_2 production from fermentation (red line) by apple fruit. The anaerobic compensation point (ACP), fermentation threshold (FT), and lower O_2 limit (LOL) are indicated, as well as the range of PO_2 applied in controlled atmosphere (CA), ultralow O_2 (ULO), and dynamic controlled atmosphere (DCA) storage. The term no O_2 stress is used to define all O_2 levels where enough O_2 is available for the fruit to have a normal respiratory metabolism, ranging from normoxia (typically 20.6% O_2 at 101.325 kPa and 20°C) and below (hypoxia). The term low-O_2 stress is used when O_2 levels are limited (severe hypoxia) or completely absent in the environment (anoxia) and the fruit needs to reduce or adapt their respiratory metabolism. The color gradients mirror the gradual transitions between no O_2 stress and low-O_2 stress and between normal respiration and reduced respiration.

Commercial storage conditions are typically set at constant safe values with O_2 levels kept above the lower O_2 limit (LOL) below which the fruit starts developing disorders (Yearsley et al., 1997; Yahia, 2009; Wright et al., 2011). Since the LOL may be slightly different every year, a safe but likely suboptimal O_2 concentration above the ACP is often maintained at the cost of higher quality losses (Wright et al., 2011; Bessemans et al., 2016).

As the static storage approach does not always provide optimal poststorage results, dynamic low-O_2 storage approaches have been developed where the O_2 conditions are adapted to the changing fruit physiology (Veltman et al., 2003; Wright et al., 2012; Bessemans et al., 2016).

While there are some studies on the effect of long-term low-O_2 storage on metabolic adaptations of apple fruit during low-O_2 storage (Saquet and Streif, 2008; Bekele et al., 2015; Brizzolara et al., 2017), and physiological disorders, (Pedreschi et al., 2007; Pedreschi et al., 2008; Pedreschi et al., 2009; Vandendriessche et al., 2013; Hatoum et al., 2014; Mellidou et al., 2014) the precise mode of action of low O_2 not is well known. Cukrov et al. (2016) have shown that "Granny Smith" apples stored at 0.4 or 0.8 kPa O_2 have a clearly different metabolic and transcriptomic profile. Brizzolara et al. (2017) demonstrated that "Granny Smith" and "Red Delicious" apples respond differently to low-O_2 storage, suggesting that the genetic background played a key role in determining and modulating the observed metabolic changes to changed O_2 levels. Hence, to further optimize low-O_2 storage, it is important to get a better understanding on the fruit's central carbon metabolism and the regulatory mechanisms involved in the responses of apple fruit to hypoxic conditions.

CENTRAL CARBON METABOLISM OF APPLE FRUIT

The following sections present data based on model species such as *Arabidopsis* and rice, which is extended with information specifically for apple fruit, whenever available.

Central Carbon Metabolism in the Absence of O_2 Stress Conditions

When O_2 is not limiting, apples produce their energy by completely oxidizing sugars through the respiratory metabolism consisting of the following pathways: glycolysis, pentose phosphate pathways (PPP), tricarboxylic acid (TCA) cycle, and the mitochondrial electron transport chain (mETC) (**Figure 2**).

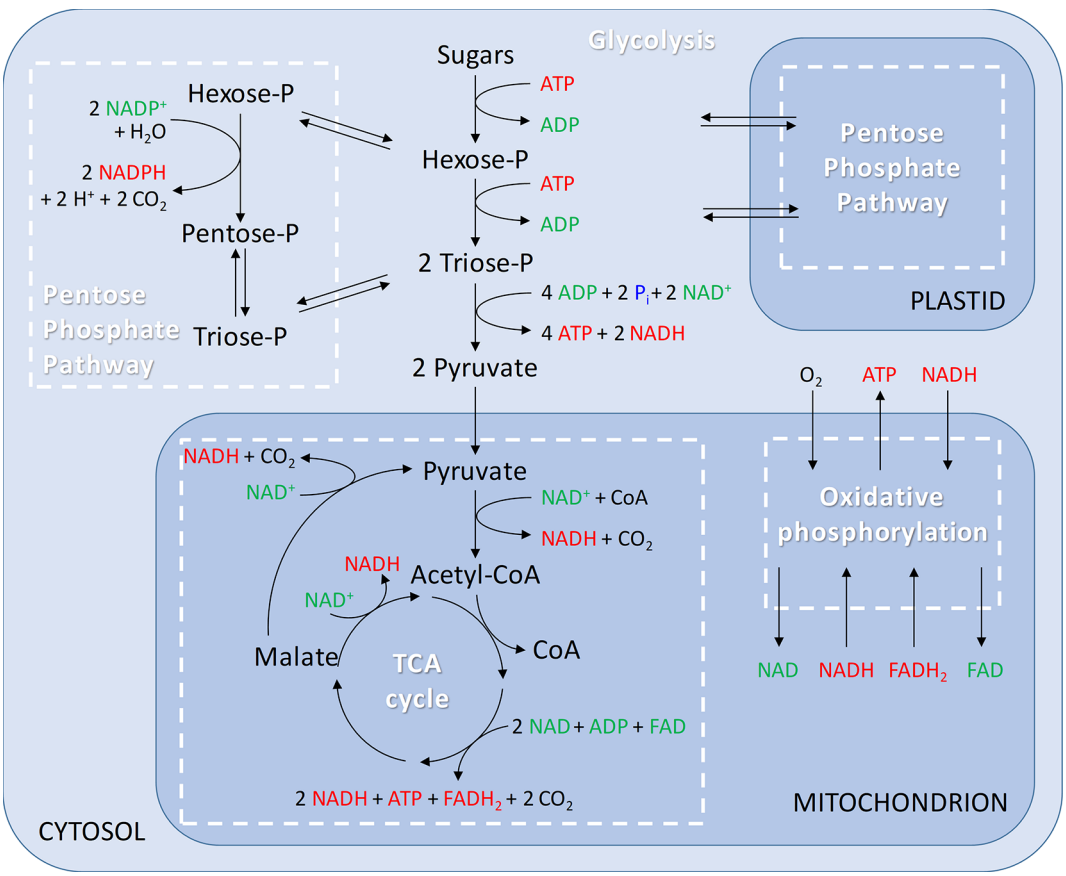

FIGURE 2 | Generalised overview of respiration including glycolysis in the cytosol; pentose phosphate pathway in the cytosol and plastids; tricarboxylic acid (TCA) cycle and oxidative phosphorylation in the mitochondria.

To appreciate their importance with regard to low-O_2 storage, these pathways will first be summarized.

Glycolysis is an O_2 independent pathway involving a series of enzymatic reactions that break down hexoses (mainly glucose and fructose) into pyruvate. To degrade sucrose *via* the glycolytic pathway, it is first cleaved by invertase (Bologa et al., 2003). The glycolysis consists of an energy consuming phase and an energy conserving phase. In the initial energy consuming phase, hexose (glucose or fructose) is phosphorylated requiring two molecules of adenosine triphosphate (ATP), and subsequently split into triose phosphates (i.e., glyceraldehyde 3-phosphate and dihydroxyacetone-phosphate). In the energy conserving phase, each triose phosphate is oxidized to pyruvate, providing two molecules of ATP and one molecule of reduced nicotinamide adenine dinucleotide (NADH). Overall, the glycolysis results in a net production of two molecules of ATP and two molecules of NADH, the major biochemical electron transporter and co-enzyme in plants. In plant cells, glycolysis can occur both in the cytosol and plastids (Plaxton, 1996; Mauseth, 2008; Taiz and Zeiger, 2010; António et al., 2016; Garrett and Grisham, 2017).

An alternative route for the cell to oxidize sugars is provided by the PPP, which takes place in the cytosol and in the plastids (**Figure 2**). The PPP is divided into an oxidative and a nonoxidative branch. The initial oxidative branch of the PPP oxidizes glucose 6-phosphate to ribulose 5-phosphate. In nonphotosynthetic cells, like apple cells, the oxidative branch of the PPP is a major source of reduced nicotinamide adenine dinucleotide phosphate (NADPH), which is used in biosynthetic processes such as fatty-acid synthesis and the assimilation of inorganic nitrogen (Neuhaus and Emes, 2000; Kruger and Von Schaewen, 2003). Furthermore, NADPH plays an important role in maintaining the redox potential necessary to protect plants against oxidative stress (Juhnke et al., 1996). Nowadays, the basic functions of the oxidative branch of the PPP are well-established (Mauseth, 2008; Taiz and Zeiger, 2010; Garrett and Grisham, 2017), but details of how the pathway operates in plants and how it influences other processes remain largely unknown. The reversible nonoxidative branch converts ribulose 5-phosphate to intermediates of the glycolysis, i.e., fructose 6-phosphate and glyceraldehyde 3-phosphate. This part of the PPP is a source of carbon skeletons for the synthesis of nucleotides, aromatic amino acids, phenylpropanoids, and their derivatives. Although both glycolysis and the PPP are involved in sugar oxidation in plants, the PPP only accounts for 15% to 30% of the hexose phosphate oxidized to glyceraldehyde 3-phosphate and CO_2 (Kruger and Von Schaewen, 2003).

The next step in the respiratory metabolism is the TCA cycle, which takes place in the matrix of the mitochondria. Pyruvate produced in the glycolytic pathway is used as a substrate to fuel the TCA cycle and is transported across the inner membrane of the mitochondria by a mitochondrial pyruvate carrier (Li et al., 2014). Alternatively, pyruvate can be generated in the matrix from malate by the action of malic enzyme (Jacoby et al., 2012). Inside the mitochondria, pyruvate is oxidatively decarboxylated by pyruvate dehydrogenase complex (PDH) generating acetyl-CoA, CO_2, and NADH (**Figure 2**). Acetyl-CoA is then incorporated in the TCA cycle by combining acetyl-CoA with oxaloacetate to form citrate. In the next steps, the two remaining carbon atoms of pyruvate are released as CO_2. Besides the production of ATP, the TCA cycle also stores energy in the form of NADH and flavin adenine dinucleotide ($FADH_2$). The intermediates of the TCA cycle also serve as substrates in the biosynthesis of amino acids, nucleic acids, and cell wall components needed for plant growth and development (Taiz and Zeiger, 2010; Harvey Millar et al., 2011; Jacoby et al., 2012).

The mETC is where the final and only O_2 consuming process of the respiratory metabolism, the oxidative phosphorylation, takes place (**Figure 2**). The mETC is located in the inner mitochondrial membrane and consist of several dehydrogenases, cytochrome oxidases, and an alternative oxidase (AOX). The reducing equivalents (NADH and $FADH_2$) and succinate, produced inside the mitochondria in the TCA cycle, transfer their electrons to NADH dehydrogenase (complex I) and succinate dehydrogenase (complex II), respectively. The mitochondria of plants contain several additional nonphosphorylating NAD(P)H dehydrogenases (ND2) on the outside, as well as on the inside of the inner membrane (**Figure 3**). These enzymes enable plant mitochondria to oxidize NADH and NADPH, formed in the glycolysis and PPP, directly from the cytosol. The reduced compounds need to be oxidized to enable the respiratory metabolism to function continuously. All the dehydrogenases (complex I, complex II, and ND2) are linked to the ubiquinone (UQ) pool. Ubiquinone is reduced by the input of electrons *via* the several dehydrogenases and again oxidized by the cytochrome oxidase pathway and/or AOX. The cytochrome pathway contains the enzymes cytochrome c reductase (complex III), cytochrome c (Cyt C), and cytochrome c oxidase (complex IV, COX). Cytochrome c reductase oxidizes ubiquinone and transfers the electrons to Cyt C, which passes on the electrons to COX. This complex reduces O_2 to two molecules of H_2O. Due to the activity of the complexes I, III, and IV, an electrochemical proton gradient is formed across the inner membrane (**Figure 3**). The ATP synthase (complex V) uses this potential energy to generate ATP from ADP and Pi by allowing protons to flow back across the membrane down the gradient. Furthermore, plants also contain an ubiquinol-oxidizing AOX. This oxidase transfers the electrons directly to O_2, thereby bypassing the proton pumping of the cytochrome pathway (complex II and COX). Therefore, energy is not conserved *via* this pathway and lost as heat (Millenaar and Lambers, 2003; Van Dongen et al., 2011; Päpke et al., 2014). Several studies show that AOX activity plays an important role in preventing and reducing ROS by avoiding an "over-reduction" of UQ and by maintaining the O_2 homeostasis (Maxwell et al., 1999; McDonald and Vanlerberghe, 2006). Under normoxia, neither AOX nor COX is active at full capacity and both have been shown to compete for the distribution of electrons (Van Dongen et al., 2011; Päpke et al., 2014).

Taking together the ATP produced by the glycolysis, TCA cycle and mitochondrial electron transport chain, a total amount of 36 molecules of ATP is formed for each molecule of glucose used as substrate.

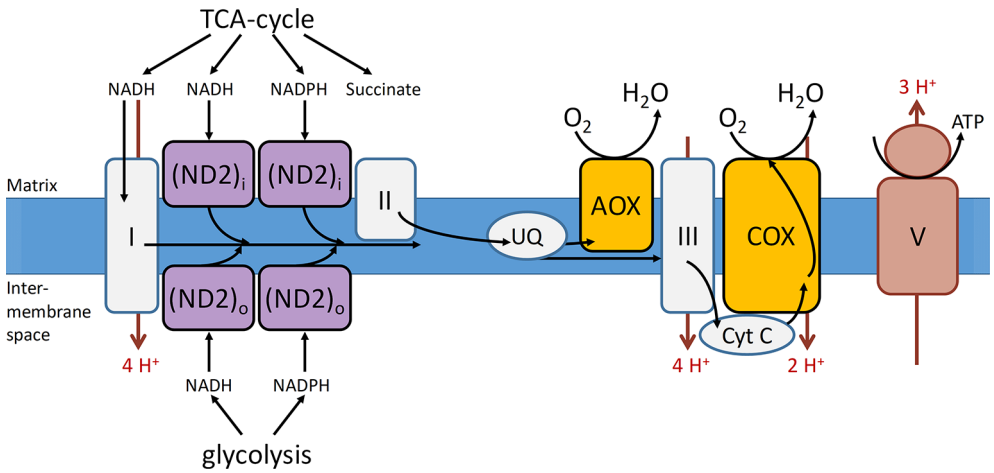

FIGURE 3 | Representation of the mitochondrial electron transport chain with the conventional and alternative reactions located in the inner mitochondrial membrane. Complexes I–III, that is, NADH dehydrogenase, succinate dehydrogenase, cytochrome c reductase, respectively; COX, cytochrome c oxidase; V, ATP synthase; AOX, alternative oxidase; cyt c, cytochrome c; ND2i and ND2o, type II NAD(P)H dehydrogenase located at the inside (i) and outside (o) of the mitochondrial inner membrane; UQ, ubiquinone pool. The electron flow is indicated with black arrows and the proton flow with red arrows (reprinted with slight modification from Päpke et al., 2014. Copyright Springer-Verlag).

Regulation of Central Carbon Metabolism in the Absence of O_2 Stress

In nonplant systems, the glycolytic flux is regulated by phosphofructokinase (PFK), with additional control exerted by pyruvate kinase (PK). In addition, multiple studies have shown that in plants, PFK is controlled through a negative feedback from phosphoenolpyruvate (PEP) (**Figure 4**; Adams and Rowan, 1970; Kobr and Beevers, 1971; Beaudry et al., 1989; Geigenberger and Stitt, 1991; Hatzfeld and Stitt, 1991; Vanlerberghe et al., 1992; Huppe and Turpin, 1994; Paul et al., 1995; Plaxton, 1996; Plaxton and Podestá, 2006; Givan, 2007; Van Dongen et al., 2011). When the cytosolic PEP levels drop due to an increased activity of PK and PEP carboxylase (PEPC), the inhibition of PFK is lifted, thus increasing the glycolytic flux again (Plaxton and Podestá, 2006). Pyruvate kinase catalyses the final reaction of the glycolysis converting ADP and PEP to ATP and pyruvate thus also plays a critical role in the regulation of glycolysis. In spite of this, transgenic studies intervening with the glycolytic enzymes have only been able to induce minor to negligible changes in the rate of respiration suggesting that the respiratory control is unlikely to be mediated by an individual enzyme only.

Similarly, transgenic studies focussing on the TCA cycle enzymes also only showed limited changes in respiration of photosynthetic plants (Van Dongen et al., 2011). However, in nonphotosynthetic plant tissue, respiration was clearly impacted by chemical inhibition of the TCA cycle enzyme 2-oxoglutarate dehydrogenase as was the amino acid metabolism (Araújo et al., 2008; Van der Merwe et al., 2009; Van der Merwe et al., 2010). This lack of clear impacts of changed enzyme expression on cellular respiration rate does not mean that the glycolysis and TCA cycle are not regulated at all. Both pathways are, for instance, influenced by the availability of sucrose (Fernie et al., 2002; Urbanczyk-Wochniak et al., 2006) and redox status fluctuations (Kolbe et al., 2006; Araújo et al., 2012). Furthermore, while both ATP and UTP are involved in the various steps of the glycolysis (see **Figure 5**), cellular respiration only responds to fluctuations in adenylate, but not uridylate metabolism (Regierer et al., 2002; Geigenberger et al., 2005) highlighting the central role of ATP as main cellular energy source. Altogether, these observations suggest that the mETC is pivotal to controlling respiration rate (Van Dongen et al., 2011).

The demand of ATP plays an important role in the regulation of the mETC. The activity of the mETC complexes I–IV is regulated by the proton motive force. A high cellular demand for ATP stimulates the ATP synthase complex increasing the flux of protons into the matrix. To compensate for this increase of proton influx, the activity of complex I, III, and IV is increased. In addition, the cell needs to control the production of ROS, which are a by-product of the mETC. Like mentioned in section 2.1, the mETC has several alternative electron donor and acceptor proteins, which need regulation (Van Dongen et al., 2011). While in mammals and yeast, the activity of COX can be regulated by exchanging subunits of COX (Burke and Poyton, 1998; Semenza, 2007), it is not clear whether a similar regulation exist in plants. Another regulatory mechanism might relate to the various phosphorylation sites on the mETC complexes, but for now the role phosphorylation might play is unknown (Bykova et al., 2003; Ito et al., 2009; Kadenbach et al., 2010). While the mETC is typically seen to operate in a linear way, the mitochondrial complexes can be ordered in so-called supercomplexes or respirasomes with specific configuration and stoichiometry (Eubel et al., 2004; Boekema and Braun, 2007; Schäfer et al., 2007). In plants, this is only known to occur for the complexes I, III, and IV (Schäfer et al., 2007). While the functional role of these supercomplexes is not yet clear it might increase the stability of individual complexes (Diaz et al., 2006) increasing the protein density of the membrane (Boekema and Braun, 2007). The supercomplexes might thus facilitate channelling electrons

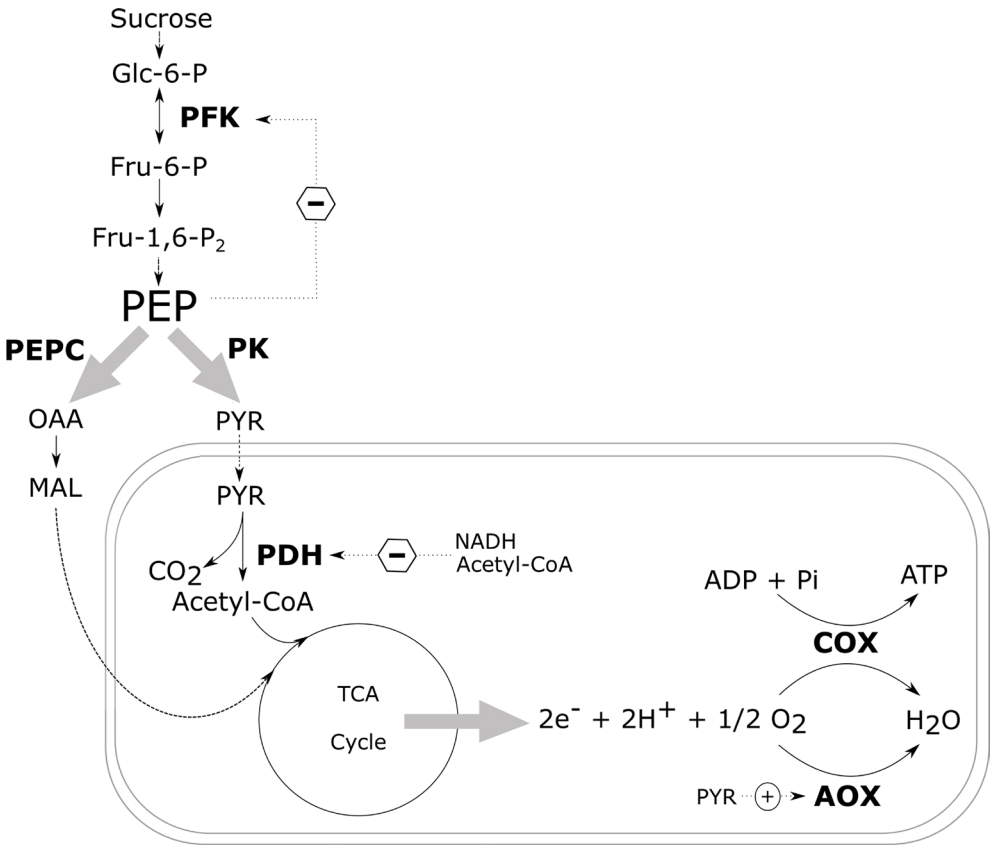

FIGURE 4 | Schematic overview of the most important regulatory control sites of the carbon metabolism. The central role of PEP in the regulation of the glycolysis *via* a negative feedback mechanism is indicated, as well as the most important control sites of the mitochondrial respiration. Dotted arrows with a circled plus and minus sign indicate enzyme activation and inhibition, respectively, by allosteric effectors. Glc-6-P, glucose-6-phosphate; Fru-6-P, fructose-6-phosphate; Fru-1,6-P_2, fructose-1,6-biphosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; OAA, oxaloacetate; MAL, malate; PFK, phosphofructokinase; PEPC, phosphoenolpyruvate carboxylase; PK, pyruvate kinase; PDH, pyruvate dehydrogenase complex; COX, cytochrome c oxidase; AOX, alternative oxidase (redrawn after Plaxton and Podestá, 2006).

between the reactive sites, affecting respiratory rate and ATP production (Van Dongen et al., 2011; Päpke et al., 2014; Schmidt et al., 2018).

Regulation of Central Carbon Metabolism Under Low-O_2 Stress Conditions

Energy-Saving Metabolic Adaptations Upon Low-O_2 Stress

Due to the respiratory activity and the diffusion properties of the fruit, O_2 levels inside a pome fruit can be several factors lower than the levels of the storage atmosphere inducing low-O_2 stress (Ho et al., 2009). This strongly affects the fruit's metabolism. One of the most direct effects of low-O_2 stress is the reduction of the energy status of the cells (Geigenberger, 2003). In an attempt to prevent, or at least postpone, the occurrence of internal anoxia and its concomitant negative consequences, plants try to save energy by adapting their metabolism (Geigenberger, 2003; Van Dongen et al., 2011; Bailey-Serres et al., 2012). One of these adaptations is the reduction of nonessential, energy-consuming processes such as the synthesis of storage products like starch, protein, and lipids (Geigenberger, 2003; Vigeolas et al., 2003; Van Dongen and Licausi, 2015). A second metabolic adaptation is to favor pyrophosphate (PPi) dependent reactions above those which use ATP as substrate (**Figure 5**; Bailey-Serres et al., 2012; Kosmacz and Weits, 2014).

There are at least three reactions known in the plant primary metabolism that have PPi-dependent alternative enzymes. The first known reaction involves the cleavage of sucrose. Instead of using invertases and hexokinases to provide hexose-phosphates from sucrose, which is a ATP consuming process, plants can use sucrose synthase (SUS) and UDP-glucose pyrophosphorylase (UGPase) to cleave sucrose. This alternative pathway is truly energy saving by using only one molecule PPi instead of two molecules ATP (Mustroph et al., 2014). *SUS* has been reported to be one of the core-responsive genes to hypoxia in apple fruit. The gene expression of SUS in apple fruit is highly sensitive to changes in O_2 concentration in the environment (Cukrov et al., 2016).

Secondly, the ATP dependent glycolytic enzyme phosphofructokinase (PFK), which catalyses the phosphorylation of fructose 6-phosphate, has a reversible PPi using variant, i.e., pyrophosphate:fructose-6-phosphate phosphotransferase (PFP).

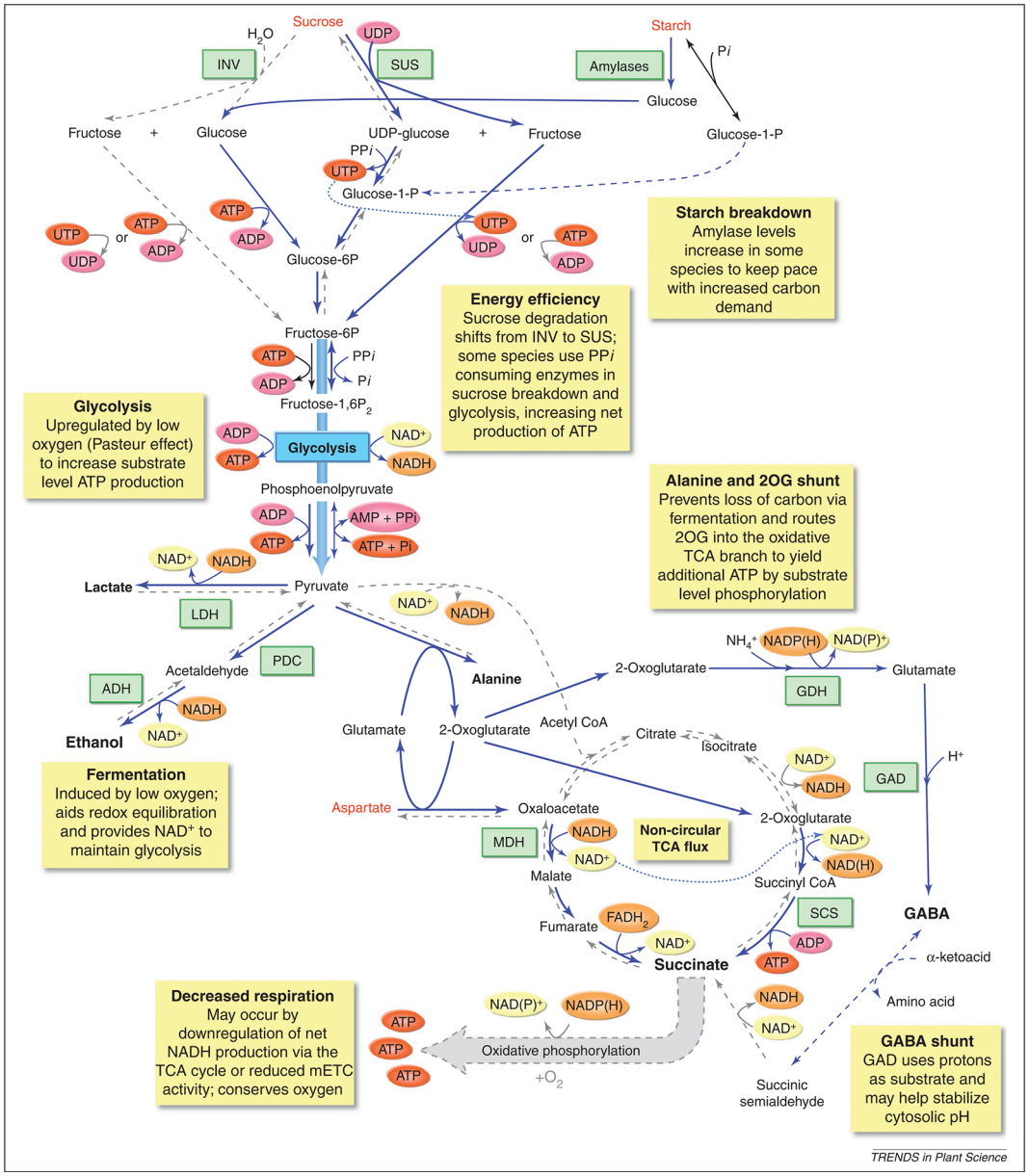

FIGURE 5 | Metabolic responses of plants to low-O_2 stress. The yellow boxes highlight a number of metabolic changes that are known to happen when O_2 availability is limited, such as enhanced sucrose-starch metabolism, glycolysis, fermentation, a modified TCA flow, an alanine and 2-oxoglutarate (2-OG) shunt, and a GABA shunt. Blue lines indicate pathways enhanced during low-O_2 stress, blue dashed lines indicate pathways proposed to be active during low-O_2 stress, and grey dashed lines indicate reaction that are inhibited during low-O_2 stress. Metabolites that increase during low-O_2 stress are shown in enlarged black font; metabolites that decrease are shown in red font. 2OG, 2-oxoglutarate; ADH, alcohol dehydrogenase; GAD, glutamic acid decarboxylase; GDH, glutamate dehydrogenase; INV, invertase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PDC, pyruvate decarboxylase; SCS, succinyl CoA ligase; SUS, sucrose synthase (reprinted from Bailey-Serres et al., 2012 with permission from Elsevier).

However, the induction of *PFP* expression during hypoxia was not observed in all species (Mustroph et al., 2014). Interestingly, *PFK* genes were observed to be expressed during hypoxia in all plant organisms studied so far (Mustroph et al., 2010), suggesting that PFK and not PFP catalyses the main glycolytic pathway under hypoxic conditions in most organisms (Mustroph et al., 2014). A rapid upregulation of PFK expression during low-O_2 stress was observed in apple fruit (Cukrov et al., 2016). However, how the *PFK/PFP* gene expression and activity is regulated in apple fruit still remains to be investigated.

Also, the last step of the glycolysis, where phoshoenolpyruvate is converted to pyruvate, might be catalysed by a PPi dependent enzyme; i.e., pyruvate-orthophosphate dikinase (PPDK), instead of pyruvate kinase (PK). Although these alternative pathways could compensate for the severe ATP deficiency during low-O_2 stress, their exact impact is not yet clear. PPi is

a side product produced during biosynthetic reactions, which are known to be downregulated during hypoxia. However, the content of PPi under hypoxia is reported to stay mostly stable, unlike ATP, suggesting PPi production from alternative sources. Furthermore, the alternative enzymes mostly catalyse reversible reactions, making it difficult to estimate which direction is the favorable one under low-O_2 stress (Mustroph et al., 2014).

When plants try to save energy, the demand for respiratory O_2 consumption decreases and the respiratory activity of the mETC slows down (**Figure 5**; Gupta et al., 2009; Zabalza et al., 2009). In this situation, plants try to make the ATP production per O_2 that is consumed as efficient as possible. Gupta et al. (2009) observed that the ratio between the capacities of COX to AOX increases when the O_2 availability goes down. This suggests that the amount of ATP produced by the reduction of one molecule of O_2 will increase. Nowadays, the role of respiratory supercomplexes in the regulation of the electron flux to either AOX or COX is being investigated (Van Dongen et al., 2011). Pyruvate can bind to AOX to activate the enzyme (**Figure 4**; Oliver et al., 2008; Zabalza et al., 2009). Therefore, it seems important to control the cellular concentrations of pyruvate during low-O_2 stress. However, in most plant species the affinity of AOX to O_2 is one or two orders of magnitude lower compared to the affinity of COX to O_2. Hence, it is unlikely that AOX competes with COX for O_2 as a substrate during low-O_2 stress (Van Dongen et al., 2011; Päpke et al., 2014).

Fermentative Metabolism

The goal of postharvest storage of fruit is to lower the O_2 levels to such a degree that the respiratory metabolism is reduces as much as possible, but without inducing too many negative effects on fruit quality. One of these negative effects is the induction of fermentation. When the low-O_2 stress induced in the tissue is too severe, the mitochondrial respiration is compromised too much, leading to insufficient supply of ATP for energy demanding processes. In an attempt to compensate for this decrease in respiratory energy production, plants increase their glycolytic flux to produce more energy with the conversion of glucose to pyruvate, a process known as the Pasteur effect (**Figure 5**).

Since apple fruit is a nonphotosynthetic organ, it needs to hydrolyse starch to provide enough sucrose to support the high glycolytic flux. The gene expression of the starch degrading enzyme, β-amylase, appears to be upregulated in "Granny Smith" apples stored under hypoxia (Cukrov et al., 2016). When the glycolytic flux increases, pyruvate starts to accumulate since it is no longer shuttled into the TCA cycle due to the decreased pyruvate dehydrogenase activity. Pyruvate dehydrogenase is inhibited by the high reduction levels of NADH/NAD^+ pool and the accumulation of acetyl-CoA (**Figure 4**; Päpke et al., 2014). However, to be able to maintain the high glycolytic flux, $NADP^+$ and NAD^+ have to be regenerated and the accumulation of pyruvate should remain limited (Geigenberger, 2003; Bailey-Serres et al., 2012; António et al., 2016). Therefore, the increase in glycolytic flux is typically coupled to fermentation.

In the fermentation pathway, pyruvate can be converted to either lactate or ethanol and CO_2, meanwhile maintaining the redox balance in the cell by the formation of NAD^+ (**Figure 5**; Perata and Alpi, 1993). Pyruvate is converted to lactate by the enzyme lactate dehydrogenase. The accumulation of lactic acid will cause cytoplasmic acidosis, which can result in cell damage (Perata and Alpi, 1993). The decreasing cytosolic pH, results in a decreasing activity of lactate dehydrogenase and activation of pyruvate decarboxylase. This enzyme converts pyruvate to acetaldehyde, which is further converted to ethanol by alcohol dehydrogenase and finally to ethyl acetate by the enzyme alcohol acyl transferase. These volatile fermentation metabolites (ethanol, acetaldehyde, and ethyl acetate) readily diffuse out of the cells into the external environment, leading to a depletion of carbon reserves and causing off-flavors in the fruit (Tadege et al., 1999; Fukao and Bailey-Serres, 2004; Pesis, 2005; António et al., 2016). The accumulation of ethanol under extreme low O_2 concentrations and/or during prolonged low-O_2 storage has been reported for multiple apple cultivars (Mattheis et al., 1991; Saquet and Streif, 2008; Bekele et al., 2015; Cukrov et al., 2016; Brizzolara et al., 2017), also in relation to the development of low O_2 induced physiological disorders (Vandendriessche et al., 2013; Hatoum et al., 2014; Lumpkin et al., 2014). Furthermore, it has been shown that the rate of ethanol accumulation increases with decreasing O_2 levels (Lumpkin et al., 2014; Cukrov et al., 2016; Brizzolara et al., 2017).

Boeckx et al. (2019) performed an extensive study on the *in vivo* regulation of postharvest fermentation in apple fruit as a function of storage temperature and time. A kinetic modelling approach was used to link measured enzyme activities of PDC and ADH to the observed changes in pyruvate, acetaldehyde, ethanol, and ethyl acetate by calculating the intermediate fluxes. This revealed that control of the ethanol pathway depended on the actual conditions applied, showing both elements of molecular and metabolic control. Prolonged exposure to low-O_2 stress resulted in a decrease in fermentation products indicating a switch to alternative pathways, possibly as an effort to minimize carbon losses (Boeckx et al., 2019).

Alternative Pathways Induced by Low-O_2 Stress

Although the induction of fermentation helps apple fruit to tolerate low-O_2 stress by mitigating the damaging effects of energy crisis, it causes acidosis of the cytoplasmic pH and depletion of the carbon reserves (Limami, 2014). Changes in amino acid metabolism may help to reduce these negative effects of fermentation. More specifically, alanine metabolism plays an important role in maintaining the glycolytic flux by converting pyruvate with the help of alanine aminotransferase (AlaAT) to alanine, providing an alternative, nondetrimental end product (**Figure 5**). Alanine is observed in a number of species, including apple (Bailey-Serres et al., 2012; Cukrov et al., 2016; Hatoum et al., 2014; Vandendriessche et al., 2013). Alanine concentrations are found to be increased in Braeburn apples after long term storage (up to 8 months) under 2.5 and 3.7 kPa CO_2 conditions (Hatoum et al., 2014). Furthermore, it was shown that alanine accumulated to different levels depending on the composition of the atmosphere (Vandendriessche et al., 2013; Cukrov et al., 2016; Brizzolara et al., 2017). This is probably due to hypoxic stress inducing the expression of the genes coding for *AlaAT*.

Cukrov et al. (2016) showed that the accumulation of *AlaAT* transcripts was extremely abundant in apples stored under 0.4 kPa O_2, but in fruit stored under 0.8 kPa O_2, *AlaAT* gene expression appeared to remain at basal levels throughout the experiment. These findings suggest a low-O_2 threshold for this gene in apple. Since the concentration of alanine was clearly higher in apples stored under 0.8 kPa O_2 as compared to normoxia, another mode of regulation has to be present (Cukrov et al., 2016). By feeding *Medicago truncatula* (cv. Paraggio) seedlings with ^{15}N-glutamate or ^{15}N-alanine, Ricoult et al. (2006) provided some evidence that under hypoxia the activity of the reversible enzyme AlaAT was directed towards alanine synthesis using glutamate as amino donor while the reaction of glutamate synthesis using alanine as amino donor was inhibited. These results show that AlaAT has a dual mode of regulation by hypoxia, both at transcriptional and posttranslational level (Limami, 2014).

As can be seen from **Figure 5**, the synthesis of alanine is accompanied by the generation of 2-oxoglutarate, which can be further metabolized in the TCA cycle *via* 2-oxoglutarate dehydrogenase and succinate CoA ligase (SCS) to form succinate and produce ATP. The amount of ATP gained *via* this pathway doubles the amount of energy produced by the glycolysis alone. Succinate will accumulate as the TCA cycle is blocked due to the O_2 limitation at the reaction catalysed by complex II of the mETC. The NAD^+ that is required for this reaction is guaranteed by the oxidation of NADH *via* malate dehydrogenase (MDH) catalysing the reaction from oxaloacetate to malate in the reversed TCA cycle. During hypoxia, oxaloacetate is produced by aspartate aminotransferase (AspAT), simultaneously producing glutamate, which is used by AlaAT as a cosubstrate for alanine synthesis (**Figure 5**). Hence, the accumulation of alanine during low-O_2 stress as a C/N storage compound produces extra ATP, thereby diminishing an energy crisis and saving carbon atoms, which otherwise would be lost *via* ethanol (Ricoult et al., 2006; Limami et al., 2008; Rocha et al., 2010; Bailey-Serres et al., 2012; Limami, 2014).

Furthermore, synthesis of alanine also limits cytoplasmic acidification by competing with lactate fermentation for the use of pyruvate (Ricoult et al., 2005; Ricoult et al., 2006). In addition, alanine may accumulate as a by-product of the GABA shunt, which is known to help stabilizing cytosolic pH (**Figure 5**). In the GABA shunt, glutamate is decarboxylated into GABA by glutamate decarboxylase (GDC). The formation of GABA is a proton consuming reaction, which increases the pH (Greenway and Gibbs, 2003). GABA can react further to succinic semialdehyde *via* GABA-T that uses pyruvate as amino acceptor under hypoxic conditions leading to the production of alanine (Limami, 2014). GABA content is found to be increased under low-O_2 stress in "Granny Smith" and "Red Delicious" apples (Cukrov et al., 2016; Brizzolara et al., 2017). Cukrov et al. (2016) found a similar trend in the accumulation patterns of alanine and GABA under hypoxia, suggesting that both pathways are coregulated in apple fruit. The same authors saw a reduction of asparagine and aspartate by 0.4 kPa O_2 but not by 0.8 kPa O_2. This could be the result from a different modulation of the carbon flux into the TCA cycle under these two stress levels (Cukrov et al., 2016). Based on a metabolomics approach, Brizzolara et al. (2017) found that "Granny Smith" and "Red Delicious" apples have a different approach to balance the levels of pyruvate and to keep on producing energy under hypoxia. "Red Delicious" apples are producing more ethanol and GABA, while "Granny Smith" apples accumulate more alanine.

During low-O_2 stress, another alternative pathway is induced to recycle NAD^+ from NADH, to be able to maintain the high glycolytic flux. This alternative pathway is the nitric oxide (NO) cycle, in which NO is either produced in the cytosol or in the mitochondrial matrix (**Figure 6**). In the latter case, nitrite is transported to the mitochondrial matrix through a yet unknown transport system, where it serves as an alternative electron acceptor at the sites of complex III and COX. The electrons necessary for this reaction are generated by the oxidation of NAD(P)H by the Ca^{2+}-sensitive NAD(P)H dehydrogenase on the inner mitochondrial membrane surface. In both cases, NO is scavenged by class-1 nonsymbiotic haemoglobins (Hb), which are found to be highly expressed during low-O_2 stress. Due to its high affinity for O_2, Hb spontaneously oxygenate to oxyhaemoglobins, which catalyse the turnover of NO to nitrate (for further details see Igamberdiev and Hill, 2004; Limami, 2014; Limami et al., 2014; Van Dongen and Licausi, 2015). Plants can protect the cells against deleterious nitrosative stress by regulating the NO levels *via* the NO cycle. Thereby, they can control the multiple functions exerted by NO, such as the interaction of NO with hormone signalling and its inhibitory effect on haeme- and Fe-S cluster-containing enzymes such as aconitase, COX, and catalases (**Figure 7**). Through its inhibitory effect on these enzymes, NO can directly affect the energy status of the cell and their defence against ROS (Blokhina et al., 2014).

In *Arabidopsis* shoots, a metabolic association between the TCA cycle and the NO cycle under low-O_2 stress was suggested, as can be seen in **Figure 7** (Blokhina et al., 2014). Under low-O_2 stress, monodehydroascorbate reductase (MDHAR) can act as a MetHb reductase (Igamberdiev et al., 2006), thereby simultaneously producing ascorbate. Based on metabolomics and microarray studies under O_2 deprivation, a novel route, besides the ascorbate-glutathione cycle, is suggested to oxidize ascorbate. In the novel route, Fe-dependent 2-ketoglutarate oxygenase (Fe2KGO) utilizes 2 ketoglutarate (2KG) and ascorbate to form succinate. Under low-O_2 stress conditions, 2KG needed for the reaction is supplied through the metabolism of alanine. The novel route *via* Fe2KGO makes it possible to bypass the NO-inactivated TCA cycle components (Blokhina et al., 2014). Also in apple fruit, Fe2KGO genes were found to be strongly upregulated under low-O_2 stress (Cukrov et al., 2016).

In conclusion, how well plants can tolerate low-O_2 stress highly depends on their ability to mitigate damaging effects of energy crisis and acidosis of cytoplasmic pH (Greenway and Gibbs, 2003).

Low-O_2 Sensing and Signalling

Regulation of the energy metabolism in plants experiencing low-O_2 stress demands an efficient and tuneable sensing mechanism (Kosmacz and Weits, 2014; Van Dongen and Licausi, 2015; Schmidt et al., 2018). In this section, the different molecular

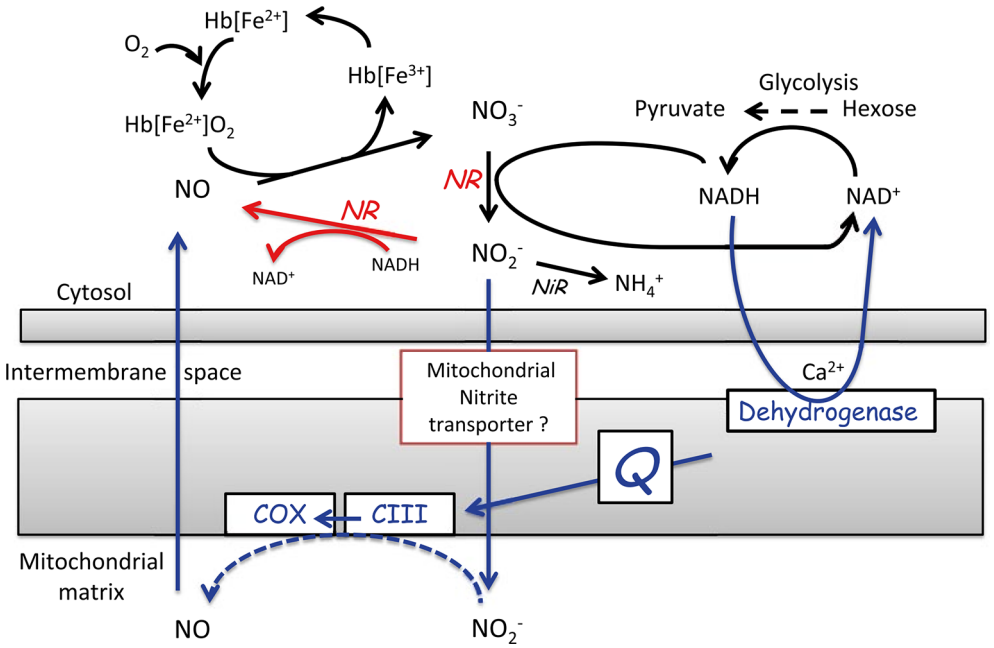

FIGURE 6 | Representation of the nitric oxide (NO) cycle. NO is either produced in the cytosol by the reduction of nitrite by nitrate reductase (NR), shown in red or NO is produced in the mitochondrial matrix at levels of O_2 below the saturation of COX, shown in blue. When NO is produced, it is assumed to be scavenged by oxyhaemoglobin [Hb(Fe_2^+)O_2] to regenerate NO_3^- and metHb [Hb(Fe_3^+)] in the NO cycle (reprinted from Limami et al., 2014. Copyright Springer-Verlag).

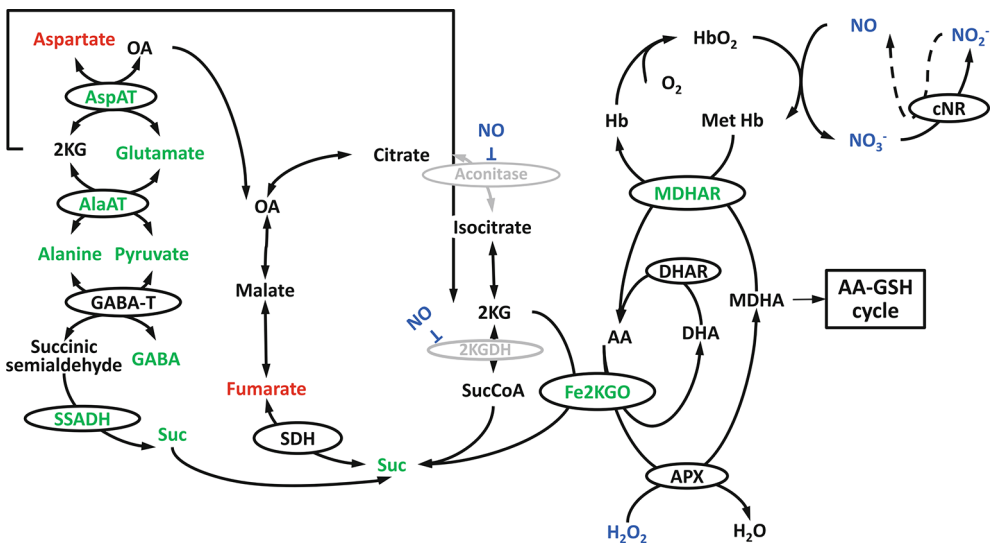

FIGURE 7 | Hypoxia induced tricarboxylic acid (TCA) cycle modification in Arabidopsis shoots: the involvement of Fe2+ dependent ketoglutarate oxidase, nonsymbiotic haemoglobins, NO, alanine metabolism and GABA shunt (reprinted from Blokhina et al., 2014 Copyright Springer-Verlag).

mechanisms plants employ to sense decreased cellular O_2 concentrations and how they respond to this is discussed.

Direct O_2 Sensing

From studies conducted on model plants like *Arabidopsis thaliana* and rice, it became clear that plants have a highly adaptive and flexible response to low O_2, regulated by a set of cardinal genes. A set of 49 specific genes known as the hypoxia-responsive genes (HGRs) are involved in a variety of important cell adaptation processes (Mustroph et al., 2009; Mustroph et al., 2010; Lee et al., 2011; Bui et al., 2015). HRG transcript accumulation has a domino effect in facilitating an efficient, but flexible metabolic reprogramming of the cell. The up-regulation of HRGs lead to the production of pyruvate decarboxylase, alcohol dehydrogenase,

lactate dehydrogenase, PFK, and alanine aminotransferase, as well as ACC synthase and ACC oxidase (Mustroph et al., 2010). These enzymes are involved in various cellular processes including carbon catabolism, anaerobic fermentation and the regulation of reactive O_2 species (ROS) production, that help the cell adapt to the limited O_2 available (Mustroph et al., 2009; Papdi et al., 2015). Increased ROS production due to a faltering mETC could cause oxidative damage to the cell. Therefore, upon severe hypoxia, scavenging enzymes are induced to prevent the accumulation of ROS, and protein chaperones and inhibitors of lipid peroxidation are induced to add to the defence (Pucciariello et al., 2012). Recently, some additional negative regulators of the adaptive metabolic response to low-O_2 stress were identified, which play a role when the plant returns to normoxic conditions (Weits et al., 2014). This highlights the importance of the reversibility and timely control of the adaptive metabolic response to low-O_2 stress (Van Dongen and Licausi, 2015). There still remains various proteins of unknown function that should be investigated further to determine their involvement and understand the pathway to its fullest (Giuntoli and Perata, 2018).

Over the past decade, research has focused on identifying a primary "O_2 sensor" that would be responsible for the regulation of the HRG in response to lower cellular O_2 concentrations (Gibbs et al., 2011; Licausi et al., 2011a). It was discovered that low O_2 levels have a direct effect on the stability, and thus activity of the transcription factor subfamily from the plant specific ethylene response factor gene family. The subfamily is referred to as group-VII ethylene response factors (ERFs) and in *Arabidopsis thaliana* consist of five family members: RAP2.2, RAP2.12, RAP2.3, HRE1, and HRE2 (Nakano et al., 2006; Licausi et al., 2011b; Bailey-Serres et al., 2012; Kosmacz and Weits, 2014; Van Dongen and Licausi, 2015; Schmidt et al., 2018).

The protein levels of ERF-VII transcription factors are regulated through an ancient, conserved branch of the ubiquitin proteasome system known as the N-end rule pathway (**Figure 8**), which acts as a safeguard mechanism that continuously targets

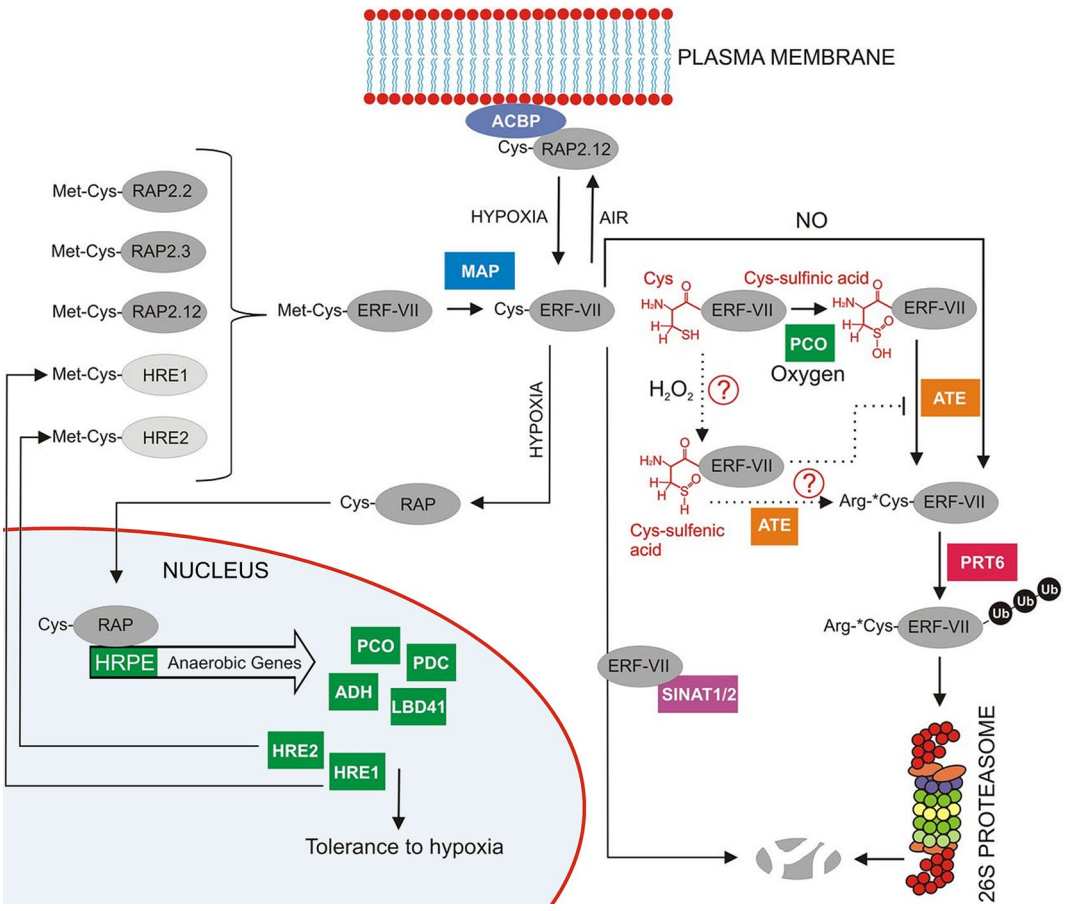

FIGURE 8 | Overview of the regulation of group-VII ERF factors in Arabidopsis as reviewed by Giuntoli and Perata (2018). RAP2.12 is under normoxic conditions located at the membrane through interaction with an acyl-coenzyme A-binding protein membrane (ACBP). Any RAP2.12 dissociated from A-binding protein (ACBP) in air will eventually be degraded by the 26S proteasome after initial Met cleavage through MAP (methionine aminopeptidase). The subsequently exposed N-terminal Cys is susceptible to oxidation through plant Cys oxidases (PCO). Also, NO can play a role in this N-end rule pathway with E3 ubiquitin ligase responsible for labelling the substrate for final degradation. When O_2 availability becomes limited, RAP2.12 no longer gets oxidised by the N-end rule pathway and is able to move to the nucleus to trigger the transcription of hypoxia responsive genes (reprinted with slight modification from Giuntoli and Perata, 2018, Copyright American Society of Plant Biologists).

the ERF-VII transcription factors for proteolysis under aerobic conditions (Gibbs et al., 2016). The N-end rule pathway regulated through a highly conserved N-terminal domain was first described by Nakano et al, in 2006. The domain is a 5-amino acid motif initiated with Methionine (Met) and Cysteine (Cys) residues. It was found to be specifically conserved in flowering plants. Ultimately, the domain acts as an N-degron, shuttling the protein into the N-end rule pathway and targeting it for proteolysis (Gibbs et al., 2011; Licausi et al., 2011b; Giuntoli and Perata, 2018).

It is known that the Cys2 from the motif has a highly reactive thiol group attached, which is sensitive to O_2. The Cys2 is therefore known as a regulatory Cys (White et al., 2017). When the initial methionine is removed from the N-terminus *via* methionine aminopeptidase (MAP), it exposes the Cys2 to the activity of plant cysteine oxidases (PCOs). In the presence of O_2 and NO, PCO oxidizes the Cys2 to sulfinic or sulfonic acid (Weits et al., 2014; White et al., 2017; Giuntoli and Perata, 2018), which acts as an acceptor for the condensation of an arginine residue by arginyltransferase (ATE). The exposed arginine residue is subsequently ubiquitinated by the proteolysis (PRT) E3 ligases and targeted to the 26S proteasome for degradation (Kosmacz and Weits, 2014; Van Dongen and Licausi, 2015; Schmidt et al., 2018). Therefore, this mechanism provides an efficient way to regulate the metabolic response under low-O_2 stress conditions; the presence of O_2 and NO causes ERF-VII destabilization, whereas their absence permits stabilization and accumulation of ERF-VII transcription factors in the nucleus (Gibbs et al., 2011; Licausi et al., 2011a; Van Dongen and Licausi, 2015; Schmidt et al., 2018). However, it is still unclear how exactly O_2 and NO act together to oxidize ERF-VII (Van Dongen and Licausi, 2015).

What has, however, become clearer is the specific oxidization method PCO proteins employ in oxidizing the cysteine residue of the N-degron. Research recently identified various characteristics of the *Arabidopsis* PCO proteins that allow them to biochemically sense the presence of molecular O_2 and efficiently oxidize Cys2 (White et al., 2018). After verifying the sensing capabilities of the protein, it was proposed that the PCO's act as O_2 sensor, instead of the previously speculated RAP2.12. The PCOs were shown to effectively regulate ERF-VII protein levels and directly influence protein stability. A direct connection between ERF-VII protein levels and PCO transcript levels could be made where it would seem that a negative feedback loop exists between RAP2.12 and PCOs. RAP2.12 is directly responsible for the up-regulation of *AtPCO*s. Increased levels of PCO proteins, consequently, lead to the active oxidation of and breakdown of ERV-II proteins *via* the N-end rule. It prevents the unnecessary buildup of the ERF-VII proteins in the nucleus. These findings open up new doors for gene manipulation and the potential to engineer increased low-O_2 stress tolerance in plants (White et al., 2017; White et al., 2018).

For the most part, research on low-O_2 stress in plants has mainly focused on the ERF-VII transcription factor RAP2.12, specifically *AtRAP2.12*. It was found to be localized at the plasma membrane under aerobic conditions (Licausi et al., 2011a) through interactions with the peripheral membrane proteins acyl-coenzyme A-binding protein 1 (ACBP1) and ACBP2 (**Figure 8**). This interaction protects the RAP2.12 protein from being shuttled into the N-end rule pathway for degradation. However, under hypoxic conditions, RAP2.12 can detach from the membrane and is transported to the nucleus where it then accumulates. The protein accumulation subsequently triggers the expression of the 49 HRGs. It has been speculated that the accumulation of RAP2.12 at the plasma membrane under normoxic conditions might be a reservoir of activators, which enables a quick induction of the HRGs and an efficient response to declining cellular O_2 levels (Kosmacz and Weits, 2014; Van Dongen and Licausi, 2015). As seen with the recent PCO-based discoveries, much about how RAP2.12 is regulated during low-O_2 stress is still unknown.

When referring to regulation of the low-O_2 stress, it also entails adapting to and managing O_2 concentration fluctuations. Plant cells should be able to adapt quickly and efficiently not only to hypoxic conditions, but also to a sudden return to normoxic conditions. When O_2 becomes available again, it is unlikely that the displacement of the ERF-VII proteins from the promotors of the hypoxia responsive genes and their subsequent degradation is sufficient to rapidly silence the expression of the core HRGs (Van Dongen and Licausi, 2015). In *Arabidopsis,* it was found that a transcriptional regulator, hypoxia response attenuator 1 (HRA1) was responsible for the repression of the upregulated HRGs by binding to RAP2.12 (Giuntoli et al., 2014). Since RAP2.12 induces the expression of HRA1, as is the case with PCO, it acts as a positive regulator while PCOs and HRA1 restrict the function of RAP2.12 in a feedback loop, depending on the availability of O_2 (Weits et al., 2014).

The contribution of each individual group-VII ERF to sensing low-O_2 stress has remained largely unknown for the remaining 4 family members (Gasch et al., 2015). Transgenic studies conducted in RAP2.12, RAP2.2 and RAP2.3 overexpressing lines indicated that all three genes are responsible for activating the important set of 49 HRGs (Papdi et al., 2015). Both RAP2.12 and RAP2.2 were responsible for gene transactivation when studied in protoplasts, indicating a redundant role (Licausi et al., 2011b; Weits et al., 2014; Papdi et al., 2015). This type of redundant transactivation of similar target genes is not uncommon for plant transcription factors and was confirmed through promoter studies. It was shown that both RAP2.12 and RAP2.2 are cardinal to the plants stress response. Both transcription factors are able to recognize and bind to a conserved 12 base pair domain that acts as a *cis*-regulatory motif for the transactivation of HRGs. Initial studies also opened up speculation surrounding the remaining two family members HYPOXIA RESPONSIVE ERF1 and 2 (HRE1 and HRE2) (Gibbs et al., 2011; Licausi et al., 2011b). Subsequent research pointed to the idea that they play minor, supportive roles in the response mechanism (Papdi et al., 2015). However, Gibbs et al. (2011) showed that not all hypoxia responsive genes are controlled by the N-end rule pathway, giving the first indication that alternative regulatory pathways should exist. Further research is still required to discover more of the potential role the additional family members might play.

In an early study of the apple genome, eight genes were assigned as coding for ERF-VII transcription factors (Girardi et al., 2013), while the first evidence of an O_2 sensing mechanism based on the N-end rule pathway and on the posttranslational regulation of ERF-VII protein stability in apple fruit is given by Cukrov et al. (2016). Unfortunately very little research has

focused on nonmodel plants and further research is necessary to fully understand the behavior of the O_2 sensing mechanism in apple fruit and its effect on the different metabolic responses upon low-O_2 stress between the different apple varieties.

Discovering the Multifunctional Role of Group-VII ERFS

As research in the field expands, more evidence of additional regulatory roles for group-VII ERFs are being discovered. It would seem that the influence and regulatory function of this subfamily expands to processes related to germination, abiotic stress tolerance, and an increased resistance against pathogen infection (Giuntoli and Perata, 2018). It is hypothesized that the additional physiological roles the group-VII ERFs take on, are mainly facilitated by the occurrences of hypoxic microenvironments throughout plant tissue.

Low-O_2-associated secondary signalling pathway has been investigated intensively where it was found that when respiration is affected by low-O_2 stress, it has an impact on many parameters and processes, including reactive O_2 and nitrogen species (ROS/RNS) homeostasis, redox status of NAD(P)H and antioxidant pools, ATP/ADP ratio, the proton-motive force, calcium, and metabolites levels, which could all trigger the mitochondrial retrograde responses (Schmidt et al., 2018; Wagner et al., 2018). It became clear that the RAP-type group-VII ERF genes are directly involved in and actively participate in both osmotic – and oxidative stress tolerance (Papdi et al., 2015). Studies showed that the group-VII ERF induced signalling could contribute to transcriptional reprogramming of the nuclei of hypoxic cells and is commonly referred to as the mitochondrial retrograde regulation. For a more detailed discussion on the role of these diverse signals in mitochondrial retrograde responses and its role in the low-O_2 response of plants, the reader is referred to the review of Wagner et al. (2018).

PERSPECTIVES AND FUTURE CHALLENGES

Although much is known about the regulation of the central carbon metabolism in response to low- O_2 stress in plant systems in general, many questions still remain to be answered, especially regarding apple fruit. The regulation of the carbon metabolism was already shown to differ between apple cultivars. In addition, due to the ongoing ripening of apple fruit during prolonged storage, the contribution of the various regulatory events are likely to change during the lifetime of an apple (Beshir et al., 2017).

Some aspects have not been studied yet in full for any particular plant system, such as the function of the supercomplexes and the regulation of the COX of the mitochondrial electron transport chain. While the PPP recently received quite some attention because of its role in retaining redox homeostasis in human diseases (Stincone et al., 2015), its role during low-O_2 stress conditions in apple fruit can only be assumed for now.

Even though the apple genome has been sequenced, the bottle neck remains the quality of the annotation (Velasco et al., 2010; Daccord et al., 2017). This makes it difficult to confirm findings on the molecular regulation found in other plant species in apple. The added challenge is that many of the commercial apple cultivars are polyploid not only making the linking of genotype to phenotype an even more challenging task, but also hindering assembling of the true biological genome from sequencing data (Kyriakidou et al., 2018).

To study the molecular control in response to low-O_2 stress, *in vivo* experiments under well controlled O_2 conditions need to be designed. In bulky organs like apples, fruit internal O_2 gradients will inevitably arise complicating interpretation of the experimental results using intact fruit. To this end, integrated mathematical models are urgently needed that combine knowledge on the physics of gas transport with expertise on molecular and metabolic control of the central carbon metabolism in apple (Ho et al., 2009; Ho et al., 2011; Ho et al., 2018).

Recently, exciting research using classical model plant systems has discovered a mechanism of O_2 sensing in plants. This work bears large relevance to the postharvest physiology of apple fruit exposed to low-O_2 storage conditions. It, therefore, is important to fully understand the behavior of apple fruit exposed to low-O_2 stress to ultimately minimise the incidence of low-O_2 related storage disorders, not just by trial and error, but through a proper understanding of the regulation of their central carbon metabolism.

AUTHOR CONTRIBUTIONS

JB wrote the first draft of the manuscript. SP and MH wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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REVIEW
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Tomato Fruit Development and Metabolism

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Tomato (*Solanum lycopersicum* L.) belongs to the Solanaceae family and is the second most important fruit or vegetable crop next to potato (*Solanum tuberosum* L.). It is cultivated for fresh fruit and processed products. Tomatoes contain many health-promoting compounds including vitamins, carotenoids, and phenolic compounds. In addition to its economic and nutritional importance, tomatoes have become the model for the study of fleshy fruit development. Tomato is a climacteric fruit and dramatic metabolic changes occur during its fruit development. In this review, we provide an overview of our current understanding of tomato fruit metabolism. We begin by detailing the genetic and hormonal control of fruit development and ripening, after which we document the primary metabolism of tomato fruits, with a special focus on sugar, organic acid, and amino acid metabolism. Links between primary and secondary metabolic pathways are further highlighted by the importance of pigments, flavonoids, and volatiles for tomato fruit quality. Finally, as tomato plants are sensitive to several abiotic stresses, we briefly summarize the effects of adverse environmental conditions on tomato fruit metabolism and quality.

Keywords: abiotic stress, fruit set, fruit ripening, genetic control, hormonal control, primary metabolism, secondary metabolism, *Solanum lycopersicum*

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INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is the second most important fruit or vegetable crop next to potato (*Solanum tuberosum* L.), with approximately 182.3 million tons of tomato fruits produced on 4.85 million ha each year (FAOSTAT, 2019). Asia accounts for 61.1% of global tomato production, while Europe, America, and Africa produced 13.5%, 13.4%, and 11.8% of the total tomato yield, respectively. Tomato yields are highly variable, ranging from more than 508 tons per ha in the Netherlands to fewer than 1.5 tons per ha in Somalia in 2017 (FAOSTAT, 2019), with an average global yield of 376 tons per ha. Tomato consumption is concentrated in China, India, North Africa, the Middle East, the US, and Brazil with tomato consumption per capita, ranging from 61.9 to 198.9 kg per capita (FAOSTAT, 2019). Tomato is a member of the Solanaceae family, which includes several other economically important crops such as potato, pepper (*Capsicum annuum* L.), and eggplant (*Solanum melongena* L.), representing one of the most valuable plant families for vegetable and fruit crops.

Tomatoes contain many health-promoting compounds and are easily integrated as a nutritious part of a balanced diet (Martí et al., 2016). In addition to consuming the fresh fruits, consumers use tomatoes in processed products such as soups, juices, and sauces (Krauss et al., 2006; Li et al., 2018b). Over the last decade, consumers have become more aware of foods as a source of health benefits and their roles in prevention of several chronic diseases and dysfunctions (Pem and Jeewon, 2015). Although a wealth of functional foodstuffs have been created to fulfil these requirements, it is important to note that the consumption of "conventional foods" such as fruits and vegetables is more effective for this purpose (Viuda-Martos et al., 2014).

The nutritional importance of tomatoes is largely explained by their various health-promoting compounds, including vitamins, carotenoids, and phenolic compounds (Raiola et al., 2014; Liu et al., 2016; Martí et al., 2016; Li et al., 2018b). These bioactive compounds have a wide range of physiological properties, including anti-inflammatory, anti-allergenic, antimicrobial, vasodilatory, antithrombotic, cardio-protective, and antioxidant effects (Raiola et al., 2014). Tomatoes are rich in carotenoids, representing the main source of lycopene in the human diet (Viuda-Martos et al., 2014). Carotenoids and polyphenolic compounds contribute to the nutritional value of tomatoes and improve their functional attributes and sensory qualities, including taste, aroma, and texture (Raiola et al., 2014; Tohge and Fernie, 2015; Martí et al., 2016). Tomatoes also have the naturally occurring antioxidants Vitamins C and E (Agarwal and Rao, 2000; Martí et al., 2016) as well as large amounts of metabolites, such as sucrose, hexoses, citrate, malate, and ascorbic acid (Li et al., 2018b).

Tomato fruit quality and metabolite biosynthesis are affected by plant growing conditions (Diouf et al., 2018). Tomato production is challenged by several problems around the world, including the scarcity of water resources, soil salinization, and other abiotic stresses (Fahad et al., 2017; Gharbi et al., 2017; Zhou et al., 2019). In particular, in countries with a Mediterranean climate, including some regions in southern Europe and North and South America, tomato cultivation is increasingly confronted with limiting conditions such as drought and salinity, which ultimately reduce the competitiveness of tomato farmers in these areas. This, in turn, impacts the integrity of the ecosystem, contributing to the relocation (abandonment) of rural sectors.

In addition to its economic and nutritional importance, tomatoes have become the model for the study of fleshy fruit development (Karlova et al., 2014; Kim et al., 2018; Li et al., 2018b). The entire tomato genome has been sequenced, serving as a rich genomic resource, and both genetic and physical maps and molecular markers are available for this species (The Tomato Genome Consortium, 2012; Suresh et al., 2014; Zhao et al., 2019). Moreover, a range of well-characterized monogenic mutants, TILLING populations, wild tomato species, recombinant inbred lines and genome editing tools are available (Eshed and Zamir, 1994; Minoia et al., 2010; Pérez-Martín et al., 2017; Li et al., 2018b; Martín-Pizarro and Posé, 2018; Tomato Genetics Resource Center, 2019; Rothan et al., 2019). Several databases contain gene expression analysis data (Fei et al., 2006; Suresh et al., 2014; Zouine et al., 2017; Shinozaki et al., 2018b), while recent progress in tomato metabolomics has provided substantial information about the primary and specialized metabolism of this species and the pathways involved in molecular biosynthesis and turnover (Luo, 2015; Tieman et al., 2017; Zhu et al., 2018).

Dramatic metabolic changes occur during tomato fruit development (Carrari and Fernie, 2006). Tomato is a climacteric fruit, meaning it undergoes a surge in respiration and ethylene production at the onset of ripening (Li et al., 2019a). As ripening progresses, tomato fruits transit from partially photosynthetic to true heterotrophic tissues through the parallel differentiation of chloroplasts into chromoplasts and the dominance of carotenoids and lycopene in the cells of the ripe fruits (Carrari and Fernie, 2006). The ripening process has evolved to make fruit palatable to the organisms that consume them and disperse their seeds. In doing so, ripening activates pathways that generally influence the levels of pigments, sugars, acids, and aroma-associated volatiles to make the fruit more appealing, while simultaneously promoting tissue softening and degradation to permit easier seed release (Matas et al., 2009).

In this review, we provide an overview of our current understanding of tomato fruit metabolism. We begin by detailing the genetic and hormonal control of fruit development and ripening, after which we document the primary metabolism of tomato fruits, with a special focus on sugar, organic acid, and amino acid metabolism. Links between primary and secondary metabolic pathways are further highlighted by the importance of pigments, flavonoids, and volatiles for tomato fruit quality. Finally, as tomato plants are sensitive to several abiotic stresses, we briefly summarize the effects of adverse environmental conditions on tomato fruit metabolism and quality.

GENETIC REGULATION OF THE DEVELOPMENT AND RIPENING OF TOMATO FRUIT

Fruit Set and Early Fruit Development

The genetic regulation of fruit development begins in the floral meristem (FM), where the architecture and organization of this tissue is determined, and continues until the later developmental stages before fruit ripening (Gillaspy et al., 1993) (**Figures 1A**, **B**). At the initial stage of tomato fruit development, the CLAVATA-WUSCHEL (CLV-WUS) feedback loop controls meristem activity and regulates FM size, which in turn determines the final number of carpels in flowers and, hence, seed locules in fruits (Rodríguez-Leal et al., 2017). The signaling peptide CLV3 directly interacts with leucine-rich repeat receptor kinases, such as CLV1 or CLV2, to activate a signaling cascade that negatively regulates the stem cell-promoting transcription factor WUS (Somssich et al., 2016). Loss-of-function mutations in any of the *CLV* genes will therefore cause stem cell over proliferation, resulting in the development of extra floral organs and larger fruits (Xu et al., 2015; Rodríguez-Leal et al., 2017); for example, the joint action of the natural mutations *fasciated* (*fas*) and *locule number* (*lc*) gave rise to large-fruited cultivars, in contrast to the

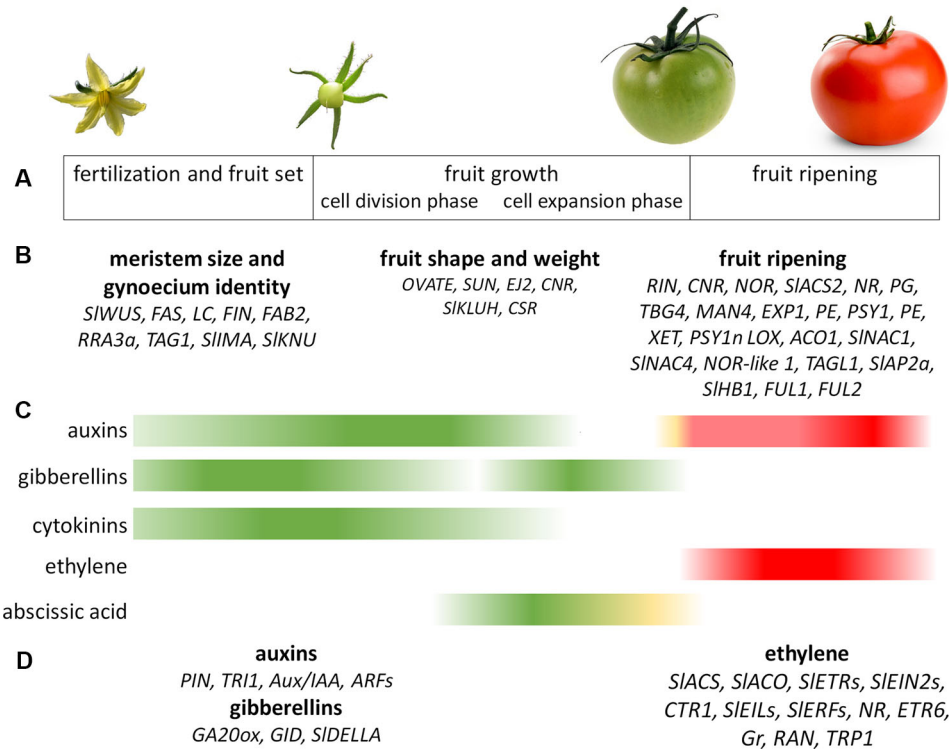

FIGURE 1 | Genetic and hormonal control of tomato fruit development. **(A)** Main stages of tomato fruit development. **(B)** Genes involved in the control of tomato fruit development that are mentioned in this article. **(C)** Main hormones involved in tomato fruit development during fruit set and fruit growth (green) and fruit ripening (red). **(D)** Genes involved in the hormonal regulation of fruit development that are mentioned in this article. The Figure summarizes data collected by Gillaspy et al. (1993); Srivastava and Handa (2005); Karlova et al. (2014) and Obroucheva (2014).

bilocular fruits of tomato wild species and most small-fruited varieties (Tanksley, 2004; Barrero et al., 2006). The *fas* mutation is a 294-kb inversion disrupting the tomato *CLV3* (*SlCLV3*) promoter (Xu et al., 2015), whereas *lc* is associated with two single-nucleotide polymorphisms in a putative CArG box regulatory element downstream of *WUS* (*SlWUS*) (Muños et al., 2011; van der Knaap et al., 2014). Furthermore, using forward genetics and CRISPR/Cas9 genome editing technology, Xu et al. (2015) identified the arabinosyltransferase genes *FASCIATED INFLORESCENCE* (*FIN*), *FASCIATED AND BRANCHED2* (*FAB2*), and *REDUCED RESIDUAL ARABINOSE 3a* (*RRA3a*) as new components of the CLV-WUS pathway. The SlCLV3 peptide must therefore be fully arabinosylated to maintain meristem size since the loss of an arabinosyltransferase cascade causes floral and fruit fasciation.

As the flower develops, the gynoecium is initiated in the fourth whorl to terminate FM activity. The MADS box transcription factor AGAMOUS (AG) is required to form the carpel primordium (Yanofsky et al., 1990). Consequently, the downregulation of *TOMATO AGAMOUS1* (*TAG1*), the tomato ortholog of *Arabidopsis thaliana AG*, gives rise to alterations in carpel development and determinacy by producing fruits that continue to develop in an indeterminate fashion (Pnueli et al., 1994; Pan et al., 2010; Gimenez et al., 2016). Furthermore, in *Arabidopsis*, *AG* turns off the stem cell maintenance program through the transcriptional repression of *WUS via* two different pathways: directly, by promoting the recruitment of Polycomb Group (PcG) proteins to methylate histone H3K27 at the *WUS* locus (Liu et al., 2011); and indirectly, by inducing the expression of a gene encoding the C2H2 zinc-finger protein KNUCKLES (KNU) (Sun et al., 2009). The induction of *KNU* expression by AG requires a time delay regulated by the epigenetic modification of histones at the *KNU* locus (Sun et al., 2014). Recently, Bollier et al. (2018) demonstrated that the AG-KNU-WUS pathway is conserved in Arabidopsis and tomato and regulates the timed termination of floral stem cell activity. In this context, the tomato mini zinc-finger protein INHIBITOR OF MERISTEM ACTIVITY (SlIMA) recruits SlKNU to form a transcriptional repressor complex together with TOPLESS and HISTONE DEACETYLASE19, which binds to the *SlWUS* locus to repress its transcription (Bollier et al., 2018). Additionally, it has been hypothesized that *lc* is a weak gain-of-function mutation that reduces or blocks the binding of TAG1 to the *SlWUS* 3′ regulatory region, which impairs the ability of TAG1 to repress *SlWUS*, resulting in the formation of larger fruits as a consequence of the development of extra carpels (van der Knaap et al., 2014).

The variation in tomato fruit morphology not only depends on CLV-WUS signaling pathway-related genes, but also on *OVATE* and *SUN*, which have a large effect on fruit shape (**Figure 1B**). The *ovate* null mutation gives rise to changes in cell division patterns during the earliest stages of gynoecium development, with more cells produced in the proximo-distal direction and fewer in the medio-lateral

direction, causing the development of elongated fruits (Ku et al., 1999; Liu et al., 2002; Rodríguez et al., 2011). In contrast, the effect of *SUN* on fruit shape is most noticeable at flower anthesis, when it begins to increase cell division along the proximo-distal axis and cell elongation immediately after fertilization (Xiao et al., 2009; Wu et al., 2011; van der Knaap et al., 2014). Thus, a profound shift in the expression of genes involved in cell division, cell wall development, and patterning processes was observed in the elongating fruit tissues of the *sun* mutant (Clevenger et al., 2015). Moreover, the MADS box gene *ENHANCER OF J2* (*EJ2*) also seems to be involved in determining fruit shape; *ej2* knockout mutants develop slightly elongated fruits together with several pleiotropic effects, such as branched inflorescences and jointless pedicels (Soyk et al., 2017).

Among the fruit weight regulators, *CELL NUMBER REGULATOR* (*CNR*) was found to underlie the *fw2.2* quantitative trait locus (QTL), acting early during the development of the gynoecium to increase ovary size (Frary et al., 2000; Guo and Simmons, 2011) and enlarge the placenta and columella fruit tissues (Cong et al., 2002; Gonzalo et al., 2009). *SlKLUH* is the causal gene for the *fw3.2* QTL and encodes a CYP450 of the 78A class (Chakrabarti et al., 2013). One single-nucleotide polymorphism in the *SlKLUH* promoter leads to its enhanced expression in meristems and young flower bud tissues; however, the increased fruit weight of these mutant plants becomes evident only after fertilization. An increased number of cell layers in the pericarp gives rise to heavier fruits with a ripening delay, which has been hypothesized to be the result of the extension of the cell proliferation stage (Chakrabarti et al., 2013). Studies in *Arabidopsis* have suggested that KLUH is involved in generating a mobile growth-promoting signal, although its exact molecular and biochemical nature is yet to be deciphered (Anastasiou et al., 2007; Adamski et al., 2009). Cell expansion in the pericarp is responsible for the dramatic increase in fruit size from a 1- to 2-mm gynoecium to a 5- to 10-cm tomato fruit (Gillaspy et al., 1993; Xiao et al., 2009). The *CELL SIZE REGULATOR* (*CSR*) gene controls pericarp cell size and underlies the *fw11.3* QTL (Huang and van der Knaap, 2011; Mu et al., 2017). *CSR* expression is restricted to fruits, starting about 5 days after pollination and decreasing at the onset of ripening. Along with the increased cell size, coexpression studies suggest that *CSR* is also involved in shoot development and phloem/xylem histogenesis; however, the molecular function of *CSR* in controlling these developmental processes remains unclear (Mu et al., 2017).

Fruit Ripening

At the end of fruit development, when seeds are mature and ready for dispersal, tomato fruits undergo ripening, a complex developmental program involving the coordinated regulation of numerous physiological and biochemical changes that determine flavor, color, texture, and aroma. These changes involve the up- or downregulation of numerous genes in various metabolic pathways (Alba et al., 2005; Fujisawa et al., 2011; Osorio et al., 2011). Multiple studies of the development and maturation of tomato fruits have facilitated the identification of specific genes that participate in ripening (Vrebalov et al., 2002; Manning et al., 2006; Giovannoni, 2007; Wang et al., 2009; Chung et al., 2010; Nashilevitz et al., 2010; Karlova et al., 2011; Pesaresi et al., 2014) (**Figure 1B**).

Tomatoes are classified as climacteric fruits, exhibiting a peak of respiration and ethylene production at the start of ripening (Alexander and Grierson, 2002). The biosynthesis and perception of ethylene are highly regulated, involving genes conserved in various plant taxa (Seymour et al., 2013). Some transcription factors modulate ethylene biosynthesis and signal transduction during fruit ripening, among which it is worth highlighting RIPENING INHIBITOR (RIN) (Vrebalov et al., 2002), COLORLESS NON-RIPENING (CNR) (Manning et al., 2006), and NON-RIPENING (NOR) (Yuan et al., 2016). RIN acts as the main regulator of fruit ripening, directly controlling the expression of target genes involved in a wide range of ripening-related events (Fujisawa et al., 2011; Qin et al., 2012). *RIN* encodes a SEPALLATA (SEP)-class MADS-box transcription factor (Vrebalov et al., 2002), which was previously considered to be an essential regulator of the induction of ripening (Vrebalov et al., 2002); however, its role in fruit ripening was recently reassessed following the publication of studies showing that *RIN*, although necessary to complete ripening, is not required for the initiation of this process (Ito et al., 2017). The *rin* mutant was found to be caused by the deletion of a genomic DNA fragment between *RIN* and *MACROCALYX* (*MC*), forming the chimeric gene *RIN-MC* (Vrebalov et al., 2002). *MC* affects inflorescence determinacy and sepal development (Vrebalov et al., 2002), and the *rin* mutant was found to be a gain-of-function mutant that produced a protein that actively represses ripening (Ito et al., 2008; Li et al., 2018a). RIN binds to the demethylated promoter regions of several genes, such as the ethylene biosynthesis genes *SlACS2* (*1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE 2*), *SlACS4*, *SlACO1* (*ACC OXIDASE 1*), the ethylene receptor *NEVER RIPE* (*NR*), and others whose products are involved in fruit softening and the transcriptional regulation of cell wall hydrolases [*POLYGALACTURONASE* (*PG*), β-*GALACTOSIDASE4* (*TBG4*), *ENDO-(1,4)-β-MANNANASE4* (*MAN4*), and α-*EXPANSIN1* (*EXP1*)] (Klee and Tieman, 2002; Ito et al., 2008; Fujisawa et al., 2011; Martel et al., 2011; Shima et al., 2013; Ito et al., 2017).

RIN also positively stimulates the expression of *CNR* (Cardon et al., 1999; Manning et al., 2006). The *cnr* mutation is the result of a spontaneous epigenetic change that increases cytosine methylation in the promoter of a *SQUAMOSA* promoter-binding protein-encoding gene, which strongly decreases gene expression and produces colorless fruits with an altered pericarp texture (Manning et al., 2006). During ripening, the *CNR* promoter is progressively demethylated, but in *cnr* mutants, the promoter remains hypermethylated, preventing RIN from binding to it (Zhong et al., 2013). In addition, CNR was involved in the positive regulation of many ripening-related genes, including *PG*, *PECTINESTERASE* (*PE*), *XYLOGLUCAN ENDOTRANSGLYCOSYLASE* (*XET*), *PHYTOENE SYNTHASE1* (*PSY1*), *LIPOXYGENASE* (*LOX*), and *ACO1* (Eriksson et al., 2004).

The *nor* mutant exhibits abnormal ripening as a result of a 2-bp deletion in the *NOR* coding sequence, leading to the early termination of protein translation (Tigchelaar et al., 1973; Martel et al., 2011; Osorio et al., 2011). NOR encodes a NAC family

transcription factor that regulates fruit ripening through a currently unclear mechanism, while mutations in this gene inhibit multiple metabolic processes and prolong fruit shelf life (Kumar et al., 2018). A study of the role of *NOR* and *RIN* in tomato fruit ripening confirmed that the *nor* mutation had a more global effect on ethylene/ripening-related gene expression than *rin*, suggesting that *NOR* might even act upstream of *RIN* in the transcriptional network controlling tomato fruit ripening (Osorio et al., 2011). In addition to *NOR*, three other NAC family genes, *SlNAC1*, *SlNAC4*, and *NOR-like1*, are known to be involved in the regulation of tomato fruit ripening (Ma et al., 2014; Zhu et al., 2014; Meng et al., 2016).

Other ripening factors, such as the MADS box TOMATO AGAMOUS-LIKE1 (TAGL1) (Vrebalov et al., 2002; Giménez et al., 2010), tomato APETALA2 (SlAP2a) (Karlova et al., 2011), and the tomato homeodomain leucine zipper homeobox protein SlHB1 (Lin et al., 2008), exercise their regulatory functions by interacting with RIN (Fujisawa et al., 2011; Qin et al., 2012; Seymour et al., 2013). *TAGL1* (also referred to as *ARLEQUIN* in some publications), a *PLENA* lineage gene orthologous to *Arabidopsis SHATTERPROOF1/2*, controls many aspects of tomato fruit ripening (Vrebalov et al., 2009; Garceau et al., 2017), including the direct activation of the expression of the ethylene biosynthesis gene *ACS2* (Itkin et al., 2009). Tomato fruits produced by *TAGL1*-silenced plants had defects in ripening without their floral organ specification being affected (Vrebalov et al., 2009; Giménez et al., 2010; Pan et al., 2010). Plants with reduced *TAGL1* expression produced fruits with a narrow pericarp and reduced firmness at the breaker stage, which remained yellow and produced significantly less ethylene than the control fruits (Vrebalov et al., 2009). The MADS box proteins TAGL1 and two homologs of FRUITFULL (FUL1/TDR4 and FUL2/MBP7) function as coregulators of RIN (Leseberg et al., 2008; Itkin et al., 2009; Vrebalov et al., 2009; Giménez et al., 2010; Martel et al., 2011; Bemer et al., 2012; Shima et al., 2013; Wang et al., 2014). Fujisawa et al. (2014) demonstrated that RIN, TAGL1, and the FUL homologs form a DNA-binding complex, probably a tetramer, which is believed to regulate tomato fruit ripening. The RIN and CNR regulators have been shown to function upstream of *SlAP2a* and to positively regulate its expression (Karlova et al., 2014), whereas *SlHB1* controls ethylene metabolism by binding to the regulatory regions of *ACO1* (Lin et al., 2008). On the other hand, transcriptomic studies have shown that *SlAP2a* participates in the control of fruit ripening as a negative regulator of several processes involved in ethylene biosynthesis, and signaling pathways, as well as in the differentiation of chromoplasts (Chung et al., 2010; Karlova et al., 2011).

HORMONAL REGULATION OF THE DEVELOPMENT AND RIPENING OF TOMATO FRUIT

Fruit Set and Early Fruit Development

Fruit set and fruit development are complex processes that require the coordination of different phytohormones (McAtee et al., 2013; Shinozaki et al., 2018b; Li et al., 2019b) (**Figures 1C, D**). From flower initiation to fertilization, the morphogenesis and growth of carpels and ovules require the spatial and temporal biosynthesis and action of auxins, cytokinins (CKs), and gibberellins (GAs) (Azzi et al., 2015). Shortly before anthesis, when the ovary has reached its mature size, abscisic acid (ABA) and ethylene work to stop growth within the ovary to maintain a temporally protected and dormant state (Gillaspy et al., 1993; Azzi et al., 2015). After the successful pollination and fertilization of the ovules, ovary growth resumes and the fruit and seeds develop concomitantly (Azzi et al., 2015). These changes are associated with a decrease in ABA and ethylene concentrations and an increase in auxin, GAs, and CKs (de Jong et al., 2009; McAtee et al., 2013; Shinozaki et al., 2015; Shinozaki et al., 2018a). GAs produced by pollen may increase auxin production in the ovary, which in turn may act as a signal for fruit set and the subsequent activation of cell division (Gillaspy et al., 1993; de Jong et al., 2009). Active fruit growth involving pericarp cell division and elongation is promoted by the biosynthesis of auxin in the developing seeds and GAs in the pericarp (Obroucheva, 2014). Auxins and GAs appear to be the predominant hormones required for tomato fruit initiation in response to fertilization, since the exogenous application of both hormones leads to fruit initiation and parthenocarpic development (de Jong et al., 2009). CKs, ethylene, ABA, brassinosteroids, and polyamines (PAs) have also been shown to play a role in fruit formation, but this is currently less well documented (Srivastava and Handa, 2005; McAtee et al., 2013; Azzi et al., 2015; Shinozaki et al., 2015; Liu et al., 2018; Shinozaki et al., 2018a).

In tomato, early fruit development is governed by the allocation of auxin to tissues and cells, which initiates signal transduction pathways (Azzi et al., 2015). The PIN-FORMED (PIN) auxin efflux transport proteins were shown to be involved in fruit set and early tomato fruit development (Mounet et al., 2012; Pattison and Catalá, 2012). Silencing *SlPIN4* resulted in the production of small parthenocarpic fruits exhibiting precocious development (Mounet et al., 2012). The auxin signaling pathway involves an auxin receptor called TRANSPORT INHIBITOR RESPONSE1 (TIR1) (Azzi et al., 2015). In the presence of auxin, TIR1 recruits the transcriptional repressors *AUXIN/INDOLE-3-ACETIC ACID* (*Aux/IAA*) and triggers their degradation by the 26S proteasome (Azzi et al., 2015), releasing the Aux/IAA-bound auxin response factors (ARFs) and initiating the auxin response through auxin-responsive element-mediated gene transcription (Azzi et al., 2015). In tomato, the misexpression of *TIR1* and specific members of the *Aux/IAA* and *ARF* gene family alters the normal flower-to-fruit transition and results in parthenocarpic fruit production (de Jong et al., 2009; Ren et al., 2011; Mounet et al., 2012; Azzi et al., 2015). However, *Aux/IAA* and *ARF* genes may have opposing functions to *TIR* regarding fruit set; the transcript abundance of *SlIAA9* and *SlARF7* decreased in *SlTIR1*-overexpressing plants, which resulted in the formation of seedless fruit (Ren and Wang, 2016; Goldental-Cohen et al., 2017). The silencing of the Aux/IAA transcriptional repressor *SlIAA17* resulted in larger fruits with thicker pericarp tissues, a phenotype caused by enhanced cell expansion (Su et al., 2014). Ren and Wang (2016) showed that *SlTIR* was regulated by GAs, auxins, ABA, and ethylene, suggesting that TIR may be a key

mediator of the crosstalk between auxin and other phytohormones. The SlARF7/SlIAA9 complex also mediates crosstalk between auxin and GA pathways to regulate fruit initiation through their interaction with the GA-signaling repressor SlDELLA (Hu et al., 2018). SlARF7/SlIAA9 complex and SlDELLA antagonistically regulate genes involved in auxin and GA metabolism while they additively coregulate genes involved in fruit growth (Hu et al., 2018).

Indeed, auxins do not act alone to trigger fruit development and fruit set; these processes are partly mediated by GAs, as part of a complex hormonal cross-talk with auxin (de Jong et al., 2009; McAtee et al., 2013; Azzi et al., 2015). Pollination triggers the upregulation of transcripts encoding GA 20-oxidases (GA20ox), which biosynthesize active GA1 and GA4 (Azzi et al., 2015). It was suggested that the expression of more than one *GA20ox* gene is required to control fruit set in tomato because the silencing of individual *GA20ox* genes did not strongly affect fruit set or development (Xiao et al., 2006; Olimpieri et al., 2011; Azzi et al., 2015). Despite this, the heterologous overexpression of citrus *CgGA20ox1* in tomato resulted in an elevated GA4 content and parthenocarpic fruit development, demonstrating the influence of GA and GA20ox activity on fruit set and development (García-Hurtado et al., 2012). The GA signal transduction pathway requires the recognition of GA by its receptor, GA INSENSITIVE DWARF1 (GID1) (Azzi et al., 2015). The GID1-GA complex interacts with the nuclear repressor DELLA to target it for ubiquitin-dependent proteolytic degradation by the 26S proteasome (Azzi et al., 2015). This removes the repression of the GA-responsive genes, which are then able to initiate GA signal transduction. Consistent with this, the silencing of the *SlDELLA* gene in tomato resulted in small, facultative parthenocarpic fruits with an elongated shape (Martí et al., 2007). The *procera* (*pro*) mutant, which carries a point mutation in the GRAS region of *SlDELLA*, has also very strong parthenocarpic capacity and shows enhanced growth of preanthesis ovaries (Jones, 1987; Carrera et al., 2012; Shinozaki et al., 2018c). The parthenocarpic capacity of *pro* is mainly associated with changes in the expression of genes involved in GA and auxin pathways (Carrera et al., 2012). A new *SlDELLA* mutant containing a single nucleotide substitution, *procera2* (*pro2*), has been recently identified and shows a potential for high fruit yield in both optimal and unfavorable growing conditions due to its facultative parthenocarpic capacity (Shinozaki et al., 2018c). Parthenocarpy is indeed an attractive trait for fruit production (Shinozaki et al., 2018c).

As mentioned previously, other phytohormones are involved in fruit set and growth. A number of ABA-deficient mutants have provided valuable insights into the role of ABA in fruit growth (Azzi et al., 2015). Phenotypic characterization of the ABA biosynthesis *not/flc* double mutant showed that its small fruits had considerably reduced ABA levels and smaller cell sizes, especially within the pericarp (Nitsch et al., 2012). It was suggested that ABA stimulates fruit growth by restricting the level of ethylene in normal fruits (Azzi et al., 2015), which may indeed induce fruit set as tomato plants treated with the ethylene action inhibitor 1-methylcyclopropene (1-MCP) produce parthenocarpic fruits (Shinozaki et al., 2015). In the same way, tomato plants carrying either of two allelic mutations in *ETHYLENE RECEPTOR1* (*Sletr1-1* or *Sletr1-2*) were insensitive to ethylene, resulting in parthenocarpy (Shinozaki et al., 2015; Shinozaki et al., 2018a). Ethylene is involved in the senescence of unpollinated ovaries and prevents fruit set by downregulating GA accumulation, acting downstream of auxin and upstream of GA in the control of fruit set (Shinozaki et al., 2018a). Exogenous CK application induces parthenocarpic fruits (Matsuo et al., 2012; Ding et al., 2013), suggesting a role for CKs during tomato fruit initiation. Cytokinins induce parthenocarpy in tomato partially through modulation of GA and auxin metabolisms (Ding et al., 2013). Moreover, transcriptomic and metabolomic studies showed that although CKs mainly control cell division during tomato fruit development, they also play a critical role in fruit-set and early growth of tomato fruits (Mariotti et al., 2011; Matsuo et al., 2012). A key role for PAs during fruit set was also suggested, with tomato genes encoding enzymes involved in PA biosynthesis, such as arginine/ornithine decarboxylase (ADC/ODC) and spermine synthase (SPMS), suggested to be particularly important during the process of fruit setting (Liu et al., 2018).

Fruit Ripening

Fruit ripening has been widely studied in tomato, with ethylene known to play a key role in this process (Osorio et al., 2013; Seymour et al., 2013; Liu et al., 2015; Borghesi et al., 2016; Shinozaki et al., 2018b; Li et al., 2019a) (**Figures 1C, D**). Two systems of ethylene biosynthesis have been proposed in climacteric fruits (McMurchie et al., 1972): System 1 is responsible for producing basal ethylene levels during fruit growth and is ethylene autoinhibitory, while system 2 operates during climacteric ripening and is autocatalytic (Liu et al., 2015). At the onset of ripening, an increase in ethylene is observed in mature green tomatoes, resulting in an eventual 100- to 300-fold increase in the ethylene concentration during fruit ripening (Karlova et al., 2014; Li et al., 2019a). Ethylene initiates a cascade of changes, which culminate in the transformation of the hard, unpalatable green tomato into an attractive, brightly colored succulent and nutritious fruit (Giovannoni, 2004; Li et al., 2019a).

Ethylene signaling can be regulated at several levels, including ethylene biosynthesis and its perception (Karlova et al., 2014; Mata et al., 2018; Li et al., 2019a). Ethylene biosynthesis involves multiple aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase enzymes and genes (Osorio et al., 2013; Karlova et al., 2014; Kou et al., 2016; Li et al., 2019a). Fourteen putative *ACS* genes and six *ACO* genes have been identified in the tomato genome (Liu et al., 2015). Among them, it has been proposed that *SlACS2*, *SlACS4*, *SlACO1*, *SlACO2*, and *SlACO4* play important roles in ethylene production during tomato fruit maturation (Cara and Giovannoni, 2008; Liu et al., 2015). Some transcription factors are known to act upstream of the ethylene biosynthesis genes to regulate fruit ripening, including RIN, SlHB-1, and the NAC transcription factors SNAC4 and SNAC9 (Liu et al., 2015; Kou et al., 2016).

Ethylene perception is mediated through ethylene receptors encoded by *ETHYLENE RESPONSE* (*ETR*) genes, which activate

a signal transduction cascade through the release of the block on *ETHYLENE INSENSITIVE2* (*EIN2*) exerted by *CONSTITUTIVE TRIPLE RESPONSE1* (*CTR1*) (Karlova et al., 2014; Liu et al., 2015; Mata et al., 2018; Li et al., 2019a). Seven *ETR* genes and four *CTR1* homologs have been identified in tomato thus far, all of which control ethylene sensitivity by balancing the turnover of the components of the ethylene signaling pathway, combining positive and negative feedback (Liu et al., 2015; Mata et al., 2018). This release then activates the *EIN3/EIN3-like* (*EIL*) primary transcription factor genes, resulting in the expression of secondary transcription factor genes encoding the ethylene response factors (ERFs) (Karlova et al., 2014; Liu et al., 2015; Mata et al., 2018). The final result of this signaling pathway is the transcriptional regulation of the target genes by the EILs or ERFs (Karlova et al., 2014). Some of the *ERF* genes have been characterized in tomato, including *SlERF1*, *SlERF.B3*, and *SlERF6* (Li et al., 2007; Liu et al., 2013; Karlova et al., 2014), but many of their functions and ethylene-responsive target genes remain unknown (Li et al., 2019a). Six *EIL* genes have been identified in tomato, although *SlEIL5* and *SlEIL6* may not be involved in tomato ripening (Liu et al., 2015). Several genes that regulate tomato ripening through the transduction of ethylene signals have been identified (Karlova et al., 2014), including the ethylene receptor genes *NR*, *ETR6*, and *GREEN-RIPE* (*Gr*) (Yen et al., 1995; Barry and Giovannoni, 2006; Kevany et al., 2007). Two other proteins, RESPONSE TO ANTAGONIST1 (RAN1) and TETRATRICOPEPTIDE REPEAT1 (TRP1), also play important roles at the receptor levels (Liu et al., 2015).

Ripening is also influenced by the balance of other hormones, including ABA, auxin, and the brassinosteroids (Seymour et al., 2013; Karlova et al., 2014; Liu et al., 2015; Shinozaki et al., 2018b; Li et al., 2019a; Shin et al., 2019). ABA is known to promote ripening, whereas auxin seems to have an antagonistic effect (Liu et al., 2015). ABA is a key intermediate regulator of tomato fruit ripening, and its levels change according to fruit development stages (Zhang et al., 2009; Borghesi et al., 2016). In tomato, the suppression of the gene that catalyzes the first step in ABA biosynthesis [9-cis-epoxy carotenoid dioxygenase (NCED1)] results in the downregulation of some ripening-related cell wall genes, such as those encoding polygalacturonase and pectin methylesterase, promoting an increase in firmness and a longer shelf life (Sun et al., 2012). ABA interacts with ethylene signaling; the expression of genes involved in ethylene biosynthesis are induced by exogenous ABA (Liu et al., 2015).

Low levels of auxins are also required at the onset of ripening, and auxin signaling declines at this stage (Gillaspy et al., 1993; Karlova et al., 2014; Shin et al., 2019); however, it seems that the ratio between indole acetic acid (IAA) and its conjugated forms is more important than the level of free IAA for the regulation of tomato ripening (Karlova et al., 2014). Indeed, the decrease of free IAA at the onset of ripening is associated with an increase in its conjugated form, IAA-Asp (Buta and Spaulding, 1994; Karlova et al., 2014). SlSAUR69 is involved in the decrease of auxin levels and/or signaling in the pericarp tissue at the onset of fruit ripening *via* the repression of polar auxin transport (Shin et al., 2019). *ARF* genes are also involved in fruit ripening; the downregulation of *SlARF4* or *SlARF2* resulted in fruits with dramatic ripening defects (Jones et al., 2002; Karlova et al., 2014; Hao et al., 2015). Auxin–ethylene interactions are crucial for the fruit ripening process, although the molecular basis of the regulatory network is still relatively unclear (Li et al., 2017; Shin et al., 2019). An antagonistic effect between auxin and ethylene has been observed during the ripening of tomatoes (Li et al., 2017), with ethylene inhibiting auxin transport, metabolism, and signaling processes, while auxin represses the expression of genes involved in ethylene biosynthesis and signaling (Chaabouni et al., 2009; Liu et al., 2015; Li et al., 2016a; Li et al., 2017). Moreover, both auxin and ethylene differentially regulate CK metabolism and signaling processes during tomato ripening (Li et al., 2017).

Brassinosteroids might also be involved in tomato ripening, as exogenous applications of this hormone can promote ripening and ethylene production in tomatoes (Karlova et al., 2014). PAs are also actively involved in climacteric fruit ripening (Liu et al., 2018); for example, putrescine levels progressively increase during fruit maturation and peak in ripe tomatoes, while spermine and spermidine levels decrease gradually until the fruits are fully ripe (Tsaniklidis et al., 2016; Liu et al., 2018). Moreover, although the expression levels of *SPMS*, *ADC*, and *ODC* were minimal during the fruit ripening process, the *SPDS* genes may play an important role during tomato fruit ripening (Liu et al., 2018).

Phytohormones also play a key role in the regulation of tomato fruit metabolism and quality (Van Meulebroek et al., 2015; Cruz et al., 2018; Li et al., 2019b). The hormones discussed above all contribute to the metabolism of tomato fruits, although ABA and ethylene play the most important roles (Li et al., 2019b). ABA had a greater effect on the regulation of the primary metabolism, while ethylene plays an important role in the transition of primary to secondary metabolism in tomatoes (Li et al., 2019b). Regarding secondary metabolism, ethylene and auxins were described as the most important regulators of carotenoid biosynthesis during tomato fruit ripening (Van Meulebroek et al., 2015; Cruz et al., 2018).

PRIMARY METABOLISM IN TOMATO FRUIT

Development of the tomato fleshy fruit occurs in three distinct phases : i) cell division phase occurs in the early days following fertilization until 10 DAA ii) cell expansion (from 10 DAA to 40 DAA) and iii) fruit ripening and maturation (**Figure 1A**). During this evolution, tomato fruits follows a transition from partially photosynthetic to complete heterotrophic metabolism. Typical morphophysiological steps are considered and include immature, mature green, breaker, pink and red ripe fruits. Although the fruit ripening is an important step determining the fruit quality and nutritional values, recent works provided evidences that the early fruit development also assumes key roles for acquisition of quality traits, including the accumulation of sugars and organic acids

(Carrari and Fernie, 2006; Beauvoit et al., 2014; Biais et al., 2014; Bauchet et al., 2017). Postgenomic approaches including analyses of fruit transcriptomes, proteomes, and metabolomes as well as multilevel studies integrating enzyme profiling generated a large set of useful data improving our knowledge on the regulation of metabolites turnover during tomato fruit development (Mounet et al., 2009; Centeno et al., 2011; Van de Poel et al., 2012; Van Meulebroek et al., 2015). Hierarchical clustering performed by Biais et al. (2014) revealed tight associations between enzyme activities and developmental phase and concluded that metabolites are more sensitive to growth conditions than enzyme activities. A global overview of the main recorded changes in metabolites recorded during fruit transition from green to red mature fruits is provided in **Figure 2**.

Carbohydrate Metabolism

Immature Green Fruit Photosynthesis

Sugars are closely related to fruit yield and quality. In tomato fruits, sugars provide sweetness and are important for the generation of turgor pressure to promote cell expansion (Kanayama, 2017). Sugars also act as signal molecules controlling fruit development and metabolism. Green fruits remain able to perform photosynthesis which can produce up to 20% of the fruit photosynthetates, the remaining part being imported by source leaves (Pesaresi et al., 2014). The light harvesting electron transfer and CO_2 fixation proteins are conserved in their active state in green fruit tissues (Matas et al., 2011). Fruit chloroplasts contain sufficient amounts of plastocyanin, ferredoxins, Rieske proteins, cytochrome *f* and cytochrome b_{559} and ribulose-1,5-biphosphate carboxylase activity is detected in the fruits (Hetherington et al., 1998). The triose phosphate and glucose phosphate transporters are active in the tomato chloroplasts. Unexpectedly, genes associated with photosynthesis are highly expressed in the locule which is in fact the main site of respiration (Lemaire-Chamley et al., 2005).

Nevertheless, the importance of green fruit photosynthesis is still a matter of debate. According to Carrara et al. (2001), tomato fruits do not show signs of CO_2 fixation, even if photochemical

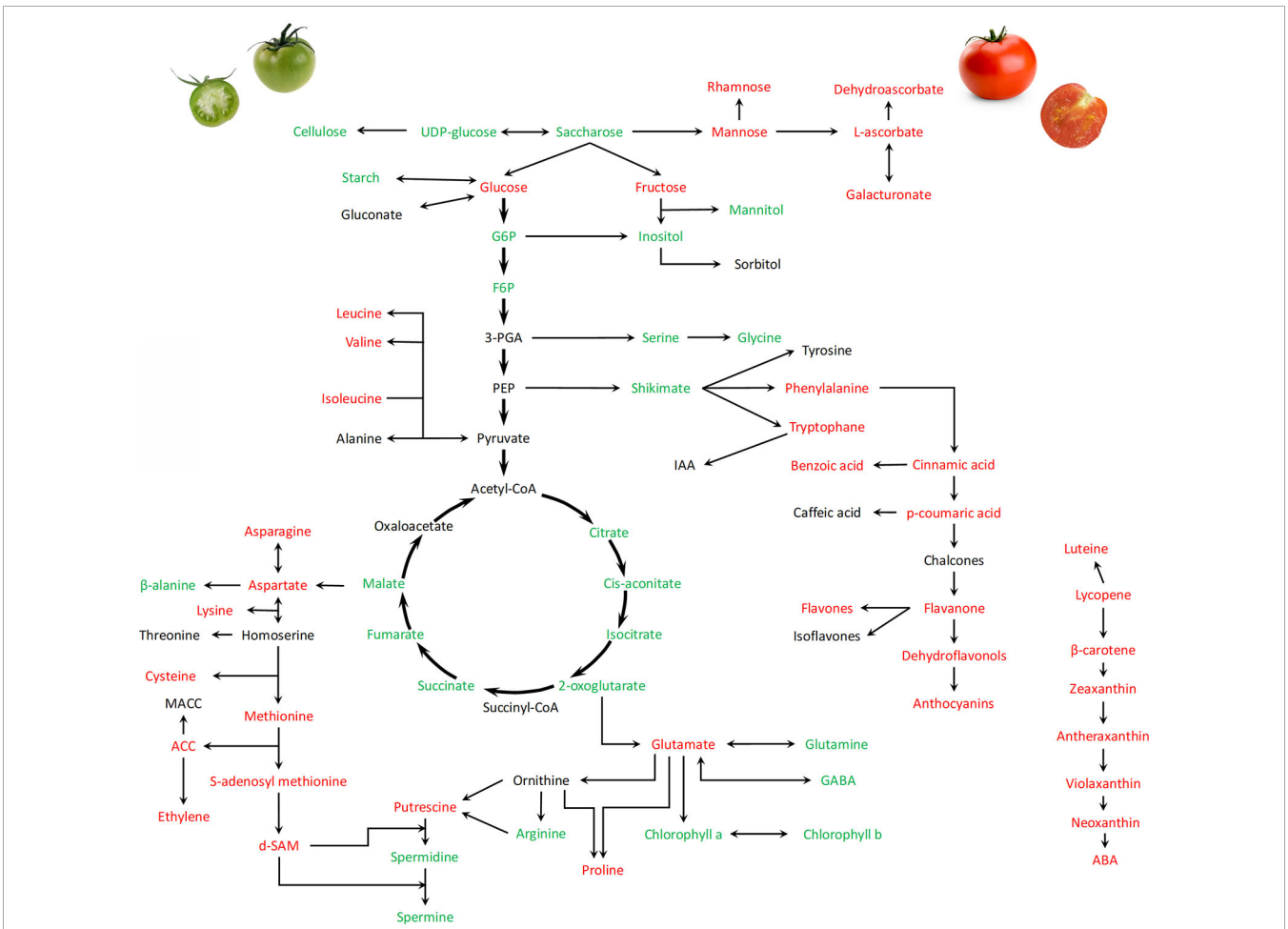

FIGURE 2 | Global overview of metabolic changes occurring during the transition from green expanding fruit to ripening processes (from 30 DAA to 60 DAA) in tomato fruit. Names of metabolites in red, green and black indicate increase, decrease or no changes, respectively. Metabolites are analyzed mainly in pericarps. The Figure summarizes data collected by Carrari and Fernie (2006); Gilbert (2009); Mounet et al. (2009); Centeno et al. (2011); Beauvoit et al. (2014); Biais et al. (2014), Van Meulebroek et al. (2015), Van de Poel et al. (2012), and Zhao et al. (2018).

activity is detectable and an effective electron transport observed. Xu et al. (1997) reported that a small fruit (fresh weight lower than 10 g) is able to perform a gross photosynthesis equivalent to a 3-cm^2 leaf blade but that this activity rapidly decreases thereafter: in heavier fruits, gross photosynthesis decreases to negligible values. These authors even assume that the aim of the photosynthetic process in maturing fruit is mainly to delete CO_2 produced by respiration rather than contributing to photosynthate production. Kahlau and Bock (2008) showed that RNA, translation and protein accumulation downregulation was observed for all plastid-encoded photosynthesis genes already in the green fruit. Hetherington et al. (1998) however demonstrated that all truss tissues, including fruits, are quite active photosynthetically. These authors interestingly demonstrated that the relative contribution of the fruit versus the leaf photosynthesis for fruit photosynthate accumulation tend to narrow under low light intensities.

A fruit specific antisense inhibition of the chloroplastic fructose 1,6-biphosphatase (FBPase) led to an obvious decrease in final weight of ripe fruits (Obiadalla-Ali et al., 2004) while, conversely, tomato lines with a fruit specific reduction in the expression of glutamate-1-semialdehyde aminotransferase (GSA) and thus a lower level of chlorophyll and photosynthetic rate, remained unaffected in terms of fruit weight (Lytovchenko et al., 2011). Ntagkas et al. (2019) recently demonstrated that phosynthetically active fruits able to respond to light may trigger ascorbate synthesis while non-photosynthetic red maturing fruits are unable to produce this antioxidant in response to light.

Auxin plays an important role for determining final fruit stage through the control of cell division and cell expansion. Auxin-responsive factors (ARF) can either activate or repress transcription of auxin-responsive genes. Combined metabolomics and transcriptomic studies of plants deficient in the expression of the tomato *Aux/IAA* transcription factor *IAA9* suggest a role for photosynthesis in the initiation of fruit development (Wang et al., 2009). Downregulation of *SlARF4* enhanced fruit firmness and increased chlorophyll content in green fruits in relation to an increased number of chloroplasts (Guillon et al., 2008). SlARF4 also has a direct impact on fruit sugar metabolism: the *SlARF4* underexpression tomato lines accumulated more starch at early stages of fruit development associated with an improved photochemical efficiency (Sagar et al., 2013). Moreover, *SlARF4* is highly expressed in the pericarp tissues of immature fruits and undergoes decline at the onset of ripening. Down-regulated tomatoes also present a higher starch content than the wild type in developing fruits which is directly related to up-regulation of several genes and enzyme activities involved in starch biosynthesis (Sagar et al., 2013).

Plastid numbers and chlorophyll content in fruits are positively correlated with photosynthesis and photosynthate accumulation and both are influenced by numerous environmental and genetic factors. In tomato fruits, the *GOLDEN2-LIKE* (*GLK*) transcription factor induces the expression of numerous genes related to chloroplast differentiation and photosynthesis (Powell et al., 2012). The genome of *S. lycopersicum* possesses two copies of this gene: *SlGLK1* is predominantly expressed in the leaves while *SlGLK2* is expressed in the fruits, especially in the area of pedicel junction (Nguyen et al., 2014). A latitudinal gradient of *SlGLK2* expression induces a typical uneven coloration in ripe fruit *SlGLK2* is preferentially expressed in the shoulder of the fruit (Sagar et al., 2013). Sl-GLK2 belongs to the GARP subfamily of the myb transcription factor and is encoded by the *UNIFORM (U)* gene (Powell et al., 2012). The *u* mutation has been widely selected in modern tomato varieties which consequently exhibit a uniform ripening attractive to consumers and suitable for industrial processing. This mutant contains less sugar and chloroplasts present a lower number of thylakoid grana. According to Nadakuduti et al. (2014), some class I *KNOTTED1-LIKE HOMEOBOX* gene (*TKN2* and *TKN4*) also influence chloroplast development in tomato fruits and act upstream of SlGLK2. A dominant gain-of-function mutation of *TKN2* induces ectopic fruit chloroplast development that resembles *SlGLK2* overexpression. More recently, Lupi et al. (2019) demonstrated that *SlGLK2* expression is partly regulated by a phytochrome-mediated light perception. Auxin appears as a negative regulator of *SlGLK2* expression and SlGLK2 enhances cytokinin responsiveness. This study also demonstrated that SlGLK2 enhances tocopherol and total soluble solid through amylase stimulation, so that selection of the *u* mutation in commercial varieties probably inadvertently compromise ripe fruit quality.

Sugar Unloading in Fruits

Sugar unloading in tomato fruit is a controlled process and its pattern is not constant during the fruit development. In green developing fruits, sugar is mainly unloaded *via* the symplasm. Numerous plasmodesmata and cell connections are present at this stage (Ruan and Patrick, 1995) but then are progressively lost. During this early phase of development, only a small amount of sucrose is unloaded by the apoplastic invertase and transported into the fruit cells by hexose transporters (Nguyen-Quoc and Foyer, 2001; Beckles et al., 2012). Although it has been demonstrated that sucrose unloads in tomato pericarp until 35 DAA, a precocious role for apoplastic invertase has however been postulated on the basis of kinetics properties explaining a moderate QTL for Brix index (Fridman et al., 2004).

Sugar Metabolism At the Cell Division Stage

In growing fruits, sucrose represents less than 1% DW while fructose and glucose are the main accumulated soluble sugars (25 and 22% DW; Gilbert, 2009). Glucose and fructose content strongly increased during early fruit developmental phase. Most studies until recent year have focused on the ripe stage but omics analysis need to be conducted throughout fruit development since several interactions may occur between the different stages (Kanayama, 2017). In green fruits, hexose phosphates are mainly used for starch synthesis until 13 DPA. Starch accumulation in pericarp and columella tissues at this early stage is a key factor determining the final soluble solid content of mature fruits (Carrari and Fernie, 2006).

The sink strength of a developing fruit depends on both sink activity and sink size, the latter being a function of both the number and the size of the fruit cells. According to Kataoka et al. (2009), gibberellic acid just after anthesis can promote an

increased sink size of individual pericarp cell through the activation of vacuolar acid invertase and neutral invertase. During the cell division phase, a high rate of mitotic activity is observed and the final cell number is determined at the end of this period. It is, at least partly, influenced by endoreduplication processes, seed number and hormonal cues. During cell division, enzymes involved in glycolysis (especially gluokinase and fructokinase) are activated. According to Biais et al. (2014), Glc-6-P is accumulating during this phase, and maintenance of a low ATP to ADP ratio and high hexose-P results in high flux through glycolysis. Pyruvate kinase and tricarboxylic acid cycle enzymes also exhibit high activities, indicating that ATP production as a priority. Beauvoit et al. (2014) postulated that the close match of the catalytic capacity to flux needs may be partly due to protein neosynthesis occuring during the early cell division phase, although protein-protein interactions and post-translational modifications may modulate enzyme V_{max} even if enzyme content remains constant.

At the end of the cell division phase, most soluble sugars accumulated in the vacuoles, together with malic and citric acid (Carrari and Fernie, 2006; Centeno et al., 2011). The osmotic potential of the vacuole consequently dropped to about -0.6 MPa and triggers water inflow in the dividing cells. Cytosolic sucrose synthase (SuSy) is involved in sucrose cleavage at the cell division stage. According to Nguyen-Quoc and Foyer (2001) cell vacuoles at this stage accumulate high concentration of hexose (up to 100 μmol g^{-1} FW) and contain equal amounts of glucose and fructose. This implies that soluble sugars must be transported to the vacuoles by specific transporters. Vacuolar proton ATPase (V-ATPase) and vacuolar proton pyrophosphatase (V-PPase) generate the electrochemical gradient to transport sugar to the vacuolar compartment (Kanayama, 2017). Amemiya et al. (2006) showed that fruit specific V-ATPase suppression in antisense-transgenic tomato reduces fruit growth and seed formation. It is noteworthy that the highest expression of the *V-PPase* gene was observed during the cell division stage and not during latter stages of fruit development (Kanayama, 2017). Sucrose loading into the vacuole by the sucrose antiport–transporter is an efficient component of vacuolar storage and there is no requirement for sucrose hydrolysis to allow vacuolar loading or unloading. The fact that regulation of sugar transporters may be influenced by endogenous sugar levels through kinases provide an additional level of complexity regarding carbohydrate subcellular distribution at the end of the cell dividing phase (Lecourieux et al., 2010).

Beside Susy, acid invertase (AI; EC 3.2.1.26) may also be involved in sucrose cleavage and this implies that sucrolytic activity occurs within the vacuole and not only in the cytosol. According to Beauvoit et al. (2014), AI even assumes most of the sucrose cleavage in dividing cells while cytosolic neutral invertase (NI) and SuSy are mainly involved during the following cell expansion phase.

Although sucrose-phosphate-synthase (SPS : 2.4.1.14) activity remains low throughout the fruit development, it may significantly contribute to sucrose re-synthesis in the cytosol, inducing a « futile » cycle between sucrose and hexose characterized by a continuous sugar exchange between cytosol and vacuoles (sucrose influx and hexose efflux) (Nguyen-Quoc and Foyer, 2001). The extent of such resynthesis however remains limited from a quantitative point of view and never exceeds 10% of the cleavage (Beauvoit et al., 2014). Seeds may somewhat control the expression of genes coding for UGPase and SPS : Rounis et al. (2015) found drastic differences in transcript accumulation and enzyme activities of both UGPase and SPS between seeded and parthenocarpic fruits but only minor differences were recorded for sugar levels.

Sugar Metabolism During the Cell Expansion Phase

Mounet et al. (2009) explored transcriptional and metabolic changes in expanding fruit tissues (12–35 DAA) using multivariate analysis and gene-metabolites correlation networks. These authors demonstrated that cell expansion during fruit development proceeds differently in mesocarp and locular tissues which clearly differ in their metabolic composition. Mesocarp represent approximatively 50% (w/v) of the fruit fresh weight and its quantitative importance remains stable throughout fruit development while the locular tissues strongly develop reaching 23% (w/v) of the fruit fresh weight at the mature green stage. Some soluble sugars (mainly Suc and UDP-Glc) are most abundant in locular tissues at the end of the cell expansion phase while others such as hexoses mainly accumulate in the mesocarp. Beside differences in terms of distribution, discrepancies may also result from the mode of expression of enzyme activities or metabolites concentration. Biais et al. (2014) indeed estimated that expressing enzyme activities per protein content minimizes the influence of vacuolar expansion comparatively to an expression on a fresh weight basis.

The cell expansion phase itself is commonly divided in two distinct steps corresponding to « early » and « late » expansion. During the early cell expansion, enzymes involved in the middle part of the glycolysis (NAD-GAPDH, P6K, enolase [EC 4.2.1.11)] are activated in a coordinated way. The main enzyme controlling starch synthesis (ADP-Glc pyrophoshorylase) is also activated in order to produce ADP-Glc for starch synthesis. Sucrose synthase activity presented its highest value during this phase and could be involved in providing UDP-Glc for cell wall cellulose synthesis. Cell expansion is mainly driven by the hexose content. Hexose accumulation in the vacuole is responsible for at least 50% of the fruit osmotic potential during the time course of cell expansion. The crucial role of hexose in cell expansion may thus explain the small fruit size produced by shaded plants.

Some enzymes exhibit a high activity during the late elongation phase and culminate at the green mature stage. This is the case for phosphoglucoisomerase (PGI; EC 5.3.1.9), ATP-phosphofructokinase (PFK; EC 2.7.1.11) and for UDP-Glc pyrophosphorylase (UGPase; EC 2.7.7.9). These enzymes are involved in recycling of hexose-P issued from starch degradation (Carrari et al., 2007). Starch accumulation in the fruit occurs during the early expansion phase while net starch degradation occurs during the late cell expansion phase. Nevertheless, all enzymes required for starch synthesis and degradation are present in the fruits at all developmental stages and there is a continuous starch synthesis and breakdown in tomato fruits. The most important enzyme for starch degradation in fruit is starch

phosphorylase which produces G1P while amylase activity remains rather low (Yelle et al., 1991). Beside regulation of ADP-Glc pyrophosphorylase, the concentration of hexose phosphate in the amyloplasts and the rate of hexose phosphate exchange between cytosol and amyloplast constitute major control points to regulate the balance between starch synthesis and starch degradation (Nguyen-Quoc and Foyer, 2001; Centeno et al., 2011).

SlARF4 represses the expression of SlAGPase gene (Sagar et al., 2013). Other transcription factors play key roles in the regulation of gene expression during cell expansion phase. Mounet et al. (2009) reported important roles for zinc finger proteins, MYB, bZIP, an ERF and a NAC transcription factors. The homeobox-Leu zipper protein HAT22 appears to be implicated in the complex regulation of the metabolic shift occurring between fruit early development and subsequent ripening. Some transcription factors assume important roles in the mesocarp while others are more specifically acting in the locular tissues (Lemaire-Chamley et al., 2005; Mintz-Oron et al., 2008; Mounet et al., 2009). Sugar signaling during the cell expansion phase may involve direct sugar-binding: hexokinase is acting as sugar sensor with dual independent functions in hexose phosphorylation and glucose sensing. Sugar signaling may also involve upstream open reading frame as reported for the sucrose-induced repression of translation in which the translation of the normal ORF of a bZIP transcription factor is repressed by sucrose (Kanayama, 2017). Sagor et al. (2016) expressed a tomato homolog of the bZIP gene lacking the uORF in fruit using a ripe fruit specific E8 promoter and strongly increased the fruit sugar concentration in the transgenic lines.

Both cell division and cell expansion phases imply the regulation of the cell wall metabolism, which also directly influences the fruit firmness and texture. Cell wall polysaccharides largely derive from sugar and sugar phosphates, and in tomato fleshy fruits mainly formed by unlignified parenchyma cells, pectic and hemicellulose polysaccharides account for nearly 95% of the cell wall. Regulation of the cell wall-related enzymes are however mainly studied in relation to the ripening phase of tomato fruit development.

Sugar Metabolism During Repining Phase and Putative Interest of Wild-Related Tomato Species

Ripening phase involves both catabolism and accumulation of key metabolites. During ripening, fruit weight still slightly increases and hexoses exhibit their highest concentration. Total protein content also increases and enzymes involved in TCA cycle and glycolysis strongly increased while glucokinase and fructokinase activities decreased. Degradation of starch hence becomes the main source of hexose-P used as substrate for respiration (Carrari and Fernie, 2006; Beckles et al., 2012; Biais et al., 2014). Sucrose-phosphate-synthase activity, which remains low during the previous expanding phases, significantly increased at the beginning of ripening phase (Biais et al., 2014). Accumulation of sucrose, however, remains limited since invertase activities also increased during ripening in the cultivated tomato species *S. lycopersicum* (Yelle et al., 1991). According to Bastías et al. (2011), ABA which increases before ethylene at the early beginning of maturation phase may be involved in stimulating the expression of genes coding acid vacuolar invertase. The ABA-responsive element binding factor SlAREB1 is indeed present in the fruit pericarp at the end of the mature green stage (Yáñez et al., 2009) and plays an important role for up-regulation of genes involved in sugar metabolism during ripening. During the breaker stage, chlorophyll content strongly declines and the dedifferentiation of chloroplasts in chromoplasts occur under the control of anterograd and retrograd mechanisms leading to the breakdown of starch granules and lysis of thylakoid membrane (Pesaresi et al., 2014). Cell walls are then degraded as a consequence of activation of rhamnogalacturonase and β-galactosidase which depolymerize branched pectins resistant to attack by endo-polygalacturonase (Carrari and Fernie, 2006). Pectin methylesterase catalyses de-esterification of pectin and are encoded by three genes, one being fruit specific and involved in shelf-life of tomato upon storage at room temperature. Fruit softening is also determined by cellulase (endo-β-1,4 glucanases) and by xyloglucan endotransglucosylase (Jiang et al., 2019).

Fructose is sweeter than other sugars and metabolic engineering was therefore specifically performed using fructokinase targets to increase fructose content in commercial tomato fruits (Odanaka et al., 2002; Kanayama, 2017). According to Schaffer et al. (1998), the trait of high fructose to glucose is independently inherited from that of sucrose accumulation. Numerous wild species differ from domesticated tomato cultivars and contain high TSS (Total Soluble Solid, a convenient proxy for sugar content) (more than 10% against 4–6% for *S. lycopersicum*). These wild species often present an increased import of sugar from source leaves, especially during the latter stage of development. Some of them (*Solanum chmielewskii, Solanum peruvianum, Solanum neorickiim*, and *Solanum habrochaites*) store large amounts of sucrose and present constitutively low invertase activities (Miron and Schaffer, 1991). Others (*Solanum cheesmanii, Solanum pennellii*, and *Solanum pimpinelifolium*) accumulate mainly glucose and fructose in relation to a high apoplastic invertase in the columella which increases the sugar gradient with the phloem (Beckles et al., 2012). Introgression lines thus constitute convenient tools to investigate the control of sugar content (Eshed and Zamir, 1994; Gur and Zamir, 2004). The line IL8-3 contains a single short segment from *S. pennellii* in the *S. lycopersicum* background. This promising line contains a high level of sugar resulting from an increased hexose content, probably as a consequence of a high activity of ADP-glucose pyrophosphorylase leading to accumulation of starch during the middle part of development, followed by an active starch remobilization during ripening (Ikeda et al., 2016).

Beside structural enzymes involved in sugar metabolism in fruits, sugar transporters also appear to play a key role in soluble sugar profile (Schroeder et al., 2013). This is especially the case for members of the *SWEET* gene family: the expression pattern of those genes frequently coincides with sugar accumulation pattern in tomato fruit (Feng et al., 2015). Two interacting

chromosomal regions introgressed from the inedible *S. habrochaites* present an almost 3-fold epistatic increase in the fructose to glucose ratio in mature fruits (Levin et al., 2000). More recently, Shammai et al. (2018) reported that introgressions of the Fgr^H allele from *S. habrochaites* into cultivated tomato increased the fructose to glucose ratio of the ripe fruit. These authors clearly demonstrated that the *SlFgr* gene encodes a plasma membrane-localized glucose efflux transporter of the *SWEET* family. Its overexpression in transgenic tomato plants strongly reduced glucose concentration and increased fructose:glucose ratio. Interestingly, no clear impact of the *Fgr* gene overexpression on the expression of sugar metabolizing genes was recorded and the relationship between glucose efflux and fructose increase still remains an open question.

Organic Acid Metabolism

Organic acid content in fruits is one of the most important properties from a commercial point of view and have a strong influence on the sensorial qualities of the product. Acid taste in tomato is attributed to citric and malic acid which constitute together more than 90% of the total pool of organic acid in harvestable fruits (Bastías et al., 2011). High sugar content and relatively high acid content are required for a favorable taste. High level of acids with low level of sugar will produce a tart tomato, while high levels of sugars and low acids will result in a bland taste (Davies and Hobson, 1981).

The cell division phase is characterized by very high rates of organic acids accumulation (from 2 to 5 nmol min^{-1} g^{-1} FW) between 4 and 15 DAA according to Beauvoit et al. (2014). It is consequently tempting to speculate that such a high level of accumulation contribute with soluble sugars to decrease the cell water potential allowing water uptake. However, beside this osmotic function, organic acids are also of paramount importance at the cellular level for various biochemical pathways. According to Carrari and Fernie (2006), manipulation of central organic acids is a promising approach to improve tomato fruit yield.

During the cell expansion phase, clear differences were recorded between locular and mesocarp tissues since most organic acids were more abundant in the former than in the latter, and this is especially the case for citrate and malate (Mounet et al., 2009). According to this study, among genes related to organic acid metabolism, 13 were differentially expressed in the two types of tissues. In both tissues, however, organic acid concentration increased between 20 and 35 DAA, mainly in locular tissues and this was correlated with an increased expression of gene coding for aconitase, a key enzyme involved in TCA cycle.

At the ripening stage, tomato climacteric fruits strongly increase ethylene synthesis and respiration, although both subsequently decreased during post-climacteric storage (Van de Poel et al., 2012). Increasing respiration implies hastening of the TCA cycle. Before the ethylene burst, a transient increase in ABA may induce an accumulation of citric and malic enzymes. At the beginning of the ripening phase, fruit preferentially accumulates citrate through stimulation of citrate synthase and the expression of a gene encoding mitochondrial citrate synthase is upregulated by SlAREB1 (Bastías et al., 2011).

Centeno et al. (2011) experimentally decreased the activities of mitochondrial malate dehydrogenase or fumarase *via* targeted fruit-specific antisense approach in tomato. These authors demonstrated that the line containing higher concentration of malate exhibited a lower starch accumulation during the cell expansion phase and lower soluble sugars at harvest. Although modification of organic acid content in the mitochondria could be relevant from modification in the TCA cycle, it has to be mentioned that mitochondrial pool represents only a small portion of the total cellular organic acid. According to Centeno et al. (2011), correlation between malate and starch concentration could be related to an altered redox status of the AGPase protein allowing an allosteric enhancement of its maximal catalytic activity.

During the ripening stage, phosphoenolpyruvate carboxykinase (PEPCK; which was almost undetectable in green fruits) is suspected to act in the dissimilation of malate/citrate to provide sugar through neoglucogenesis. This hypothesis was confirmed by Huang et al. (2015) who analyzed the effect of an excessive PEPCK in transgenic lines overexpression SlPEPCK by either the constitutive CaMV35S or the fruit-specific *E8* promoter. Soluble sugars increased while malate content decreased in both lines, confirming the participation of gluconeogenesis in sugar/acid metabolism during fruit ripening. Similarly, Schouten et al. (2016) recently confirmed that an important part of malate is converted to hexose

Amino Acids Metabolism

Total concentration of free amino acids in tomato fruits varies between 2.0 and 2.5% on a dry weight basis. The most quantitatively important are Glu, Asp and GABA (γ-aminobutiric acid) (Sorrequieta et al., 2010; Snowden et al., 2015). GABA is a four carbon non-protein amino acid which assumes important functional properties in reducing blood pressure in the human body (Zhao et al., 2018). It is also an important metabolite in plants and control cytosolic pH under acid load *via* the GABA shunt pathway. It is present at high concentration at the green mature stage but then progressively declines during ripening processes (Klee and Giovannoni, 2011). Threonine also declines during ripening and could be metabolized to pyruvate involved with glyceraldehyde 3-phosphate in the synthesis off isopentenyl pyrophosphate acting as a precursor of carotenoids. Most of the other free amino acids increased during ripening while the protein content decreased in relation to an increment in exopeptidase activity and non-specific protease activity pattern (Sorrequieta et al., 2010).

The recorded increase is especially important for glutamate whose concentration may be as high as 10 mmol kg^{-1} FW in mature fruits. Such an increase is partly due to stimulation of glutamate dehydrogenase (aminating reaction) and α-ketoglutarate-dependent γ-aminobutyrate transaminase. Cultivated *S. lycopersicum* has quite higher glutamate content than wild

species (Schauer et al., 2005). Since glutamate is a direct precursor of chlorophyll, its accumulation in ripening fruit may be, at least partly, regarded as the consequence of downregulation of chlorophyll synthesis. Mature green fruit contain Fd-GOGAT putatively involved in glutamate synthesis but this enzyme was not detected in red mature fruits where glutamate accumulates (Sorrequieta et al., 2010). Considering the importance of glutamate in phloem sap, transfer of this amino acid from the source leaves to the maturing fruits could not be excluded. (Snowden et al., 2015) considered that GABA may be interconverted in Glu and Asp and provided evidences that these amino acids must be stored in the vacuoles. These authors identified SlCAT9 as a candidate protein for tonoplast transporter exporting GABA from the vacuole and importing Glu and Asp.

Aromatic amino acids also increase and are of special interest since they constitute precursor of flavor volatiles during the ripening process. Valine increased in relation to a stimulation of dihydroxy acid dehydratase (Mounet et al., 2009). MYB and bZIP transcription factors were shown to affect amino acid metabolism (Mounet et al., 2009). (Zhao et al., 2018) recently demonstrated that TAGL1, which play a major role in fruit development (see above), also directly influences fruit metabolism in relation to an increase in seven amino acids (tyrosine, glutamic acid, valine, phenylalanine, proline, leucine and isoleucine).

SECONDARY METABOLISM IN TOMATO FRUIT

Pigments and Flavonoids

The onset and progression of ripening in tomato is typically associated with changes in the external color of the pericarp, reflecting the accumulation of carotenoid and flavonoid pigments (Shinozaki et al., 2018b). Tomato fruits typically provide the principal dietary source of carotenoids in many Western diets (Carrari and Fernie, 2006). The characteristic red tomato color is a result of the accumulation of the carotenoid lycopene in both the fruit skin and pulp (Seymour et al., 2013; Borghesi et al., 2016; D'Ambrosio et al., 2018). During tomato ripening, the concentrations of carotenoids increase by between 10- and 14-fold, mainly due to the accumulation of lycopene (Fraser et al., 1994), which increases as the fruit matures (Tamasi et al., 2019). Alterations in the pigment accumulation patterns have also been observed in several spontaneously occurring tomato mutants (Carrari and Fernie, 2006); for example, the recessive mutant *high pigment* (*hp*) produces fruits with two times more carotenoids than wild-type fruits and increased levels of other antioxidants (Yen et al., 1997; Bino et al., 2005; Carrari and Fernie, 2006).

Carotenoid biosynthesis has been studied extensively in tomato, and major steps in the pathway have been identified (Seymour et al., 2013). Light signaling and plant hormones, particularly ethylene and auxins, have been identified as important regulators of carotenoid biosynthesis during tomato fruit ripening (Cruz et al., 2018). Almost all the enzymes acting in the carotenoid biosynthesis pathway have been cloned, and metabolic engineering approaches have been developed to enhance pigment quantity and quality (Carrari and Fernie, 2006; Alseekh et al., 2015; D'Ambrosio et al., 2018). The first committed step of carotenoid biosynthesis is the formation of phytoene, which is dependent on the catalytic activity of phytoene synthase. Phytoene then undergoes two desaturation reactions to form ζ-carotene, catalyzed by phytoene desaturase, which in turn is desaturated to neurosporene and finally lycopene. Lycopene is then either cyclized at both ends of the molecule by lycopene b-cyclase to form β-carotene, or cyclized at one end by lycopene b-cyclase and at the other by lycopene e-cyclase to form α-carotene. These cyclic carotenoids can then be converted to xanthophylls.

Tomatoes also accumulate semipolar metabolites, such as flavonoids, phenolic acids, and alkaloids, which are important health-promoting compounds (Bovy et al., 2007; Tohge and Fernie, 2015; Ballester et al., 2016; Tohge et al., 2017; Tamasi et al., 2019; Wang et al., 2019). To identify the genes responsible for their biosynthesis, QTL analyses were performed in different populations of introgression lines between *S. lycopersicum* and wild tomato species such as *S. chmielewskii* and *S. pennellii* (Alseekh et al., 2015; Ballester et al., 2016; Liu et al., 2016; Alseekh et al., 2017). The flavonoids represent a large family of low molecular weight polyphenolic secondary metabolites, which are grouped into several classes based on their aglycone structure (Bovy et al., 2007; Ballester et al., 2016). The main flavonoid classes are the flavones, flavonols, flavanones, flavanols, anthocyanidins, and isoflavones (Bovy et al., 2007; Tohge et al., 2017). More than 500 different forms of flavonoids are present in tomato, with the most major being the chalcone naringenin chalcone and various sugar conjugates of the flavonols quercetin and kaempferol, including rutin (Bovy et al., 2007; Ballester et al., 2016; Tamasi et al., 2019). In tomato fruits, the accumulation of flavonoids is restricted to the peel, with only traces found in the flesh, which comprises approximately 95% of the whole fruit (Schijlen et al., 2008; Bovy et al., 2010; Ballester et al., 2016). As a result, in a typical tomato cultivar such as Moneymaker, quercetin levels rarely go above 10 mg kg^{-1} fresh weight (Bovy et al., 2010). Usually, cultivated tomatoes lack high levels of anthocyanins, while some wild tomato species (*S. chilense* and *S. cheesmaniae*) have much higher levels, giving a purple tone to the skin of certain organs (Seymour et al., 2013; Borghesi et al., 2016; Wang et al., 2019). The main phenylpropanoids found in tomato are chlorogenic and caffeic acids (Tamasi et al., 2019).

Flavonoids, along with other phenylpropanoids, are biosynthesized from phenylalanine. Three enzymes [phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumaroyl CoA ligase (4CL)] convert phenylalanine into 4-coumaroyl CoA, the activated intermediate for the various branches of phenylpropanoid metabolism (Zhang et al., 2015). Chalcone synthase (CHS) is the first enzyme involved in the phenylpropanoid/flavonoid pathway and converts 4-coumaroyl CoA into naringenin chalcone (Ballester et al., 2016; Tohge et al., 2017). Most of the biosynthetic genes involved in the flavonoid pathway and the transcription factors regulating them have been identified (Adato et al., 2009; Bovy et al., 2010; Ballester et al., 2016; Tohge et al., 2017; Li et al., 2018b; Wang et al., 2019). These insights have been used to develop genetic engineering strategies to increase the flavonoid contents of tomatoes, since this species accumulates

limited amounts of phenolic antioxidants relative to its content of lipophilic antioxidants such as carotenoids (Carrari and Fernie, 2006; Bovy et al., 2007; Bovy et al., 2010; Zhang et al., 2015; Tohge et al., 2017; Wang et al., 2019).

Alkaloids are generally considered to be antinutritional factors in our diet (Friedman, 2002). Breeding efforts have focused on reducing their levels in foods, but some of these substances still remain in our daily diet (Friedman, 2002). More than 100 glycoalkaloids have been found to be present in the tomato clade in various tissues and accessions (Tohge and Fernie, 2015). The main alkaloids present in tomato are α-tomatine and dehydrotomatine, which are often concurrently analyzed as tomatine (Friedman, 2015; Ballester et al., 2016; Tamasi et al., 2019). Immature green tomatoes contain up to 500 mg of tomatine per kilogram of dry weight, while the levels in red tomatoes are much lower (up to about 5 mg kg^{-1}) (Friedman, 2015; Tamasi et al., 2019). The tomatine contents of cherry tomatoes (grape tomatoes, minitomatoes) are several fold greater than those of the larger standard tomato varieties (Friedman, 2015; Tamasi et al., 2019). In tomato fruits, the bitter-tasting α-tomatine is present at high levels in early developmental stages, but its levels decrease upon ripening due to its conversion into its acetyl glucosylated forms lycoperoside G and F or esculeoside A, which are not bitter (Tohge and Fernie, 2015; Ballester et al., 2016). Dehydrotomatine is 10 times less abundant than α-tomatine in immature fruits (Tamasi et al., 2019). Despite their negative impact on nutrition and their toxicity, glycoalkaloids found in Solanaceous plants, such as α-tomatine, and their hydrolysis products were shown to have anticancer properties (Friedman, 2015).

Volatiles

Volatile metabolites biosynthesized during tomato ripening are responsible for fruit flavor and aroma (Carrari and Fernie, 2006; Ballester et al., 2016; Shinozaki et al., 2018b). More than 400 volatiles have been detected in tomatoes, but a smaller set of 15 to 20 are made in sufficient quantities to have an impact on human perception (Baldwin et al., 2000; Mathieu et al., 2009; Zanor et al., 2009). These volatile compounds are generally derived from various precursors, including fatty acids, carotenoids, and amino acids (Tieman et al., 2006; Zanor et al., 2009; Bauchet et al., 2017). The principal contributors to the ripe tomato flavor are cis-3-hexanal, cis-3-hexanol, hexanal, 3-methylbutanal, 6-methyl-5-hepten-2-one, 1-pentan-3-one, trans-2-hexanal, methyl salicylate, 2-isobutylthiazole, and β-ionone (Carrari and Fernie, 2006). There are differences of many orders of magnitude between the abundance of the various volatile compounds, with concentrations ranging from several micrograms per gram of fresh weight for the most abundant, such as (*Z*)-3-hexenal or hexanal, to nanograms per gram and even lower levels detected for β-damascenone or β-ionone (Rambla et al., 2014; Tieman et al., 2017). The levels of almost any volatile compound also vary substantially between varieties and accessions (Rambla et al., 2014). Modern commercial varieties contain significantly lower amounts of many of the important flavor chemicals than older varieties since it was not the focus of breeding programs (Tohge and Fernie, 2015; Bauchet et al., 2017; Tieman et al., 2017). Volatiles display a variable pattern of heritability, suggesting a high sensitivity to environmental conditions (Bauchet et al., 2017). Moreover, not all volatile compounds confer positive taste attributes to tomato (Carrari and Fernie, 2006). An example is the identification of *malodorous*, a wild tomato species allele affecting tomato aroma that was selected against during domestication (Tadmor et al., 2002).

QTL analyses, genome-wide association studies, and targeted metabolome quantifications were conducted in several cultivars and accessions of cultivated tomato, wild relatives, and inbred lines to identify tomato volatiles and their associated genetic loci (Saliba-Colombani et al., 2001; Tieman et al., 2006; Mathieu et al., 2009; Zanor et al., 2009; Alseekh et al., 2015; Ballester et al., 2016; Liu et al., 2016; Alseekh et al., 2017; Bauchet et al., 2017; Tieman et al., 2017). These studies revealed the complex and distinct regulation of metabolites in tomato subspecies (Rambla et al., 2014; Bauchet et al., 2017), demonstrating that there is ample genetic scope to improve the volatile composition of commercial varieties (Rambla et al., 2014).

As mentioned previously, several classes of volatiles exist in tomato. Volatiles derived from fatty acids constitute a class of compounds containing the most abundant volatiles produced in tomato fruits: the C6 volatiles 1-hexanol, (Z)-3-hexenal, (E)-2-hexenal, or hexanal, and the C5 volatile 1-penten-3-one (Rambla et al., 2014). These compounds are classified as green-leaf volatiles due to their characteristic fresh aroma of cut grass (Rambla et al., 2014). The production of these compounds increases as the fruit ripens (Klee, 2010). A second class are volatiles derived from amino acids. A significant number of volatile compounds considered important for the tomato aroma are derived from amino acids (Rambla et al., 2014). These volatiles can be grouped into two categories: phenolic and branched-chain compounds. Phenolic volatiles include a variety of compounds derived from the amino acid phenylalanine, while branched-chain volatiles have particularly low molecular weights and high volatility (Rambla et al., 2014). Additional classes are ester and terpenoid volatiles. Few esters are found in the volatile fraction of tomato (Rambla et al., 2014), while volatile terpenoids are among the most abundant volatiles in tomato vegetative tissues, but only a few of them, such as limonene, linalool, or α-terpineol, are present in the ripe fruit (Rambla et al., 2014), Volatile terpenoids can be classified into two groups, the monoterpenoids (C10) and sesquiterpenoids (C15), both of which are biosynthesized from the five-carbon precursors isopentenyl diphosphate and dimethylallyl diphosphate (Rambla et al., 2014). Carotenoid-derived volatiles are produced at low levels in ripe fruit but are important in our perception of tomato flavor due to their very low odor thresholds (Vogel et al., 2010; Rambla et al., 2014). This is particularly true for β-ionone or β-damascenone, which can be detected ortho-nasally at concentrations of 0.007 and 0.002 nL L^{-1}, respectively (Buttery et al., 1989). Volatile compounds first accumulate in a conjugated nonvolatile form, such as a glycoside, before being released during the ripening process (Rambla et al., 2014; Tohge and Fernie, 2015). The accumulation of the appropriate glycosidases in a separate subcellular location would allow the immediate liberation of high

amounts of the aglycone when the enzyme and the conjugate glycosylated form come into contact with each other (Rambla et al., 2014).

EFFECTS OF ABIOTIC STRESS ON TOMATO FRUIT METABOLISM

Tomato is one of the most cultivated vegetable species but its productivity is impaired by a wide range of abiotic stresses (Gerszberg and Hnatuszko-Konka, 2017). The presence of adverse environmental factors like extreme temperatures, salinity or drought affects tomato yield as a consequence of reduced fruit number and fruit size but it also affects fruit quality (Moretti et al., 2010; Li et al., 2012; Gerszberg and Hnatuszko-Konka, 2017). It has been shown that moderate stress conditions may improve fruit quality through higher concentration of flavor compounds (Zheng et al., 2013; Albert et al., 2016a; Albert et al., 2016b). In several studies, the concentrations of sugars, organic acids, vitamin C, phenolic compounds and carotenoids increased in tomato fruits in response to water deficit, salinity, or heat (Saito et al., 2009; Patanè et al., 2011; Zushi and Matsuzoe, 2015; Albert et al., 2016b; Flores et al., 2016; Marsic et al., 2018). However, increased CO_2 levels increased fruit production but decreased fruit quality (Mamatha et al., 2014). Nevertheless, metabolic modifications in tomato fruits in response to abiotic stress may be cultivar-dependent (Sánchez-Rodríguez et al., 2012a; Sánchez-Rodríguez et al., 2012b; Zushi and Matsuzoe, 2015; Albert et al., 2016b; Albert et al., 2016a; Flores et al., 2016; Marsic et al., 2018). **Table 1** summarizes some recent studies regarding the impact of abiotic stress occurring during plant growth on primary and secondary metabolism of tomato fruits. Modification of fruit metabolism was mainly investigated in response to salinity and drought (**Table 1**). The effect of salinity was investigated in various cultivars under hydroponic culture with NaCl concentrations varying between 0 to 100 mM and salinity overall increased the concentrations of sugars, organic acids, amino acids, pigments and antioxidants (Saito et al., 2009; Schnitzler and Krauss, 2010; Zushi and Matsuzoe, 2015; Flores et al., 2016; Marsic et al., 2018). The effect of drought was investigated under both greenhouse and field conditions. Most studies reported an increase in sugars and organic acids in response to drought in a wide range of tomato accessions (Patanè et al., 2011; Murshed et al., 2013; Zheng et al., 2013; Shao et al., 2014; Albert et al., 2016b; Albert et al., 2016a) while others reported less strong effects (Atkinson et al., 2011; Sánchez-Rodríguez et al., 2012; Wei et al., 2018). The effect of drought on the concentration of secondary metabolites was more

TABLE 1 | Effect of abiotic stress occurring during plant growth on primary and secondary metabolite production in tomato fruits.

Metabolites	Salinity	Drought	Heat	Cold	CO_2 increase
		Primary metabolites			
Sugars					
Soluble solid Content	↑	↑		=	↓(↑)
Total soluble Sugars	↑	↑(=*)	↑	=	↑
Fructose	↑	↑(↓, =*)			
Glucose	↑	↑(↓, =*)			
Saccharose	↑	(=*)			
Organic acids					
Citric acid	↑(=*)	↑(=*)		=	↓
Malic acid	↑(=*)	↑(=*)		=	↓
Glutamic acid	↑(=*)				
Quinic acid	↑(=*)				
Amino acids					
Arginine	↑*				
Histidine	↑*				
Isoleucine	↑*				
Threonine	↑				
Serine	↑				
Proline	↑				
Phenylalanine	↑				
		Secondary metabolites			
Pigments					
Carotenoids	↑(=)	↑(=*)	↓		↓
Lycopene	↑*(=)	↑(↓*)	↓		↓
β-carotenoid	↑	↑(↓*)	↓=		
Antioxydants					
Total		↑			
Polyphenols	↑*	↑(=*)			↓
Flavonoids	↑*	↑(=,↓*)			↓
Ascorbic acid	↑(=*)	↑(=*)	↓		↑=
References	(Saito et al., 2009; Schnitzler and Krauss, 2010; Zushi and Matsuzoe, 2015; Flores et al., 2016; Marsic et al., 2018)	(Atkinson et al., 2011; Patanè et al., 2011; Sánchez-Rodríguez et al., 2012; Murshed et al., 2013; Zheng et al., 2013; Shao et al., 2014; Albert et al., 2016a; Albert et al., 2016b; Wei et al., 2018)	(Li et al., 2012; Hernández et al., 2015)	(Kläring et al., 2015)	(Moretti et al., 2010; Mamatha et al., 2014; Wei et al., 2018)

*↑ : Increase; ↓: Decrease; =: No modification; *Cultivar-dependent; () Effect observed only in one study or few cultivars.*

cultivar-dependent (Atkinson et al., 2011; Sánchez-Rodríguez et al., 2012). In contrast to salinity and drought, heat mainly decreased the concentration of pigments and ascorbic acid in tomatoes (Li et al., 2012; Hernández et al., 2015) and increased CO_2 levels decreased carotenoid, polyphenol and flavonoid concentrations but increased ascorbic acid concentration in tomatoes (Mamatha et al., 2014). All these compounds play an important role in the final nutritional and commercial quality of tomato and depend on genetic, environmental, agronomic and post-harvest factors (Flores et al., 2016). Several studies based on the influence of these factors on fruit composition have been carried out with the aim of increasing tomato quality (Flores et al., 2016).

In addition to the environmental conditions to which plants are subjected during their growth, post-harvest conditions may also affect fruit quality and metabolism. The impact of low temperature storage on tomato quality has been extensively investigated (Sevillano et al., 2009; Luengwilai et al., 2012; Cruz-Mendívil et al., 2015; Wang et al., 2015; Raffo et al., 2018; Zhang et al., 2019). Among others, early harvesting and cold storage negatively affect tomato flavor and decrease the levels of aroma compounds (Wang et al., 2015; Raffo et al., 2018). Indeed, metabolomics data showed that 7 amino acids, 27 organic acids, 16 of sugars and 22 other compounds had a significantly different content in cold-stored tomatoes and transcriptomics data showed 1735 differentially expressed genes due to cold storage (Zhang et al., 2019). Some pre-treatments have been proposed to improve tomato fruit resistance to cold stress such as ozone exposition, high CO_2 treatment, UV-C hormesis, oxalic acid application and heat treatment (Moretti et al., 2010; Luengwilai et al., 2012; Mattos et al., 2014; Charles et al., 2015; Cruz-Mendívil et al., 2015; Li et al., 2016b; Sangwanangkul et al., 2017; Raffo et al., 2018). These treatments provide protection from chilling in part by altering levels of fruit metabolites (Luengwilai et al., 2012; Wang et al., 2015; Sangwanangkul et al., 2017).

CONCLUSIONS

In this review, we focused on the tomato fruit development and metabolism. Tomato has long been the model for the study of fleshy fruits and the emergence of "omics" approaches (phenomics, genomics, transcriptomics, proteomics, and metabolomics) has largely contributed to improve our understanding of the genetic, hormonal and metabolic networks that govern tomato fruit development and metabolism. Tomatoes are climacteric fruits with high level of health-promoting compounds. As important as yield improvement and stress resistance, enhancement of tomato fruit quality has gained extensive attention. Improvement of tomato flavor and quality is a challenge for the coming years. The sequencing of tomato genome and genome-wide association studies provide genetic insights into the genetic control of tomato flavor and gives a roadmap for flavor improvement. Moreover, several techniques can now be exploited for breeding superior tomato varieties in the context of current changing climatic conditions.

AUTHOR CONTRIBUTIONS

MQ and SL designed the outline of the manuscript. MQ, SL, FY-L, TA, and J-PM contributed to writing and revisions of the manuscript. SB and RB-G contributed to figure design and revisions of the manuscript. All authors read and approved the final manuscript.

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Metabolite Storage in *Theobroma cacao* L. Seed: Cyto-Histological and Phytochemical Analyses

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Cocoa (*Theobroma cacao* L.), an economically important tropical-fruit crop as source of chocolate, has recently gained a considerable attention; its seeds contain a large amount of different bioactive compounds that have attracted interest because may be beneficial to humans by improving cardiovascular health, by cancer chemo-preventive effects and also through neuroprotective activities. The morphological and anatomical characteristics of cocoa seeds are closely related to the aroma and to the nutritional properties. This study aimed to provide more information about the storage of some metabolites in the various components of cocoa seed by microscopical and phytochemical analyses. Polyphenols, sterols, tocopherols and fatty acids were detected in different portions of the seeds (teguments, cotyledons, embryo axis and pulp). Quali and quantitative differences were observed and a characteristic polyphenol pattern was detected in the different portions of the seed; cytological analysis demonstrated the presence of these compounds in big vacuolated polyphenolic cells. Among the analyzed fatty acids, the stearic and oleic acids were the most abundant in all the seed components (teguments, cotyledons and embryo axis). Fatty acids, usually found in the form of esters, thioesters and amides, represent one of the storage substances of cocoa seed probably localized in lipid globules, which in our observations occupied almost the entire volume of small isodiametric cells of cotyledon mesophyll. In the cocoa seeds we observed also a different distribution of sterols: β-sitosterol and Δ5-avenasterol were the most abundant, above all in the embryo axis; stigmasterol and campesterol were less present in embryo axis and more abundant in teguments; campestanol level was again higher in teguments but lower in cotyledons. The specific localization of different kind of sterols was probably related to a peculiar function. Our experiments demonstrated that all seed components contribute to the metabolites storage, but with interesting differences in the localization and amount of each metabolite.

Keywords: antioxidant, cotyledons, histology, lipids, polyphenols, teguments

INTRODUCTION

Cocoa (*Theobroma cacao* L.) belongs to the family Sterculiaceae. It is an economically important tropical-fruit crop, mainly known as the source of chocolate and was introduced to Europe during the 16th century.

The fruit of cocoa tree is a pod, or cherelle, which shape and colour can differ among morphogenetic groups. Each pod holds 20 to 60 seeds, or beans, embedded in a white pulp, and is constituted by a thick epicarp, of variable hardness and with a pigmented epidermis, a thin and hard mesocarp, more or less woody, and finally the endocarp (Bertazzo et al., 2013). The endocarp is composed by big tubular cells with large intercellular spaces which, in the ripe seeds, collapse and form the so-called pulp. Pulp is rich in water and nutrients, contains 10–15% of sugar, is characterized by a low pH (3.6–4.0) and plays an important role during the seed fermentation, contributing to the flavour development (Hui et al., 2006).

The potential health implications of biologically active substances present in cocoa seeds are well documented. They are rich in natural antioxidants, such as polyphenols and tocopherols. Thanks to this antioxidant property, many of these compounds, especially flavonoids, exhibit also a wide range of pharmacologic effects, including antibacterial, antiviral, anti-inflammatory, antiallergic, antithrombotic and vasodilatory actions (Cook and Samman, 1996; Batista et al., 2016). The high levels of flavanols are responsible for the bitterness of cocoa that represent a fundamental aspect of the organoleptic and palatability characteristics of chocolate, and contribute to cocoa health benefits (Jalil and Ismail, 2008).

Epicatechins (that are classified as flavan-3-ols, based on their structure) are the most abundant cocoa phenolic components; they mainly include monomeric (–) epicatechin and (+) catechin (as well as oligomeric and polymeric proanthocyanidin flavanols), gallocatechin and epigallocatechin (Ortega et al., 2008).

Epicatechin represents approximately 35% of polyphenol content of unfermented Forastero cocoa beans (Othman et al., 2010). The antioxidant properties are also related to the presence of tocopherols and tocotrienols, which reduce oxidative stress and delay the progress of a variety of degenerative disorders, such as cardiovascular diseases and cancer. In addition, they have been shown to regulate cellular signalling, cell proliferation and gene expression (Sen et al., 2007). The total tocopherol content in cocoa beans is reported to be in a range of 100–300 mg/Kg fat (Carpenter et al., 1994; Shukla et al., 2005), values that are similar to those generally observed for wheat germ oil (Beliz and Grosch, 1999). The predominant isomer in cocoa bean is the gamma-tocopherol and a different distribution of the four isomers in seed parts of *Theobroma subincanum* were described by Bruni et al., 2002.

Precursors of tocopherols and polyphenols are produced from the plant primary biosynthetic shikimate and acetate pathways. The main products of these pathways are the aromatic amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) and acetyl-coenzyme A (CoA), respectively. They represent the starting molecules for the biosynthesis of a wide range of secondary metabolites (Tzin et al., 2012).

Other interesting classes of cocoa bioactive constituents are represented by phytosterols (collective terms comprising saturated sterols, also known as stanols) and fatty acids. Phytosterols are typical plant lipids which have a structural similarity with cholesterol and inhibit its intestinal absorption, contributing to a lower total plasma cholesterol and low-density lipoproteins levels. In cocoa seeds, the content of plant sterols is 2–3 mg/g fat, with an abundance of β-sitosterol and stigmasterol (Staphylakis and Gegiou, 1985) . Fatty acids are organized as triacylglycerol (TAG), the majority of these TAG's being 2-oleyl glycerides (O) of palmitic (P) and stearic (S) acids (POP, POS, SOS) (Simoneau et al., 1999; Segall et al., 2005). This TAG structure directly affects the way chocolate behaves in the manufacturing process and the characteristics of the final product (texture, viscosity, melting behaviour, flavour and taste) (Afoakwa, 2010). The fatty acids content depends on the variety and region of cultivation of cocoa beans (Bruni et al., 2002; Torres-Moreno et al., 2015).

The biosynthesis of fatty acids requires as precursor the acetyl-CoA, that also represents the starting point of the mevalonate pathway from which arise the secondary metabolites, sterols/phythosterols.

The presence of above-mentioned compounds (as polyphenols, phytosterol and fatty acids) is not the only trait influencing the nutritional characteristics and aroma of cocoa beans, their morphological and anatomical traits, as the permeability of the seed coat, are also very important aspects. Andersson et al. (2006) suggested that water and solutes flow through seed coat during the fermentation process was strongly related to the flavour quality. The acetic acid produced during the fermentation moves thought the seed coat to contribute to formation of flavour precursors in the cotyledons. The uptake of acetic acid is also influenced by the fruit pulp and the inner pulp epidermis. The cyto-histological characteristics of cotyledons parenchyma are also important; the size of cells can influence the diffusion of acetic acid into the seed, because cells with higher volume can provide larger spaces for chemical reactions (Biehl, 1973; Biehl et al., 1977). After a comparison between different clones and varieties of cocoa, Elwers et al. (2010) suggested that 'the larger polyphenol and storage cells of Criollo seeds may contribute to the unique quality of this fine flavour cocoa'. Considering the important role of histo-anatomical traits, microscopy studies can be very useful to understand how metabolic processes are compartmentalized in plant tissues (Walker et al., 2001). The aim of this study is to better investigate the cyto-histological characteristics of cocoa seeds related to their composition in secondary metabolites, such as polyphenol and tocopherols. To our knowledge, there are in literature many data about the histological characterization of cocoa seeds (Martini et al., 2008; Elwers et al., 2010), as well as biochemical studies about its phenolics content and antioxidant capacity (Aprotosoaie et al., 2016; Endraiyani et al., 2016; Wang et al., 2016), but a specific localization of the phytochemicals in the different structures of seed and fruit has not been carried out

yet. Metabolite localization was focused until now in the cotyledon structure, but, as previous described, also other structure of seed and fruit are important for the flavour development and nutritional characteristics. Knowledge about chemical composition and metabolite storage in the fruit wall of seed tegument can be also useful for the possible use of residual biomass originated during cocoa processing and for the presence of anticancer agents in these non-edible portions of cocoa (Zainal et al., 2014). Our research supplies a more complete picture of metabolites localization in all portions of cocoa seed and fruit.

MATERIALS AND METHODS

Plant Material

Samples were collected from fresh fruits of cocoa, harvested at maturity, coming from the municipality of Tucupita, Delta Amacuro state, Venezuela, and kindly donated to our University. The cocoa plantations of the Delta are established essentially in the tropical humid forest, in fertile alluvial soils with problems of poor drainage and in some units of tropical dry forest, with humid edaphic associations (Reyes and Capriles De Reyes, 2000; Gómez and Azócar 2002; Ministerio del Poder Popular para la Ciencia y Tecnología (MPPCT), 2006), in this area in general the soils have drainage problems and are flooded at certain times of the year (Corporación Venezolana de Guayana (CVG), 2006).

Seeds were separated from the rest of the fruit and their different components were progressively isolated. When gently removed from the fruit, seeds conserve mucilage, a portion of the modified endocarp; seeds with mucilage represent the first sample of this study (SM). In other seeds, mucilage was removed and seeds without mucilage were considered (S); the last samples were represented by the only teguments (T), embryo axis (EA) and cotyledons (C) isolated from seeds. All the samples were pulverized with liquid nitrogen and silicon dioxide in a mortar.

Cyto-Histological Observations

Cocoa seeds were collected from a fresh fruit of cocoa; portions of fruit pericarp, seed T, C and embryo were fixed in 3% (w/v) glutaraldehyde in 0.075 M cacodylate buffer, pH 7.2, for 24 h. The samples were then washed three times for 7 min in 0.075 M cacodylate buffer, pH 7.2, post-fixed in 1% (w/v) OsO_4 in the same buffer for 1 h, dehydrated in increasing concentrations of ethanol and finally embedded in epoxy resin (Epon, 2-dodecenylsuccinic anhydride and methylnadic anhydride mixture) (Reale et al., 2017, with modifications). Semi-thin sections (1–2 μm), obtained with an ultramicrotome (OmU2, Reichert, Heidelberg) after staining were observed under a light microscope (BX53; Olympus, Tokyo, Japan).

Periodic Acid Schiff's Reaction

Semi-thin sections were treated with 0.5% periodic acid for 30 min at 40°C, washed with tap and demineralized water and covered with Schiff's reagent for 15 min (O'Brien and McCully, 1981). Sections were then washed rapidly with tap water, two times for 3 min with SO_2 water and two times for 10 min with demineralized water. Sections were also counter-stained with 1% (w/v) amido black in 7% acetic acid for protein. The presence of proteins was indicated by a blue colour, whilst starch grains appeared magenta.

Toluidine Blue Staining

Semi-thin sections were covered with 0.5% (w/v) toluidine blue in 2% $NaHCO_3$ buffer. Toluidine blue has a high affinity for acidic tissue components and stains nucleic acids blue and polysaccharides purple (Feder and O'Brien, 1968).

Toluidine Blue O Staining

Semi-thin sections were stained with 0.5% (w/v) toluidine blue O in 0.1 M phosphate buffer, pH 7.2. The metachromatic stain develops a green-blue colour when associated with polyphenolic compounds, whilst turns pink with pectic substances and purple with nucleic acids and protein (Feder and O'Brien, 1968).

Vanillin Staining

Semi-thin sections were deresinated, stained for 15' with vanillin (10% w/v) in ethanol mixed with 12 M HCl (2:1, v/v) (Earp et al., 2004), and mounted in glycerin. Vanillin turns up red upon binding to flavan-3,4-diols and flavan-4-ols (catechins), which are present either as monomers or as terminal subunits of proanthocyanidins.

Chemicals

All the chemicals were of analytical grade and, unless otherwise specified, were purchased from Merck (Darmstadt, Germany).

Chemical Analyses

For chemical analysis the considered samples (SM, S, T, C, EA) were collected from different seeds, pooled and analyzed in triplicates.

Analysis of Total Polyphenolic Compounds Content

The total phenolic compounds were extracted from defatted cocoa samples (SM, S, T, C, EA) (through exhaustive extraction with *n*-hexane) with a mixture of acetone and water 80:20 (v/v) in an ultrasonic bath for 15 min. After centrifugation the supernatant was filtered, and this procedure was repeated twice. The supernatants were combined, the acetone was evaporated under vacuum and the residue was analyzed for the total phenolic contents using Folin-Ciocalteu's procedure (Singleton and Rossi, 1965). The total phenolic contents were calculated as a gallic acid equivalent (GAE) from a calibration curve of GA standard solutions and expressed as mg of GAE/g of sample.

Antioxidant Activity of Cocoa Polyphenol Extracts

The extracts were analyzed for their total antioxidant activity using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method (Miliauskas et al., 2004). The $ABTS^+$ radical was generated by oxidation of ABTS with potassium persulfate, then

1 ml of extract was added to the radical solution and the absorbance measured at 734 nm. Standard Trolox solutions were evaluated against the radical in order to obtain the calibration curve. The results of the antioxidant activity of cocoa extracts are expressed in terms of Trolox equivalent antioxidant capacity (TEAC) as mMol Trolox/g of sample.

High-Performance Liquid Chromatography-Analysis of the Extracts for Polyphenols and Tocopherols Identification

The samples of the previous aqueous extract derived from T, C and seed (with and without mucilage, SM and S) were analyzed by high-performance liquid chromatography (HPLC) for the identification of the polyphenolic compounds. Analyses were performed on a Perkin-Elmer PE 200 system (autosampler, binary pump and UV-Vis detector) equipped with an Inertsil 5 ODS-3 (250 mm x 4.6 mm i.d. x 5 μm, Varian) at a flow rate of 1.5 ml min^{-1}; the injection volume was 20 μl, and detection was made in a spectrum from 280 to 530 nm for the different classes of compounds. The mobile phase consisted of (A) 1% acetic acid in water and (B) acetonitrile (80): water 1% acetic acid (20). The program was as follows: isocratic with solvent (B) for 20 min, linear gradient to 100% (A) for 15 min, 15 min linear gradient to solvent (B). The compounds were identified by comparing the retention times and areas with those of appropriate standards used for the calibration curve.

For the HPLC-analysis of the tocopherols in the T and C, these vegetable parts were suspended in 5 ml of methanol and sonicated (Branson 2800, Danbury, USA) for 20 min, the mixture was then filtered, rinsed with the solvent and evaporated under vacuum. The methanolic fractions were processed by a second extraction with 5 ml of n-hexane and dried under vacuum. HPLC analyses were performed on a Perkin-Elmer PE 200 system (autosampler, binary pump and UV-Vis detector) equipped with a LiChrosorb Si60 (250 mm x 4 mm i.d. x 5 μm column Phenomenex), the mobile phase was 0.05% isopropanol/hexane at a flow rate of 1 ml/min, the wavelength was 295 nm and the injection volume 20 μl. Tocopherols peaks from samples were identified by comparing the retention times with those of authentic reference compounds (tocopherols mix pure standards, Sigma-Aldrich). The compounds were quantified by an external standard method, using a calibration curve. For each extract, quali-quantitative analyses were performed in triplicates.

The lack of a sufficient amount of sample has not allowed to perform these analyses in the embryo.

Analysis of Fatty Acids

In the samples T, C, EA the lipid fraction (from hexane extraction) was subsequently derivatized into fatty acid methyl esters (FAMEs) by mild alkaline methanolysis. FAMEs were extracted twice with 2 ml aliquots of hexane:chloroform (4:1 v/v) and the pooled aliquots were dried under N_2 at room temperature and redissolved in hexane-containing methyl nonadecanoate (C19:0) as internal standard. FAMEs were analyzed by gas chromatography-flame ionization detector (GC-FID) detector (Trace 2000, Thermo-Fisher) equipped with a 100% dimethyl-polysiloxane non-polar column (50 m length, 0.25 mm i.d. and 0.25 mm film thickness, Agilent J&W) and a split/splitless injector (1/10 ratio). The temperatures of the injector and detector were 220 and 250°C, respectively. The oven was temperature-programmed from 60°C (5 min) to 300°C (5 min) at a rate of 25°C/min. The injection volume was 1 μl. Preliminary peak identification was carried out by comparison of retention times with known standards. Relative amounts of given fatty acids were calculated from their respective chromatographic peak areas and the relative percentage of each fatty acid was related to the total peak areas of both saturated and unsaturated fatty acids.

Analysis of Phytosterols

In the samples T, C, EA the hexane extract was saponified with 2 M methanolic potassium hydroxide for 1 h at 70°C in ultrasonic bath, then was added 2 ml H_2O and 3 ml hexane, vortexed and the two phases separated. Sterols were extracted from aqueous phase for three times. The organic phases were collected, added with anhydrous sodium sulphate and evaporated to dryness. Samples dissolved in 50 μl of $CHCl_3$ were derivatized as corresponding trimethylsilyl ether by adding 150 μl of bis (trimethylsilyl)trifluoroacetamide and heated for 1 h at 60°C. The samples were evaporated to dryness under a gentle stream of N_2 and then redissolved in 1 ml of $CHCl_3$. GC-FID (Trace 2000, Thermo-Fisher) analyses were performed with a fused-silica capillary column (30 m length × 0.25 mm i.d. × 0.25 μm film thickness, Agilent J&W). The oven was temperature-programmed from 180°C (3 min) to 280°C (5 min) at a rate of 10°C/min and to 300°C (10 min) at a rate of 10°C/min. The temperatures of the injector and detector were 250 and 280°C, respectively. The sample volume was 1 μl with a split injection (1/20). Preliminary peak identification was carried out by comparison of retention times with known standards and external calibration was carried out.

Statistical Analysis

Data were checked for normality and homoscedasticity; afterwards, they were analyzed by one-way analysis of variance (ANOVA) or T-Student test. Mean separations were performed using the least significant difference (LSD) test at P = 0.05. Analyses were conducted in R environment (R Core Team, 2017).

RESULTS

Cyto-Histological Observations

Cocoa pod was characterized by a thin epicarp, a fleshy mesocarp and the endocarp. In the mesocarp, large cells rich in water were alternated with smaller polyphenolic cells (**Figures 1A–D**), in which vesicles of polyphenolic substances were localized in the periphery of cells; numerous gaps were also observed. In the endocarp, big tubular cells with large intercellular spaces were detected; the periodic acid–Schiff (PAS) and toluidine blue O staining showed the presence of pectins (coloured in magenta)

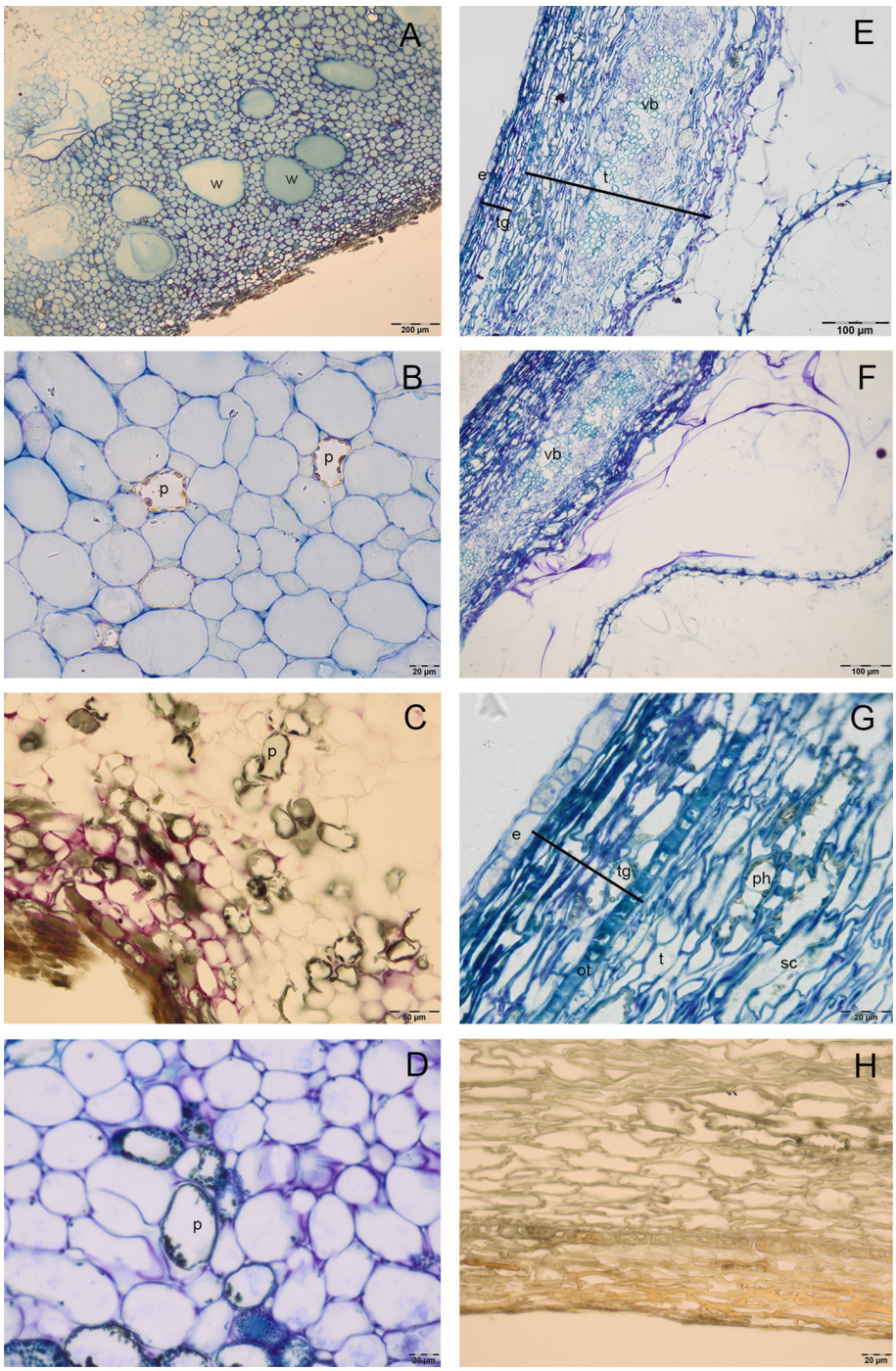

FIGURE 1 | Semithin sections of cocoa fruit and seed. **(A–D)** Fleshy mesocarp of pod, stained with toluidine blue **(A**, **B)**, vanillin **(C)** and toluidine blue O **(D)**, in which large cells rich in water (w) and polyphenolic cells (p) are detected. **(E–F)** Big tubular cells of endocarp with cell walls rich in pectins, which appeared coloured in magenta after toluidine blue O **(E)** and PAS staining **(F)**; seed tegument constituted by testa (t) and tegmen (tg, in the mesophyll of testa it is possible to distinguish numerous vascular bundles (vb). **(G)** Single layered endosperm (e) adheres to seed tegument, in the tegmen we can identify an outer epidermis (ot), composed by sclerified cells (sc) and more layers of crashed cells; polyphenolic substances (ph) are evident in the region of sclerified cells of testa. **(H)** Tegument stained with vanillin: no epicatechin or catechin presence was detected.

in the wall of these cells (**Figures 1E**, **F**). Endocarp cells formed, in ripe seeds, a mucilaginous pulp that adhered tightly to seed coat (**Figures 1E**, **F**). In the seed coat, testa was constituted by an exotesta, a single layer of elongated cells, a mesophyll, in which there were numerous vascular bundles, and lignified stellate cells (**Figures 1F**, **G**). In tegmen it was possible to distinguish the outer

epidermis, composed by one layer of sclereid cells with a thickened wall, many layers of crushed cells and the inner single-layered epidermis. In T polyphenolic substances were evident above all in the region of lignified stellate cells of testa (**Figure 1G**). The secondary endosperm was constituted by a single layer of cells, with lightly thickened outer walls (**Figure 1G**); it adhered to the seed coat and to numerous folding of the cotyledon tissue. In the T (**Figure 1H**) or mesocarp (**Figure 1C**), the vanillin staining did not reveal the presence of catechin, epicatechin or proanthocyanidins.

The C were foliaceous and densely folded near the EA, then became fleshy and darker for the anthocyanins synthesis (**Figures 2A**, **B**); they were formed by a two single-layered epidermis and a mesophyll rich in reserve substances. In the cotyledon mesophyll it was possible to distinguish two kind of cells: big vacuolated cells, containing vesicles of different size, which can be identified as polyphenolic cells; smaller isodiametric cells, rich in cytoplasm (**Figures 2C–F**). Fresh sections showed the presence of anthocyanins in some of the big vacuolated cells, which appeared in some cases also rich in phenolic compounds after staining with toluidine blue O (**Figure 2E**). Vanilline staining demonstrated the localization in some of these cells of catechin and epicatechin (**Figures 2C**, **F**). In the small isodiametric cells, PAS staining demonstrated the presence of starch grains (magenta colour) (**Figure 2D**); in the same cells lipid bodies were also detected (**Figure 2D**).

In the embryo axis, constituted by isodiametric tightly stuck cells arranged in parallel lines, the toluidine blue staining sharpened the presence of 5/6 superficial layers of polyphenolic

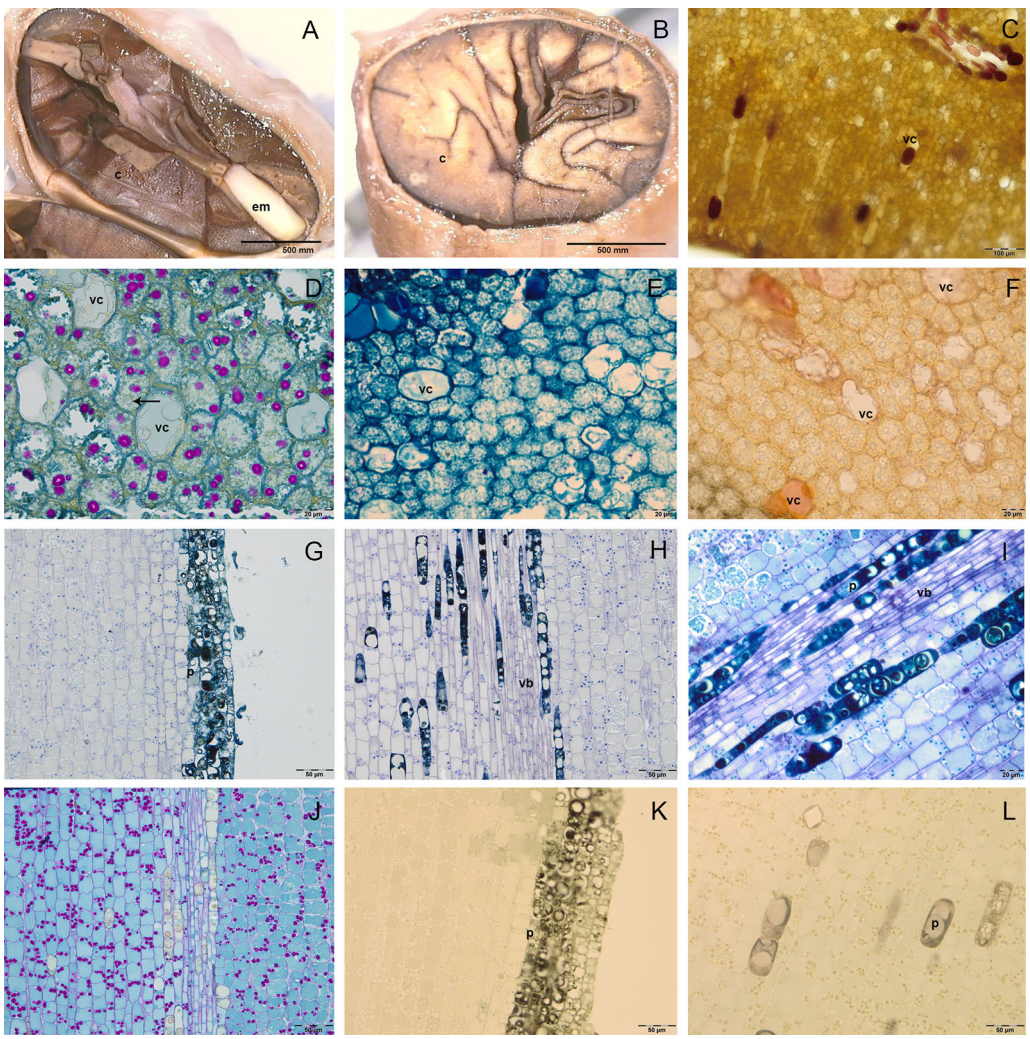

FIGURE 2 | Semithin **(D–L)** and fresh **(A–C)** sections of cocoa fruit and seed. **(A–B)** Fresh section of cocoa seeds: embryo axis (em) and brown cotyledons (c) are evident; cotyledons are foliaceous near the embryo axis and then become fleshy and densely folded. **(C)** Fresh section of cotyledons with big vacuolated cells (vc) rich in anthocyanins. **(D–F)** Semi-thin sections of cotyledons stained with periodic acid–Schiff (PAS) **(D)**, toluidine blue O **(E)**, vanillin **(F)**; in cotyledons mesophyll, big vacuolated cells (vc) appeared rich in epicatechin after vanillin staining **(F)**, whilst small cells showed lipid drops (black arrows) and starch grains coloured in magenta after PAS staining. **(G–L)** Semi-thin sections of embryo axis, stained with toluidine blue **(G**, **H)**, toluidine blue O **(I)**, PAS **(J)**, vanillin **(K**, **L)**; polyphenolic cells (p) are evident in the superficial layers **(G)** and near the vascular bundles (vb) **(H**, **I)**; the presence of starch grains (magenta colour) is evident **(J)**; no catechin or epicatechin were detected **(K**, **L)**.

cells (**Figure 2G**), which were observed also near to vascular bundles located in the center of embryo axis (**Figure 2H**). The polyphenolic content of these cells was confirmed also by staining with toluidine blue O (**Figure 2I**). In the same portions, PAS staining demonstrated also the presence of starch grains (**Figure 2J**). No catechin or epicatechin were detected through vanillin staining (**Figures 2K, L**).

Phytochemical Analysis

The distribution of polyphenols, tocopherols, fatty acids and sterols were also evaluated in the different seed parts with the aim to better characterize them and to confirm the histological data. Particularly relevant was the significantly highest abundance of total polyphenols in the T, data confirmed by the results of the antioxidant activity (TEAC value) of the different cocoa extracts (**Figure 3**). The content of polyphenols and TEAC were significantly lower in S (seed without mucilage) than in the SM (seed with mucilage) sample, showing that the mucilage contributes greatly to the content of these phytochemicals and to the antioxidant activity. The individual phenolic content was investigated in the samples T, C, S, SM. As shown in **Table 1**, whole seed samples, S and SM, were qualitatively richer in phenolic compounds than the other isolated seed components. Moreover, in S and SM samples there was a significantly higher abundance of chlorogenic acid, hydroxycinnamic, vanillic and ferulic acid.

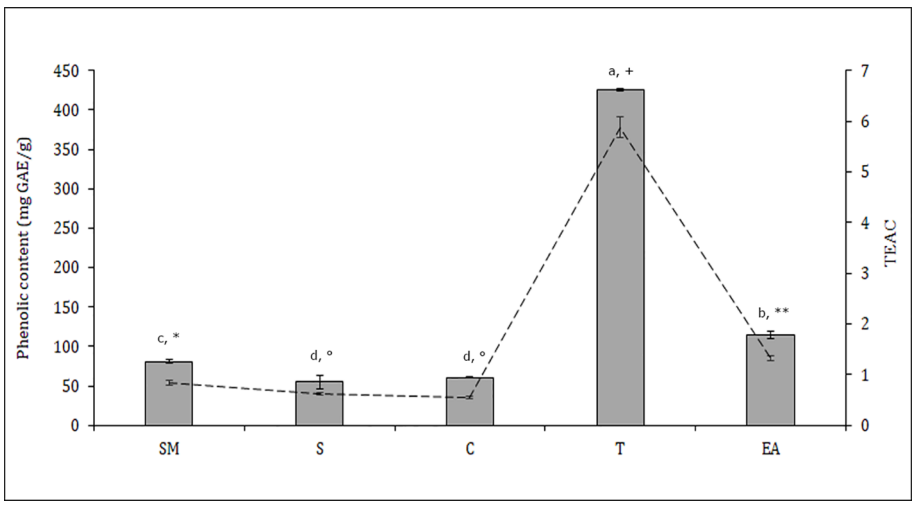

FIGURE 3 | Total phenolic content (bars) and antioxidant activity Trolox equivalent antioxidant capacity (TEAC) (dashed line) in different *Theobroma cacao* seed samples; T, tegument; C, cotyledons; SM, whole seed; S, seed without mucilage; EA, embryo axis (see *Materials and Methods*). The values represent means ± SD of three replicates. Different letters and symbols indicate statistically significant differences according to LSD test ($p \leq 0.05$) for phenolic content and TEAC, respectively.

TABLE 1 | Phenolic compounds content in different *Theobroma cacao* seed components.

Phenolic compound	T	C	SM	S
Chlorogenic acid	n.d.	8.3 ± 0.80 ^{f,†}	10.52 ± 0.84 ^{f,‡}	11.32 ± 0.63 $^{d,\bullet}$
Ferulic acid	n.d.	12.45 ± 1.26 ^{c,†}	22.62 ± 1.65 $^{c,\bullet}$	18.51 ± 1.51 ^{b,‡}
Caffeic acid	18.7 ± 0.94 $^{a,\bullet}$	8.92 ± 0.58 ^{e,†}	13.45 ± 2.54 ^{e,‡}	4.51 ± 0.64 ^{g,*}
p-Cumaric acid	10.8 ± 1.84 ^{b,‡}	7.54 ± $^{1.2\ g,\dagger}$	14.52 ± 1.64 $^{d,\bullet}$	10.21 ± 2.15 ^{e,‡}
p-Hydroxycinnamic acid	n.d.	13.07 ± 1.5 ^{b,†}	23.41 ± 3.54 $^{b,\bullet}$	13.71 ± 1.82 ^{c,‡}
p-Hydroxybenzoic acid	n.d.	10.41 ± 2.1 $^{d,\bullet}$	0.65 ± 0.17 ^{k,†}	2.61 ± 0.50 ^{i,‡}
Syringic acid	6.21 ± 0.58 $^{c,\bullet}$	n.d.	4.25 ± 0.08 ^{g,‡}	3.43 ± 0.20 ^{h,†}
Vanillic acid	3.04 ± 0.06 ^{d,†}	n.d.	3.65 ± 1.24 ^{h,‡}	6.14 ± 0.42 $^{f,\bullet}$
Protocathetic acid	2.61 ± 0.05 $^{d,\bullet}$	1.8 ± 0.04 ^{†,h}	2.85 ± 0.10 $^{i,\bullet}$	2.16 ± 0.40 ^{j,‡}
Catechin	n.d.	0.61 ± 0.02 ^{i,†}	1.21 ± 0.06 $^{j,\bullet}$	0.96 ± 0.08 ^{k,‡}
Epicatechin	n.d.	34.25 ± 1.34 $^{a,\bullet}$	32.15 ± 2.54 ^{a,†}	33.4 ± 0.95 ^{a,‡}
Quercetin	n.d.	n.d.	n.d.	n.d.

T, tegument; C, cotyledons; SM, whole seed; S, seed without mucilage (see Materials and Methods). Results are expressed as mean milligram/gram sample ± SD of three replicates. In each column, different letters indicate statistically significant differences according to least significant difference (LSD) test ($p \leq 0.05$); in each row, different symbols indicate statistically significant differences according to LSD test ($p \leq 0.05$). n.d., not detected.

Caffeic and syringic acids were, instead, more abundant in the T; caffeic acid together with p-cumaric and protocathetic acid were the only components present in all the analyzed samples. Catechin and epicatechin were not detected in the T but present in C, SM and S; particularly epicatechin was the most abundant compound isolated in S, SM and C sample (e.g., 34% of the total phenols in C). Quercitin was not detected in any analyzed samples. The total tocopherols content was analyzed in the single components of seed, T and C samples; it was higher in C with respect to the T (1.84 ± 0.07 *vs.* 1.43 ± 0.04 mg/g). The distribution of the single isomers can be different in these two parts of the seed; this is true for α, β and δ-tocopherol but not for γ-tocopherol (**Figure 4**); indeed β and δ-tocopherol were higher in C whilst the T was richer in α-tocopherol.

The phytosterols characterization of seed parts showed a qualitative homogenous distribution with a quantitative predominance of β-sitosterol in all the three seed components (T, C, EA) with respect to the others (**Table 2**). In our samples the concentrations of the single compounds were significantly higher in T in comparison to the other seed parts, except for β-sitosterol and Δ5-avenasterol that were detected in significantly larger amount in the embryo-axis (**Table 2**).

The fatty acids profiles showed a homogeneous content of saturated (SFA; palmitic, stearic and arachidic) and unsaturated (UFA, palmitoleic, oleic, linoleic and linolenic) fatty acids among the different seed components (**Table 3**). The oleic acid C18:1 was, in all samples, the most representative unsaturated fatty acid, whilst stearic was the most abundant SFA. The SFA content were always higher than UFA; the ratio S/U (saturated *vs.* unsaturated) was in a range of 1.46–1.67.

DISCUSSION

Recently, the interest about cocoa has increased due to the demonstrated benefit activity of cocoa extracts; they showed cardioprotective effects but also seemed to reduce inflammations and cholesterol levels (Engler and Engler, 2004; Cooper et al., 2008; Tokede et al., 2011). The composition of these extracts has been investigated during the different phases of seed development, but few information is yet available about their distribution in different fruit and seed components.

Our data outlined that antioxidant molecules, as tocopherols and polyphenols, were differentially distributed in cocoa fruit

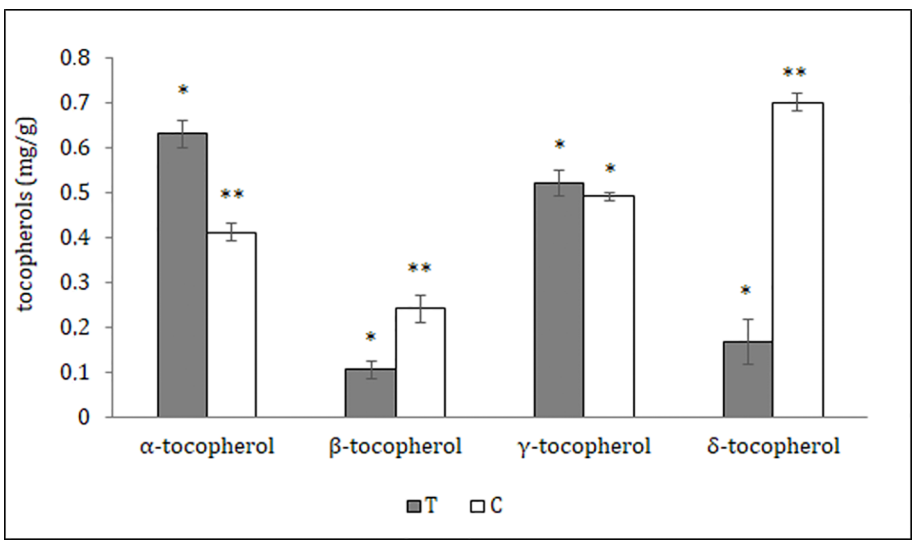

FIGURE 4 | Tocopherols content of different *Theobroma cacao* seed parts; T, tegument; C, cotyledons (see *Materials and Methods*). The results are expressed in milligram/gram sample ± SD of three replicates. Different symbols indicate statistically significant differences according to least significant difference test ($p \leq 0.05$).

TABLE 2 | Sterols content in different *Theobroma cacao* seed components.

Sample	Campesterol	Campestanol	Stigmasterol	β-Sitosterol	Δ5-Avenasterol
T	11.2 ± 1.2 $^{a,\square}$	15.8 ± 0.6 ^{a,*}	18.6 ± 2.6 ^{a,†}	62.3 ± 3.5 $^{b,\bullet}$	29.6 ± 0.5 ^{b,‡}
C	5.62 ± 0.8 $^{b,\square}$	8.6 ± 1.1 ^{c,*}	16.2 ± 0.6 ^{b,†}	52.3 ± 5.1 $^{c,\bullet}$	23.6 ± 3.5 ^{c,‡}
EA	1.36 ± 0.3 $^{c,\square}$	12.1 ± 1.8 ^{b,†}	8.6 ± 1.7 ^{c,*}	72.3 ± 6.42 $^{a,\bullet}$	42.3 ± 1.4 ^{a,‡}

T, tegument; C, cotyledons; EA, embryo axis (see Materials and Results are expressed as mean milligram/gram sample ± SD of three replicates. In each column, different letters indicate statistically significant differences according to least significant difference (LSD) test ($p \leq 0.05$); in each row, different symbols indicate statistically significant differences according to LSD test ($p \leq 0.05$).

TABLE 3 | Fatty acids content in different *Theobroma cacao* seed components.

Sample	Palmitic acid C16:0	Palmitoleic acid C16:1	Stearic acid C18:0	Oleic acid C18:1	Linoleic acid C18:2	Linolenic acid C18:3	Arachidic acid C20:0
T	19.62 ± 0.6 ^{b,†}	9.52 ± 0.8 $^{b,\square}$	49.56 ± 1.6 $^{b,\bullet}$	41.92 ± 1.2 ^{b,‡}	1.27 ± 0.2 $^{a,\blacklozenge}$	0.23 ± 0.05 $^{a,\blacklozenge}$	13.03 ± 0.2 ^{a,*}
C	22.21 ± 1.2 ^{a,†}	5.23 ± 0.7 $^{c,\square}$	43.56 ± 2.1 ^{c,‡}	46.95 ± 2.7 $^{a,\bullet}$	0.36 ± 0.02 $^{b,\blacklozenge}$	0.28 ± 0.02 $^{a,\blacklozenge}$	11.23 ± 0.4 $^{*\ a,}$
EA	24.68 ± 0.9 ^{a,†}	13.55 ± 1.8 ^{a,*}	52.36 ± 1.8 a$^{\bullet}$	37.9 ± 1.8 ^{c,‡}	1.06 ± 0.03 $^{a,\blacklozenge}$	0.38 ± 0.08 $^{a,\blacklozenge}$	11.23 ± 1.2 $^{a,\square}$

T, tegument; C, cotyledons; EA, embryo axis (see Materials and Methods). Results are expressed as relative percentage ± SD of three replicates. In each column, different letters indicate statistically significant differences according to least significant difference (LSD) test ($p \leq 0.05$); in each row, different symbols indicate statistically significant differences according to LSD test ($p \leq 0.05$).

portions [i.e., pulp or mucilage (endocarp), T and C]. The highest polyphenols diversity was observed in the whole seed (with and without mucilage), and a characteristic polyphenol pattern was observed in different portion of the seed. Caffeic acid and p-cumaric acid are involved in lignin synthesis, so the higher amounts of these phenols detected in T respect to C can be probably related to a high presence of cells with lignified walls. In C phenolic compounds were located in big vacuolated polyphenolic cells; in some of these cells the presence of anthocyanins and epicatechin, was also highlighted vanillin staining confirmed the absence of epicatechin and catechin in the T, indeed, as evident in phytochemical analysis, epicatechin is the predominant one in C and absent in the T. Catechin and epicatechin are known to be the most abundant flavonoid compounds in cocoa powder; they play a role in plant immunity, growth regulation and support nutrient uptake and photosynthesis (Natsume et al., 2002; Jalil and Ismail, 2008; Prakash et al., 2019).

The presence of phenolic compounds in pulp was demonstrated by Endraiyani et al. (2016), which outlined the thermolability of these substances, but our cytological observations did not confirm that: the osmium and toluidine blue staining did not highlight phenols in the pulp tissue, probably because they are dissolved in the big vacuoles. Polyphenolic substances were, instead, observed in periphery cells of mesocarp, where they appeared reorganized in round vesicles, probably due to fixing in glutaraldehyde (Martini et al., 2008). They were also well evident in the embryo axis, where they localized in the external layers and near the vascular tissues. Even if also non-phenolic substances can be responsible for the antioxidant activity, phenolic content could be used as an indicator of antioxidant activity (TEAC), which was the highest in the T. In T, polyphenolic cells were evident in the testa but the histological localization of few polyphenolic cells in our samples did not seem to justify the high presence detected by chemical analysis. Moreover, TEAC was higher in SM than in S: this is probably connected to a higher presence of ferulic acid, caffeic acid, cumaric acid and syringic acid in the seed with mucilage. Other important components of cocoa beans are represented by fatty acids and phytosterols; the content of saturated and unsaturated fatty acids, bound in triglycerides, influence cocoa butter hardness and consequently its commercial value.

Among the analyzed fatty acids, the stearic and oleic acids were the most abundant in all the seed components (T, C and embryo axis). In particular the content of stearic acid is higher in embryos with respect the other seed parts whereas the amount of oleic acid is higher in C. Stearic acid is particularly appreciated, as it did not seem to increase the level of total cholesterol and low-density lipoprotein (LDL)-cholesterol in serum unlike the other saturated acid (Yu et al., 1995). In embryos the C16:0 and C18:0 were the predominant SFA, and this represent an advantage because the yield of ATP molecules during complete oxidation is higher than UFA.

Fatty acids, either free or as part of complex lipids, are important constituent of membranes or other cellular structures but also represent one of the storage substances of cocoa seed probably localized in lipid globules. In cocoa, as observed by Bayés-García et al. (2019), lipid droplets or lipid globules represent almost the 50% (w/w) in fresh cacao beans and serve as important reservoirs of lipids, but also as substrates for multiple cellular processes; in our observations they occupied almost the entire volume of small isodiametric cells of cotyledon mesophyll.

The cocoa seeds were also rich in plant sterols, which had protective effects on the oxidation of lipids as result of synergistic interactions with tocopherols (Caporaso et al., 2018); they also regulate the fluidity and permeability of plant cell membranes, in a similar manner as cholesterol in mammalian. Moreover, these compounds have a beneficial effect on the human health as they seemed to contribute to reduce the level of LDL cholesterol in blood serum (Wollgast and Anklam, 2000; Andújar et al., 2012). In cocoa seeds, we observed a different distribution of the analyzed sterols: β-sitosterol and Δ5-avenasterol were the most abundant, above all in the embryo axis; stigmasterol and campesterol were less present in EA and more abundant in T; campestanol level was again higher in T but lower in C. The specific localization of different kind of sterols was related probably with a peculiar function. Stigmasterol, for instance, is generally not involved in the regulation of membrane characteristics, but in cell proliferation and proton pumping (Hartmann, 1998). Tocopherol also showed a differential distribution in T and C. Content, composition and presence of tocopherols varies widely in different plant tissues, moreover, both plant growth and development affect the levels of tocopherol content and

composition, which changes for example during senescence, chloroplast to chromoplast conversion, fruit ripening and seed development (Arrom and Munné-Bosch, 2010; Falk and Munne-Bosh, 2010).

CONCLUSIONS

This study provides for the first time a whole picture of the distribution of many important compounds in cocoa, as until now only the localization of single chemicals in specific seed structures had been investigated (Dangou et al., 2002; Martini et al., 2008; Elwers et al., 2010). New information about cyto-histological characteristics of cocoa seeds were also reported, as the storage role of big vacuolated cells present in C tissue. These cells can accumulate different compounds; anthocyanins and epicatechins were for example detected only in some of them.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

LR conceived the work. MC and LR performed microscopy analyses. CZ performed chemical analyses. MC performed statistical analyses. LR drafted the manuscript and MC and CZ critically revised the draft. All the authors approved the version of the manuscript to be published.

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REVIEW
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Primary Metabolism in Fresh Fruits During Storage

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The extension of commercial life and the reduction of postharvest losses of perishable fruits is mainly based on storage at low temperatures alone or in combination with modified atmospheres (MAs) and controlled atmospheres (CAs), directed primarily at reducing their overall metabolism thus delaying ripening and senescence. Fruits react to postharvest conditions with desirable changes if appropriate protocols are applied, but otherwise can develop negative and unacceptable traits due to the onset of physiological disorders. Extended cold storage periods and/or inappropriate temperatures can result in development of chilling injuries (CIs). The etiology, incidence, and severity of such symptoms vary even within cultivars of the same species, indicating the genotype significance. Carbohydrates and amino acids have protective/regulating roles in CI development. MA/CA storage protocols involve storage under hypoxic conditions and high carbon dioxide concentrations that can maximize quality over extended storage periods but are also affected by the cultivar, exposure time, and storage temperatures. Pyruvate metabolism is highly reactive to changes in oxygen concentration and is greatly affected by the shift from aerobic to anaerobic metabolism. Ethylene-induced changes in fruits can also have deleterious effects under cold storage and MA/CA conditions, affecting susceptibility to chilling and carbon dioxide injuries. The availability of the inhibitor of ethylene perception 1-methylcyclopropene (1-MCP) has not only resulted in development of a new technology but has also been used to increase understanding of the role of ethylene in ripening of both non-climacteric and climacteric fruits. Temperature, MA/CA, and 1-MCP alter fruit physiology and biochemistry, resulting in compositional changes in carbon- and nitrogen-related metabolisms and compounds. Successful application of these storage technologies to fruits must consider their effects on the metabolism of carbohydrates, organic acids, amino acids and lipids.

Keywords: low temperature, controlled atmospheres, modified atmospheres, hypoxia, ethylene, 1-methylcyclopropene, fruit composition, post-harvest

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INTRODUCTION

Harvested fruits are treated with a range of postharvest technologies to mantain quality by delaying ripening and senescence, preventing the incidence of physiological and pathological disorders, and avoiding water loss and physical damage. The main pillar of fruit storage is the use of refrigeration. Temperate fruit crops are commonly stored at temperatures close to freezing (0–1°C), while those of tropical or subtropical origin must be kept at higher storage temperatures (7–15°C) to avoid losses due to the development of chilling injury (CI) symptoms. These symptoms can be manifested as altered ripening behavior and external (peel) and/or internal (flesh) damages (browning, pitting, breakdown, discoloration), being more evident after subsequent removal from cold storage and maintenance at room temperarure. Appropriate storage temperatures can extend storage life by about 2–4 weeks for crops such as apricots, sweet cherries, and peaches, and up to several months for apples, pears, and kiwifruits.

Storage protocols that reduce oxygen and/or increase carbon dioxide concentrations in combination with low temperature are mainly used for fruits such as apples, pears, and kiwifruits. Similar conditions are also applied with modified atmosphere (MA) storage, usually applied as MA packaging (MAP) for minimally processed (fresh cut) products and to a lesser extent as whole fruits. For controlled atmosphere (CA) storage, the atmosphere composition is strictly monitored and adjusted in gas tight rooms by control systems, whereas in MA the changes in oxygen and carbon dioxide concentrations within the package are a function of factors such as the respiration rate of the produce as affected by cultivar, ripening stage, weight, and temperature in combination with packaging film characteristics. Optimum CA/MA storage regimes for different fruit types have been mainly developed empirically based on their quality after storage.

The main effects of low temperature and the CA/MA storage alone or in combination are associated with respiration, ethylene biosynthesis and its action, and other metabolic processes, thereby decreasing the rates of change that occur during post-harvest ripening, including color (chlorophylls, carotenoids, and flavonoids), texture (softening as a result of cell wall disassembly and reduced cell turgor), and flavor (taste and aroma as a result of starch degradation, sugar-acid metabolism, and synthesis of aromatic volatiles). These effects can apply regardless of whether the fruit is non-climacteric or climacteric, but for the latter fruit types are important for reduction of ethylene production. Ethylene has a key physiological role during the ripening process, a genetically regulated stage of development of climacteric fruits that is highly complex and coordinated by hormonal metabolism. In addition to physical methods, a chemical antagonist of ethylene, 1-methylcyclopropene (1-MCP), is used on specific fruit types. 1-MCP effects vary depending on the species, cultivar, maturity and ripening stage, and factors such as 1-MCP concentration, treatment duration and temperature, and post-treatment storage conditions. It is commercially applied to several commercially important fleshy fruits, such as apples and pears

Storage technologies have profound effects on primary metabolism with marked consequences on the composition and the overall flavor of the commodity and, hence, the commercial life and the consumer acceptance. The objective of this review is to provide a perspective of these effects on the metabolism of carbohydrates, organic acids, amino acids, and lipids in fruits.

LOW-TEMPERATURE STORAGE

Low temperature is the main method used to extend storage and market life of fruits. By reducing general metabolism, cold storage protocols delay ripening and senescence processes through the control of the respiration rate. Low temperature is effective in decreasing the catalytic activities of different enzymes, including those involved in the different steps of respiration. The changes induced by temperature in a biological system are measured by using the Q10 temperature coefficient. This coefficient indicates the rate of reactions as a result of a temperature increase of 10°C (Atkin and Tjoelker, 2003). For most of harvested fruit crops, within a temperature range of 5–25°C, Q10 values associated with respiration are, in general, between 2.0 and 3.0. This indicates that when lowering storage temperature from 20 to 10°C the respiration is decreased by a factor of 2–3, with positive consequences in terms of shelf/commercial life of the produce. The optimum low-temperature and storage duration are highly variable based on the fruit type and are applied keeping in mind two principles: (1) low temperatures slow down general metabolism and reduce the rate of compositional changes; and (2) temperatures lower than a specific threshold and/or a prolonged cold storage induce physiological disorders. For the non-chilling sensitive fruit types, the storage temperatures are set just above their freezing point. Other types, e.g., tropical or sub-tropical fruits, must be stored at higher temperatures, typically between 7 and 15°C. Factors such as cooling rates and precooling techniques also play a key role in determining the success of cold storage (Valero and Serrano, 2010). As a general effect, low-temperature storage upregulates stress-responsive genes, blocks signal transduction of ethylene-related processes, and affects both primary and secondary metabolism (Yun et al., 2012; Lin et al., 2018). Changes at the level of secondary metabolism during low-temperature storage have been extensively studied, mostly focusing on how cell wall alterations affect abnormal ripening (Brummell et al., 2004). Despite the well-documented CI-induced activation of genes that are involved in different metabolic pathways (Gonzalez-Aguero et al., 2011), information about the effects of cold storage on fruit primary metabolism remains limited.

Carbohydrate Metabolism

Carbohydrates influence the sensitivity of plant tissues and organs, including fruits, to low temperature. Besides being an important fruit component markedly impacting the overall flavor, soluble carbohydrates may have several beneficial effects

in protecting plants against stresses, including cold storage regime, and the relationship between CIs and carbohydrate metabolism was investigated in an array of fruit types. Special focus has been addressed to commercially important fruits, such as apples, pears, kiwifruits, and bananas that, at physiological maturity, are characterized by high starch contents that are converted to sugars during storage. An emerging research area is the impact of carbohydrate composition and contents on the sensitivity of fruits to low temperatures, although results are inconsistent (Zhao H. et al., 2019). Furthermore, over the recent years, a number of studies reported the effects of applications of chemicals on CI development in fruits in relation to sugar metabolism and/or changes in carbohydrate content. Excellent examples of sugar metabolism during cold storage of two distinct fruit types (peach, mandarin), which are characterized by abnormal ripening after cold storage, are available.

Carbohydrate metabolism has been extensively studied in cold-stored peaches with differential responses of individual sugars, mainly associated with CI symptoms, evident as browning. Enhanced chilling tolerance in peaches has been associated with higher sucrose contents, resulting from the balance between its degradation and biosynthesis, which may contribute to membrane stability (Wang et al., 2013). Brizzolara et al. (2018) found that "Red Haven" peaches had higher contents of sucrose and sugar alcohols such as sorbitol and maltitol after cold storage and reduced susceptibility to CI compared with "Flaminia" and "Regina di Londa" fruits. Induction of chilling tolerance of nectarines stored at near freezing temperatures (−1.4 °C) has been associated to reduced activities of sucrose metabolism-associated enzymes that resulted in higher sucrose contents (Zhao H. et al., 2019). Sound peaches were characterized by higher activities of hexokinase, fructokinase, and energy metabolism-associated enzymes and higher content of sucrose and lower contents of fructose and glucose were associated with reduced CI induced by glycine betaine treatment (Wang L. et al., 2019).

In citrus, the carbohydrate changes occurring in the flavedo along with fruit maturation do not appear to be related to the chilling tolerance in cold stored "Fortune" mandarins (Holland et al., 1999). Pre-storage heat conditioning (3 days at 37°C) enhanced chilling tolerance of the fruits, favoring sucrose, but not hexose, accumulation. This effect was attributed to heat-induced increase in the activities of sucrose-synthesizing enzymes, such as SPS and SuSy (Holland et al., 2005). Heat treatment limited the decline in sucrose content of flavedo tissue during cold storage, evidenced by the substantially higher amounts of sugars in heat-conditioned compared with non-conditioned fruits (Holland et al., 2002). Heat conditioning led to loss of glucose, fructose, and starch in fruit kept subsequently at 2°C, suggesting that only sucrose is actively involved in the heat-induced chilling tolerance of citrus fruits.

Collectively, the role of sugar synthesis during cold storage on the incidence of CI symptoms should be evaluated on a species basis and also based on the nature and symptomology of each chilling related disorder. Comparative studies can be convincing, but it is not always certain that the variation among cultivars is smaller than variations between chilling-tolerant and chilling-sensitive cultivars; simple comparisons of two cultivar responses should be discouraged.

Organic Acid Metabolism

There is increasing evidence that organic acids act not only as intermediates in carbon metabolism but also as key components in response to environmental stress factors (Lopez-Bucio et al., 2000). In most fruits, the organic acid pool decreases during ripening, and low temperature storage limits the rate of titratable acidity (TA) loss: this is imputed to the reduced metabolism, in particular respiration. In different kiwifruit cultivars, Cha et al. (2019) reported significantly lower TA values in fruit kept at 18° C compared to 10 and 5°C storage.

Considering the fate of specific organic acids during cold storage, a genotype-dependent behavior is present. Bustamante et al. (2016) showed that six different peach genotypes displayed a decrease in 2-oxoglutarate (2-OG) and succinate contents during refrigerated storage. Decreases of malic and quinic acid contents were observed in myrtle fruits stored at 2 and 10°C (Angioni et al., 2011; Mulas et al., 2013), indicating a possible shift of metabolic activity toward the biosynthesis of secondary metabolites such as anthocyanins. An increasing number of reports highlight the link between postharvest treatments performed prior to cold storage and organic acid metabolism. Zhou et al. (2019) demonstrated that UV-treated peaches stored at 1°C were characterized by a down-regulation of aconitase and NADP-malic enzyme activities and gene expression levels, but higher levels of citrate synthase and NAD-malate dehydrogenase, resulting in a reduced degradation of citric and malic acids. Interestingly, pre-cold storage hot air treatment (40°C, 48 h) in ponkan orange promoted citric acid degradation, attributed to regulation by ATP citrate lyase (ACL) and γ-aminobutyric acid (GABA) pathways (Gao et al., 2018).

Amino Acid Metabolism

Amino acid metabolic responses of fruits to cold storage are species- and storage condition-specific. For example, Micro-Tom tomatoes stored at 4°C resulted in a rise in Glu, Gln, Asp, and Asn contents (Gonzalez et al., 2019). Cold storage also increased endogenous substrate proteolysis, azocaseinolytic activity, and free amino acid contents, but their specific roles have not been elucidated (Re et al., 2012). Similar approaches have been employed for other species such as kiwifruit, where cold storage resulted in increased Thr, Ile, and Val contents, but not of Gln and Asn (Salzano et al., 2019). In peaches, the beneficial effect of heat pre-conditioning to alleviate CI symptoms was linked with the modification of metabolites, including sugars, polyamines, and amino acid precursors of the phenylpropanoid pathway (Lauxmann et al., 2014). Bustamante et al. (2016) reported that cold storage of six peach cultivars at 0°C resulted in increased GABA, Asp, and Phe contents, and a genotype-dependent tolerance of peach cultivars to CIs was associated with higher amino acid contents (Brizzolara et al., 2018).

Lipid Metabolism

Fatty acids are essential cell membrane components, constituting a selectively permeable barrier which represents an accessible fluid medium for lipophilic molecules/complexes and a matrix for enzymes that catalyze different metabolic reactions. Stress conditions alter membrane lipids, especially their level of unsaturation, altering membrane functioning, leading to ion leakage and cellular decompartmentalization (Marangoni et al., 1996). However, information about lipid metabolism of fruit during refrigerated storage is limited.

In peaches, the genotype affects the relative amounts of plastidic glycerolipid and triacylglyceride forms, possibly indicating their use as a source of energy during fruit senescence (Bustamante et al., 2018). Posphatidylethanolamine, phosphatidylcholine (PC), and digalactosyldiacylglycerol (DGDG) contents are possible markers of cold tolerance, given the important role played by the membranes in the development of CI symptoms. Wang Y. et al. (2019) found increased DGDG contents in blueberries after 30 and 60 days of storage at 0°C. Other lipids such as phosphatidic acid (PA) are accumulated in pineapple fruit during blackheart development at 10°C (Zhou et al., 2014). Accumulation of PA could be linked with previously observed increased activity of phospholipase D (PLD) in cold-stored fruits, as PLD hydrolyzes structural phospholipids such as PC to PA and a free-head group such as soluble choline (Wang, 1999). Sheng et al. (2016) and Shi et al. (2018) also measured increased PLD enzyme activities and transcript levels in cold-stored pears. Similarly, chilling injured "Honeycrisp" apples with soggy breakdown had elevated contents of glycerol and TAGs (Leisso et al., 2015).

CA AND MA STORAGE

An extensive amount of information is available regarding the responses of different fruit types to reduced oxygen and elevated carbon dioxide concentrations (Yahia, 2009; Gross et al., 2016; Thompson et al., 2018). At commercial level, CA storage is widely applied to extend storage potential of commercially important commodities such as apples, pears, and kiwifruits.

For apples, traditional CA storage regimes (oxygen concentrations above 1 kPa) is being replaced by use of ultra-low oxygen (ULO) concentrations (<1 kPa). Dynamic CA (DCA) allows use of much lower oxygen concentrations. Measurement of physiological/biochemical responses of fruit to low oxygen by chlorophyll fluorescence (Prange et al., 2005), ethanol production (Gasser et al., 2008) and the respiratory quotient (RQ) (Bessemans et al., 2016) is allowing storage closer to the anaerobic compensation point (ACP).

The effects of low-oxygen storage depend on the intensity and duration of the applied stress on fruit metabolism. The fruit type, cultivar, maturity and ripening stage, and pre-harvest conditions are also important factors affecting behavior of harvested fruits. Most of the fundamental information available concerning the primary metabolic changes in CA-stored fruits (mainly apples and, to a lesser extent, pears) derives from studies where both oxygen and carbon dioxide concentrations were altered. Therefore, it is not always easy to discriminate between the effects of the two conditions, especially where synergistic or additive effects occur, e.g. the responses of specific components and reactions of the tricarboxylic acid-cycle, and the fermentation products (acetaldehyde, ethanol, and ethyl acetate) (Kanellis et al., 2009).

Carbohydrate Metabolism

A major effect of low oxygen concentrations in fruit responses is the switch from the aerobic to anaerobic metabolism to compensate for energy deficits. Severe oxygen deficiency reduces mitochondrial respiration resulting in limited ATP availability for energy-demanding processes. Fruit metabolism responds to this energy crisis by increasing substrate level ATP production through different processes, including the catabolism of soluble sugars and, in some species, of starch. For example apples, during the advanced developmental stages, accumulate starch that decreases during CA storage (Gorin et al., 1978). In rice seed hypoxia induces the up-regulation of β-*amylase* genes that are activated to satisfy the increased carbon demand by glycolysis (Planchet et al., 2017). β-*Amylase* is also induced by low oxygen in apples (Cukrov et al., 2016). Futile cycles involving sucrose/hexose interconversion are considered the main mechanisms producing the specific sugar types in fruit tissue in normoxia (Nguyen-Quoc and Foyer, 2001). Low oxygen concentrations affect sugar-metabolism-related enzyme activities and gene expression in fruits. However, contents and the fate of simple sugars (glucose, fructose) and sucrose in fruit under CA conditions are still not yet fully clarified. Different responses may be a function of different oxygen and carbon dioxide concentrations, temperatures, and storage durations as well as effect of genotype. The decline of sucrose contents after harvest is slower in CAs than in air for apples (Zhu et al., 2013) and peaches (Lara et al., 2011), and associated with lower invertase but higher SuSy activities (Zeng et al., 1999; Geigenberger, 2003). *SuSy* gene expression is highly induced in apples under hypoxic conditions, as also observed in the model species *Arabidopsis* (Mustroph et al., 2010), representing a key responsive gene to hypoxia in fruit tissues (Cukrov et al., 2016). *SuSy* induction has been related to the activation of alternative pathways that use inorganic pyrophosphate (PPi) instead of ATP phosphorylation reactions to compensate for severe ATP deficiency. In apples kept from 0.4 to 3.0 kPa oxygen, fructose and glucose do not show significant changes within 30–60 days of storage, probably due to similar rates of consumption and synthesis (Bekele et al., 2016; Cukrov et al., 2016). To meet the energy demand under low oxygen concentrations, an increase in carbohydrate flux through glycolysis occurs with the conversion of glucose-6-phosphate to pyruvate (Pasteur effect). The oxygen concentrations activating the responses leading to the Pasteur effect vary depending on fruit type and the ACP. The conversion of fructose-6-phosphate to fructose-1,6-biphosphate is catalyzed by phospho-fructo kinase (PFK) that is rapidly induced under hypoxia in apples. In "Granny Smith" apples, PFK is highly responsive to subtle changes in oxygen concentration (Cukrov et al., 2016), and, similarly to SuSy, uses PPi instead of ATP to

save energy (Bailey-Serres et al., 2012). PFK most likely represents a key element for re-setting carbon metabolism under oxygen deficiency through an induction of the glycolytic pathway in rice (Mustroph et al., 2013). Both PFK and pyruvate kinase (PK) are highly and rapidly responsive to subtle changes of oxygen concentrations in apple tissues (Brizzolara et al., 2019). $NADP^+$ and NAD^+ must be regenerated to maintain the glycolytic flux and the concentration of pyruvate, which cannot be used in Krebs cycle in the absence of oxygen, must remain low (Bailey-Serres et al., 2012; António et al., 2016). To decrease its concentration and further produce ATP, pyruvate is redirected toward the fermentation pathways that produce lactate, acetaldehyde and ethanol with, at the same time, the maintenance of the redox balance in the cell by the formation of NAD^+.

Ethanol production/accumulation occurs in apples stored under both static and DCA (Saquet and Streif, 2008; Cukrov et al., 2016; Brizzolara et al., 2017), and also in relation to the onset of low oxygen-related physiological disorders (Vandendriessche et al., 2013; Lumpkin et al., 2014). The metabolic responses including the activation of the fermentation pathway and the accumulation of related metabolites in apples stored under low oxygen/high carbon dioxide concentrations is highly variable depending on a number of factors, including the genotype, climatic conditions and agronomic practices, pre-treatments before storage, and CA protocols (Tonutti, 2015; Zanella and Sturz, 2015; Boeckx et al., 2019). "Granny Smith" and "Red Delicious" apples have different ACPs and accumulation patterns of ethanol, acetaldehyde, and lactate under static and DCA conditions; "Red Delicious" accumulates fermentative metabolites at higher oxygen concentrations (0.9 kPa) than "Granny Smith," highlighting significant genotype differences (Brizzolara et al., 2017).

While accumulation of lactate is negligible during fermentative metabolism in CA-stored apples (Saquet and Streif, 2008), the transient increase of acetaldehyde and ethanol contents characterizes the early responses of apple fruits to oxygen concentrations below the specific ACP for a given cultivar. Interestingly, after an increase of fermentation metabolites, their concentration decreases after prolonged exposure to low oxygen stress (Cukrov et al., 2016; Brizzolara et al., 2017; Boeckx et al., 2019). The physiological mechanism responsible for this behavior remains to be elucidated.

The reduction of acetaldehyde to ethanol catalyzed by ADH is a key biochemical step in plant responses to hypoxic conditions. Specific *ADH* family members can be identified as core-responsive genes to hypoxia in apples. In fact, *ADH* genes are extremely reactive to changes in oxygen concentrations showing fine-tuned regulated expression of different members belonging to this multigene family (Cukrov et al., 2016; Brizzolara et al., 2019). However, the regulatory mechanisms of fermentative metabolism in fruits in response to low oxygen concentrations are complex. Based on kinetic modeling of responses of apple slices to low oxygen concentrations, two mechanisms were suggested by Boeckx et al. (2019): (1) the molecular control of the transcript and protein levels of PDC, ADH, and LDH and (2) the metabolic control of these enzymes by changing cytosolic pH set-points, cofactors, and substrate levels. These authors suggest that the fermentative metabolism is highly dependent on factors that modulate reaction rates, with both molecular and metabolic control systems being activated when the overall metabolic rate is fast.

Mannose and melibiose contents increased under low oxygen conditions. (Hatoum et al., 2014; Brizzolara et al., 2017). Together with other sugar alcohols, melibiose plays an osmoprotection role and it is involved in stress responses, accumulating in stress-tolerant species, and is a stress markers in plants (Hill et al., 2013). Increased sugar alcohols have been observed in different fruits kept at low oxygen concentrations (Pedreschi et al., 2009). Given their antioxidant potential, such compounds may act directly as protectant molecules, or in complementing carbon storage reserves under sugar shortages (Moing, 2000).

Organic Acid Metabolism

A general decrease of organic acid concentrations in plant tissues may be a survival strategy aimed at reducing the flux toward nitrogen metabolism to keep on producing substrates for the glycolysis (Geigenberger, 2003; Galili, 2011; Ampofo-Asiama et al., 2014). Organic acids are key elements of the adaptive response of fruit tissue to low oxygen stress, although the overall pattern of organic acids in fruits stored under low oxygen and high carbon dioxide concentrations has still to be defined in detail. Succinate, a key metabolite in hypoxic responses, increases under low oxygen stresses in model systems (Bailey-Serres et al., 2012). Succinate also accumulates in apples and pears under high carbon dioxide concentrations (Vandendriessche et al., 2013; Bang et al., 2019), and this is associated with the inhibition of succinic dehydrogenase activity (Gonzalez-Meler et al., 1996). Accumulation of succinate has been correlated with the development of carbon dioxide-related physiological disorders (Pedreschi et al., 2008), although succinate can accumulate in the presence of the antioxidant diphenyamine, which prevents carbon dioxide injury (Fernandez-Trujillo et al., 1999). The effects of low oxygen concentrations in fruit tissues seem to be slightly different from those detected in rice and *Arabidopsis.* Lower levels of succinate have been detected in "Granny Smith" fruits kept at 0.4 kPa compared with those maintained at 0.8 kPa oxygen (Brizzolara et al., 2019), while "Red Delicious" fruit had higher succinate contents under static conditions at 0.9 kPa oxygen compared with 0.2–0.55 kPa oxygen (Brizzolara et al., 2017). One hypothesis is that GABA is catabolized to succinic semialdehyde (SSA) and then to succinate, with the latter reaction being catalyzed by SSA dehydrogenase (SSADH) in air-stored fruit. Under hypoxia, SSADH activity is likely reduced by increases in reducing potential and adenylate energy charge, thus resulting in the increase of GABA and the decrease of succinate. The effects of low oxygen/high carbon dioxide concentrations in fruits in relation to succinate metabolism need to be further elucidated.

The fate of malate is also affected by low oxygen concentrations. In general, the lower the oxygen concentration

during storage, the greater the malic acid contents are maintained (Hatoum et al., 2016). Hypoxia induces aerobic respiration processes and affects specific TCA enzymes (Ampofo-Asiama et al., 2014). Normally, a decrease in malate content occurs in pome fruits after prolonged storage under hypoxia, which can be attributed to advanced ripening, and higher malate contents in the inner cortex compared with the outer cortex is probably the result of the lower respiration rate of this tissue due to reduced oxygen availability (Hatoum et al., 2016).

Fumarate contents increase under CA conditions, especially with higher carbon dioxide concentrations (Vandendriessche et al., 2013; Hatoum et al., 2016), perhaps as a result of down-regulation of fumarase, which catalyzes the hydration of fumarate to malate (Pedreschi et al., 2007). CA storage also may contribute to higher fumarate contents by promoting the conversion of oxaloacetate from phosphoenolpyruvate through the reversal of TCA reactions (Pedreschi et al., 2009).

In contrast, citrate contents in fruits are stable during air storage, but decline in CAs (Flaherty et al., 2018a). Citrate accumulation could result from the efflux of the vacuolar reserves, where most citrate is stored (Etienne et al., 2013). Citrate contents are also linked to glutamate fate since this latter is formed from 2-oxoglutarate (2-OG) and ammonia through the activity of cytosolic glutamate dehydrogenase, while 2-OG is produced from stored citrate (Sweetlove et al., 2010).

Amino Acid Metabolism

In addition to the activation of the fermentative pathways to sustain glycolysis in the absence of mitochondrial respiration, nitrogen metabolism is profoundly affected by oxygen deprivation.

Amino acids linked to the TCA cycle are an integral component of respiratory metabolism and changes in their contents represent one of the main responses of plants to oxygen stress. Tolerant plants (e.g. rice) generally accumulate amino acids such as Ala, Ser, and Gly when subjected to low oxygen stress (Shingaki-Wells et al., 2011). The accumulation of Ala and GABA appears to be common responses of plants to hypoxia (Narsai et al., 2011). It has been hypothesized that Ala formation is important for removal of accumulated pyruvate under oxygen deficiency, thus indirectly contributing to pH homeostasis by competing with lactate dehydrogenase (Mustroph et al., 2014).

The contents of many different amino acids, such as Ala, Asp, GABA, Pro, Ser, and Thr in apples are modulated by oxygen concentrations during CA storage, with pyruvate-derived compounds and the expression of related metabolic genes affected differently by low oxygen concentrations (Cukrov et al., 2016).

Ala, which has been found to be a main hypoxia-related metabolite accumulating in several plant species (Bailey-Serres et al., 2012; Diab and Limami, 2016) including apples (Vandendriessche et al., 2013; Hatoum et al., 2014; Cukrov et al., 2016; Brizzolara et al., 2017), derives from pyruvate transamination *via* alanine aminotransferase (AlaAT) activity coupled to two different possible reactions: the production of 2-OG from glutamate or the generation of SSA from GABA (Hyun et al., 2013). The GABA shunt is less efficient at generating 2-OG and, thus, it is possible that alanine is derived from Glu under low oxygen concentrations, and that plant cells use it as storage form of pyruvate. Hatoum et al. (2016) reported increases of alanine contents in "Braeburn" apples after 8 months of CA storage. In "Granny Smith" apples Ala content increases very rapidly after low oxygen stress is imposed but, differently from ethanol, a less marked effect of oxygen concentration is present (Cukrov et al., 2016). *AlaAT* gene expression is highly induced by hypoxic stress in apple cortex and a rapid re-adjustment of transcription occurs in relation to oxygen concentrations applied under DCA conditions (Brizzolara et al., 2019).

GABA appears to play a key role in energy metabolism and defence against different abiotic stresses in fruits (Shang et al., 2011; Yang et al., 2011; Wang et al., 2016). GABA represents a key compound in the interface between C and N metabolism under energetically demanding stresses possessing a pivotal role in stress responses, due to its double role of acting directly as protectant compound and functioning as signalling molecule used by plant tissue to tune stress responses (Michaeli and Fromm, 2015; Takayama and Ezura, 2015; Diab and Limami, 2016). GABA synthesis is enhanced when the cytosolic pH decreases (Hyun et al., 2013), hence its production could also be related to the fate of the organic acids in the cell, possibly being a tissue- or treatment-specific event also uncoupled from the response to low oxygen concentrations itself. The accumulation of GABA is highly dependent on the oxygen concentrations (Cukrov et al., 2016) and enhanced concentration of GABA are considered as a marker of hypoxia in apples and pears (Pedreschi et al., 2009; Cukrov et al., 2016). In addition, GABA catabolism is highly reactive and sensitive to oxygen and carbon dioxide changes. Decreases of GABA content have been detected in apple cortex tissues within 24 h of oxygen shift from 0.4 to 0.8 kPa (Brizzolara et al., 2019) and two apple GABA transaminase (GABA-T) genes are rapidly up-regulated in apples moved from CA to normoxic conditions (Trobacher et al., 2013). GABA contents have been also reported to increase in strawberries and tomatoes during postharvest treatment with 20 kPa CO_2 (Deewatthanawong et al., 2010a; Deewatthanawong et al., 2010b).

Overall, Ala and GABA production are important adaptive process allowing carbon and nitrogen storage, as well as acting as osmoprotectants under stress conditions, and balancing a rapid decrease in carbohydrates. Ala production under hypoxic stress represents an adaptive carbon allocation strategy that maintains the glycolytic flux, limits pyruvate accumulation and, at the same time, maintains carbon resources in the cell (Rocha et al., 2010). In fact, the fermentative pathway and ethanol production result in NAD+ regenration thereby allowing glycolysis to proceed to sustain ATP production, but the pathway drains carbon for the production of metabolically useless dead-end products (Limami, 2014). In addition, the interconversion of other amino acids normally takes place at cellular level acting as a tool for adapting

cell metabolism/homeostasis to different environments. Therefore, hypoxia affects the fate also of other compounds belonging to this chemical class. Among them, Glu, Pro, as well as Ile, Thr, and Ser production/accumulation increase, while Asp and Asn contents showed a decrease in fruits stored under low oxygen concentrations (Lee et al., 2012; Hatoum et al., 2014; Bekele et al., 2016; Cukrov et al., 2016; Brikis et al., 2018). These results are mainly in agreement with the role of these amino acids as precursors for Ala and GABA production (Oliveira and Sodek, 2013).

Lipid Metabolism

Lipid metabolism in fruits under CA conditions is not well studied. Lipid biosynthesis is characterized by several pathways involving enzymes that require oxygen for their activity, such as the ones involved in the biogenesis of sterols and the desaturation of fatty acids (Harwood, 1988; Ohlrogge and Browse, 1995), and which may be affected by hypoxia. Indeed, lipid biosynthesis and desaturation have been since long time recognized to decrease in plants subjected to low oxygen concentrations (Brown and Beevers, 1987).

An early study postulated that phospholipids accumulated more slowly in apples stored in CA than in air (Bartley, 1986), while Brackmann et al. (1993) found lower peel fatty acid content in CA- than air-stored apples. CA storage affected the biosynthetic mechanisms of these compounds, especially linoleic acid. How different oxygen concentrations exert their effect on such pathways is still unclear. Production of unsaturated fatty acids, such as linoleic acid, requires oxygen and this partly explains their lower content under CA conditions. However, lipid biosynthesis could also be reduced because CA conditions inhibit respiration and general metabolism, thereby reducing the available energy equivalents, such as NADPH, that are needed for fatty acid synthesis (Ho et al., 2013). Delaying CA storage of apples results in higher contents of fatty acids, and polar lipids, including phospholipids after storage (Saquet et al., 2003). In addition to the energy issue, another possible cause of the observed decrease of lipids under low oxygen conditions involves ethylene production and action, which are lower after CA storage: in apples a pre-storage treatment with the inhibitor of ethylene biosynthesis, aminoethoxyvinylglycine, decreased ATP and fatty acid levels under storage in normoxia (Halder-Doll and Bangerth, 1987).

Higher PCs and phosphatidylethanolamines (PEs) and lower arachidonic acid contents, the latter a product of PC and PE metabolism, have been observed in apple cortex one day after a partial re-oxygenation of the fruit when compared with apples stored constantly at low oxygen regimes (Brizzolara et al., 2019). This study reported that after oxygen re-supply the up-regulation of two genes encoding phospholipase A2 and the down-regulation of two phospholipases (C2 and D delta) occurred, indicating that phospholipid metabolism is highly reactive to slight changes in oxygen concentrations within the storage rooms. These results also pointed out that a transient burst of phospholipid metabolism could play an important role in the early responses of apple cortex cells subjected to oxygen re-supply, as well as in the generation of regulatory signals. Phosphoglycerides are main components of cell membranes, and mitochondria membranes are particularly rich in PC and PE (Harwood, 1987). Lipid metabolism in plants is altered under stress conditions: Xie et al. (2015) demonstrated that in hypoxic *Arabidopsis,* rosettes are characterized by increases in unsaturated glycerolipid species, PA, as well as oxidized membrane lipids, and decreases of phosphatidylglycerols (PGs), PCs, and PEs.

ETHYLENE EFFECTS ON PRIMARY METABOLISM OF HARVESTED FRUITS

The role of ethylene has long been recognized as a critical factor regulating ripening and senescence, and subsequently the storage life, of an array of horticultural commodities (Abeles et al., 1992; Saltveit, 1999; Tucker et al., 2017). Ethylene can affect the quality of these crops either when produced endogenously, or exogenously either applied to positively affect a biological factor, or as a contaminant. Low temperature and CA/MA affect product quality beneficially by decreasing metabolic activity in part by reducing ethylene production and action.

Fruits have traditionally been separated into two distinct groups, climacteric and non-climacteric. In climacteric fruits (and fruit vegetables) such as apple, avocado, banana, pear, and tomato, ripening is associated with an increase in respiratory activity that is associated with autocatalytic production of ethylene. Climacteric fruit, if harvested when mature, continue to ripen. In contrast, the respiration rates of non-climacteric fruits such as citrus, strawberry, and grape, decline during ripening with no autocatalytic ethylene production, and coordinated ripening changes do not take place after harvest. However, both climacteric and non-climacteric fruits respond to ethylene, but in very distinct ways that can be used as diagnostic tools (Wills and Golding, 2016). When progressively higher ethylene concentrations are applied to climacteric fruits, the size of the respiratory peak remains similar, but the timing of the respiratory maximum (and ripening) is accelerated even after treatment stops. In a non-climacteric fruit, respiration rates are proportionally greater as the ethylene concentration is increased but timing of the peak is not affected, and respiration decreases as soon as ethylene is removed. The rate of ripening is not necessarily a function of ripening type: non-climacteric fruits such as sweet cherries and strawberry can ripen rapidly, while citrus ripen slowly; climacteric fruits such as avocados and pears ripen rapidly, while certain apple cultivars ripen slowly.

However, a strict distinction between climacteric and non-climacteric fruits is less clear than originally thought (Paul et al., 2012). Fruit types that have both ripening patterns include melon and plums (Abdi et al., 1997; Fernandez-Trujillo et al., 2008; Farcuh et al., 2019). It has long been thought that the ripening of climacteric fruits is regulated by ethylene while that of non-climacteric fruits is regulated by abscisic acid (ABA) (Jia et al., 2011; Jia et al., 2013). However, it is clear that ethylene and ABA,

as well as other hormones (e.g. auxins), are involved in ripening of both types of fruits (Manganaris et al., 2011; Li et al., 2019). Climacteric and non-climacteric types also share many aspects of ethylene perception and signaling and interestingly, the EThylene Receptor (*ETR*) gene is more abundant in climacteric fruit than non-climacteric fruits, and ETR accumulates earlier in the latter (Chen et al., 2018).

The involvement of ethylene in primary metabolism has been explored in many ways that include investigations into metabolism of normally ripening fruits, use of mutants or transgenic approaches, ethylene treatment of fruits with ethylene [directly as a gas or by use of ethylene releasing agents such as 2-chloroethylphosphonic acid (ethephon)], and ethylene analogues such as propylene. Ethylene antagonists such as silver and 1-MCP also have been used; of the two, 1-MCP has been especially useful for researchers due to its non-toxic nature, gaseous form at physiological temperatures, and its effectiveness at low concentrations (Sisler and Serek, 2003; Watkins, 2015). Although ethylene concentrations around fruits can also be lowered by methods such as ventilation, avoidance, and absorbers and oxidizers (Martinez-Romero et al., 2007), there is little information about their effects on primary metabolism.

In general, application of ethylene to climacteric fruits accelerates the rate of ripening (Saltveit, 1999), while 1-MCP decreases the rate of ripening, and in some cases can totally inhibit it (Watkins, 2006; Watkins, 2015). However, 1-MCP induces variable responses in different climacteric fruit species and even in cultivars of the same species (Watkins, 2015). Ethylene biosynthetic and signal transduction pathways of apples and peaches are differentially affected by 1-MCP action (Dal Cin et al., 2006). 1-MCP can also affect various ripening/senescence processes of non-climacteric fruits (Huber, 2008; Li D. et al., 2016; Kafkaletou et al., 2019).

Non-targeted genomic, proteomic, and metabolomic approaches have revealed a number of changes in primary metabolism in response to ripening, exogenous ethylene, and ethylene inhibitors (Giovannoni et al., 2017; Li et al., 2017; Zhao X. et al., 2019). In nectarines, about 30% of the transcriptome corresponded to genes involved in primary metabolism and response processes related to ethylene, auxin, and other hormones (Ziliotto et al., 2008). In 1-MCP-treated fruits, altered transcript accumulation was detected for some genes with roles in ripening-related events including sugar metabolism. Changes in proteins of apples during ripening revealed enzymes involved in gluconeogenesis, C-compounds and carbohydrate metabolism, electron transport/energy production, and malic acid metabolism. Proteins involved in several multiple metabolic pathways, including glycolysis and the pentose-phosphate pathway were down-regulated, especially during the climacteric burst in respiration and during the senescent stage (Shi et al., 2014). In peaches, 34% of differential protein expression in response to 1-MCP and ethephon were associated with pathways involved in energy and general metabolism (Zhang et al., 2012). In CA-stored "Empire" apples, most carbohydrates and organic acids were not appreciably affected by 1-MCP treatment, but levels of sorbitol and some amino acids were elevated toward the end of storage in treated fruit (Lee et al., 2012).

Carbohydrate Metabolism

Most research on the effects of ethylene on starch metabolism in fruit has been performed on bananas, and to a lesser extent on apples and kiwifruits. Starch degradation and sucrose synthesis are both developmentally regulated at transcriptional level (Janssen et al., 2008; Xiao et al., 2018; Zhang et al., 2018; Yan et al., 2019). Examples include kiwifruits, where analysis of the regulatory effects of differentially expressed genes identified a zinc finger TF, DNA BINDING WITH ONE FINGER (AdDof3), which showed significant transactivation on the *AdBAM3L* (β-amylase) promoter (Zhang et al., 2018). In apples, ethylene biosynthesis is inhibited in the antisense-suppressed MADS8as-9 line; ethylene application to this line partially stimulated ripening, with starch degradation and other late ripening processes not being complete (Ireland et al., 2014).

Starch hydrolysis in bananas was enhanced by treatment with propylene or ethylene (Saraiva et al., 2018). Starch hydrolysis in apples can be dependent or independent of ethylene concentration, whether exogenous or endogenous (Thammawong and Arakawa, 2007; Doerflinger et al., 2015a). Postharvest 1-MCP effects on starch hydrolysis in apples also can be cultivar-dependent (Fan et al., 1999; Neuwald et al., 2010; Thammawong and Arakawa, 2010; Doerflinger et al., 2015b). In bananas, 1-MCP delays hydrolysis in a dose- and cultivar-dependent manner (Nascimento et al., 1997; Mainardi et al., 2006). Variation of responses to ethylene in different fruit types and cultivars may be explained by differences in sensitivity (Johnston et al., 2009); initiation of starch hydrolysis is ethylene sensitive and therefore even a very small increase in ethylene concentration can trigger the onset of starch hydrolysis. However, once initiated its hydrolysis is relatively independent of ethylene concentrations within the fruit tissue. Also, relationships between ethylene and starch metabolism are not straightforward, as starch degradation in bananas, for example, is coordinated with that of cell wall softening (Shiga et al., 2011).

Simultaneous synthesis and degradation of sucrose during postharvest storage of fruits has been well described (Duque et al., 1999; Zhu et al., 2013). A link between ethylene and sucrose metabolism is suggested by increased gene transcript and enzymatic activity of SPS by exogenous ethylene treatment or during postharvest ripening (Duque et al., 1999; Choudhury et al., 2008; Lombardo et al., 2011). Sucrose treatment of tomato fruits advanced ripening, and increased expression of genes involved in both sugar biosynthesis and degradation (Li L. et al., 2016). The *ETR* transcripts accumulated earlier in non-climacteric than in climacteric fruits, and this expression coincided with the onset of sugar accumulation (Chen et al., 2018). Investigations with "Micro-Tom" tomatoes and five hormone mutants (namely *dpy*, *not*, *dgt*, *epi*, and *pro*) indicated that ethylene plays an important role in regulation of sugar accumulation (Li et al., 2019).

A feature of 1-MCP-treated apples is the low sucrose contents compared with the controls (Defilippi et al., 2004; Bekele et al., 2015). Bekele et al. (2015) suggested that low sucrose was due to temperature mediated activation of sucrose degrading enzymes and suppression of SPS activity by 1-MCP treatment, whereas in untreated fruit, the breakdown of sucrose did not result in accumulation of glucose due to its utilization to sustain a high respiration rates. However, 1-MCP treatment increased *SuSy* expression associated with the sucrose–sucrose cycle in "Royal Gala" apples (Storch et al., 2017). It was suggested that in the absence of ethylene, the fruit resumes a set of metabolic activities that were suppressed during ripening in the presence of ethylene. Sucrose contents are also lower in fruit from trees with downregulated 1-aminocyclopropane-1-carboxylic acid (ACC)-synthase or ACC-oxidase enzyme activity, but contents could be restored to similar levels as wild type after exposure to ethylene (Defilippi et al., 2004).

Although grapes as non-climacteric fruits are thought to be ethylene independent, 1-MCP treatment resulted in lower sucrose accumulation in berries than in untreated fruit (Chervin et al., 2006). Decreased sucrose accumulation was associated with a down-regulation of two sucrose transporters (*SUC11* and *SUC12*), whose expression is triggered at veraison when grape berries start to accumulate sugars. In citrus, 1-MCP repressed genes involved in starch synthesis and degradation and increased the expression levels of SuSy genes (Estables-Ortiz et al., 2016). In grapes, and another non-climacteric fruit, strawberry, it has been proposed that sucrose functions as a signal that acts upstream of the ABA signaling pathway, thus playing an important role in the regulation of fruit ripening (Jia et al., 2013; Jia et al., 2017).

A mutant series of Japanese plums exhibits three distinct ripening patterns: climacteric, suppressed-climacteric, and non-climacteric (Minas et al., 2015). "Santa Rosa," which is climacteric, and its bud-sport mutant "Sweet Miriam," which is non-climacteric, have been used to investigate sugar metabolism. At the ripe stage, non-climacteric fruits accumulate higher sorbitol than that of climacteric fruits (Kim et al., 2015; Farcuh et al., 2017; Farcuh et al., 2018). Higher sorbitol contents in "Sweet Miriam" were associated with decreased activity and expression of NAD^+-dependent sorbitol dehydrogenase and sorbitol oxidase and increased sorbitol-6-phosphate dehydrogenase activity, as well as increased sucrose catabolism (Kim et al., 2015). Enhanced sorbitol synthesis and lower sucrose, glucose, and fructose contents were also found during on-tree ripening of the non-climacteric "Sweet Miriam" than in the climacteric "Santa Rosa" (Farcuh et al., 2017); contents of the minor sugars galactinol, raffinose, *myo*-inositol, and trehalose also increased in "Sweet Miriam," while that of galactose was higher in "Santa Rosa." The effects of ethylene on sugar metabolism were studied using propylene and 1-MCP (Farcuh et al., 2018). Ethylene increased biosynthesis of sucrose while decreasing that of sorbitol. Decreased and increased sucrose and fructose accumulation may result from sorbitol and sucrose catabolism in climacteric and non-climacteric fruits, respectively. Ethylene resulted in galactose accumulation in "Santa Rosa"; galactose has been also reported to stimulate ethylene production in tomato fruits (Kim et al., 1987).

Organic Acids

A general pattern of organic acid contents of fruits during development, is an initial accumulation of acids followed by a decrease of contents either by increasing fruit mass or metabolism as a function of synthesis, degradation, and compartmentalization. The acids are sequestered in the vacuoles and released to provide substrate for increased respiration during ripening of climacteric fruits, meet higher energy demand in non-climacteric fruits, and produce hexoses by gluconeogenesis (Beruter, 2004; Sweetman et al., 2009). Influx and efflux of malic acid from the vacuole may vary over time (Walker et al., 2015; Famiani et al., 2016b). In peaches, contributions of malic and citric acids to metabolism are negligible, and it is likely that gluconeogenesis occurs during ripening (Famiani et al., 2016b). In ripening of grapes, a non-climacteric fruit, decreased malic acid contents were associated with metabolism, but to a lesser extent compared with sugars (Famiani et al., 2016a). Overall, there is a diversity of responses of different fruits to ethylene and the contribution of stored organic acids to metabolism is not well understood. Organic acid metabolism is not directly linked to respiratory and climacteric characteristics of the fruit, however, interactions between organic acid metabolism and hormone signaling provide useful insights for future research (Batista-Silva et al., 2018).

Detailed studies exploiting 1-MCP to investigate organic acid metabolism are recent. In apples, 1-MCP slowed the decreases of malic and citric acid contents during ripening at 20°C by regulating organic acid metabolism (Liu et al., 2016). In addition, up-regulation of *Md PEPC* and *MdcyMDH* expression, higher PEPC and cyNAD-MDH activities, and the decreased malate degradation *via* limiting *MdPEPCK* expression with lower activity of PEPCK were reported. 1-MCP treatment up-regulated acid transport genes, including *MdVHA-A*, *MdVHP*, and *Ma1*, resulting in higher malic acid contents in the vacuole (Liu et al., 2016). In Asian pears, decreasing malic acid contents during ripening were associated with down-regulation of genes associated with malic acid metabolism, lower cyNAD-MDH, and higher cyNADP-MDH activity (Wang et al., 2018). 1-MCP-treated fruits had higher malic acid contents, upregulated gene expression, higher NAD-dependent malate dehydrogenase activity, and lower NADP-ME-dependent malate dehydrogenase activity.

Amino Acids and Lipids

Amino acid contents typically decrease during ripening and storage, often associated with secondary pathways that produce aroma volatiles, particularly esters, because of the close linkages with ethylene production during ripening (Giovannoni et al., 2017). 1-MCP treatments of fruits can maintain higher amino acid contents (Lee et al., 2012; Zhang et al., 2014; Bekele et al., 2015) that could result from a reduced utilization in metabolic

processes. Amino acids may also be involved in fruit responses to stress and development of physiological disorders associated with 1-MCP treatment through their role as substrate for compounds such as GABA (Flaherty et al., 2018b). They are also directly linked to many secondary metabolic processes, most notably production of aroma volatiles, several groups of which are related to amino acid and fatty acid metabolism.

Loss of membrane function with changes in membrane lipids, particularly their degree of unsaturation, is a feature of ripening and senescence (Marangoni et al., 1996). These changes lead to altered membrane properties and result in defects such as ion leakage and loss of cellular compartmentalization, and therefore represent an obvious consequence of ethylene production. The literature on these changes outside of the effects of imposed postharvest treatments such as low temperature and CA/MA is limited. While 1-MCP has been used to investigate the effects of ethylene on lipids it is not surprising that the results typically show slowing down of changes associated with ripening and senescence. In fruits such as kiwifruit, pear, pitaya, and tomato, 1-MCP inhibited lipid associated changes such as increases of membrane permeability, lipid peroxidation, the decrease of unsaturated fatty acids and *PLD* gene expression (Dek et al., 2018; Huang et al., 2019; Tao et al., 2019; Xu et al., 2019).

FINAL REMARKS AND FUTURE PERSPECTIVES

Storage protocols are based on the application of controlled stresses or treatments aimed at delaying genetically programmed fruit ripening and the onset of senescence by affecting primary metabolism.

This review highlights a number of issues specifically dealing with the effects of three main post-harvest physical and chemical factors on the primary metabolic processes of stored fruits. The effects of these protocols depend on a number of factors including the intensity and duration of the applied protocol, the fruit type and cultivar, the maturity and ripening stage at harvest, as well as pre-harvest conditions. Considering the impact of pre-harvest factors, the information available concerns solely the effects of specific treatments and protocols (e.g. spraying with chemicals, controlling crop load, fertilization, water management) on the fruit responses in terms of shelf-life, technological parameters and the incidence of decay and disorders, with no specific information regarding primary metabolic pathways.

An extensive literature on ethylene and our understanding of its involvement in ripening of both non-climacteric and climacteric fruits has been developed. Transgenic and 1-MCP treatments have become powerful tools to investigate the role of ethylene on primary metabolism. Segregation of fruits based on ripening patterns are less strict than previously assumed and cross talk among hormones other than ethylene, has been identified. The interactions between ethylene signaling and sugars also is an especially active area, while there is a paucity of information about organic acids, amino acids and lipids. Studies concerning postharvest treatments such as low temperature and the effects of CA/MA have centered more on injuries associated with these treatments rather than on the effect of these treatments on non-injurious metabolism. High variability of responses associated with different genotypes to an array of postharvest treatments, often used in an integrated manner, renders difficult to identify primary metabolic changes occurring in fruit tissues in contrast to model systems.

The increasing availability of genome sequencing of different genotypes, and the development of "omics" techniques are providing tools to overcome these limitations, and thereby better understand and clarify the fundamental mechanism regulating the postharvest metabolic responses. These tools are also providing opportunities to exploit findings that are, so far, mainly based on model systems. This is the case of low oxygen and high carbon dioxide postharvest stress physiology studies. Recent reports describe the role of specific transcription factors (TFs) such as ethylene-responsive factors (ERFs), in synergy with WRKY and MYB elements, in controlling the expression of *PDC* gene promoter in persimmons under high carbon dioxide/hypoxia (Zhu et al., 2018; Zhu et al., 2019). Specific and multiple TFs of different clades/classes and a TF regulatory network are involved in the responses to such storage conditions that induce marked changes of primary metabolism gene expression. *ADH* and *PDC*, belonging to the core responsive genes of plants to low oxygen conditions (Mustroph et al., 2009), are controlled by ERFs in *Arabidopsis*, through a fine-tuned mechanism (N-end rule pathway, NERP) of oxygen sensing (Gibbs et al., 2011; Licausi et al., 2011). ADH and PDC enzymes are involved in primary (fermentative) metabolism and play a major role in the determination of fruit flavor and aroma. In apples, a specific ERF protein (MdRAP2.12) has been shown to differentially accumulate at different oxygen concentrations, suggesting that the oxygen-sensing mechanisms described in *Arabidopsis* are also present in apple fruit (Cukrov et al., 2016). The variable responses in terms of the primary metabolism compound (pyruvate, alanine, ethanol) accumulation observed in different apple cultivars under hypoxic conditions might be the result of the selective activation and specific organization of ERF-based sensing mechanisms (Brizzolara et al., 2017).

An additional field of investigation involves the tolerance of fruits to cold stress occurring during storage. Cold-stored fruits re-direct their metabolism, starting from changes in gene expression, with different levels of tolerance to cold stress depending on the genetic background. A number of cold-responsive (COR) genes have been identified in vegetative tissues of model species, some of them involved in physiological and biochemical changes during the process of cold acclimation and tolerance. COR genes have been identified also in different fruit species such as peaches where their expression has been analyzed in relation to different behavior of CI sensitive and CI non-sensitive cultivars during refrigerated storage (Bustamante et al., 2016; Nilo-Poyanco et al., 2019).

Selective changes in expression of genes related to energy and stress response, amino acid, carbohydrate, lipid, and specialized metabolism have been observed (Zhang et al., 2010; Pons et al., 2014; Pons et al., 2016) with C-repeat-binding factors (CBFs) apparently playing a key role in modulating the expression (Liang et al., 2013).

Elucidation of these regulatory mechanisms will widen our understanding on primary metabolic, and associated secondary, responses of fruit tissues of harvested fruits, and also help to optimize storage protocols with benefits in terms of reduced losses and improved consumer satisfaction.

AUTHOR CONTRIBUTIONS

All authors planned the structure of the review, contributed in writing the article, read and approved the submitted version.

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frontiers in Plant Science

REVIEW
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Metabolism of Stone Fruits: Reciprocal Contribution Between Primary Metabolism and Cell Wall

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Cell wall turnover and modification in its composition are key factors during stone fruit development and patterning. Changes in cell wall disassembly and reassembly are essential for fruit growth and ripening. Modifications in cell wall composition, resulting in the formation of secondary cell walls, are necessary for producing the most distinctive trait of drupes: the lignified endocarp. The contribution of primary metabolism to cell wall synthesis has been investigated in detail, while the knowledge on the contribution of the cell wall to primary metabolites and related processes is still fragmented. In this review, starting from peculiarities of cell wall of drupes cells (in mesocarp and endocarp layers), we discuss the structure and composition of cell wall, processes related to its modification and contribution to the synthesis of primary metabolites. In particular, our attention has been focused on the ascorbate synthesis cell wall-related and on the potential role of cyanogenic compounds in the deposition of the secondary cell wall.

Keywords: endocarp, ascorbic acid, cyanogenic compounds, lignin, cell wall turnover

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INTRODUCTION

Cell wall metabolism is an integral part of the primary metabolism since the cell wall is the primary carbon sink in many plant tissues. The majority of C is stored into the cell wall polysaccharides making this cellular component the most important biomass on earth. In addition to polysaccharides (cellulose, hemicelluloses, and pectins), cell walls are composed by other, quantitatively minor but functionally important components, such as polyphenols and proteins, many of which are glycosylated (Somerville et al., 2004). Plant cell walls composition is highly variable not only among species but also within an individual plant at both the tissue and cell levels (Zhang et al., 2014). Moreover, cell walls are classified as primary walls, which are surrounding the cell driving its growth and consequently also its morphology, and secondary walls, whose rigidity and strength is necessary to fulfill specialized cell functions (Somerville et al., 2004). In stone fruits (or drupes), cell wall changes resulting in the formation of secondary cell walls are particularly important because they are necessary for producing the most distinctive trait of drupes: the lignified endocarp.

PRIMARY METABOLISM AND PRIMARY CELL WALL

Primary Cell Wall Composition and Its Metabolism During Drupe Growth and Ripening

Similarly to other fleshy fruit, an active cell wall turnover is essential for a correct fruit development and ripening in *Prunus* spp. More than 50 cell wall-related genes encoding for lignocellulose-degrading enzymes and nonenzymatic protein (e.g., expansins, EXP) (Goulao and Oliveira, 2008; Mercado et al., 2011; Gapper et al., 2013), as well as components of subcellular structures (e.g. cytoskeleton), have been claimed to be involved in the cell wall turnover (Bashline et al., 2014). Drupe growth is the result of coordinated cell divisions and expansion processes in which cell turgor pressure, cell wall biosynthesis and its remodeling play a fundamental role. During fruit ripening, cell wall disassembly, combined with a decrease of cell turgor pressure, is the main process responsible for fruit softening. In this context, before discussing how the cell wall changes during fruit development and ripening and its contribution to primary metabolism, it is necessary to provide some information about the cell wall composition of *Prunus* spp. fruit.

Parenchyma cells with a thin primary wall are the main cell type present in fleshy fruit tissues. In dicotyledonous plants, the primary cell wall is composed roughly by equal part, ranging around 30%, of cellulose, hemicelluloses, and pectins, plus 1–10% of structural proteins. Fruit cell walls have also high content of water (Posé et al., 2018).

A detailed description of each component is not the main goal of this review, however, here it will be summarized the newest results on cell wall component reached by employing the Atomic Force Microscopy (AFM), an imaging tool for studying food macromolecules and colloids (Gunning and Morris, 2017). The advantage of AFM, in comparison to techniques based on high-resolution scanning electron microscopy, is the avoidance of cell wall polymers coalescing because it is not necessary to dehydrate the sample before the analysis. Thanks to this characteristic, AFM, in the last decade, has opened a new vision of structural features of cell wall components, particularly polysaccharides.

Among polysaccharides, cellulose is the main component of the primary cell wall. It is composed of a repetitive unit formed by residues of β-(1–4) linked D-glucose. These are arranged in fibrils. By using AFM, Niimura et al. (2010) demonstrated that cellulose microfibrils present in peach fruit are ultra-thin (diameter ranging between 1 and 2 nm). Based on this characteristic, peach cellulose nanofibrils can be classified as dietary fiber, and therefore, they can heavily contribute to the nutritional value of peach fruit.

Xyloglucan (XyG) is the most abundant hemicellulosic component, this polymer is embedded in an amorphous pectin matrix composed of polygalacturonides together with other less abundant components, such as phenols, structural proteins, enzymes, and a variety of receptors and sensors (Goulao and Oliveira, 2008; Posé et al., 2018). Differences in the thickness of hemicellulose chains have been related to differences in fruit texture. Chen et al. (2009) reported that tick hemicellulose chains were more abundant in cherries with crisp texture than the softer ones.

Pectin is a heterogeneous polysaccharide mainly composed of D-galacturonic acid (GalA) (Fishman et al., 2004; Yang et al., 2009; Wang et al., 2018). Visualization of the pectin sample isolated from the fruit by AFM has confirmed the great nano-structure heterogeneity. In peach, plum, and apricot fruit, pectins are naturally aggregated in large (1–3 nm) and branched fiber (Yang et al., 2009; Liu et al., 2017; Mierczynska et al., 2017). The presence of long pectin chains (longer than 1000 nm) is usually associated with a consistent texture/relatively high firmness, while fruits with thinner and shorter pectin chains (e.g. strawberry and tomato) undergo to a rapid softening. A reduction of the complexity of pectin nanostructure occurs during the fruit softening of Prunus ssp., as observed by Zhang et al. (2008) in Chinese cherry. In this study, differences in the structure of chelated pectins that were presently moving from the first growth phases to ripening have been detected by AFM, being pectins from unripe fruit longer and wider than those from ripe fruit.

Modifications of cell wall composition and structure are the foundation of changes in fruit firmness and texture during ripening. Some changes in cell wall components ultrastructure appear to be common (e.g. the hemicellulose depolymerization), but others occur in a specie-specific manner or are totally absent. For example, a slightly decrease of cellulose content occurs during ripening in most fruits, although this event is often uncoupled with the increase of crystalline cellulose in ripe fruit (Posé et al., 2018). On the other hand, although in cell wall galactose (Gal) and arabinose (Ara) level declines in ripe fruit of most species, Gal and Ara loss does not take place in plum, and Ara reduction is minor or absent in apricot. In peach, XyG depolymerization is among early events during softening (Brummell et al., 2004; Posé et al., 2018), while other fleshy fruit such as apple (Percy et al., 1997) the ripening proceeds in absence of the XyG depolymerization or this event is cultivar-dependent as in strawberry (Rosli et al., 2004).

Fruit softening is a very important event during ripening (Payasi et al., 2009) and it, primarily, results from both the decline in cell wall strength and cell-to-cell adhesion caused by modification of mechanical properties of cell wall and the depolymerization of pectins forming the middle lamella, respectively. These modifications together with the turgor pressure decline, that is associated with an increase in the concentration of apoplastic solutes (Wada et al., 2008), lead to fruit juiciness and texture softening (Toivonen and Brummell, 2008).

Pectins are the cell wall components showing the highest structural modifications during ripening; however, their role in fruit firmness and softening is still extremely controversial (Paniagua et al., 2014). These changes are an early solubilization and loss of neutral side chain, and, later on, a depolymerization mainly by polygalacturonases (PGases) (Goulao, 2010). Pectin solubilization may occur when cohesion of pectin molecules is weakened by the loss of neutral sugars in the form of neutral galactose-rich side-chains of rhamnogalacturonans 1 (RG-I).

Neutral side chains from RG-I might aggregate pectins either by physical interaction with other cell wall polymers or by binding to hemicellulose and cellulose (Zykwinska et al., 2008). In the Colorless non-ripening (Cnr) tomato fruit, the deposition of (1→5)-α-L-arabinan, which is the constituent the branched sidechain of RG-I, is impaired resulting in a reduced length and the low esterification degree of pectin (Orfila et al., 2001). The consequence of the altered Cnr fruit pectins structure is the lack of pericarp swelling and the presence of large intercellular space in the inner pericarp in comparison with the wild type fruit. Loss of cell adhesion has been also observed between cells of leaf parenchyma and abscission zone of transgenic apple plants overexpressing a PG gene. The overexpression of PG leads to a formation of pectins with shorter chains in comparison to those observed in the wild-type plants (Atkinson et al., 2002). These observations confirm that the cell adhesion strength is related to the composition of pectins forming the middle lamella (Jarvis, 1984). Later on, a more detailed analysis of cell wall microstructure of Cnr fruit parenchyma cells located in the interface with neighboring cells highlighted the presence of xylan and xyloglucan (Ordaz-Ortiz et al., 2009).This result indicates that hemicellulose polymers are structural cell wall component involved in the cell adhesion/cell separation process. Depolymerization of pectins during ripening is largely due to result of a sequential and coordinated action of several pectin-metabolizing enzymes such as PGases, pectinmethylesterases (PME) and pectinlyases (PL) (Brummell et al., 2004; Morgutti et al., 2006). Among PGs, endo-polygalacturonase (endo-PG) plays a central role in the depolymerization of cell wall pectins of peach fruit; however, endo-PG is essential for the achievement of a melting flesh (MF) fruit texture, due to the loss of cell adhesion, but not for reducing fruit firmness. The localization of endo-PG isoforms at the middle lamella of the cell wall of MF fruit supports this role (Morgutti et al., 2006). In addition, in non-MF (NMF) no endo-PG was detected and consequentially no loss of cell adhesion was observed. On the basis of these observations, the role of endo-PG activity on the reduction of fruit firmness has been debunked because of NMF peaches are able to soften and, at the same time, change of symplast/apoplast water status has been suggested as the main mechanism through which peach fruit firmness is regulated. A re-thinking of the main role of pectin depolymerization in the fruit softening (Wang et al., 2018) has been proposed for other fleshy fruits including strawberry and apple also on the basis of observation carried out after the silencing of PL (Posé et al., 2013) and PG (Atkinson et al., 2012), respectively. In both species the silencing of pectolitic enzymes lead an increase in cell-to-cell adhesion together with slight depolymerization of pectins and an improvement of fruit firmness and textural proprieties, without affecting other fruit quality traits.

Level of cell wall hydrolases change accordingly with the variation of the transcription of the corresponding genes of these enzymes (particularly PGases and PL) in ripening fruit, as pointed out by several transcriptome studies (Trainotti et al., 2006; Pan et al., 2016; Pei et al., 2019). In ripening peaches, Pei et al., 2019 also reported the up-regulation of xyloglucan endotransglucosylase/hydrolases (XTHs), responsible for the reduction of mass of wall-bound xyloglucans and consequently able to increase the cell wall extensibility. Worthy of note is the fact that the action of XTHs is induced by xyloglucan oligosaccharides (XGOs) and that, during peach fruit ripening, Pei et al. (2019) observed the down-regulation of two esterase/lipase proteins (GELPs) known for their action against XGOs.

Contribution of Cell Wall Disassembly to Primary Metabolites in Ripening Fruit

Cell wall degradation during ripening contributes substantially to the change level of primary metabolites fundamental for the human diet. It is high probable, that the quantity of these metabolites is strictly related to the composition and structure of polymers of the primary cell walls and the middle lamella as well as the disassembly mechanisms that can differ among species and within them among cultivars. However, at the moment, studies on the contribution of cell wall disassembly have been focused on the impact of pectin depolymerization on the ascorbate level (AsA, vitamin C). The biosynthetic pathway of AsA in plants can be represented with a complex network in which different pathways are converging: D-mannose/L-galactose (D-Man/L-Gal) (Wheeler et al., 1998), L-glucose (Wolucka and Van Montagu, 2003), myo-inositol (Lorence et al., 2004) and D-galacturonic acid (D-GalUA) (Agius et al., 2003), which is a component of pectins (**Figure 1**). Which pathway predominates is dependent on the species, tissue and stage of development (Walker and Famiani, 2018). The degradation of pectins releases methyl-galacturonate (Smirnoff et al., 2011), which is then converted into D-GalUA by a (pectin) methyl esterase (Paciolla et al., 2019) and successively into L-galactonic acid by D-galacturonate reductase (GalAR), firstly isolated in strawberry (Agius et al., 2003). An aldonolactonase (Alase), up to now isolated and well characterized only in Euglena (Ishikawa et al., 2008), converts L-galactonic acid into L-galactono-1,4-lactone, which is the last precursor of vitamin C (**Figure 1**). The D-Man/L-Gal pathway has been reported for many fruit-bearing plants, such as kiwifruits, acerola, peach, and tomato (Badejo et al., 2009; Bulley et al., 2009; Imai et al., 2009; Ioannidi et al., 2009), but these evidences are only clear for developing fruits, while there is still obscure how the AsA pool size is controlled during fruit maturation. A study carried out on microtomato fruit, based on feeding experiment with potential AsA precursors, suggests that it could be activated a switch from D-Mann/L-Gal to Ga1UA pathway moving from immature to ripe fruit (Badejo et al., 2012). In peach, genes involved in the conversion of sugar pool into L-Galactose were showing different expression pattern although the majority of them were highly expressed in the early phases of fruit development (**Figure 1**).The expression level of L-galactose dehydrogenase (GDH) and L-galactono-1,4-lactone dehydrogenase (GalLDH), the most important genes involved in the D-Man/L-Gal pathway were showing a biphasic expression profile with maximal at early stage and, at lower extent, during ripening phase. Transcript accumulation of GDH and GalLDH and AsA content, expressed per gram fresh weight basis, were related in the early period of fruit development, whereas this relationship was less evident in the last phase of fruit development in which AsA was at the lowest content (Imai et al., 2009). This

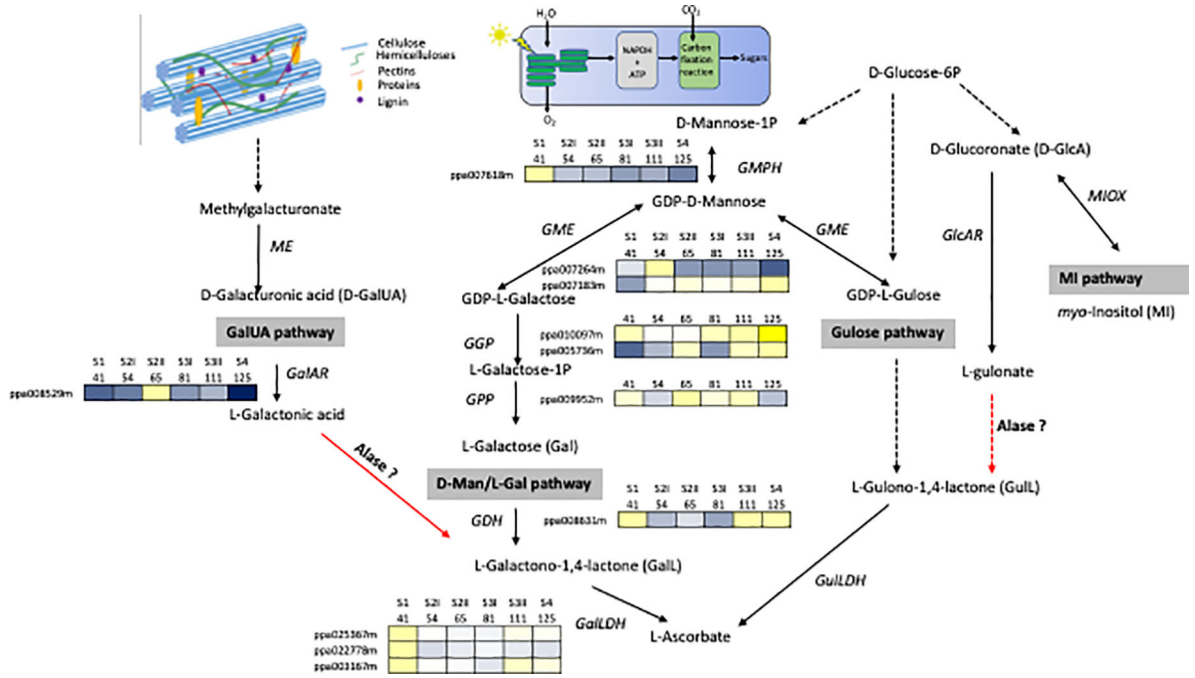

FIGURE 1 | Network for the biosynthesis of AsA. The four possible pathways include the D-galacturonic acid (GalUA), L-galactose (Gal), L-gulose, and myo-inositol (MI). Enzymes catalyzing reaction are Alase, aldonolactonase; GalLDH, L-galactono-1,4-lactone dehydrogenase; GalAR, D-galacturonate reductase; GDH, L-galactose dehydrogenase; GlcAR, D-glucuronate reductase; GME, GDP-D-mannose -3′,5′-epimerase; GulLDH, L-gulono-1,4-lactone dehydrogenase; ME, methyl esterase, MIOX, myo-inositol oxygenase; GMPH, GDP-D-mannose pyrophosphorylase; GGP, GDP-L-galactose phosphorylase; GPP, L-galactose 1-P phosphatase. Broken arrows show more than one enzymatic reaction step. Red arrows indicate steps still missing in higher plants. Transcripts profiles of GMPH, GME, GGP, GPP, GDH, GalLDH, GalAR genes during peach fruit development (cv Fantasia) have been retrieved from Gene Expression Omnibus (GEO) database under accession number GSE71561. Each gene is identified by the transcript code (peach genome version 1, see at https://www.rosaceae.org/organism/24333). Transcript levels significantly decreased were displayed in blue, while transcript levels significantly increased were displayed in yellow. The brightness of each color corresponded to the magnitude of the difference when compared with the average value.

result opens the possibility that in peach, as suggested in tomato, a switch between the AsA biosynthetic pathways can occur at ripening. Experiment feeding with D-GalUA of peach ripe fruit induced an increase of in the reduced form of AsA (Imai et al., 2009). At the moment, for GalUA pathway it is available only the expression of GalAR that shows the lowest expression at ripe stage, no information is available for Alase. Investigations are necessary to demonstrate if this mechanism may be present in peach or other Prunus species. For this goal, the major constrain is the lack of identified orthologs to the *Euglena* Aldolactonase in higher plants. However, a quantitative trait locus (QTL) analysis allowed the identification of five regions and two of them included genes annotated with terms related to the known D-Man/L-Gal and AsA/glutathione pathways (Stevens et al., 2007). Based on this result, it is probable that genes encoding the unidentified enzymes for the D-GalA pathway could reside in the rest of the candidate loci.

Partial cell wall degradation at ripening also leads to a massive release of sugars, which in plants are recycled for providing energy and building units for a large number of processes, including synthesis of protein and sugar accumulation. An indirect evidence of this causal relationship was obtained in fruit of transgenic plant of tomato in which PME transcripts were almost abolished by an antisense approach (Tieman et al., 1992). In particular, fruits from transgenic tomato plant were richer in soluble neutral sugar (sucrose) than wild-type fruits.

PRIMARY METABOLISM AND FORMATION OF THE LIGNIFIED ENDOCARP

Both the end-products and intermediates of primary metabolism are precursors of secondary metabolites (Douglas, 1996). The amino acid phenylalanine (Phe) is an example of the interconnection between primary and secondary metabolism because Phe can be a protein building block or a precursor of lignin, a secondary metabolite essential for plant growth, development, and defense (Pascual et al., 2016). Here, we first describe the structural characteristics of a lignified endocarp and then we analyze the role of primary metabolism in its formation.

Structural Characteristics of Drupe Pit

The lignified endocarp (or pit) is a distinctive trait of mature drupe fruits, but its formation occurs relatively early in fruit development, and its subsequent lignification takes place in phase

II of the double sigmoid fruit-growth curve, in which the mesocarp growth is suspended (Bonghi et al., 2011; Rapoport et al., 2013). This alternate pattern of growth between the different fruit tissues suggests the presence of cyclic events of competition for assimilates among fruit tissues and seed (Bollard, 1970; Opara, 2000). During stage II, when the endocarp is lignifying, the endosperm, throughout the absorption of nucellus, grows largely and later on, the embryo development is sustained by metabolites stored in the endosperm (Bassi and Ryugo, 1990; Ognjanov et al., 1995; Walker et al., 2011; Famiani et al., 2012). Therefore, one view is that the cost of embryo growth and endocarp lignification, in terms of assimilates, results in a temporary cessation of mesocarp growth. However, it is also possible that the temporary cessation in growth is brought about either largely or in part by genetic information that controls development (Pavel and DeJong, 1993b). The pit hardening is a progressive event as suggested by both anatomical observations and chemical analysis of lignin deposition. In the olive fruit, the time occurring for pit hardening takes, after bloom, a period ranging from 5 weeks up to 3 months (Hartmann, 1949; Lavee, 1986). In nectarine, the evaluation of force required to crush the endocarp pointed out that, although sclerification occurred slowly, the degree of hardness increased sharply around 12 to 13 weeks post-flowering (King et al., 1987). However, in peaches, the timing of this event is more related to the ripening time and shows large differences among early, mid, and late ripening peach cultivars (Pavel and DeJong, 1993a; Yamaguchi et al., 2002). In particular in early ripening peach and plums cultivars, the second exponential growth phase (Stage III) of fruit starts before the endocarp has completely lignified (Kritzinger et al., 2017). Additional factors in the variation of pit hardening timing are the tree water status (Rapoport et al., 2004; Lavee et al., 2007) and temperature (Dardick and Callahan, 2014; Souza et al., 2019).

Endocarp expansion ceases with the deposition of a thick, lignified secondary wall in endocarp cells (King, 1938; Dardick et al., 2010; Rapoport, 2010). In peaches, it has been reported that the onset of wall thickening and lignification of endocarp cells starts at the stylar end (Hayama et al., 2006; Dardick et al., 2010) and, then, proceeds toward the stem end of the fruit (Sterling, 1953; Lilien-Kipnis and Lavee, 1971). The presence of lignin in the peach endocarp was firstly reported by Ryugo (1963) in the early 1960s. This observation has been supported by a subsequent study in which the accumulation of lignin precursors (phenol bodies) was observed in endocarp cells (Masia et al., 1992). The lignin biosynthesis results from a sequential involvement of phenoloxidase, peroxidase, and laccases (Alba et al., 2000; Dardick et al., 2010). In peach endocarp cells, phenoloxidase was detected mainly in the ionically bound cell wall protein fraction suggesting its role in the polymerization of lignin precursor. This localization could suggest the engagement of this enzyme in the early changes of cell wall undergoing lignification, such as the polymerization reactions of oligolignols, occurring at the end of the first stage of development. Peroxidase and laccases seem more related to the late stage of sclerefication process by aiding the cross-coupling between the growing polymers. To support this vision, the activity of peroxidase and laccase increases concurrently with the rise of lignin content detected during the second stage of fruit development.

Additional information on lignin deposition in cell wall during drupe development and patterning have obtained from a Slow Ripening (SR) peach phenotype in which fruit development is apparently stopped during the stage III (Bonghi et al., 2011), and the flesh shows a very slow rate of softening accompanied by a low level of ethylene (Brecht and Kader, 1984). A metabolomic analysis of SR fruit, pointed out a strong accumulation of phenylpropanoids (in particular lignin and its precursors) in the mesocarp paralleled by the expression of phenylpropanoids biosynthetic genes (Botton et al., 2016). This evidence, together with microscopic analysis, suggests that SR mesocarp behaves like an endocarp. The comparison of the expression profile of genes responsible for endocarp identity in SR and Fantasia allowed the identification of an additional regulator of endocarp lignification named FLESHY, similar to Arabidopsis HECATE3 (Botton et al., 2016). In SR fruit, FLESHY shows a transient increase in the mesocarp while remaining at a very low level in Fantasia mesocarp. Therefore, FLESHY has been claimed to play a crucial role in determining the fruit tissue patterning of the peach fruit.

In *Prunus* spp, there is a strong variability of endocarp phenotypes, the most part of them have been obtained by using traditional breeding. Dardick and Callahan (2014), reported that almond shells were found to differ according to endocarp thickness, hardness, and bitterness. The seed of some peach, apricot, and plum varieties is easily exposed to pests and diseases as a consequence of the unsealed endocarp. This defect, named "split pit", is the result of a down-regulation of phenylpropanoid biosynthetic genes (Zhang et al., 2017). Environmental conditions (Engin et al., 2010), cultivation practices (Claypool et al., 1972) and the ripening time, play a role in the development of split pit. In particular, early maturing peach and plum cultivars are usually more susceptible to stone splitting, because their stones do not harden properly for resisting the growing forces of the rapidly expanding fruit flesh (Tani et al., 2009).

A natural phenotype isolated in a wild-type population of plum was called "Stoneless" for its incomplete development of the endocarp layer that results in a partially naked seed (Callahan et al., 2009). The stoneless phenotype is strongly affected by the environment conditions since a complete endocarp can develop in years with hot spring temperatures, while in cooler years very little stone is present. The absence of endocarp tissue suggests that this mutant does not contain a complete endocarp layer (Dardick and Callahan, 2014).

Contribution of Primary Metabolism to the Formation of a Lignified Endocarp

It has been reported that the activity of the most enzymes involved in primary metabolism are repressed during lignin and flavonoid biosynthesis in the endocarp layer (Dardick et al., 2010). However, this contrasts with the observation by Hu et al. (2012), which found the lignin content is positively related with the Pyruvate Dehydrogenase (PDH E1α) protein level, a well-characterized

enzyme complex that links two of the most important metabolic pathways of primary metabolism: glycolysis and TCA cycle (Tovar-Méndez et al., 2003). In addition, another PDH gene (a sub-unit called PDH E1β) has been identified as a member belong to a regulon that is induced in correspondence of lignin deposition in the peach endocarp layer (Dardick et al., 2010). One interpretation of these conflicting observations is that the expression of enzyme abundance was on a per DW basis and during lignification there is a large increase in the DW content of the tissue; and thus the decrease is just a dilution effect (Famiani et al., 2015). In support of this in both plum and cherry endocarp, a large number of enzymes involved in primary metabolism are abundant (or actually increase in abundance) on a per FW basis during lignification (Walker et al., 2011; Famiani et al., 2012). On the other hand, the relevant impact of endocarp lignification on fruit primary metabolism is suggested by the rerouting of several primary metabolites toward lignin biosynthesis. A decrease of protein synthesis has been observed during the very early phase of peach development, which follows the use of free amino acids as substrates for the synthesis of phenylpropanoids required for endocarp lignification (Lombardo et al., 2011; Rodriguez et al., 2019). Amino acids, phenylalanine in particular, are also precursors of cyanide glucosides such as prunasin, which are nitrogen-containing secondary metabolites that strongly accumulate in Prunus fruit. Cyanide glucosides have the ability to produce highly toxic hydrogen cyanide (HCN) when cleaved by mandelonitrile lyase. Differential expression of a putative mandelonitrile lyase gene has been observed in apricots having endocarp with different thicknesses and lignin content (Zhang et al., 2017). Previous reports have demonstrated that HCN can generate reactive oxygen species (ROS) (Oracz et al., 2009). Accumulation of ROS has been observed in tissues, including endocarp, undergoing

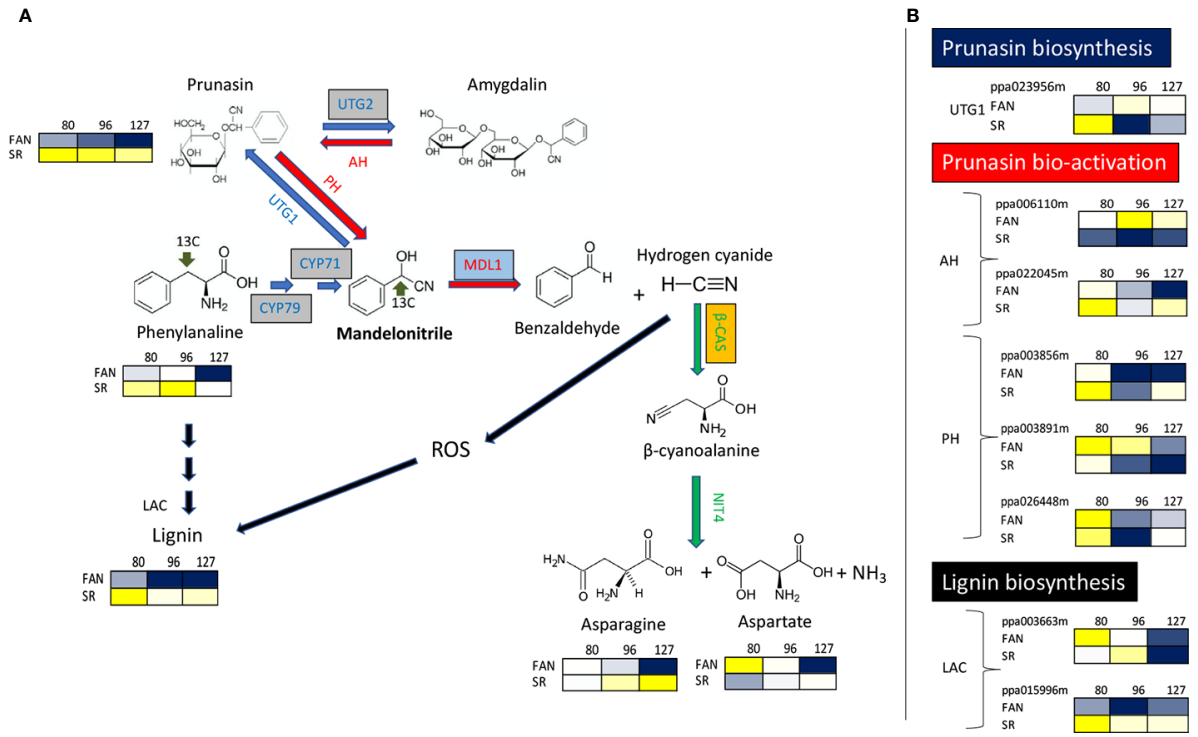

FIGURE 2 | The metabolic pathways for synthesis, bio-activation, and detoxification of the cyanogenic glucosides prunasin and amygdalin in the peach mesocarp of Fantasia (FAN) and slow ripening (SR). **(A)** Biosynthetic enzymes/genes (blue arrows) are: CYP79 and CYP71 (Cyt P450 monooxygenases); UGT1 (UDPG-mandelonitrile glucosyltransferase); and UGT2 (UDPG-prunasin glucosyltransferase). Bio-activation enzymes/genes (red arrows) are: AH (Amygdalin hydrolase); PH (prunasin hydrolase); MDL1 (mandelonitrile lyase). Detoxification enzymes/genes (green arrows) are: β-CAS (β-cyanoalanine synthase), NIT (Nitrilase 4). Lignin biosynthetic enzymes/genes (black arrows) LAC (Laccases). Levels of Phenylalanine, Prunasin, Asparagine, Aspartate at three fruit developmental stages (80 DAFB, late S2; 96 DAFB, early S3; 127 DAFB, pre-climacteric S4) assessed in both the genotypes (Fantasia= FAN, and slow ripening= SR). Metabolites significantly decreased were displayed in blue, while metabolites significantly increased were displayed in yellow. The brightness of each color corresponded to the magnitude of the difference when compared with average value. **(B)** Expression profile of genes involved in biosynthesis and bio-activation of cyanide glucosides, and in the last step lignin biosynthesis evaluated in FA and SR mesocarp at the three developmental stage reported in A. Each gene is identified by the transcript code (peach genome version 1, see at https://www.rosaceae.org/organism/24333). Transcript levels significantly decreased were displayed in blue, while transcript levels significantly increased were displayed in yellow. The brightness of each color corresponded to the magnitude of the difference when compared with the average value. Metabolites and transcripts level were retrieved from Botton et al. (2016). CYP79, CYP71, and UTG2 genes, boxed in grey, have been characterized only in almond seeds and their putative orthologs have been identified in peach (Thodberg et al., 2018). Transcripts of MALD1, boxed in light blue, have been detected in the endocarp layer of apricots (Zhang et al., 2017). Enzyme activity of β-CAS, boxed in orange, has been determined in peach endocarp (Hu et al., 2012; Rodriguez et al., 2019).

lignification (Liu et al., 2017). The detoxification of hydrogen cyanide, and consequently a potential reduction of ROS, is catalyzed by β-cyanoalanine synthase (Blumenthal et al., 1968; Machingura et al., 2016). It has been reported that during early phases of fruit development (up to 59 DAFB corresponding to S2) β-cyanoalanine synthase protein level shows a decreasing trend (Hu et al., 2012) or it almost stable (Rodriguez et al., 2019) during the lignification of endocarp, while it is increasing in the mesocarp (Hu et al., 2012; Rodriguez et al., 2019). These pieces of evidence suggest that endocarp lignification is accompanied by an increase of ROS precursors due to an increase of cyanide glucosides and reduced or stable detoxification of the action of hydrogen cyanide. To support this view, in the lignin-rich mesocarp of SR peach mutant it was observed a higher level of prunasin, paralleled by the accumulation of genes involved in its biosynthesis, in comparison to wild type peaches (**Figure 2**; Botton et al., 2016).

In addition, the interconversion between nucleotide sugars is affected by the lignification process on fruit primary metabolism. In peach, xylans are the most important component of hemicelluloses in the secondary wall as observed in other dicotyledonous plants (Harper and Bar-Peled, 2002). UDP-xylose is used for the backbone of xylans and its conversion from UDP-D-glucuronate is mediated by UDP-D-glucuronate carboxylase (UDP-GlcA DCX). The peach UDP-GlcA DCX was strongly over-expressed during endocarp lignification, while it remained at lower levels in the mesocarp (Hu et al., 2012).

FUTURE PERSPECTIVES

New investigations methods on the architectural and composition of cell walls, such as AFM and optical imaging approaches (for more detail see Sarkar et al., 2009), can aid in the understanding of cell wall modification occurring throughout the fruit patterning and development. This information is essential to correctly address the manipulation of the biosynthesis of primary metabolites used in cell wall building with the goal of rerouting them toward other biosynthetic pathways. Up to now, the most interesting advancements in this direction are regarding the manipulation of carbon flux for modifying cell wall polysaccharides composition and consequently fruit firmness and composition. A study has been carried out by silencing the tomato galacturonosyltransferase 4 (GAUT4), a member of enzyme family responsible for the pectin biosynthesis, showed that silenced fruits had an altered pectin composition, which coincided with an increase in firmness (De Godoy et al., 2013). Authors suggested that in silenced plants a shift in source to sink carbon partitioning occurred *via* the modulation of resource allocation *via* cell wall polysaccharides and raffinose metabolisms. For fruit trees, and in particular those harboring stone fruit, genetic transformation is still a long way for the difficulty to regenerate transformed plants (Prieto, 2011). However, it is possible by using agricultural practices to modify the carbon flux as demonstrated by covering with plastic film tangerine trees (Jin et al., 2018). Tangerine fruits collected from trees cultivated under plastic film were sweeter and softer. Authors suggested that the higher sugar accumulation in fruit may depend on the redistribution of carbohydrate toward fruit as indirectly supported by the parallel increase of sugar transporters gene expression in shaded trees. On the contrary, the modification of the water-soluble pectin and the protopectin content in shaded fruit resulted from the alteration of GAUTs and pectinesterases transcript profiles.

In conclusion, there are all premises for putting the reciprocal contribution between primary metabolism and cell wall into perspective to obtain better fruit as underlined by Beauvoit et al. (2018).

AUTHOR CONTRIBUTIONS

All authors have contributed significantly to the work and approved it for publication.

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MdMYC2 and MdERF3 Positively Co-Regulate α-Farnesene Biosynthesis in Apple

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α-Farnesene, a sesquiterpene volatile compound plays an important role in plant defense and is known to be associated with insect attraction and with superficial scald of apple and pear fruits during cold storage. But the mechanism whereby transcription factors regulate apple α-farnesene biosynthesis has not been clarified. Here, we report that two transcription factors, MdMYC2 and MdERF3 regulated α-farnesene biosynthesis in apple fruit. Dual-luciferase assays and Y1H assays indicated that MdMYC2 and MdERF3 effectively trans-activated the *MdAFS* promoter. EMSAs showed that MdERF3 directly binds the DRE motif in the *MdAFS* promoter. Subsequently, overexpression of *MdMYC2* and *MdERF3* in apple calli markedly activated the transcript levels of *MdHMGR2* and *MdAFS*. Furthermore, transient overexpression of *MdMYC2* and *MdERF3* in apple fruit significantly increased *MdAFS* expression and hence, α-farnesene production. These results indicate that MdMYC2 and MdERF3 are positive regulators of α-farnesene biosynthesis and have important value in genetic engineering of α-farnesene production.

Keywords: apple, α-farnesene, α-farnesene synthase, transcriptional regulation, MdMYC2, MdERF3, genetic engineering, terpenes biosynthesis

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INTRODUCTION

Plants produce a large number of metabolites that are essential for ecological interactions and terpenes are natural products of the largest and structurally most diverse class (Reddy et al., 2017). Terpenes play an important role in the communication between plants and the environment, between plants and animals, and between plants and plants; they can attract pollinators and seed spreaders, and they can act as defense agents against herbivores and pathogens. In addition, they protect plants from abiotic stress phenomena, such as high light intensity, high temperature, and oxidative stress, among others. Terpenes are also useful nutrients in human diet and are used as chemotherapeutic agents for their antitumor activities (Dudareva et al., 2006).

α-Farnesene is an abundant compound in apple peel, which has an important function in plant defense (Nieuwenhuizen et al., 2014). The oxidative product of α-farnesene, are widely considered as a principal cause of scald, the physiological disorder of apple and pear, which intensify when fruits are transferred to ambient temperature for transportation to the market (Huelin and Coggiola, 1970; Ingle and D'Souza, 1989; Rowan et al., 1995). Overexpression of α-farnesene synthase in pathogen-sensitive soybeans enhances plant antimicrobial activity and α-farnesene has also been considered as a potential biofuel precursor (Lin et al., 2017).

There are three kinds enzymes involved in α-farnesene metabolism: 1) 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), which initiates synthesize sesquiterpenes (Rupasinghe et al., 2001; Zhang et al., 2020); 2) farnesyl diphosphate synthase (FPS), which catalyzes the conversion of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) to farnesyl diphosphate (FPP), the substrate of α-farnesene synthesis; 3) α-farnesene synthase (AFS), which catalyzes the final rate-limiting step in α-farnesene biosynthesis (Pechous and Whitaker, 2004; Gapper et al., 2006); But in plant the regulatory mechanisms of α-farnesene biosynthesis remains largely unknown.

The synthesis of terpenes in plants is regulated by many transcription factors, including MYC, AP2/ERF, bZIP, WRKY. MYC2 is important for JA response to secondary metabolite accumulation. *AtMYC2* binds the promoters of *AtTPS11* and *AtTPS21* for regulating the synthesis of sesquiterpenes in *Arabidopsis thaliana* (Hong et al., 2012). In *Artemisia annua*, *AaMYC2* binds the G-boxlike motifs within the promoters of genes *CYP71AV1* and *DBR2* (Shen et al., 2016). In *Salvia miltiorrhiza*, *SmMYC2a* and *SmMYC2b* play important roles in regulating the biosynthesis of phenolic acids (Zhou et al., 2016). Ethylene response factors have also been well characterized for their roles in regulating the production of terpenes. *AaERF1* and *AaERF2* bound to the promoter of *AaADS* for inducing artemisinin synthesis (Yu et al., 2012). *TcERF15*, respectively, act as activator of tasy gene of taxol biosynthesis in *Taxus chinensis* (Zhang et al., 2015). In Newhall sweet orange fruit, the transcription factor *CitERF71* directly binds the *CitTPS16* promoter, therefore probably has a function in the transcriptional regulation of *E*-geraniol production (Li et al., 2017).

Although transcription factors related to the metabolic pathway of terpenes have been reported in recent years, there are few reports on transcription factors related to the synthesis of terpenes in apples. At present, regardless of the metabolic engineering research of α-farnesene or the biological research of apple superficial scald, transcription factors regulating the mechanism of α-farnesene biosynthesis have not been reported. By screening transcription factors involved in secondary metabolic regulation, we studied whether these transcription factors are participated in regulating the expression of α-farnesene synthase, thereby affecting the biosynthesis of α-farnesene.

Our results revealed that transcription factors MdMYC2 and MdERF3 effectively activated the promoter region of *MdAFS*, which is the terminal enzyme gene in the α-farnesene biosynthesis pathway; additionally, they activated the expression of the *MdAFS* gene, and ultimately promoted the accumulation the α-farnesene.

MATERIALS AND METHODS

Plant Materials and Treatments

Leaves obtained from five-year-old apple (Malus domestica Borkh. cv. White winter pearmain) trees were used in this study. The apple trees were cultivated in a culture room at Shandong Agriculture University. The "Orin" apple calli were used for genetic transformation and were grown at 24°C under dark conditions.

"White winter pearmains" apple leaves were treated with 100 μM MeJA and 50 mg L^{-1} Ethephon (Sigma-Aldrich), with 0.1% (v/v) ethanol as the mock. Samples were taken after 0, 2, 4, 6, and 12 h to analyze gene expression.

Fruits harvested at 140 days after full bloom were divided into four groups. The first group was not treated. The second group was treated with methyl jasmonate for 5 min. The third group was used for Ethephon treatments for 30 s. The fourth group was treated with 1-MCP for 12 h. All fruits were stored at room temperature (24°C) and 0°C for 4 weeks, with sampling every week during the storage period. The samples were frozen immediately in liquid nitrogen and then stored at -80°C.

Real-Time Quantitative PCR

Total RNA was isolated and first-strand cDNAs were synthesized, respectively, using a total RNA isolation system and First-strand cDNA Synthesis Kit (Tiangen, Beijing, China). All qRT-PCR assays were used in a CFX96 Real-time system (BIO-RAD) according to manufacturer instructions. Three independent biological replicates were carried out for each sample. Primer sequences used for real-time quantitative PCR were described in **Supplementary Table S1**.

Apple Calli Transformation

The ERF3 and MYC2 transgenic apple calli were obtained from Professor Hao's laboratory (An et al., 2016; An et al., 2018). The constructed recombinant plasmids were introduced into *Agrobacterium tumefaciens* LBA4404. 15-day-old "Orin" apple calli were infected with *A. tumefaciens* for 20 min which were carrying recombinant plasmids, and the apple calli were cultured on agar solidified MS medium for 2 days at 24 °C in darkness. Then, the apple calli were transferred to selective medium containing 35 mg L^{-1} hygromycin and 300 mg L^{-1} carbenicillin.

Dual-Luciferase Assay

Full-length *MdMYC2* and *MdERF3* sequences were amplified with the primers described in **Supplementary Table S1** and were inserted into pBI 121 vectors. The promoter of *MdAFS* (1,500 bp) was constructed in the pGreenII 0800-LUC vector. All constructs were individually transformed into *Agrobacterium* GV3101 and stored as glycerol stocks at -80°C. *Agrobacterium* cultures were prepared with infiltration buffer (10 mM MES, 10 mM $MgCl_2$, 150 mM acetosyringone, pH 5.6) to an OD600 of 1.0. The mixtures of transcription factor and promoter were infiltrated into tobacco leaves by needleless syringes. A living imaging apparatus was used for luminescence detection. For each transcription factor-promoter interaction, at least three independent experiments were performed with four replicates in each experiment.

Yeast One-Hybrid Assay

Y1H was used to detect verification of interaction between transcription factor and AFS as described by An et al. (2016). The MdMYC2 and MdERF3 gene was cloned into the pGADT7

vector and the promoter fragment of MdAFS were inserted into the pAbAi vector.

Electrophoretic Mobility Shift Assays (EMSA)

EMSAs were performed as described previously (Li et al., 2017). The LightShift™ Chemiluminescent EMSA Kit (Thermo, USA) was used in EMSA experiment. Oligonucleotide probes were synthesized and labeled with biotin. Biotin-labeled probes were incubated with MdERF3-GST protein in a binding buffer for 25 min, and the free and bound DNAs were separated in an acrylamide gel.

Transient Overexpression in Apple

Fruit injection assays were carried out as described previously (Li et al., 2012). The overexpression viral vectors MdMYC2-IL60-2 and MdMERF3-IL60-2 were generated and the mixed vectors were injected into the fruit peels. Two days after infiltration, the peel near the infiltration point was collected for volatile analysis.

Volatile Compounds Analysis by GC-MS

Volatile analysis was carried out as in our previous study (Deng et al., 2015). The fresh apple peels were ground in liquid nitrogen and 0.3 g was extracted with 5 ml of extraction buffer in a sealed container. 10 μl of 3-Nonanone (0.4 g L^{-1}) was an internal standard. The volatile compounds were collected by solid phase microextraction (SPME) and analyzed using GCMS-QP2010 with a FID detector (Shimadzu, Tokyo, Japan).

Statistical Analysis

Statistical analysis of the data was performed with SPSS. Data points represent the mean values ± standard deviation of three biological replicates. Differences were considered statistically significant when * $P < 0.05$ and ** $P < 0.01$.

RESULTS

JA and Ethephon Treatments Promote the Expression of MdAFS and Increase α-Farnesene Content in Apple

In order to study the mechanism of regulation of α-farnesene synthesis, we first analyzed the promoter of *MdAFS*, which is the terminal enzyme gene in the α-farnesene synthesis pathway. In the *MdAFS* promoter, many potential *cis*-acting elements associated with hormone-related responses were identified, such as MeJA, Ethylene, and ABA (**Supplementary Table S2**). JA has been widely used in regulating plant growth and secondary metabolism, and significant progress has been achieved in regulating the accumulation of terpenoid secondary metabolites by its use. Ethylene is an important hormone and ethylene treatment reportedly involves in volatile synthesis and ethephon facilitates the release of the ethylene. To examine whether the *MdAFS* and α-farnesene were induced by phytohormones, "White winter pearmains" leaves and fruits were treated with MeJA and Ethephon. The results indicated that the expression of *MdAFS* was significantly upregulated after these treatments. When apple leaves were treated with MeJA, the maximum expression level of *MdAFS* occurred at 6 h post treatment (**Figure 1A**). The response of *MdAFS* to Ethephon treatment was peaked within 12 h post treatment (**Figure 1A**) and the α-farnesene content in the apple leaves increased with the increase of treatment time, reaching the highest in 12 h (**Supplementary Figure 1A**). Concomitantly, α-farnesene content was markedly higher than in controls following MeJA and Ethephon treatments, but markedly lower treated with 1-MCP relative (**Figures 1B, C**).

Expression Profile of MdMYC2 and MdERF3 Correlated With MdAFS

It is reported that MYC2 and ERF play a significant roles in ethylene and JA signaling pathways. Combined with the previous promoter analysis and PlantCare software analysis results, we found that promoters contained MYCCONSENSUSAT (MYC), G-box, DRE and ERE motifs, which were reported to be the binding sites of MYC2 and ERF transcription factors, implying these transcription factors might involve in transcriptional regulation of MdAFS. Therefore, we selected transcription factors MdMYC2 and MdERF3 to test their correlation to MdAFS. As shown in **Figures 1D, E**, MdMYC2 and MdERF3 showed the same accumulation pattern as MdAFS in response to both MeJA and Ethephon treatments. The expression levels of MdMYC2 promoted at 2 h after MeJA treatment and peaked at 6 h; Further, the expression of MdERF3 peaked at 12 h. Gene expression analysis of *MdAFS*, *MdMYC2*, and *MdERF3* during room temperature storage of apple fruits under MeJA, ETH, and 1-MCP treatments showed the same results (**Supplementary Figures 1B–D**).

MdMYC2 and MdERF3 Enhance the Transcription of MdAFS

We conducted a firefly luciferase (Luc) complementation imaging assay to test if MdMYC2 and MdERF3 could regulate the expression of *MdAFS*. As predicted, these two transcription factors showed trans-activation effects on the *MdAFS* promoter (**Figure 2**). Studies have shown that transcription factors MdMYC2 and MdERF3 can bind G-box and DRE elements to regulate the expression of downstream genes (Li et al., 2016; Li et al., 2017). Y1H assays were performed to test whether *MdMYC2* and *MdERF3* could bind promoters of *MdAFS*. Thus, G-box and four repeated DRE motifs were integrated into yeast cells. We found that, indeed, the two transcription factors were capable of binding both the G-box and the DRE motifs. In addition, to conform the binding results, we performed an EMSA with the MdERF3 together with 25 bp promoter fragments of *MdAFS* containing the DRE motif. The DRE motif of the *MdAFS* promoter was recognized by *MdERF3* (**Figure 3**). These results indicated that MdMYC2 and MdERF3 effectively activated the α-farnesene biosynthetic gene *MdAFS*, and MdERF3 directly bound the DRE motif in the *MdAFS* promoter.

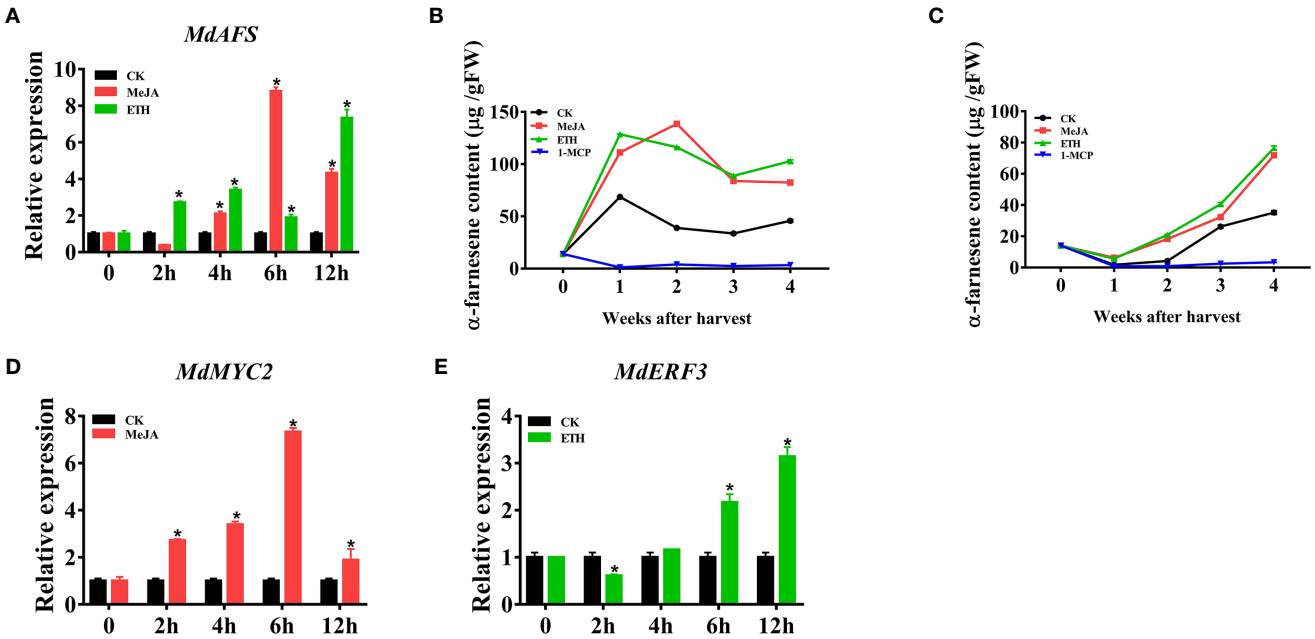

FIGURE 1 | (A) Expression of the *MdAFS* gene in apple leaves under MeJA and ETH treatments. **(B, C)** Effects of MeJA, ETH and 1-MCP on production of α-farnesene. White Winter Pearmain apples were treated with JA, ETH, and 1-MCP, and stored at room temperature and 0℃ for 4 weeks to detect the content of α-farnesene. **(D, E)** Effect of MeJA and ETH treatments on *MdMYC2* and *MdERF3* expression in apple leaves. Data points represent the mean values ± standard deviation of three biological replicates * indicates $P < 0.05$ compared to CK.

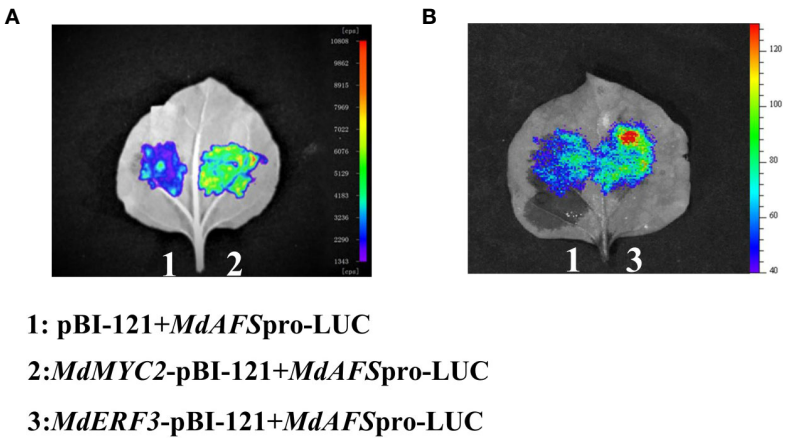

FIGURE 2 | The firefly luciferase (Luc) complementation imaging assays. *Agrobacterium* GV3101 strain harboring different constructs was infiltrated into tobacco leaves. Luminescence signals in the infiltrated region were measured 48 h after infiltration. **(A)** 1: pBI-121+MdAFSpro-LUC, 2: MdMYC2-pBI-121+MdAFSpro-LUC. **(B)** 1: pBI-121+MdAFSpro-LUC, 2: MdERF3-pBI-121+MdAFSpro-LUC.

MdMYC2 and MdERF3 Overexpression Upregulate MdAFS in Stable Transgenic Apple Calli

To further examine the regulation of *MdAFS* by *MdMYC2* and *MdERF3*, overexpression and antisense constructs were transformed into "Orin" apple calli. The expression levels of *MdAFS* gene in the wildtype and transgenic calli were analyzed by qRT-PCR. As shown in **Figure 4**, we found that when *MdMYC2* expression was upregulated by ~15-fold, *MdAFS* expression was upregulated by ~10-fold in the calli overexpression *MdMYC2*. The expression levels of *MdAFS* were significantly lower in *MdMYC2*-antisense calli than in control calli. Similarly, *MdERF3*-overexpressing calli significantly upregulated expression of the *MdAFS* gene, which was increased over 13-fold. The α-farnesene content of calli were measured, and the results showed that the content of over-expressed *MdMYC2* and *MdERF3* calli were

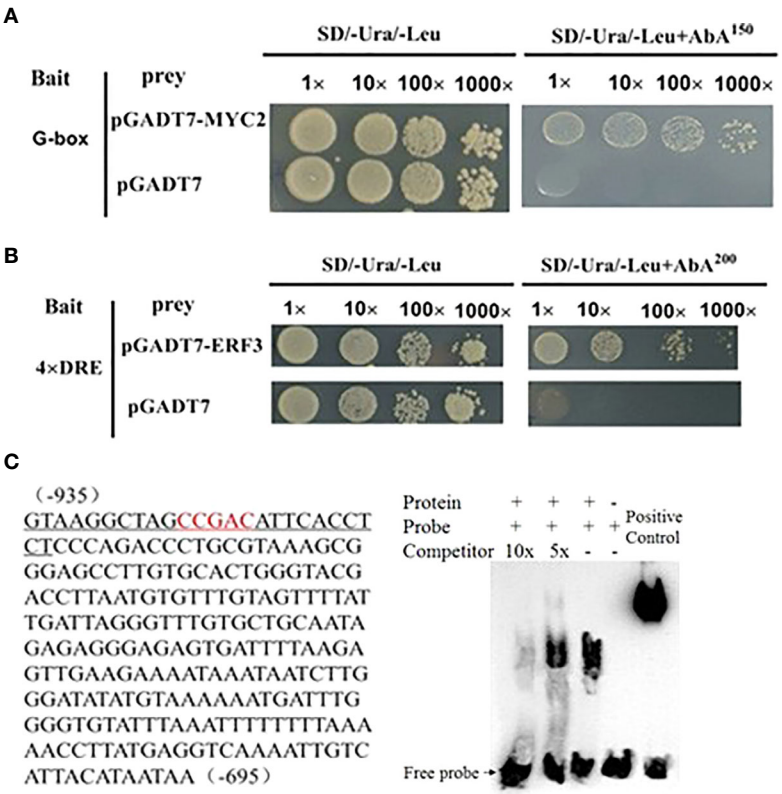

FIGURE 3 | MdMYC2 and MdERF3 bind to the *MdAFS* promoter. **(A, B)** Yeast one-hybrid analysis of MdMYC2 and MdERF3 binding to *MdAFS* promoter. **(C)** EMSA indicating that MdERF3 directly bound to the *MdAFS* promoter at CCGAC *in vitro*. Biotin-labeled probes were incubated with MdERF3 protein and then separated in an acrylamide gel. The competitors were unlabeled probes.

significantly higher than that of wildtype, and the α-farnesene content in antisense calli was the lowest (**Supplementary Figure 2**). These results showed that *MdMYC2* and *MdERF3* could positively regulate the expression of *MdAFS*.

Transient Overexpression of MdMYC2 and MdERF3 in Apple Peels Increased the Production of α-Farnesene

There are technically and experimentally challenging in generation and testing of transgenic apple fruit (Kotoda et al., 2006). The assay was chosen to examine the role of *MdMYC2* and *MdERF3* in the biosynthesis of α-farnesene in apple fruits. The α-farnesene content of the peels infiltrated with *MdMYC2* was 8.97 μg g^{-1}, respectively, representing a marked increase relative to the peels (7.4 μg g^{-1}) infiltrated with empty vector (**Figure 5**). At the same time, apple peels infiltrated with *MdERF3* exhibited a significant increase by ~1.7-fold in the level of α-farnesene content (**Figure 6**).

MdMYC2 and MdERF3 Affected Other Key Enzymes HMGR and FPPS in the Synthesis Pathway of α-Farnesene in Transgenic Apple Calli

Transcription factors not only regulate single enzyme genes in a pathway, they also regulate the co-expression of multiple genes, thereby regulating the synthesis of specific secondary metabolites. It has been reported that the expression level of the *MdHMGR2* gene is positively correlated with α-farnesene and ethylene production in apples during low temperature storage (Rupasinghe et al., 2001). Nevertheless, studies on the *FPPS* gene in apples are scarce. Only two genes, *MdFPPS1* and *MdFPPS2*, have been cloned from apples (Yuan et al., 2013), and the similarity between them is as high as 99%. In addition, the regulation of *MdFPPS* on the synthesis of α-farnesene has not been clarified. However, our results demonstrated that overexpression of MdMYC2 and MdERF3 maybe affect the expression of the α-farnesene synthesis pathway genes *MdHMGR2* and *MdFPPS* (**Figure 7**).

DISCUSSION

Terpenes are the largest and most diverse class of chemicals among the volatile compounds produced by plants (Tholl, 2015). In addition to their phyto-ecological benefits, terpenes are also of great economic value to humans, as they can be widely used in flavors, agriculture and in the chemical industry (Bouvier et al., 2006). α-farnesene was first found in apple and was found to play a role in plant defense. Farnesene is the precursor of the biofuel

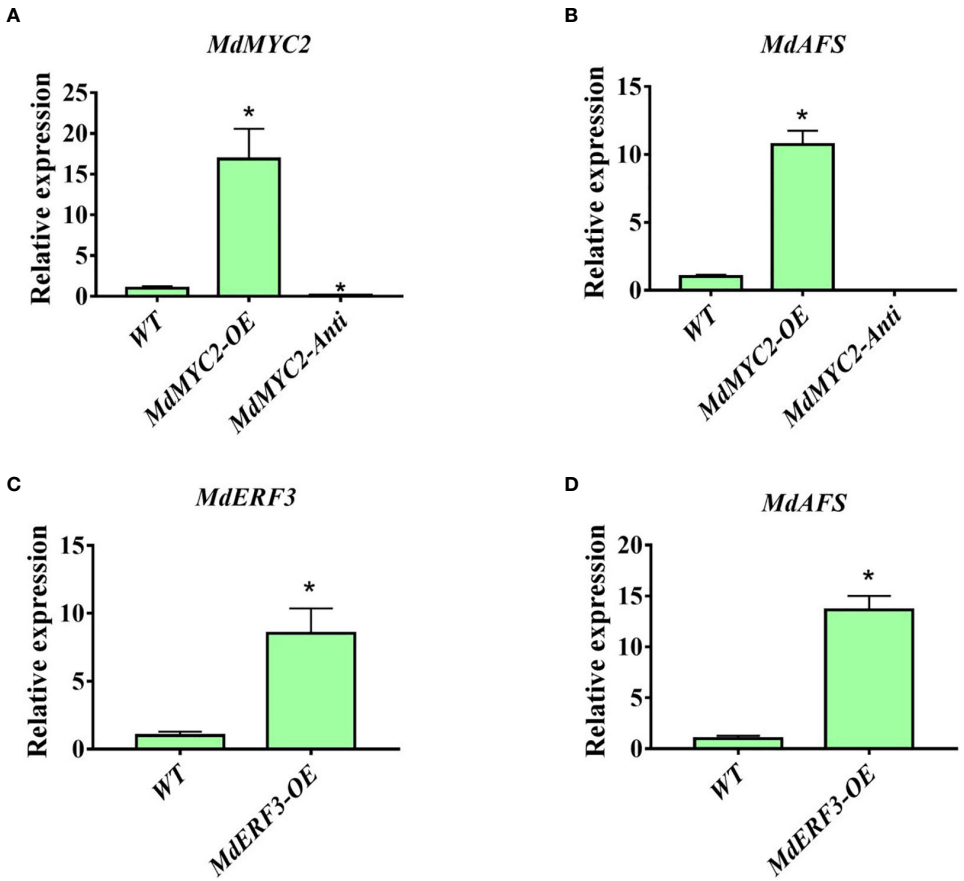

FIGURE 4 | Expression analysis of *MdAFS*, *MdMYC2*, and *MdERF3* in transgenic apple calli. **(A)** Expression level of *MdMYC2* in apple calli overexpressing and silencing *MdMYC2*. **(B)** Expression level of *MdAFS* in apple calli overexpressing and silencing *MdMYC2*. **(C)** Expression level of *MdERF3* in apple calli overexpressing *MdERF3*. **(D)** Expression level of *MdAFS* in apple calli overexpressing *MdERF3*. Standard errors were calculated from three sets of biological replicates. * indicates $P < 0.05$ compared to WT.

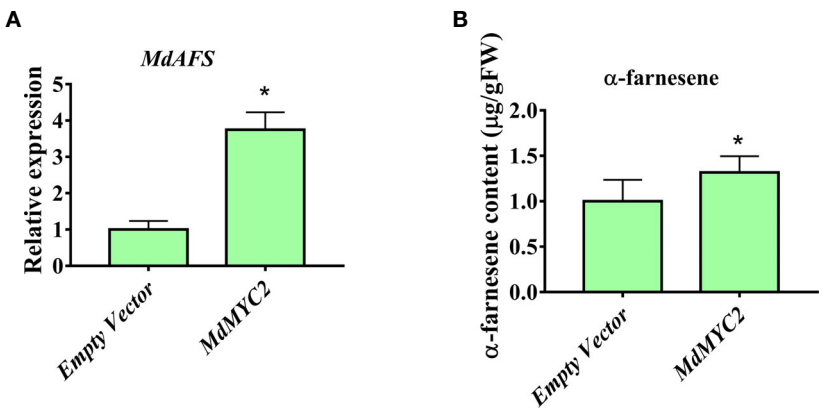

FIGURE 5 | Transient expression of *MdMYC2* in apple peel. **(A)** Relative *MdAFS* gene expression in apple peel injected with Empty Vector and *MdMYC2*. **(B)** α-farnesene content in peel infiltrated with *MdMYC2*. More than three apples were injected for determination, and the data were the average value obtained. * indicates $P < 0.05$ compared to Empty Vector.

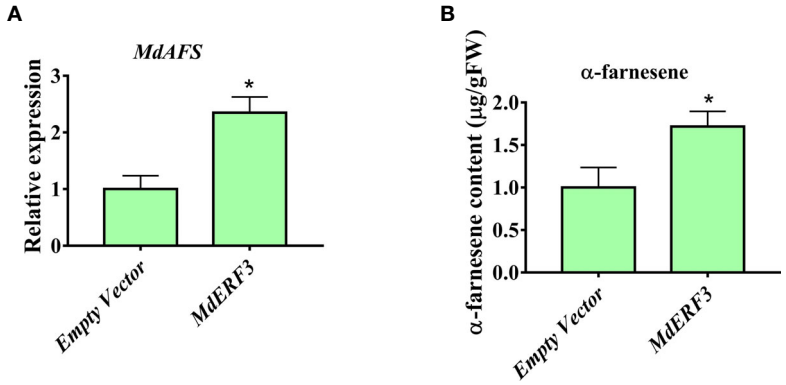

FIGURE 6 | Transient expression of *MdERF3* in apple peel. **(A)** Relative *MdAFS* gene expression in apple peel injected with Empty Vector and MdERF3. **(B)** α-farnesene content in peel infiltrated with MdERF3. More than three apples were injected for determination, and the data were the average value obtained. * indicates $P < 0.05$ compared to Empty Vector.

farnesane, which has broad market value, as it has attracted extensive attention of the society in recent years (Peralta-Yahya et al., 2011). However, sesquiterpenes including α-farnesene are naturally produced in limited quantities (Wallaart et al., 1999). Therefore, metabolic engineering of organisms is an alternative and attractive way to produce these rare and valuable compounds.

Currently, heterologous production of α-farnesene can be carried out in the host of *Escherichia coli* and *Yarrowia lipolytica*. In *E. coli*, the heterologous expression of α-farnesene synthase from fruits made production of α-farnesene in bacterial (Zhu et al., 2014). Recently, the feasibility of producing α-farnesene in metabolically engineered *Y. lipolytica* was demonstrated for the first time (Yang et al., 2016).

In recent years, there have been many studies on transcriptional regulation, as the use of transcription factors is one of the effective methods to increase the yield of terpenoid secondary metabolites. Moreover, transcription factors can activate or inhibit the expression of several key enzymes in plant secondary metabolic biosynthetic pathways by interacting with cis-acting elements in target gene promoters, thus, they effectively start or close secondary metabolic biosynthetic pathways, and regulate the biosynthetic process of specific secondary metabolites, thereby effectively affecting their accumulation.

MYC transcription factors are the most widely separated and thoroughly studied bHLH transcription factors. Their conserved domains regulate the expression of target genes by combining with E-Box (CANNTG) or G-Box (CACGTG) elements of target promoters (Pires and Dolan, 2010). At the same time, MYC family members participate in plant growth and development, resistance to environmental stress, JA and other signal transduction processes, and also in the regulation of secondary metabolic pathways (Gao et al., 2015). MYC transcription factors have been isolated from species such as *Catharanthus roseus*, *Taxus*, *Artemisinin*, *Arabidopsis thaliana*, tomato, and apple.

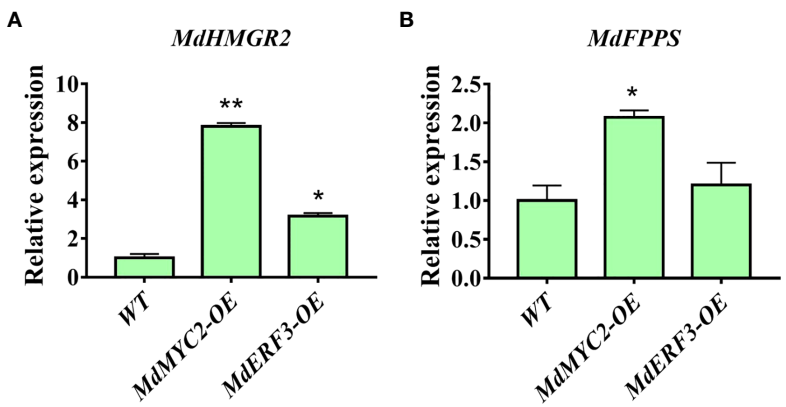

FIGURE 7 | Expression analyses of *MdHMGR2* and *MdFPPS* in apple calli. **(A)** Expression analysis of *MdHMGR2* in overexpressing *MdMYC2* and *MdERF3* apple calli. **(B)** Expression analysis of *MdFPPS* in overexpressing *MdMYC2* and *MdERF3* apple calli. Each value is expressed as mean ± SE (n = 3). * indicates $P < 0.05$ compared to WT and ** indicates $P < 0.01$ compared to WT.

Among MYC transcription factors found in plants, MYC2 is the most in-depth studied that plays an important role in the JA-mediated signal regulation pathway in plants. MYC2 can enhance plant resistance to insects by positively regulating JA-induced insect-resistant genes. In *Arabidopsis thaliana*, MYC2 regulates JA-mediated resistance to insect pests, and tolerance to oxidative stress by enhancing ascorbic acid redox cycle and flavonoid biosynthesis (Dombrecht et al., 2007). In addition, MYC2 is a positive regulator of JAs-mediated secondary metabolite synthesis. In tobacco, NtMYC2a and NtMYC2b positively regulate the JA response gene PMT to promote nicotine formation (Zhang et al., 2012). In *Salvia sclarea*, WRKY and MYC2 transcription factors which are controlled by MJ elicitation, coactivate MEP-biosynthetic genes and accumulation of abietane diterpenes (Alfieri et al., 2018).

APETALA2/Ethylene (AP2/ERF) transcription factors play an important role in regulating plant growth, development and maturation, responses to biotic and abiotic stress, and secondary metabolism (Agarwal et al., 2006). The ERF subfamily responds primarily to abiotic stress either dependently or independently of plant hormones such as ethylene and various biotic stress phenomena, such as pathogens and insect attack (Lai et al., 2014). ERF transcription factors reportedly can bind specifically GCC-box or DRE/CRT elements. The terpenes can be regulated by transcription factors, including AP2/ERF. ZmERF58 is able to directly bind the promoter of *ZmTPS10* to synthesize E-β-farnesene and E-α-bergamot in maize (Li et al., 2015). *NtERF32* and related ERF genes are important non-*NIC2* locus related to transcriptional regulators of nicotine and total alkaloid formation (Sears et al., 2014). Further, ORA59, which is the AP2/ERF-domain transcription factor, and two GCC box binding sites that enables the *PDF1.2* gene to respond to the JA and ET signaling pathways (Zarei et al., 2011).

However, although there are several transcription factors being involved in terpene biosynthesis, the transcriptional regulation of the α-farnesene remains unclear. Similarly, it has been reported that jasmonic acid and ethylene, two important plant hormones, coordinate to regulate plant growth, development and tolerance to pathogens. Indeed, EIN3/EIL1, two important transcription factors in ethylene, reportedly mediate the signal interaction between jasmonic acid and ethylene; hence, EIN3/EIL1 is also a positive regulator of the jasmonic acid signaling pathway that regulates plant root development and resistance responses (Zhu et al., 2011). Thus, the question may be asked, is there a common transcription factor regulating α-farnesene synthesis in the JA and ET signaling pathways?

In this study, we began with the regulation of these two hormones on α-farnesene. We found that whether stored at room temperature or at low temperature, JA and ET increased and 1-MCP decreased α-farnesene biosynthesis in apple fruit (**Figures 1B, C**). ERF and MYC2 are not only the key regulators in JA signaling pathway, but also the nodal factors connecting JA, ET, and other signal hormones. In addition, most steroid and alkaloid biosynthesis regulation is related to ERF and MYC2. Consistently with previous studies, we selected MdMYC2 and MdERF3 transcription factors for research, finding that these two transcription factors showed a similar expression pattern to *MdAFS* (**Figure 1**). This suggested that transcription factors MdMYC2 and MdERF3 might share a putative common regulatory mechanism of α-farnesene biosynthesis in plants. Further investigation of the transcriptional regulation of *MdAFS* gene by MdMYC2 and MdERF3, together with results of the dual-luciferase assay, the Y1H assay, and EMSA, led to the conclusion that MdMYC2 and MdERF3 is participated in transcriptional regulation of the *MdAFS* gene (**Figures 2** and **3**). In addition, to study the role of MdMYC2 and MdERF3, transient overexpression experiments were carried out. Apple peel infiltrated with MdMYC2 and MdERF3 showed a marked increase α-farnesene (**Figures 5** and **6**). Additionally, we found that transcription factors MdMYC2 and MdERF3 could regulate the expression of key enzyme genes *MdAFS*, *MdHMGR2*, and *MdFPPS* in the synthesis pathway of α-farnesene in stable transgenic apple calli (**Figures 4** and **7**). This indicated that MdMYC2 and MdERF3 effectively improved the synthesis of α-farnesene by activating the co-expression of multiple genes in the α-farnesene biosynthetic pathway. Consistently, it has been reported that MdMYC2 enhanced the transcription of MdERF3 by binding its promoter (Xu et al., 2017). We analyzed the transcript level of *MdERF3* in apple calli after overexpressing *MdMYC2*, and the results showed that the expression of *MdERF3* was increased in the overexpressing *MdMYC2* apple calli (**Supplementary Figure 3**). Although we failed to verify that MdMYC2 binds directly the promoter of

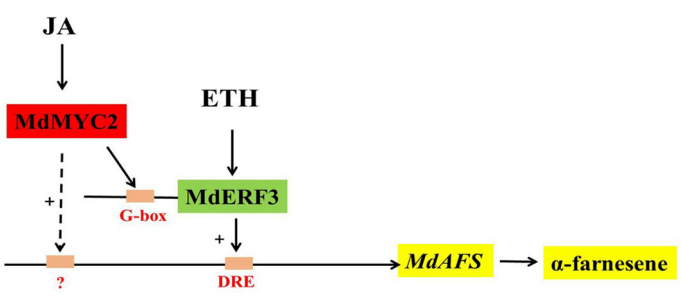

FIGURE 8 | Model of synthesis of α-farnesene regulated by MdMYC2 and MdERF3.

gene *MdAFS*, our studies showed that transcription factor MdMYC2 promoted the synthesis of α-farnesene by regulating the expression of *MdAFS*. Based on these results and earlier studies, we propose the hypothetical working model shown in **Figure 8** to explain the synthesis of α-farnesene and the involvement of MdMYC2 and MdERF3 in its regulation.

It has been suggested that superficial scald development in apple and pear is related to α-farnesene and its oxidation products, namely, conjugated trienols (CTols), which accumulate progressively in the fruit peel during storage (Bordonaba et al., 2013). Previous investigations showed that l-MCP treatment induced scald resistance while inhibiting ethylene-dependent ripening. This indicated that the ethylene metabolic pathway may also be involved in the process of superficial scald (Karagiannis et al., 2018). Additionally, analysis of "Granny Smith" apples suggested that ethylene-related transcription factors might regulate *AFS* transcription during low temperature and then regulate the synthesis of α-farnesene and the occurrence of fruit superficial scald (Busatto et al., 2018). This study provided new insights into the regulation of α-farnesene and the mechanism of superficial scald.

In summary, this is the first report on the mechanism of regulation of the biosynthesis of α-farnesene at the transcriptional level. Our results indicate that MdMYC2 and MdERF3 exert their regulatory effects as positive regulators of α-farnesene biosynthesis-related genes. Further, our study identified key candidate genes and new strategies for using metabolic engineering methods to achieve high yields of α-farnesene.

DATA AVAILABLITY STATEMENT

The datasets generated for this study are available on request to the corresponding authors.

AUTHOR CONTRIBUTIONS

Designed the experiments: QW, YH, and YZ. Performed the experiments: QW and HL. Analyzed the data: QW, HL, MZ, and SL. Wrote the paper: QW, HL.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.512844/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | (A) α-farnesene content in apple leaves under MeJA and ETH treatments. **(B)** Expression of the MdAFS gene in apples stored at room temperature for 4 weeks under MeJA, ETH and 1-MCP treatments. **(C)** Expression of the MdMYC2 gene in apples stored at room temperature for 4 weeks under MeJA, ETH and 1-MCP treatments. **(D)** Expression of the MdERF3 gene in apples stored at room temperature for 4 weeks under MeJA, ETH, and 1-MCP treatments.

SUPPLEMENTARY FIGURE 2 | α-farnesene content in apple calli overexpressing *MdMYC2*, *MdERF3* and silencing *MdMYC2*.

SUPPLEMENTARY FIGURE 3 | Analysis of *MdERF3* transcript level in apple calli after overexpression *MdMYC2*.

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frontiers in Plant Science

REVIEW
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Stone Fruit as Biofactories of Phytochemicals With Potential Roles in Human Nutrition and Health

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Phytochemicals or secondary metabolites present in fruit are key components contributing to sensory attributes like aroma, taste, and color. In addition, these compounds improve human nutrition and health. Stone fruits are an important source of an array of secondary metabolites that may reduce the risk of different diseases. The first part of this review is dedicated to the description of the main secondary organic compounds found in plants which include (a) phenolic compounds, (b) terpenoids/isoprenoids, and (c) nitrogen or sulfur containing compounds, and their principal biosynthetic pathways and their regulation in stone fruit. Then, the type and levels of bioactive compounds in different stone fruits of the Rosaceae family such as peach (*Prunus persica*), plum (*P. domestica*, *P. salicina* and *P. cerasifera)*, sweet cherries (*P. avium*), almond kernels (*P. dulcis*, syn. *P. amygdalus*), and apricot (*P. armeniaca*) are presented. The last part of this review encompasses pre- and postharvest treatments affecting the phytochemical composition in stone fruit. Appropriate management of these factors during pre- and postharvest handling, along with further characterization of phytochemicals and the regulation of their synthesis in different cultivars, could help to increase the levels of these compounds, leading to the future improvement of stone fruit not only to enhance organoleptic characteristics but also to benefit human health.

Keywords: chlorogenic acid, flavonoids, anthocyanins, carotenoids, postharvest, volatiles, health-promoting, cyanogenic compounds

HEALTH PROMOTING PROPERTIES OF FRUIT PHYTOCHEMICALS

Here, a brief description of the main secondary organic compounds found in plants, their principal biosynthetic pathways, and their biosynthesis regulation in stone fruit is provided. Then, a review about the levels and types of secondary metabolites found in different stone fruit is presented. The factors that have been identified as being involved in defining the levels of these compounds in stone fruit are finally presented. It is concluded that the identification of the key regulatory points in the

biosynthesis of these compounds or in their chemical modification to produce more compounds with better activity, as well as the identification of pre- and postharvest managements that could increase their levels, will aid in the future improvement of stone fruit for the benefit of human health.

Phytochemicals known as secondary metabolites possess diverse physiological properties, being involved in sensory attributes (aroma, taste and color) and in defense against pathogens, different kind of stresses and/or injuries (Tomas-Barberan et al., 2001; Montevecchi et al., 2013). Besides, the secondary metabolites of plants are highly beneficial to consumers. In this regard, in recent decades, consumers have become more aware of the relationship between diet and diseases. Today, there is a broad consensus that increased consumption of fruits and vegetables contributes to improving health and well-being by reducing the risk of diseases, such as cardiovascular diseases and some forms of cancer (Riboli and Norat, 2003; Hung et al., 2004). The Joint FAO/WHO (Food and Agriculture Organization/World Health Organization) report on diet, nutrition, and prevention of chronic diseases recommended in 2003 the intake of a minimum 400 g of fruits and vegetables per day, ideally 800 g, for the prevention of chronic diseases such as cancer, diabetes, heart disease and obesity (WHO Technical Report Series, 2003).

The health promoting properties of fruits and vegetables are due to the presence of some vitamins (such as A, C, E, and folates), dietary fibers, and secondary metabolites, some of which are unique of plants. Phenolic compounds, among which flavonoids are the most ingested during daily life from products of plant origin (Chun et al., 2007), are important secondary organic metabolites in terms of their health-promoting properties, with possible preventive role in neurological disorders and potential protection against chronic diseases (Weng and Yen, 2012; Silva and Poganik, 2020). In addition, different biological activities have been described for chlorogenic acid, a phenolic compound, which include anti-inflammatory (Liang and Kitts, 2016), anticancer (Liu Y. J. et al., 2013), antioxidant power, including the inhibition of lipid oxidation (Sasaki et al., 2010), antilipidic, antiepileptic, neuroprotective (Aseervatham et al., 2016), antidiabetic (Meng et al., 2013), and antihypertensive (Suzuki et al., 2006; Zhao et al., 2012). This phenolic compound has also beneficial effects in disorders related to the metabolic syndrome (Bhandarkar et al., 2019a; Bhandarkar et al., 2019b). Moreover, it possesses antimicrobial activity against a wide range of organisms, including bacteria, yeasts, molds, viruses, and amoebas, and thus, it could be used as an antimicrobial agent (Santana-Gálvez et al., 2017) for the preservation of food products. In addition, anthocyanins, another group of polyphenols, prevent tumor development by inhibiting cancer cells proliferation when tested *in vitro* and *in vivo*. Anthocyanins also exhibit anti-inflammatory activity, present neuroprotective, anti-obesity, antidiabetic activities and are proposed to prevent cardiovascular disease (extensively reviewed in Li et al., 2017). With respect to caroteonids, their role in human nutrition is well established as precursors of vitamin A and due to their antioxidant activity. Their protective function in liver health is well documented and reviewed by Elvira-Torales et al. (2019). Other metabolites that have received attention are the cyanogenic glycosides, which possess anticancer properties (Fukuda et al., 2003, Jaswal et al., 2018).

Different studies have indicated that stone fruits are particularly rich in important phytochemicals, which constitute an extra benefit to their pleasant taste and flavor. These metabolites include phenolic compounds and terpenoids, among others. Peach, plums, cherries have an important antioxidant activity due to their phenolic content (Kim et al., 2003b; Cevallos-Casals et al., 2006; Serra et al., 2011). *In vitro* studies have demonstrated the antimicrobial activity of plum and peach extracts (Cevallos-Casals et al., 2006; Belhadj et al., 2016). *In vitro* and *ex vivo* studies also showed the anti-inflammatory properties of *P. persica* extracts (Gasparotto et al., 2014). Pharmacological studies showed that *P. persica* has antihypertensive properties (Kim et al., 2019), and it influences the central cholinergic system (Kim et al., 2003c). Moreover, intake of peaches protects rat tissues from nicotine toxicity (Kim et al., 2017). Almond intake reduces cardiovascular disease risk by modulating plasma lipoproteins (extensively reviewed in Berryman et al., 2011), contributes to satiety (Hull et al., 2015), delays lipid bioaccessibility (reviewed in Grundy et al., 2016), and decreases inflammation and oxidative stress (reviewed in Kamil and Chen, 2012). Apricot has also an important therapeutic and nutritional value. Among its health promoting activities, antimicrobial, antimutagenic, cardio-protective, hepato-protective, and antioxidant properties have been described (Ozturk et al., 2009; Erdogan-Orhan and Kartal, 2011; Yurt and Celik, 2011; Chen et al., 2020).

MAIN SECONDARY ORGANIC COMPOUNDS FOUND IN PLANTS: GENERAL DESCRIPTION OF THE BIOSYNTHETIC PATHWAYS AND REGULATION WITH EMPHASIS IN STONE FRUITS

Secondary metabolites can be grouped into three major classes: (a) phenolic compounds, (b) terpenoids/isoprenoids, and (c) nitrogen or sulfur containing compounds. These phytochemicals are derived from main primary pathways (glycolysis, the tricarboxylic citric acid (TCA) cycle, the pentose phosphate pathway, aliphatic and aromatic amino acids and the shikimic acid pathway) (**Figure 1**) (Aharoni and Galili, 2011). The shikimate pathway is a key route that conducts the synthesis of tyrosine, phenylalanine, and tryptophane (for review, see Tzin and Galili, 2010; Vogt, 2010; Maeda and Dudareva, 2012). This synthetic route starting from phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) connects primary metabolism to aromatic amino acid biosynthesis. This pathway, together with its intermediate metabolites (*i.e.* chorismate), provides precursors for the biosynthesis of folates, quinones, phytohormones,

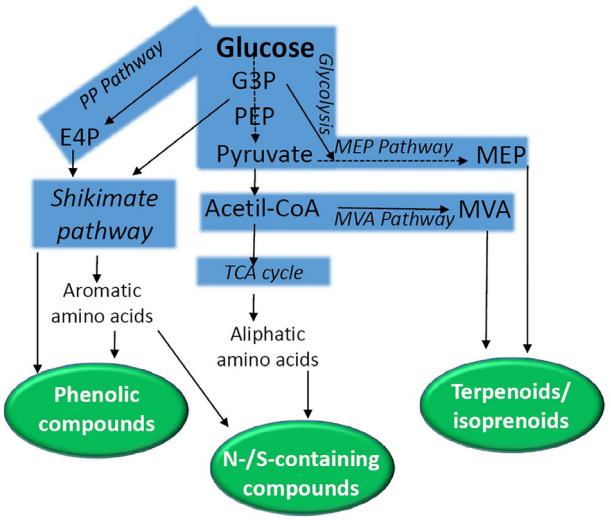

FIGURE 1 | A simplified scheme of the biochemical pathways conducting the synthesis of secondary metabolites in plants. Natural products are grouped into phenolic compounds, N-/S-containing compounds, and terpenoids. E4P, erythrose 4-phosphate; G3P, glyceraldehyde 3-phosphate; MEP, 2-*C*-methyl-d-erythritol 4-phosphate; MEV, mevalonate; PP, Pentose phosphate; PEP, phosphoenolpyruvate; TCA, tricarboxylic acid.

alkaloids, indole glucosinolates, flavonoids, hydroxycinnamic acids, lignins, and lignans.

Phenolic Compounds

Phenolic compounds are synthesized from shikimic/phenylpropanoid and the phenylpropanoid–acetate–malonate pathways and encompass a large group of monomeric and polymeric phenols and polyphenols. Phenylalanine is the precursor of a wide range of volatiles including phenylpropenes, phenylpropanes, phenethyl derivatives, and benzenoids (Gonda et al., 2018).

Phenylpropanoids are simple phenolic compounds with a benzene ring and a lateral chain, which serve as precursors of compounds such as benzoic acid derivatives, flavonoids, coumarins, stilbenes, lignans and lignins, and condensed tannins (Oksana et al., 2012). The phenylpropanoid pathway initiates with phenylalanine synthesized in the shikimic acid pathway, which generates cinnamate by the action of phenylalanine ammonia lyase (PAL, **Figure 2**). The consecutive action of cinnamic acid 4-hydroxylase (C4H) renders 4-coumarate that finally gives 4-coumaroyl-CoA by the action of 4-coumarate-CoA ligase (4CL).

Phenolic acids include derivatives of benzoic acid (C_6–C_1) such as hydroxybenzoic acids and of cinnamic acid (called hydroxycinnamic acids, C_6–C_3) (Walker and Famiani, 2018). The hydroxybenzoic acids 4-hydroxybenzoic acid (4-HBA), vanillic acid (3-methoxy-4-hydroxy) and protocatechuic acid (3,4-dihydroxy) are constituents of lignin (Pietta et al., 2003). On the other hand, gallic acid is present in hydrolysable and condensed tannins. Hydroxycinammic acids include caffeic (3,4-dihydroxycinnamic acid), ferulic (4-hydroxy-3-methoxycinnamic acid), sinapic acids (3,5-dimethoxy-4-hydroxy) and *p*-coumaric (4-hydroxy) acid, and their conjugates, mainly as esters of quinic acid (chlorogenic acids).

Chlorogenic Acid

Chlorogenic acid (5-O-caffeoylquinic acid) is an ester of caffeic acid and (−)-quinic acid from the hydroxycinnamic acid family. It can be found in different foods and herbs (Santana-Gálvez et al., 2017; Walker and Famiani, 2018).

Early studies proposed the direct synthesis of this compound through transesterification from caffeoyl-CoA (synthesized from

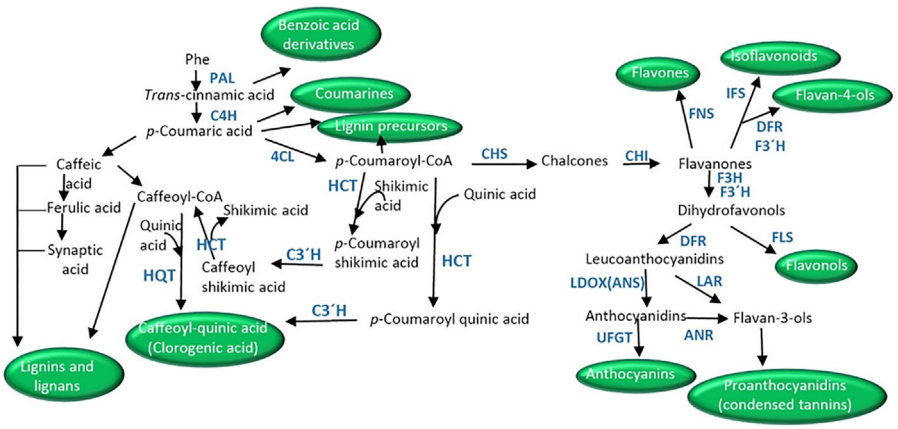

FIGURE 2 | Schematic of biosynthetic pathways derived from phenyalnaline leading to the synthesis of coumarins, benzoic acid derivatives, clorogenic acid, lignans, lignins, and the different flavonoids (isoflavonoids, flavonols, flavones, flavonls, anthocyanins, proanthocyanidins). ANR, anthocyanidin reductase; CHI, chalcone isomerase; CHS, chalcone synthase; C4H, cinnamate-4-hydoxylase; C3′H, *p*-coumarate 3′-hydroxylase; 4CL, *p*-coumaroyl CoA ligase; DRF, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; FNS, flavone synthase; F3′H, flavonoid 3′-hydroxylase; FLS, flavonol synthase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; HQT, hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase; IFS, isoflavone synthase; LDOX/ANS, leucoanthocyanidin dioxygenase/anthocyanidin synthase; LAR, leucoanthocyanidin reductase; PAL, phenylalanine ammonia lyase; UFGT, UDP-flavonoid glucosyl transferase.

caffeic acid in the reaction catalyzed by *p*-coumarate 3-hydroxylase, C3H) and quinic acid by the action of hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT, Stöckigt and Zenk, 1974, **Figure 2**). Nevertheless, the main route in higher plants is the 3′-hydroxylation of *p*-coumaroyl quinic acid by the cytochrome P450 monooxygenase *p*-coumarate 3′-hydroxylase or *p*-coumaroyl ester 3′-hydroxylase (C3′H, Niggeweg et al., 2004; Abdulrazzak et al., 2006; **Figure 2**). *p*-Coumaroyl quinic acid is synthesized from *p*-coumaroyl-CoA and quinic acid by the activities of a hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT). In addition, the action of C3′H on *p*-coumaroyl shikimate (synthesized from *p*-coumaroyl-CoA and shikimate by HCT) to give caffeoyl shikimic, which then by the action of HCT (Hoffmann et al., 2003) gives shikimic acid and caffeoyl-CoA has also been described. Caffeoyl-CoA then is the substrate of HQT in the first reaction (**Figure 2**).

Flavonoids

Flavonoids consist of a fifteen-C phenylpropanoid core (the flavan skeleton) formed by two aromatic rings connected by a heterocyclic pyran ring (C_6–C_3–C_6). Depending on the oxidation and number of unsaturations of the pyran ring, flavonoids are classified into different groups named as flavones, flavanones, isoflavones, flavonols, 3-deoxy flavonoids, and anthocyanins (Andersen and Markham, 2005).

The general pathway of flavonoid synthesis branches from the phenylpropanoid and malonic pathways (**Figures 1** and **2**). The first committed step is catalyzed by the enzyme chalcone synthases (CHS) which synthesizes 4,2′,4′,6′-tetrahydroxychalcone (chalcone) by condensing *p*-coumaroyl-CoA with malonyl-CoA (Jez and Noel, 2000). Chalcones are the main precursors of all flavonoids (Dixon and Steele, 1999). The product of CHS is later isomerized into flavanones (*e.g.*, naringenin or eriodictyol) by chalcone isomerase (CHI) (Winkel-Shirley, 2001). Flavanones are the precursors of flavones in the reaction catalyzed by flavone synthase (FNS) (Martens and Mithofer, 2005). Alternatively, flavanones give rise to dihydroflavonols (dihydrokaempferol), precursors of anthocyanins and proanthocyanidins, by the action of flavanone 3-hydroxylase (F3H). Dihydroflavanols can be routed to flavonols' synthesis by the action of flavonol synthase (FLS) (Pelletier et al., 1997). On the other hand, the action of flavonoid 3′-hydroxylase (F3′H) or flavonoid 3′5′-hydroxylase (F3′5′H) transforms dihydrokaempferol into dihydroquercetin or dihydromyricetin, respectively.

The existence of large number of flavonoids is the consequence of the different modifications on the main compounds by the action of different glycosyltransferases, methyltransferases, and acyltransferases (Saito et al., 2013). Flavonoid aglycones can be glycosylated at positions C-3, C-5, and C-7. The ability to glycosylate flavonol, anthocyanidin, and anthocyanin aglycones depends on the type of glycosyltransferase (Saito et al., 2013). Instead, flavonoid methyltransferases (FMTs) methylate flavonols giving isorhamnetin (Tohge et al., 2007).

Enzymes of the flavonoid biosynthetic pathway are grouped forming metabolons channelizing the different intermediates into the different routes (Winkel-Shirley, 1999). Although being cytosolic, the enzymes of the flavonoid pathway are bound to the cytoplasmic face of the endoplasmic reticulum by interacting with cytochrome P450 proteins (Saslowsky and Winkel, 2001). In addition, enzymes of the pathway such as CHS, CHI (Saslowsky et al., 2005), and FLS (Kuhn et al., 2011) have also been found in the nucleus.

Anthocyanins

Anthocyanins are water-soluble pigments belonging to the flavonoids and one of the main compounds responsible for coloration in plants (Tanaka et al., 2008). Their accumulation in the vacuole gives red, orange, blue, and purple color to different plant tissues and organs (Grotewold, 2006). Fruit color is a key quality trait, in which anthocyanin accumulation is involved.

Anthocyanins are synthesized as part of the flavonoid pathway through the action of dihydroflavonol 4-reductase (DFR, Heller et al., 1985; Reddy et al., 1987, **Figure 2**). The enzyme converts the dihydroflavonols or eriodictyol, a flavanone, to leucoanthocyanidins. The next step is catalysis by the action of a leucoanthocyanidin dioxygenase/anthocyanidin synthase (LDOX/ANS) which finally gives the anthocyanidins (Nakajima et al., 2001). A great number of anthocyanidins have been described in plants, of which delphinidin, pelargonidin, cyaniding, and luteolinidin are the most abundant. Anthocyanidins are the precursors of 2,3-*cis*-2R,3R-flavan 3-ols (known as proanthocyanidins or condensed tannins; *i.e.* epicatechin) by the reaction catalyzed by anthocyanidin reductase (ANR, Xie et al., 2003). Condensed tannins contribute to the astringent flavor of fruits. Leucocyandins can conduct the synthesis of catechin, another proanthocyanidin, by the action of leucoanthocyanidin reductase (LAR). Chatechins are the units of the polymeric proanthocyanidins constituting a subgroup of flavonoids. Chatechins can be galloylated by esterification with gallate in the 3-position of the C-ring (He et al., 2009). Alternatively, anthocyanidins can be modified by glycosylation to yield different anthocyanins (**Figure 2**). UDP-flavonoid glucosyl transferases (UFGT) catalyze the glycosylation of flavonoids using a UDP-sugar at 3-, 5-, 7-, 3′-, or 4′-OH positions (Tohge et al., 2005; Bowles et al., 2006; Pang et al., 2008; Yonekura-Sakakibara et al., 2009; Montefiori et al., 2011). In addition, acylation and methylation also increase anthocyanin variability (Nakayama et al., 2003; Matsuba et al., 2010; Miyahara et al., 2012; Miyahara et al., 2013). Anthocyanin modification increases anthocyanin stability and water solubility due to intramolecular and/or intermolecular stacking (Springob et al., 2003).

Anthocyanin synthesis occurs in the cytosol through the flavonoid biosynthetic enzymes associated with the cytoplasmic face of the endoplasmic reticulum (Winkel-Shirley, 1999). Once formed, anthocyanins are stored in the vacuole to prevent oxidation (Marrs et al., 1995). Glutathione S-transferases (GSTs), transporters, and vesicles are involved in their transport into the vacuole (Grotewold, 2004). Depending on the species, anthocyanins are found uniformly distributed inside the vacuole or accumulated in discrete sub-vacuolar structures (Grotewold and Davis, 2008) anthocyanoplasts, intravacuolar pigmented globules' (cyanoplasts, Nozue et al., 1993) or anthocyanic vacuolar inclusions (Markham et al., 2000).

The anthocyanin biosynthetic pathway is transcriptionally controlled by MYB and basic helix–loop–helix (bHLH) transcription factors, together with the WD40 proteins characterized by seven regions of 40 amino acids rich in tryptophan and aspartic acid (Quattrocchio et al., 1999; Hernandez et al., 2004). The ternary complex regulates the steps of the pathway in a spatial and temporal way during plant development (Hichri et al., 2011), and it is called MBW complex (Gonzales et al., 2008). bHLH and WD40 act as co-activators of MYBs, which may repress or activate anthocyanin biosynthetic genes (Koes et al., 2005; Dubos et al., 2008). bHLH proteins are essential to enhance the MYB-induced anthocyanin synthesis. NAC proteins are also involved in the regulation of anthocyanin synthesis in *Arabidopsis* (Morishita et al., 2009). Different factors such as light, temperature, and hormones modulate anthocyanin synthesis (Jeong et al., 2004; Takos et al., 2006; Steyn et al., 2009; Zhang et al., 2012). Anthocyanin biosynthesis is also developmentally controlled as demonstrated by the variation of promoter methylation of *MdMYB10*, the master regulator of the anthocyanin pathway in apple (El-Sharkawy et al., 2015).

Studies about the regulation of the biosynthesis of some secondary compounds have been started to emerge in the Rosaceae family (Lin-Wang et al., 2010). In Japanese plum (*Prunus salicina*), RNA seq analysis allowed the identification of genes involved in anthocyanin synthesis. In the late stages of fruit maturation, when anthocyanin content increases, the expression of several genes, such as *PsPAL*, *PsC4H*, *Ps4CL*, *PsCHS*, *PsCHI*, *PsF3H*, *PsF3′H*, *PsDFR*, *PsANS/LDOX*, *PsUFGT*, and *PsGST*, is increased. Fang et al. (2016) found that *MYBs* expression correlated with structural genes. While c39005.graph_c0 (homologous to AtMYB113) positively correlated with anthocyanin biosynthetic genes, c29499.graph_c0 (homologous to AtMYB73) and c32850.graph_c0 (homologous to AtMYB102) showed a negative correlation. With respect to bHLH transcription factors, 36695.graph_c0 (homologous to AtTT8) positively correlated with anthocyanin contents, and 33382.graph_c0 (homologous to AtbHLH14) decreased with ripening. In addition, a gene encoding a NAC (c27539.graph_c0, AtNAC100) was also up-regulated and positively correlated with the expression of genes involved in anthocyanin synthesis (Fang et al., 2016).

In prunus, levels of anthocyanins are responsible for the color and vary depending on the genotype, external factors, and organs. Moreover, Cheng et al. (2015) revealed that *PsPAL, PsCHS, PsCHI, PsF3H, PsDFR, PsLDOX*, and *PsUFGT* are up-regulated by ethylene treatment and repressed by 1-MCP. They showed that *PsMYB10* is involved in this regulation. More recently, Niu et al. (2017) demonstrated that at high temperatures, anthocyanin levels are regulated not only by the synthesis but also by their degradation. Furthermore, they showed that hydrogen peroxide triggers anthocyanin enzymatic degradation *via* catalysis by a vacuolar peroxidase. Furthermore, Cheng et al. (2014) showed the expression of *PpUGT78A1, PpUGT78A2, PpUGT78B*, and *PpUGT79B*, encoding flavonoid 3-O-glycosyltransferase, varies among tissues and development, conducting the different patterns of anthocyanin accumulation.

Different studies were conducted to get insight into anthocyanin level regulation exploring the eventual redundancy of TFs during peach ripening (Lin-Wang et al., 2010; Ravaglia et al., 2013; Rahim et al., 2014). Analysis of MYB10 sequences reveals that R2R3 sequences from Rosaceae are highly conserved (Lin-Wang et al., 2010). Of the six MYB10s detected in peach genome (Verde et al., 2013) only *PpMYB10.1-3* is expressed in the fruit (Rahim et al., 2014). Transient luciferase assays in *Nicotiana benthamiana* probed that *PpMYB10.2* from peach (as well as those from pear, European plum, cherry-plum, cherry and apricot, denominated *PcMYB10, PdmMYB10, PcfMYB10, PavMYB10, ParMYB10*, and *PprMYB10*, respectively) induced anthocyanin synthesis activating Arabidopsis DFR-promoter when co-expressed with a bHLH (Lin-Wang et al., 2010). In addition, in the presence of bHLH, PpMYB10.2 also transactivated a reporter gene driven by a peach *UFGT* promoter (Ravaglia et al., 2013). *PpMYB10.1* and *PpMYB10.3* were shown to correlate with anthocyanin content in the peel, mesocarp, and mesocarp around the stone of peach fruit and with anthocyanin structural genes CHS, F3H, DFR and UFGT (Rahim et al., 2014). According to Tuan et al. (2015) *PpMYB10.1* is a key factor regulating anthocyanin levels in red-skinned peach (Japanese peach cultivar 'Akatsuki') and that it activates *PpUFGT* transcription. They proposed that *PpMYB10.2/3* could have a different role than pigment regulation since in 'Mochizuki' and 'Akatsuki' cultivars the transcript levels of *PpMYB10.2* and -3 do not correlate with anthocyanin accumulation. Moreover, *PpMYB10.2* was expressed in leaves that do not exhibit anthocyanin accumulation. In addition, Zhou et al. (2014) also indicated that PpMYB10.2 is not involved in anthocyanin synthesis in leaves. On the other hand, *PpMYB10.4* is expressed in leaves, and it controls anthocyanin accumulation in this organ. *PpMYB10.4* is mapped within the interval of the red allele that controls peach leaf color (Zhou et al., 2014). Studies in flowers reveal that *PpMYB9*, is an activator highly expressed in peach flowers and it regulates UFGT gene expression. In the presence of PpbHLH3, PpMYB9 is able to induce anthocyanin synthesis when expressed in tobacco leaves. It is proposed that the *PpMYB9* genes have diverged in functions from the MYB10 genes (Zhou et al., 2016). PpMYB17-20 are repressors, which levels varied during the different stages of flower development (Zhou et al., 2016).

Among three different bHLH candidate genes (based on homology with Arabidopsis), *PpbHLH3* allowed the induction of the pigments when *PpMYB10.1 or PpMYB10.3* were expressed in tobacco. Analysis of the genome reveals that *PpMYB10.1-3* are located within 80 kb on pseudomolecule 3, within the two closest markers (CC2 and CC12A) to the *anther color* trait, and thus highlighting the role of these transcription factors in the anthocyanin synthesis regulation (Rahim et al., 2014). On the other hand, Zhou et al. (2015b) analyzing the segregation of the blood-flesh trait attribute (due to anthocyanin accumulation) in a peach landrace Dahongpao showed that *PpMYB10.1* did not co-segregate with this trait (Zhou et al., 2015b). However, by heterologous expression in tobacco, they found that BLOOD (BL), a NAC transcription factor, and PpNAC1 act together to

transactivate the *PpMYB10.1* gene. *PpSPL1*, a SQUAMOSA promoter-binding protein-like transcription factor (SPL), represses this transactivation. Another R2R3-MYB gene regulating PA synthesis in peach is *PpMYB7*, which activates the transcription of *PpLAR1* but not *PpANR*. *PpMYB7* promoter has an ABA-dependent DRE2 element and it can be activated by the basic leucine-zipper 5 TF PpbZIP5 *via* the ABA signaling (Zhou et al., 2015a).

In peach, anthocyanin accumulation is also influenced by light quality (including UV-B, UV-A, blue light and (far) red light) and depends on the genetic background (Liu T. et al., 2015). The expression of bHLH3 also correlated with induction of anthocyanin synthesis in peach peel under UV exposure (Zhao et al., 2017). Light effect is likely mediated by PpHYH that is also homologous to *A. thaliana* HY5, rather than by *PpHY5* (Zhao et al., 2017). In addition, ppa009438m, ppa009380m, ppa026582m, ppa007883m, and ppa025263m encoding PpNACs responded to UV-B light. Nevertheless, *BL* and *PpNAC1* did not respond to UV-B treatment in 'Hujingmilu' and 'Yulu' cultivars (Zhao et al., 2017). Therefore, the levels of anthocyanin in peach fruit are complexly controlled by the coordination of a set of transcription factors.

Other transcription factors such as PpMYBPA1 (ppa009439m) and Peace (ppa023768m) that is phylogenetically related to PpMYBPA1, have been identified in RNAseq studies and related to the regulation of proanthocyanin (PA) biosynthesis in peach fruit (Wang et al., 2013). *PpMYBPA1*, as in the case of *PpMYB7*, can be activated by PpbZIP5 *via* the ABA signaling (Zhou et al., 2015a). In flowers, PpMYBPA1 and Peace were to be expressed at bud stage and were shown to activate *PpLAR* and *PpANR* promoters (Zhou et al., 2016).

While many advances have been conducted to elucidate the transcription factors controlling anthocyanin accumulation in peach, less information is available with respect to apricot. Some cultivars have blushed skin due to the presence of cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, and peonidin-3-O-rutinoside. Thus, anthocyanin presence and accumulation depend on the cultivar. In apricot, *PaMYB10* also correlated with anthocyanin accumulation in blushed apricots and with *PaMYB10, PaPAL, PaCHS, PaCHI, PaF3H, PaDFR, PaLDOX*, and *PaUFGT*. The overexpression of this MYB transcription factor in fruits of Luntaixiaobaixing cultivar conducted the red coloration of the skin. *PaMYB10* was found to be located with Linkage group 3 (G3) related to skin color (García-Gómez et al., 2019). In addition, it was probed that anthocyanin accumulation in apricot was also influenced by light, as fruit bagging affects the coloration of the skin (Xi et al., 2019). Sugars were also correlated with the content of cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside; as it the case of sorbitol, glucose, fructose and sucrose (Huang et al., 2019). Further studies are required to decipher the way in which sugars affect anthocyanin synthesis in apricot.

In sweet cherry, the expression of *PacCHS, PacCHI, PacF3H, PacDFR, PacANS, and PacUFGT* correlated with anthocyanin accumulation (Liu et al., 2013). The regulation of this pathway has started to emerge. Sequencing of cDNAs encoding enzymes involved in the synthesis of anthocyanins or their regulators (bHLH and WD40) confirms the conservation at sequence level within the Prunus genus (Starkevič et al., 2015). Up to now, *PavMYB10, PavMYBA*, and *PavMYB1* have been studied in sweet cherries (Lin-Wang et al., 2010; Shen et al., 2014; Starkevič et al., 2015; Jin et al., 2016). The first transcription factor modulating the pathway characterized was PavMYB10, which was probed to correlate with anthocyanin accumulation in fruit (Lin-Wang et al., 2010). Two sub-variants of *PaMY10.1, PaMYB10.1-1* and *-3*, were later studied. By studying fruits at different stages of development from different cultivars of *P. avium*, Starkevič et al. (2015) indicated that *PaMYB10.1-1* parallels that of anthocyanin accumulation and that *PaMYB10.1-3* is expressed at low levels in fruit. Nevertheless, when transiently expressed in tobacco, *PaMYB10.1-3* was able to induce pigment synthesis. It was also shown that PavMYB10.1 has three alleles *PavMYB10.1a-to -c*. These alleles are responsible for the variation in color of the fruit. *PavMYB10.1a* regulates the expression of *PavANS* and *PavUFGT* by binding with *PavbHLH* and *PavWD40* (Jin et al., 2016). Jin et al., 2016 proposed that PavMYB10.1 is a DNA molecular marker for the color of the skin.

With respect to bHLHs, *PabHLH3 and -33* are expressed in fruit. While PabHLH3 co-activates anthocyanin biosynthesis in the presence of MYB, PabHLH33 acts as a co-repressor (Starkevič et al., 2015). In addition, *PavMYBA*, encoding for a R2R3-MYB TF from red-colored sweet cherry, was studied. Transient assays demonstrated that this factor binds to bHLHs and activates the expression of *PavDFR, PavANS*, and *PavUFGT*. In addition, ABA modulated the synthesis of anthocyanins in cherry fruit, with *PavMBA* involved in this process (Shen et al., 2014).

Terpenoids

The terpenoids or isoprenoids constitute a large group of metabolites derived from C_5 isoprene (2-methyl-1,3-butadiene) formed by "head-to-tail" conjugation. Terpenoids fulfill varied biological functions in plants, from essential roles as electron transport chain components, pigments (carotenoids, and chlorophylls), elements of membrane structure and function (phytosterols), hormones (*i.e.* gibberellins, strigolactones, brassinosteroids, abscisic acid, isopronoids cytokinins), protein glycosylation (dolichols), to defense (antimicrobial/anti-insect) and attractants (volatile signals) (Tholl, 2015; Tarkowská and Strnad, 2018; Block et al., 2019). Volatile terpenoids (*i.e.* R-limonene) are the major class of volatile compounds in plants and are predominantly isoprenes, mono- and sesquiterpenes. Volatile terpenoids have been found in roots, stems, leaves, fruits, seeds and to higher extent in flowers. In fruits, these terpenoids contribute to aroma production (Dudareva et al., 2013; Abbas et al., 2017).

Biosynthesis and functions of isoprenoids have been well characterized and extensively reviewed (Tholl, 2015; Yazaki et al., 2017; Tarkowská and Strnad, 2018). According to their number of units they can be divided into mono- (C_{10}), sesqui- (C_{15}), di- (C_{20}), sester- (C_{25}), tri- (C_{30}), tetra- (C_{40}, carotenoids), and polyterpenes (>C_{40}) (Tholl, 2015). The combination of isoprene units, in the

form of dimethylallyl diphosphate (DMAPP) and its isomer isopentenyl diphosphate (IPP), conducts the synthesis of the building blocks geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP, **Figure 3**). These precursors are synthesized by two distinct pathways: the mevalonate (MVA) and the nonmevalonate pathways. The last route is also called the 2-*C*-methyl-d-erythritol 4-phosphate (MEP) or the 1-deoxy-d-xylulose 5-phosphate pathway (DOXP). MVA derives from mevalonate synthesized from acetyl-CoA, while MEP derives from pyruvate. GPP, FP, and GGPP are the precursors in the synthesis of mono-, sesqui- and diterpenes, respectively (Tholl, 2015). Then, terpene synthases (TPSs) and cytochrome P450s are the main enzymes that generate the huge terpenoid diversification using mono-, sesqui-, and diterpenes. Other enzymes also contribute to the array of chemically diverse terpenoids.

To mention an example of biosynthesis, carotenoids are synthesized from GGPP using phytoene synthase (PSY) which condenses two molecules of GGPP to render 15-cis phytoene. The following enzymes in the route are phytoene desaturase (PDS), 15-cis-ζ-carotene isomerase (ZISO), ζ-carotene desaturase (ZDS), and carotenoid isomerase (CRTISO) giving *trans*-lycopene (Liang et al., 2018). Lycopene is the precursor of many molecules of biological importance, the δ-, γ-, α-, ϵ-, and β-carotenes. Carotenes are also important precursors; lutein and xanthopylls derive from α- and β-carotene, respectively (for further details, see Liang et al. (2018)). Apocarotenoids are the result of the oxidative cleavage of carotenoids. Examples of apocarotenoids are the derivatives of β-ionone that have pleasant scent and aroma in fruits and flowers (Hou et al., 2016). While many efforts have been dedicated to the elucidation of carotenoid metabolism in plant species, little is known about the regulation (Pott et al., 2019).

Regulation of carotenogenesis has been exhaustively investigated in tomato fruit and Arabidopsis, and it has been also comprehensively reviewed (Llorente et al., 2017; Stanley and Yuan, 2019). Transcription factors such as phytochrome interacting factors (PIFs), ethylene response factors (ERFs), MADS ripening inhibitor factor (RIN) are some of the many TFs that modulate carotenoids synthesis. Both environmental conditions (*i.e.* light) and developmental cues control the carotenoid biosynthetic genes (Llorente et al., 2016; Sun et al., 2018). The control of carotenoid accumulation is also exerted at posttranscriptional and posttranslational levels (Stanley and Yuan, 2019). Biosynthesis, degradation, and storage mediate carotenoids homeostasis as well (Li and Yuan, 2013).

The first study of regulation of carotenoids' synthesis regulation in apricot was conducted comparing varieties with contrasting colors. It showed similar regulation of the synthesis of the colorless carotenoids phytoene and phytofluene due to ethylene upregulation of PSY1 and PDS. In this study, while great differences in β-carotene levels were found in two varieties, accumulation of ZDS was found in both varieties (Marty et al., 2005). Further, RNAseq analysis revealed that structural genes like PDS1, carotenoid cleavage dioxygenase (CCD)1 and 4, violaxanthin de-epoxidase 1 (VDE1) and zeaxanthin epoxidase (ZEP) are the main regulatory points for carotenogenesis (Zhang

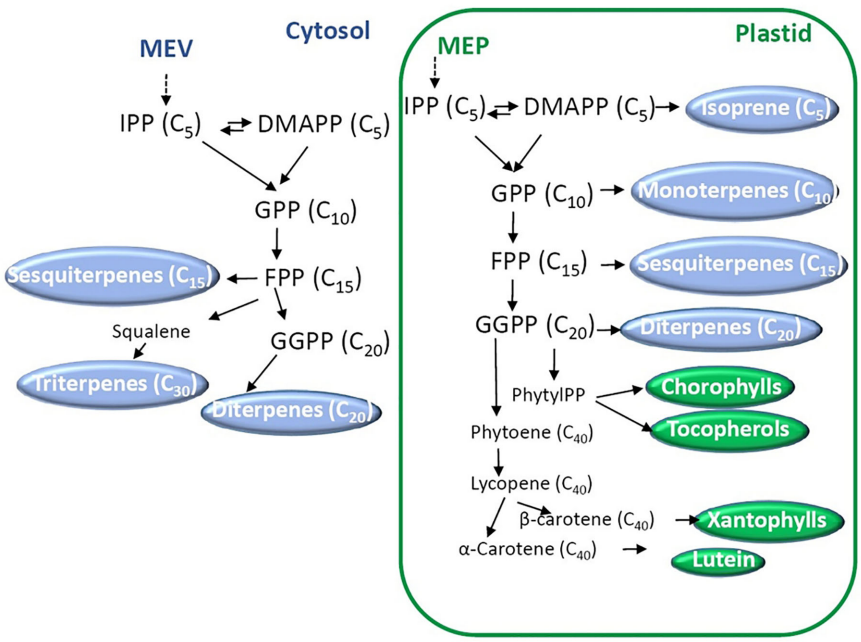

FIGURE 3 | Biosynthesis of terpenoids. Two main pathways starting from mevalonate (MEP) and 2-*C*-methyl-d-erythritol 4-phosphate (MEP) occur in the cytosol and the plastids, respectively. Dimethylallyl diphosphate (DMAPP) and its isomer isopentenyl diphosphate (IPP) are the isoprene units that conduct the synthesis of the building blocks geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP). The number of carbons of each molecule is indicated between brackets.

et al., 2019). Network analysis indicates the presence of two modules for gene expression for carotenoids synthesis in apricots. A set of transcription factors integrating hormones such as ethylene and brassinosteroids (ERF4/5/12, AP2, AP2-like and BZR1), developmental factors (MADS14, NAC2/25, MYB1R1/44, GLK1/2 and WRKY6/31/69) and light (PIF3/4 and HY5) possibly modulate carotenoid accumulation during ripening (Zhang et al., 2019).

In peach, studies regarding carotenoid accumulation in the mesocarp have been focused on carotenoid cleavage. CCDs control carotenoid degradation, and therefore color accumulation. *PpCCD4* encodes a carotenoid dioxygenase. High transcript levels increase carotenoid degradation producing colorless compounds and thus, rendering white flesh. *PpCCD4* is also associated with synthesis of carotenoid-derived volatiles (Brandi et al., 2011; Adami et al., 2013; Falchi et al., 2013). In the mentioned studies and in Cao et al. (2017), although carotenoid synthesis is linked to their biosynthetic enzymes, correlation between biosynthetic genes' expression and carotenoid levels was not found. What is clear is that the levels of *PpCCD4* increase in the flesh and the pulp of white varieties during development and ripening. Yellow varieties are characterized by a combination of higher levels of *PpPDS* and lower accumulation of *PpCCD4* with respect to the white ones (Cao et al., 2017). In addition, while blue light treatment applied during storage was effective to stimulate the transcription of carotenoid biosynthetic genes in both yellow and white varieties, it was able to induce carotenoid accumulation only in yellow but not in white peach, indicating that in white peach the levels of main carotenoid biosynthetic genes are not the primary factor regulating carotenoid amounts (Cao et al., 2017).

Nitrogen- and Sulfur-Containing Secondary Metabolites

The third group of secondary metabolites is large and diverse. It includes alkaloids, glucosinolates, and cyanogenic glucosides.

Glucosinolates are nitrogen- and sulfur-containing metabolites generally restricted to the *Brassicales* (Grubb and Abel, 2006). Glucosinolates are diverse and their derivatives participate in plant defense and contribute to flavor and aroma (Grubb and Abel, 2006).

Alkaloids are nitrogenous compounds of low molecular weight derived mostly from amino acids (Ziegler and Facchini, 2008). This is a diverse group with a wide range of biological functions. According to their structure, alkaloids can be classified into "true" alkaloids with a heterocyclic nitrogen, protoalkaloids (no cyclic nitrogen), and pseudoalkaloids (*e.g.*, steroidal and diterpene alkaloids, caffeine) (Waterman, 1998). The different biosynthetic pathways are in accord to their diversity in structure (Ziegler and Facchini, 2008).

Cyanogenic glycosides participate in plant defense and are widely distributed in the plant kingdom (Zagrobelny et al., 2008). The basic structure is a carbon backbone derived from an amino acid and a glycosilated cyanohydrin. The participation in plant defense mechanisms relays on the hydrolytic release of hydrocyanic acid potentially toxic to herbivores (HCN) (Drochioiu et al., 2008). The occurrence of cyanogenic glycosides has been described in species of the Fabaceae, Poaceae, Rosaceae, and Asteraceae, and also in conifers and ferns (Gleadow and Møller, 2014). Seeds from stone fruits of the Rosaceae family are rich in diglucoside (R)-amygdalin (Swain et al., 1992). In addition to kernels, the monoglucoside prunasin is also found in leaves, roots, stems (Dicenta et al., 2002; Sánchez-Pérez et al., 2008). Both compounds are synthesized from phenylalanine. The sequential action of two cytochrome P450 enzymes belonging to the CYP79 and CYP71 families catalyze the synthesis of mandelonitrile (Yamaguchi et al., 2014), which is then glucosylated by a UDP-glycosyltransferase (UGT) to form prunasin. This compound is then glucosylated again to form amygdalin.

In *Prunus dulcis*, two genes *PdCYP79D16* and *PdCYP71AN24* encoding CYP79 and 71 and three genes encoding UGTs involved in amygdalin (*PdUGT94AF1* and *PdUGT94AF2*) and prunasin synthesis (*PdUGT94AF3*) were identified. The functionality and activities of the proteins were tested by expression in *Nicotiana benthamiana*. Comparisons of sweet and bitter almonds, which differentially accumulate amygdalin, indicated that the expression of *PdCYP79D16* and *PdCYP71AN24* is very low or null in the tegument of the sweet genotype and high in the bitter almonds (Thodberg et al., 2018). More recently, using map-based cloning and a segregating population for sweet kernel trait Sánchez-Pérez and colleagues (2019) identified that bHLH2 controls the expression of *PdCYP79D16* and *PdCYP71AN24*. A mutation in bHLH2 avoids the transcription of both genes, and thus mandelonitrile, amygdalin and prunasin are not synthesized. Selection of this mutation is proposed to have allowed the domestication of almond (Sánchez-Pérez et al., 2019).

STONE FRUITS AS A SOURCE OF PHYTOCHEMICALS: A DESCRIPTION OF SECONDARY ORGANIC COMPOUNDS' COMPOSITION OF MAIN STONE FRUITS

Peach

Phenolic compounds are the main source of antioxidant capacity in peaches (Gil et al., 2002). They also participate in the visual appearance (pigmentation and browning) (Lee et al., 1990) and in the taste (astringency) of fruits (Tomás-Barberán et al., 2001) and therefore could be used to evaluate the quality of the fruit. In peaches, carotenoids are responsible for the yellow color of the pulp, and therefore, their concentration is low in white pulp fruits (Gil et al., 2002). Peaches contain anthocyanins (cyanidin-3-glucoside and cyanidin-3-rutinoside), flavan-3-ols (catechin—the main monomeric flavan-3-ol-, epicatechin, epigallocatechin, and procyanidins), flavonols (quercetin-3-O-rutinoside or rutin, quercetin-3-glucoside, quercetin-3-galactoside, kaempferol-3-rutinoside), hydroxycinnamic acids (chlorogenic and neoclorogenic acids) (Campbell and Padilla-Zakour, 2013; Dabbou et al., 2017), and the main carotenoids include β-carotene and xanthophylls (mono- or di-hydroxylated carotenoids), zeaxanthin, β-cryptoxanthin and violaxanthin (Tomas-Barberan

et al., 2001; Campbell and Padilla-Zakour, 2013; Dabbou et al., 2017), and lutein (Oliveira et al., 2016). Amounts of some of the most representative phytochemicals present in the pulp of peach of different cultivars at commercial maturity are shown in **Table 1**.

More than 100 volatile compounds have been identified in peach fruit. Volatile organic compounds define fruit aroma and participate, together with organic acids and sugars, in fruit taste. Aroma is a central trait that influences the fruit quality perception by consumers (Bruhn et al., 1991). In addition, volatile composition gives significant information regarding healthful composition of food since they are synthesized from essential nutrients (Goff and Klee, 2006). Peach volatiles have been classified into alcohols, aldehydes, carboxylic acids, non-cyclic esters, terpenoids, ketones, and lactones (Wang et al., 2009; Sánchez et al., 2012). The most abundant are C_6 compounds, esters, benzaldehyde, linalool, $C13_3$ norisoprenoids, and lactones. The amounts of benzaldehyde, linalool, δ-decalactone and (E)-2-hexenal in four peach cultivars are presented in **Table 2** to show their variability among cultivars.

Lactones such as γ-decalactone, c-jasmolactone c-octalactone, c-dodecalactone, δ-decalactone and 6-pentyl-α-pyrone have been described as the key odorants to the pleasant aroma of peach fruit, with γ-decalactone most highly associated with the "peach-like" note. In addition, the esters (Z)-3-hexenyl acetate, (E)-2-hexen-1-ol acetate, and ethyl acetate add to the "fruity" notes. Moreover, linalool and β-ionone and other terpenoids contribute to the "floral" notes (Horvat et al., 1990; Derail et al., 1999; Eduardo et al., 2010).

During industrial processing of peach to produce juices, marmalades, concentrates and canned fruit, peel and stone are removed and discarded. Likewise, most of the times that fruit is freshly consumed; the peel is also discarded because of the use of chemicals and pest contamination or digestion problems. Nevertheless, both peels and kernels are a source of bioactive compounds. Indeed, many phytochemicals are more abundant in the fruit peel than in the edible fleshy parts. For example, phenolics (Tomás-Barberán et al., 2001), carotenoids, and ascorbic acid double their amount in the peel than in the pulp

TABLE 1 | Amounts of some of the most representative phytochemicals present in the pulp of peach, plum, apricot, and cherry at commercial maturity.

	Prunus persica		***Prunus salicina***		***Prunus armeniaca***		***Prunus avium***	
Neochlorogenic acid	Annongshuimi	2.6[c]	Keckemetska ruza	1.19[l]	Early Magic	18.1[i]	Burlat	21.7[s]
	Huyou0 002	23.2[c]	Madjarska najbolja	1.42[l]	Beltsville Elite B70197	215.4[i]	Saco	190[s]
	Huyou0 018	7.8[c]	Velika rana	1.22[l]	NY101	179.4[i]	Summit	40.4[s]
	Sweet cap	5.8[d]	Bebecou	1.37[m]	Brite pearl	183[j]	Badascony	4.74[t]
	Ealy May Crest	5.0[d]	Nafsika	0.41[m]	September red	24.1[j]	Early Van Comact	11.9[t]
	O'Henry	4.1[d]	Niove	1.57[m]	Spring Bright	36.7[j]	Vigred	6.50[t]
	Big Top	2.3[e]	Z 109/58	3.8[n]	Laetitia	39.6[k]	Della Marca	0.96[u]
	Royal Glory	1.5[e]	Rojo passion	10.68[n]	Ruby Red	40.1[k]	Lapins	6.17[u]
	Red Haven	4.7[e]	Z 505/2	6.4[n]	African delight	39.1[k]	Moretta	7.31[u]
Chlorogenic acid	Annongshuimi	4.1[c]	Keckemetska ruza	1.47[l]	Brite pearl	277[j]	Burlat	3.65[s]
	Huyou0 002	31.1[c]	Madjarska najbolja	2.29[l]	September red	39[j]	Saco	12[s]
	Huyou0 018	23.3[c]	Velika rana	1.93[l]	Spring Bright	84.3[j]	Summit	9.73[s]
	Sweet cap	8.9[d]	Bebecou	1.01[m]	Early Magic	0.9[i]	Badascony	1.12[t]
	Ealy May Crest	8.5[d]	Nafsika	0.46[m]	Beltsville Elite B70197	9.5[i]	Early Van Comact	2.26[t]
	O'Henry	5.6[d]	Niove	2.2[m]	NY101	21.0[i]	Vigred	1.07[t]
	Big Top	0.21[e]	Z 109/58	6.9[n]	Laetitia	NDk	Della Marca	22.83[u]
	Royal Glory	0.44[e]	Rojo passion	11.0[n]	Ruby Red	ND[k]	Lapins	149.89[u]
	Red Haven	0.35[e]	Z 505/2	7.6[n]	African delight	2.5[k]	Moretta	68.4[u]
Rutin	Annongshuimi	ND[c]	Bebecou	9.37[m]	Brite pearl	27.2*	Burlat	3.06[s]
	Huyou0 002	ND[c]	Nafsika	5.65[m]	September red	41.8*[j]	Saco	13.69[s]
	Huyou0 018	ND[c]	Niove	7.73[m]	Spring Bright	56.9*[j]	Summit	2.81[s]
	Sweet cap	0.25[d]	'Keckemetska ruza	2.02[l]	Early Magic	6.3[i]	Badascony	5.78[t]
	Ealy May Crest	0.13[d]	Madjarska najbolja	1.55[l]	Beltsville Elite B70197	4.3[i]	Early Van Comact	3.53[t]
	O'Henry	0.14[d]	Velika rana	2.17[l]	NY101	5.0[i]	Vigred	5.68[t]
	Big Top	1.8[e]	LE-2927	12.16*	Laetitia	6.6[k]	Della Marca	5.13[u]
	Royal Glory	0.99[e]	Salah-Jerevan	5.41°	Ruby Red	7.9[k]	Lapins	51.97[u]
	Red Haven	0.8[e]	Chuan Zhi Hong	56.9°	African delight	7.4[k]	Moretta	41.4[u]
Cyanidin-3- rutinoside	Annongshuimi	3.5[c$]	Z 115/26	1.6[n]	Early Magic	18.9[i]	Burlat	28.5[s]
	Huyou0 002	1.3[c$]	Rojo passion	4.4[n]	Beltsville Elite B70197	25.7[i]	Saco	24.5[s]
	Huyou0 018	3.8[c$]	Z 505/2	2.9[n]	Longjohn	33.0[i]	Summit	20.1[s]
	Sweet cap	0.028[d]			Brite pearl	3.5*[j]	Badascony	12.8[t]
	Ealy May Crest	0.011[d]			September red	4.6*[j]	Early Van Comact	8.06[t]
	O'Henry	1.148[d]			Spring Bright	12.7*[j]	Vigred	13.5[t]
	Big Top	0.47[e$]			Laetitia	1.48[k]	Della Marca	2.05[u]
	Royal Glory	0.67[e$]			Ruby Red	2.81[k]	Lapins	389.9[u]
	Red Haven	0.42[e$]			African delight	1.51[k]	Moretta	268.2[u]

The amounts of chlorogenic and neochlorogenic acids (hydroxycinnamic acids), quercetin-3-O-rutinoside or rutin (flavonol) and cyanidin-3-rutinoside (anthocyanins) are indicated in different cultivars and expressed in mg/100 g of fresh weight.

[c]Zhao et al., 2015; [d]Dabbou et al., 2017; [e]Manganaris et al., 2017; [i]Kim et al., 2003a; [j]Tomas-Barberan et al., 2001; [k]Venter et al., 2013; [l]Dragovic-Uzelac et al., 2007; [m]Roussos et al., 2011; [n]Ruiz et al., 2005; [c$]Schmitzer et al., 2011; [s]Gonçàlves et al., 2004; [t]Usenik et al., 2008; [u]Martini et al., 2017.

TABLE 2 | The most abundant or representative volatiles (benzaldehyde, linalool, trans-linalool oxide, γ-decalactone, δ-decalactone, hexanal, (E)-2-hexenal, hexanol, 2-Hexen-1-ol and hexyl acetate) in the pulp of different cultivars of peach, plum, apricot, and cherry at commercial maturity are expressed in μg/kg of fresh weight.

Prunus persica		
2-Hexenal	Western red	1,381.1[a]
	Chongyanghong	2,332[&b]
	Zaohongxia	3,228[&b]
	Wuyuehuo	7078[&b]
Benzaldehyde	Western red	61.9[a]
	Chongyanghong	1,187.8[&b]
	Zaohongxia	2,918.4[&b]
	Wuyuehuo	1,080.4[&b]
Linalool	Western red	50.2[a]
	Chongyanghong	285.9[&b]
	Zaohongxia	680.2[&b]
	Wuyuehuo	759.3[&b]
δ-decalactone	Western red	16.3[a]
	Chongyanghong	1,904.5[&b]
	Zaohongxia	1,368.5[&b]
	Wuyuehuo	1,843.0[&b]
Prunus armeniaca		
(E)-2-Hexenal	Palstey	4,635[p]
	Moniqui	26,800[p]
	Rouge du Roussillo	8,752[p]
	Early Blush Rutbhart	133[q]
	Spring Blush EA3126TH	59[q]
	PBS 28-58	153[q]
	K604-19	ND[r]
	K113-40	ND[r]
	K33-81	ND[r]
Linalool	Palstey	3,019[p]
	Moniqui	1,021[p]
	Rouge du Roussillo	864[p]
	Early Blush Rutbhart	80[q]
	Spring Blush EA3126TH	256[q]
	PBS 28-58	43[q]
	K604-19	671[r]
	K113-40	365[r]
	K33-81	150[r]
δ-decalactone	Palstey	2526[p]
	Moniqui	37,310[p]
	Rouge du Roussillo	21,024[p]
	Early Blush Rutbhart	454[q]
	Spring Blush EA3126TH	105[q]
	PBS 28-58	281[q]
	K604-19	1,424[r]
	K113-40	3[r]
	K33-81	50r
Hexyl acetate	Palstey	5,244[p]
	Moniqui	1,250[p]
	Rouge du Roussillo	13,140[p]
	Early Blush Rutbhart	54[q]
	Spring Blush EA3126TH	2[q]
	PBS 28-58	3[q]
	K604-19	ND[r]
	K113-40	ND[r]
	K33-81	4[r]
Prunus salicina		
2-Hexenal	Horvin	72[g]
	Range	1.03[h]
1-Hexanol	Showtime	861[f]
	Laetitia	1,370[f]
	Primetime	1210[f]
	Horvin	1,739.7[g]
	Range	2.11[h]
Hexanal	Showtime	1,500[f]
	Laetitia	1,850[f]
	Primetime	2,420[f]
	Horvin	Traces[g]
	Range	7.67[h]
trans-Linalool oxide	Primetime	185[f]
	Laetitia	262[f]
	Showtime	726[f]
	Horvin	102.2[g]
Prunus avium		
(E)-2-Hexenal	Van	412.71[v]
	Vista	45.53[v]
	0-900Ziraat	269.50[v]
	Canada	2.56[w]
	Ferrovia	6.36[w]
	Lapins	2.60[w]
Benzaldehyde	Van	10.10[v]
	Vista	7.69[v]
	0-900Ziraat	20.52[v]
	Canada Giant	0.45[w]
	Ferrovia	0.50[w]
	Lapins	1.06[w]
Hexanal	Van	122.96[v]
	Vista	25.75[v]
	0-900Ziraat	144.66[v]
	Canada Giant	0.71[w]
	Ferrovia	2.72[w]
	Lapins	0.62[w]
2-Hexen-1-ol	Van	172[v]
	Vista	14.64[v]
	0-900Ziraat	83.77[v]
	Canada Giant	1.02[w]
	Ferrovia	1.04[w]
	Lapins	0.78[w]

[&]the ripening stage is not specified in the literature; ND, not detected; [a]Aubert et al., 2014; [b]Zhu and Xiao, 2019; [f]Cuevas et al., 2016; [g]Pino and Quijano, 2012; [h]Chai et al., 2012; [p]Guichard and Souty, 1988; [q]Aubert and Chanforan, 2007; [r]Gómez et al., 1993; [v]Hayaloglu and Demir, 2015; [w]Vavoura et al., 2015.

(Gil et al., 2002). Dabbou et al. (2017) showed that irrespective of the ripening stage, hydroxycinnamic acids, total flavonols, and total anthocyanins are higher in the peel than in the pulp. In contrast, the relative content of carotenoids depended on the harvesting stage.

Peach kernels provide seed oil and fatty acids (Wu et al., 2011). In addition, kernels are a source of a wide range of metabolites of nutritional importance. The following secondary metabolites have been identified in peach kernels: protocatechuic acid, *p*-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid, dihydroxybenzoic acid, chlorogenic acid, *p*-coumaric acid, ferulic acids, dithiothreitol, rutin, caffeic acid, procyanidin B2, hydrocinnamic acid, procatechol, catechin, gentisic acid, kuromanin chloride, vanillic acid, epicatechin gallate, sinapinic acid, and ellagic acid (Wu et al., 2011 and Koprivica et al., 2018), with catechin being prevalent.

Much effort has been dedicated to the characterization of the volatilome of peach as well as the profiling of other secondary

metabolites in stone fruits (Horvat et al., 1990; Visai and Vanoli, 1997; Derail et al., 1999; Eduardo et al., 2010; Sánchez et al., 2012). In addition, in the case of volatiles, some QTLs have been identified (Sánchez et al., 2014).

Apricot

Apricot is a good source of carotenoids (Zaghdoudi et al., 2015), fatty acids, and sterols, volatile compounds, glycosides and polyphenols (Erdogan-Orhan and Kartal, 2011). Lutein, α- and β-carotenes are the main carotenoids of apricot fruit (Erdogan-Orhan and Kartal, 2011; Katayama et al., 2011). β-carotene accounts for 60–70% of the total carotenoid content (Dragovic-Uzelac et al., 2007). Apricot is also a rich source of the carotenoid's precursors phytoene and phytofluene (Biehler et al., 2011). These carotenoids have been largely ignored in the context of agro-food and health and are the major abundant dietary carotenoids (Mapelli-Brahm et al., 2017). Violaxanthin is also present in apricot (Katayama et al., 1971). Polyphenols found in apricot include catechin, hydroxycinnamic acids (chlorogenic, neochlorogenic acids, *p*-coumaric, **Table 1**), epicatechin, epigallocatechin, kaempferol-3-rutinoside, quercetin-3-glucosides, and rutin (**Table 1**, Dragovic-Uzelac et al., 2007; Campbell et al., 2013). Recently, some other minor phenolic compounds were identified, such as hyperoside, narcissin, and naringenin (Di Vaio et al., 2019). Among anthocyanins, cyanidin-3-O-rutinoside (the most abundant, **Table 1**), cyanidin-3-O-glucoside and peonidin-3-O-rutinoside have been detected in some accessions (Bureau et al., 2009). *p*-Coumaric acid has strong antioxidant potential and besides, it has been identified in apricot fruit, and its amounts and those of coumaroyl hexose are very low (Dragovic-Uzelac et al., 2005). Chlorogenic (caffeoyl quinic acid) is an abundant polyphenol in apricot (Dragovic-Uzelac et al., 2005). Caffeic acid has also been detected (Campbell et al., 2013).

About 200 different volatile compounds have been described in apricots (El Hadi et al., 2013). The main volatile phytochemicals include aldehydes, alcohols, ketones, esters, terpenes, and hydrocarbons. The most abundant are hexanal, (E)-2-hexenal, linalool, 1-hexanol, ethyl octanoate, and hexyl acetate (**Table 2**). These volatiles are recognized as major contributors to apricot scent (Aubert and Chanforan, 2007; González-Agüero et al., 2009). The aldehydes hexanal and (E)-2-hexenal display the higher concentrations and have been shown to decrease during ripening. Terpene compounds (*i.e.*, linalool) and alcohols (*i.e.* 1-hexanol) are less abundant than aldehydes, and decrease with ripening (**Table 2**, González-Agüero et al., 2009).

Plums

Plums may be good sources of natural antioxidants. Plums contain high amounts of polyphenolic compounds, which include anthocyanins, hydroxycinnamates, flavan 3-ols and flavonols. The most common and predominant are chlorogenic acid, neo-chlorogenic acid, catechin, epicatechin, and quercetin-3-rutinoside (Rutin) (**Table 1**, Kim et al., 2003; Tomas-Barberán et al., 2001). However, differences in contents were found in different species of plum, such as *Prunus domestica*, *Prunus salicina*, and *Prunus cerasifera* (Moscatello et al., 2019). Other flavonol glycosides such as cyanidin-3-glucoside and cyanidin-3-galactoside have also been described. Anthocyanins are found in fresh plums predominantly as rutinoside derivatives, such as cyaniding-3-rutinoside (keracyanin, **Table 2**), cyanidin-3-glucoside (kuromanin), and peonidin 3-rutinoside (Raynal et al., 1989; Kim et al., 2003). It is important to mention that anthocyanins have not been detected in yellow plums (Kim et al., 2003). Although plums are not the richest source of carotenes, they contain neoxanthin, lutein, and violaxanthin (Biehler et al., 2011).

Thirty-six different volatile compounds have been identified in Japanese plums (Lozano et al., 2009). In general, they are grouped in esters and lactones, with hexanal, butyl acetate, (E)-2-hexenal, butyl butyrate, hexyl acetate, linalool (2,6-dimethyl-2,7-octadien-6-ol), γ-decalactone and γ-dodecalactone being the most abundant. Hexanal, provides the plum-like aroma (Gómez et al., 1993 and references therein). Levels of some representative volatiles are shown in **Table 2**.

Not only the number of identified volatiles is shorter in plum than in apricots and peach, but also, they have been found in lower amounts (Gómez et al., 1993). Particularly, while the concentrations of C_6 compounds (hexanal, (E)-2-hexenal, hexanol, (Z)-3-hexen-1-ol), and their esters are higher in plums than in apricots, apricots are richer in aromatic compounds (**Table 2**). Hydrocarbons were more frequent in plums than in apricots (Gómez et al., 1993).

Almond Kernels

Bitter almonds have important quantities (3–9%) of amygdalin, which releases hydrocyanic acid and benzaldehyde upon enzymatic hydrolysis (Wirthensohn et al., 2008) and are mainly used in the production of flavor extracts. In turn, sweet almond is consumed as a whole nut, blanched or peeled in the form of a healthy snack or ingredient (Yada et al., 2011). Polyphenols contribute to color and to the moderate astringency. The most predominant polyphenols are proanthocyanidins, hydrolysable tannins, and flavonoids. The amount of some representative polyphenols in different *P. armeniaca* cultivars is presented in **Table 1**.

Almond major proanthocyanidins include epicatechin and catechin. Epiafzelechin is a minor proanthocyanidin. Tannins render gallotannins and ellagitannins after hydrolysis. More than 25 flavonoids have been described in almonds including anthocyanidins (derived from the hydrolysis of proanthocyanidins), flavan-3-ols (catechin, dihydrokaempferol, dihydroquercetin, epicatechin, epicatechin gallate, epicatechin glycoside, and gallocatechin gallate), flavan-3-ols (dihydrokaempferol, catechin, and epicatechin), flavonols (isorhamnetin, kaempferol, quercetin and their 3-O-glucosides, galactosides, and rutinosides), and flavanones (eriodictyol, naringenin, and 7-O-glucosides), and a biflavone. Flavonols are the most abundant flavonoid class in almond. Phenolic acids, lignans, isoflavones, and stilbenes are less represented polyphenols (Bolling, 2017). Almonds are rich in unsaturated lipids and in α-, δ-, β-, and γ-tocopherol (Franklin et al., 2017).

Volatiles isolated from almond include more than 20 compounds such as alkylfuranones, n-alkanes, cyclopentadiene and aromatic compounds, such as benzaldehyde, methyl phenol,

benzyl alcohol, and some alkylbenzenes. Among the dominant components is benzaldehyde, which has been reported as a predominant volatile of kernel oils from the botanical family Rosaceae and is associated with a marzipan-like flavor, benzyl alcohol, and methyl benzene (**Table 2**). Octane, n-tridecane, n-tetradecane, and hexadecane are the n-alkanes identified (Picuric-Jovanovic and Milovanovic, 1993).

On the other hand, almond skin is a source of bioactive polyphenols and thus of antioxidant activity. About 30 phenolic compounds have been identified which include flavan-3-ols (the more abundant comprising proanthocyanidins, catechin, epicatechin), flavonol glycosides (kaempferol-3-O-rutinoside, kaempferol-3-O-glucoside, isorhamnetin-3-O-rutinoside, isorhamnetin-3-O-glucoside, and quercetin-3-O-glucoside), hydroxybenzoic acids (*p*-hydroxybenzoic acid, vanillic acid, and protocatechuic acid) and aldehydes (protocatechuic aldehyde), flavonol aglycones (kaempferol, quercetin, and isorhamnetin), flavanone glycosides (naringenin-7-O-glucoside and eriodictyol-7-O-glucoside), flavanone aglycones (naringenin and eriodictyol), hydroxycinnamic acids (trans-*p*-coumaric acid and chlorogenic acid), and dihydroflavonol aglycones (dihydroquercetin) (**Table 1**, Garrido et al., 2008).

Sweet Cherries

Cherries are rich in phenolic compounds, mainly represented by hydroxycinnamates, anthocyanins, flavan-3-ols and flavonols (**Table 1**, Gonçalves et al., 2004).

The main anthocyanins in cherries are cyaniding-3-glucoside, cyanidin-3-rutinoside (Gonçalves et al., 2007; Goulas et al., 2015). While pelargonidin-3-O-rutinoside (Mozetic et al., 2002) and peonidin-3-O-rutinoside occur at low levels in some cultivars (Gonçalves et al., 2007), they were not detected in others (Goulas et al., 2015). Cyanidin-3-rutinoside represents 90% of the total anthocyanin content (Usenik et al., 2008). Sweet cherries are a good source of phenolic acids such as hydroxycinnamic acid derivatives (neochlorogenic acid, *p*-coumaroyl quinic acid and chlorogenic acid) (Liu et al., 2011) Flavonoids detected in sweet cherries include catechin, epicatechin, rutin, quercetin, quercetin-3-rutinoside, quercetin derivative, and kaempferol derivative (Chockchaisawasdee et al., 2016). Fingerprinting conducted by Goulas et al. (2015) revealed that in sweet cherries the levels of hydroxycinnamates are higher than those of flavonoids. Fresh sweet cherry fruit volatiles include alcohols, aldehydes, ketones, hydrocarbons/terpenes and esters, with aldehydes, alcohols, and esters being the most represented (**Table 2**). Hexanal, (E)-2-hexenal, benzaldehyde, (E)-2-hexen-1-o1, ethyl acetate, and hexanoic acid ethyl ester give the typical sweet cherry scent.

PRE- AND POSTHARVEST TREATMENTS AFFECTING THE PHYTOCHEMICAL BIOSYNTHESIS AND COMPOSITION IN STONE FRUIT

Apart from the studies related to transcription factors involved in the regulation of secondary organic compounds in stone fruit, studies on peach, cherry, and apricot reveal that the composition and concentration of the different phytochemicals vary among the cultivars (Tomas-Barberan et al., 2001; Gil et al., 2002; Cantín et al., 2009; Di Matteo et al., 2017). However, still very few studies have investigated the profiles and content of phenolic and volatiles in several cultivars (Di Vaio et al., 2008; Wang et al., 2009; Di Vaio et al., 2015; Di Matteo et al., 2017). Moreover, the molecular basis for the differences in phytochemical content among varieties has not been investigated in detail yet.

In addition, other factors, like the rootstock (Remorini et al., 2008), the water supply (Tavarini et al., 2011), the growing region, climatic conditions, plant density, nutrient management and agronomic practices (Dragovic-Uzelac et al., 2007; Buendía et al., 2008; Álvarez-Fernández et al., 2011), the state of maturation and various factors in the postharvest stage, and handling (Gil et al., 2002; Dragovic-Uzelac et al., 2007; Tavarini et al., 2011; Scordino et al., 2012; Dabbou et al., 2016; Jia et al., 2009) influence fruit quality and the content of secondary organic compounds and thus consumers acceptance (Minas et al., 2018). Regarding the geographic growing regions, the effect of altitude in the skin pigmentation of peach fruit has been studied. Orchards from higher altitudes showed higher content of total phenols, flavonoids, carotenoids, and anthocyanins in comparison with those grown at lower altitudes (Karagiannis et al., 2016). The content of other metabolites such as quercetin-3-glucoside in apricot does not vary with the ripening stage (Campbell et al., 2013); however, it has been shown that fruit:leaf ratio has an impact on the phytochemical composition of the fruit. For example, fruit thinning, the adjustment of the fruit number of the tree, during pit hardening of apricot improved the phytochemical (total phenolics) content in some cultivars (Roussos et al., 2011). Other practices such as nitrogenous fertilization also affects the phenolic, flavonoid, and anthocyanin contents as it is the case of peach fruit (Vashisth et al., 2017). Depending on the fruit species and the phytochemical considered, the level of secondary organic metabolites might vary upon developmental and ripening stage. For example, in apricots, while the content of some phenolic compounds was constant during ripening, the amounts of others varied (Dragovic-Uzelac et al., 2007). In addition, the content of carotenoids increased during ripening in three different cultivars tested at two different locations (Dragovic-Uzelac et al., 2007). In consequence, the harvest time might affect the health-beneficial properties of the fruit. In this respect, research conducted in plums shows the evolution of the content of different secondary organic metabolites such as carotenoids, total phenolics, anthocyanins during development and ripening on tree of different cultivars (Díaz-Mula et al., 2008; Jiang et al., 2019). Nevertheless, these works showed contradictory results *i.e.*, while Díaz-Mula et al. (2008) described a decrease in phenolics over development of plum; Jiang et al. (2019) showed an increase of phenolic compounds. Later, Moscatello et al. (2019) studying the evolution of sugars, organic acids, and bioactive compounds over development, ripening and overripening, showed that metabolite content is influenced by dilution effects due to the expansion of the fruit. Thus, while phenolics exhibit a net decrease when expressed in terms of fresh or dry weight basis, the net content per fruit

increases. Therefore, during plum fruit growth and development there is a net increase in the amount of these bioactive compounds due to synthesis.

On the other hand, the application of different postharvest abiotic stress in horticultural crops has proven to be a simple and effective technology to induce the accumulation of secondary metabolites with a wide range of applications in dietary supplements, functional foods, pharmaceutical markets, cosmetics and agrochemicals. Thus, the use and/or generation of wounds, ultraviolet light, modified atmospheres and phytohormones (ethylene and methyl jasmonate, *etc.*) in fresh fruits and vegetables induces the accumulation of antioxidants (Cisneros-Zevallos, 2003; Jacobo-Velázquez and Cisneros-Zevallos, 2012). More recently, emerging technologies such as ultrasound have been used to generate abiotic stress to induce the accumulation of phenolic compounds (Jacobo-Velázquez and Cisneros- Zevallos, 2018). Particularly in peaches, it was found that the effectiveness of the treatment with UV-B in the modulation of the concentration of phenolic compounds and the expression of the genes involved in the synthesis of phenylpropanoids was genotype-dependent (Scattino et al., 2014; Zhao et al., 2017). UV-A and UV-B treatment increased the content of flavonoids, ascorbate, and cyanidin-3-O-glucoside, and thus, it increased the antioxidant activity of treated peach (Sgherri et al., 2015). In line with these results, bagging also affects the accumulation of nutraceutic compounds. Yellow paper prevents the penetration of blue and UV light resulting in poor coloration of the skin (Liu et al., 2015). In contrast, white non-woven polypropylene allows the accumulation of anthocyanin in the skin. Liu et al. (2015) showed increased expression of the TFs *PpMYB* 10.1, *PpMYB* 10.2, and *PpMYB* 10.3 and their partners *PpbHLH3* and *PpWD40-1* in peach covered with white non-woven polypropylene with respect to fruit covered with yellow paper. Enhanced expression of regulatory genes resulted in higher levels of *PpCHS*, *PpDFR*, and *PpUFGT* in peach bagged using non-woven polypropylene. UV-C treatment during the postharvest storage is also effective in inducing anthocyanin accumulation in cold stored peach (Zhou et al., 2020). In sweet cherries, UV-C applied to fruit after harvest and then cold stored also resulted in an increase in anthocyanins, flavonoids, and total phenolics (Michailidis et al., 2019). Temperature during the postharvest also affects total phenolics and anthocyanin levels. While cold storage at 1–2°C decreases total phenolic content (with neochlorogenic and p-coumaroylquinic acids been the most abundant) in four cultivars of sweet cherries harvested at ripe stage, storage at 15 ± 5°C increases them. In contrast, anthocyanin increased up to fivefold at both temperatures in both ripe and in partially ripe cherries (Gonçalves et al., 2004). On the other hand, after 15 days of fruit storage at 1°C, Esti et al. (2002), also testing two cultivars harvested at commercial maturity indicated a considerable decrease in anthocyanin content. With respect to apricot, heat stress was effective to raise the antioxidant capacity (Madrau et al., 2009).

Preharvest and postharvest oxalic acid (OA) treatment has been successfully applied to stone fruits to increase their nutraceutical properties. In sweet cherries, treatments with OA during development (Martinez-Espla et al., 2014; Martinez-Espla et al., 2019) or during the postharvest followed by 50-day cold storage (Valero et al., 2011) were effective to induce total phenolics, anthocyanins, and antioxidant activity. In peach, OA application 15 days before harvest improved total flavonoids, phenolics, and antioxidant activity in cold stored peach for 28 days (Razavi and Hajiloub, 2016). In addition, in plum (*Prunus salicina* Lindl. 'Black Splendor'), OA applied as a foliar spray during fruit development successfully increased total phenolics and total antioxidant activity in harvested fruit and after cold storage (35 days at 2°C + 1 day at 20°C, Serrano et al., 2018).

Postharvest treatment on several Prunus fruit with methylcyclopropene (1-MCP), an inhibithor of ethylene perception (Sisler and Serek, 1997), provoked a delay of color development (Valero et al., 2005). Application of 1-MCP resulted in a negative effect on the expression of genes involved in the biosythentic pathway of carotenoids (Marty et al., 2005; Ziliotto et al., 2008) and anthocyanins (Niu et al., 2017) thus, supporting the regulatory role of ethylene in the accumulation of carotenoids and anthocyanins in this species.

FUTURE PERSPECTIVES FOR IMPROVING TYPE AND LEVELS OF SECONDARY PHYTOCHEMICALS IN STONE FRUIT

Despite the well-known beneficial effects of fruits and vegetables in human health, consumption is still low. Therefore, great efforts should be made to increase the level of health promoting compounds in plant foods by both molecular and non-molecular methods. During the last years, important advances with regard the regulation of secondary metabolism, together with the coordination with primary metabolism, have been achieved in model species such as arabidopsis and tomato. Modification of the expression of individual TFs modifies the levels of enzymes involved not only in secondary but also in primary metabolism suggesting supercoordinated gene expression networks between primary and secondary metabolisms (Aharoni and Galili, 2011). In this respect, it has been proposed that the manipulation of primary metabolism (source of precursors) is a promissory strategy to alter secondary metabolite content (Pott et al., 2019).

In stone fruit, even though in the recent years there has been an increase in genetic (genetic linkage maps and markers associated to quality traits) and omics (genome sequences, transcriptomes, metabolomes, proteomes and volatilomes) resources (Aranzana et al., 2019), there is still limited knowledge about the main factors regulating the accumulation of secondary organic metabolites. Upcoming research of pre- and posthavrest treatements on phytochemical composition should also consider the differences in development and ripening stages of the fruit. More studies should be performed in the future because, as stated in the present review, there is large evidence that stone fruits are rich in secondary organic metabolites, and thus, they may become biofactories of health promoting compounds. The manipulation of primary metabolism to alter secondary metabolite content (Pott et al., 2019) is also a strategy to be considered in stone fruits.

Besides, several studies have highlighted the importance of epigenetic influence in plant ontogenesis, flowering time, heterosis, and fruit ripening process in other species. Much of the information regarding genetic control and epigenetic regulation has been obtained in tomato. The main actors (TF) involved in the transition from fruit growth to ripening have been identified (Giovannoni, 2007), and epigenetic regulation of the targets of these master regulators has also been described in tomato (Giovannoni et al., 2017). Regarding postharvest, epigenetic regulation of the senescence process has been explored in tomato, strawberry, and citrus (Farinati et al., 2017). In comparison, in stone fruits information is scarce, but it is starting to emerge (Fresnedo-Ramírez et al., 2017; Ma et al., 2018). Future studies are really needed and will help in the elucidation of epigenomic dynamics and the epigenetic mechanisms in stone fruit, which may also control secondary organic metabolite production and its relationship with stress condition exposures.

Finally, apart from a deeper knowledge about the molecular mechanisms related to the regulation of the levels of secondary organic compounds in stone fruits, the phytochemical content of the large number of different stone fruit varieties should be deeply characterized. Besides, the molecular mechanisms underlying the differences in phytochemical contents among varieties of the same stone fruit species should be identified. These results could be used in breeding programs to enhance nutritional value of stone fruits, which may aid, along with improved pre- and postharvest handling strategies, in the enlargement of the health promoting compounds of stone fruit for consumers' benefits in the years to come.

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All authors contributed to the article and approved the submitted version. However, MD and ML had a major role in the design and writing of the article.

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REVIEW
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Stone Fruits: Growth and Nitrogen and Organic Acid Metabolism in the Fruits and Seeds—A Review

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Stone fruits of the Rosaceae family consist of several distinct parts, and these include the flesh, woody endocarp, and seed. To understand the metabolism of these fruits, it is necessary to have knowledge of both their structure and growth characteristics. The nitrogen metabolism of the different tissues of stone fruits is interlinked. For example, there is an import and storage of nitrogenous compounds in the endocarp that are then exported to the seed. Moreover, there are links between the metabolism of nitrogen and that of malic/citric acids. In this article, the structure and growth characteristics, together with the import/export, contents, metabolism, and functions of nitrogenous compounds and organic acids in the different parts of stone fruits and their seeds are reviewed.

Keywords: endocarp metabolism, fruit growth, nitrogen compounds, organic acids, seed metabolism

INTRODUCTION

The term stone fruits commonly refers to certain species of the genus *Prunus* which is a member of the rose family (*Rosaceae*) (Looney and Jackson, 2006). These fruits include almonds (*P. dulcis*), apricots (*P. armeniaca*), sweet cherries (*P. avium*), sour or tart cherries (*P. cerasus*), several species of commercial plums, the most important being the European plums (*P. domestica*) and the Asian or Japanese plums (*P. salicina*), peaches and nectarines (*P. persica*). The term stone fruits derives from the woody endocarp (stone or pit) which characterises the fruits of these species. The edible portion of stone fruits consists of the fleshy epicarp and mesocarp which encloses the stony endocarp. The exceptions are almonds and certain apricots whose seeds are consumed (Looney and Jackson, 2006). Botanically, the fruit of stone fruits is classified as a drupe.

Organic acids and nitrogenous compounds are abundant constituents of stone fruits and their seeds (Walker et al., 2011a; Famiani et al., 2015). Organic acids affect the taste of the flesh and skin of stone fruits and also have several underlying metabolic roles, which include both acting as an osmoticum that contributes to generating turgor pressure and links with nitrogen metabolism (Famiani et al., 2016; Walker and Famiani, 2018). Nitrogenous compounds play several roles in

metabolism, and these include acting as the building blocks of proteins and serving as precursors that are used in the synthesis of compounds such as lignin; and the latter process is particularly pronounced in the endocarp (Walker et al., 2011a; Famiani et al., 2012). The endocarp is a specialized part of the pericarp: during development it becomes woody and gives rise to the stone which encloses and protects the seed (Romani and Jennings, 1971). The seeds of stone fruits are composed of a number of distinct tissues, including the seed coat which distributes imported assimilates to the developing internal storage tissues (Walker et al., 2011a; Famiani et al., 2012). The nitrogen metabolism of the flesh, endocarp, and seed are linked, and this is because there is an import of nitrogenous compounds into specific tissues of the fruit (e.g., endocarp), storage of this material, and subsequently export to other parts of the fruit and also to the seed (Walker et al., 2011a; Famiani et al., 2012).

In this article, the import, contents, metabolism, and functions of nitrogenous compounds and organic acids that are abundant in the flesh, endocarp, and seeds of stone fruits are reviewed. In addition, the development and structure of the whole fruit, endocarp, and seed are considered.

IN ORDER TO UNDERSTAND THE METABOLISM OF STONE FRUITS THEIR STRUCTURE AND GROWTH CHARACTERISTICS MUST BE CONSIDERED

Structure and Growth Characteristics

Stone fruits all consist of a fruit wall (pericarp) which usually encloses a single seed. The fruit wall is derived from the ovary and consists of three layers: the skin (epicarp) which encloses the flesh (mesocarp), and this then encloses the stone (endocarp) (**Figure 1**) (Romani and Jennings, 1971; Bollard, 1971; Brady, 1993). Recent studies suggest that simple genetic changes in a small number of genes that control development has brought about the evolution of the woody nature of the endocarp (Dardick and Callahan, 2014).

On the basis of changes in either their fresh weight (FW) or volume, the growth pattern of either the whole fruit or the flesh can often be depicted by a double-sigmoidal curve. The first period of rapid growth as depicted by this curve is defined as stage I, the period of reduced growth is stage II and the second period of more rapid growth is stage III (**Figure 2**) (Lilleland, 1930; Lilleland, 1933; Pavel and DeJong, 1993b; Zuzunaga et al., 2001; Baldicchi et al., 2015). Stages I and III can be further subdivided. Thus, peach stage I can be subdivided into stages Ia and Ib and stage III can be subdivided into stages III and IV; with stage IV beginning when the growth rate of the flesh slows down and the fruit is close to its maximum size (Chalmers and van den Ende, 1975; Scorza et al., 1991; Zanchin et al., 1994). These subdivisions are advantageous when considering certain aspects of the growth and metabolism of peach fruits (Scorza et al., 1991; Tonutti et al., 1997). However, in this review, we use the traditional division into three stages, because these subdivisions have not been applied to all stone fruit species (**Figure 2**). In general, the following applies to all the stone fruits considered in this review. During stage I each part of the pericarp increases greatly in size and the stone and seed approach their maximum dimensions (size). During stage II the increase in the size of the flesh slows down, and the endocarp hardens to form the stone and it reaches its maximum dry weight (DW) (**Figure 2**). During stage III there is a large increase in the volume of the flesh and it ripens. In addition, the bulk of both the sugar content of the flesh and the storage reserves of the seeds are accumulated, and their DW increases (**Figure 2**) (Lilleland, 1933; Sterling, 1953; Marshall, 1954; Hawker and Buttrose, 1980; Bassi and Ryugo, 1990; Zanchin et al., 1994; Famiani et al., 2012; Falchi et al., 2013; Baldicchi et al., 2015). An exception is almond whose flesh does not expand during stage III (Hawker and Buttrose, 1980). The length of stage

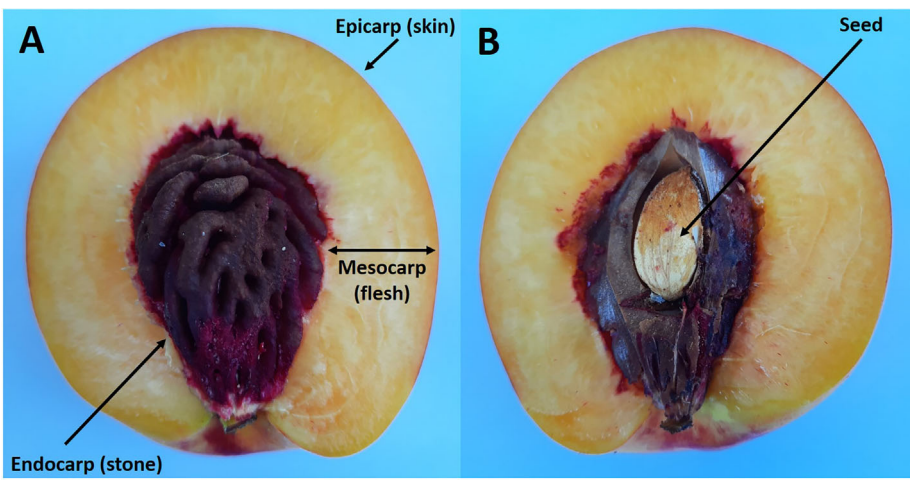

FIGURE 1 | Longitudinal section of a ripe nectarine fruit showing the flesh and stone **(A)**. Same fruit but with the endocarp cut to reveal the enclosed seed **(B)**.

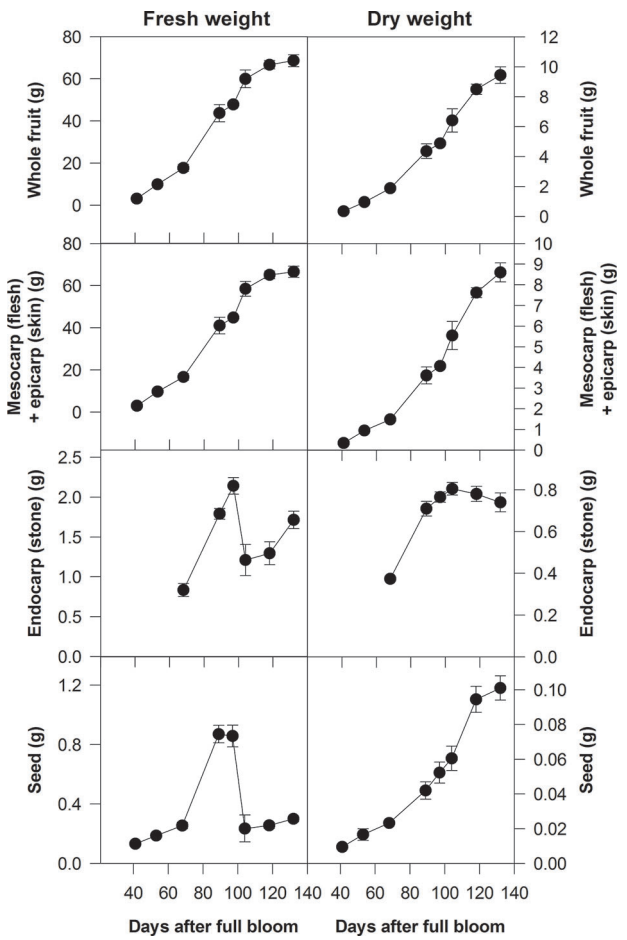

FIGURE 2 | Fresh and dry weights of whole fruits of Ozark Premier plum and their component parts at different stages of development (from Famiani et al., 2012). Based on changes in their fresh weight, the growth patterns of whole fruits and their flesh plus skin were double sigmoidal: stage I was up to about 90 days after full bloom (DAB), stage III was after 100 DAB and stage II was between about 90 and 100 DAB. Bars represent the standard errors (n = 15).

II depends on the variety of plum, peach, or cherry, and for some, it is very short, which gives rise to a sigmoidal pattern of growth (Lilleland, 1933; Pavel and DeJong, 1993a; Zuzunaga et al., 2001). Pavel and DeJong (1993b) evaluated the factors that could be responsible for the double sigmoidal pattern of growth and concluded that it might be the result of cultivar-specific developmental genetic information. By contrast, Dardick and Callahan (2014) proposed that the competition for assimilates between different parts of the fruit could be responsible for the double sigmoidal pattern of growth. More recently, these views have been perhaps reconciled by a study that compared Rosaceaeous fruits that showed either a single sigmoidal (pears) or double sigmoidal (peaches and strawberries) growth pattern (Pei et al., 2020). Thus, the block in fruit enlargement observed in peaches at stage II and in strawberry at the color-break stage is due to a diversion of assimilates and hormones toward endocarp lignification and anthocyanin biosynthesis, respectively. In contrast, the lack of dramatic changes occurring during pear fruit development allows the use of both hormones and assimilates for fruit enlargement, which resulted in the single sigmoid pattern. To support this view, a comparison of the three fruit transcriptomes during development allowed the identification of a set of genes differentially expressed in pears during the enlargement phase that are not expressed in the other two species. These genes included several transcription factors such as zinc finger proteins (ZFPs), which control cell size during plant organogenesis, and bHLHs (basic helix-loop-helix proteins), which regulate cell extension by transducing auxin signalling.

Relative Growth of the Different Parts of the Fruit and Seed

In peach and apricot, fruit growth has also been analyzed in terms of the relative growth rate (RGR) of its different parts. In peach, this showed that the double-sigmoidal growth pattern corresponded to two phases of sink activity. During the first phase, RGR decreased logarithmically, while during the second RGR was relatively constant (DeJong and Goudriaan, 1989). The endocarp + seed had a higher RGR than the mesocarp during the first phase, while the RGR of the mesocarp was higher when the RGR of the whole fruit started to become relatively constant (Pavel and DeJong, 1993b). The transition between these two phases appeared to correspond to the beginning of sugar accumulation in the mesocarp and epicarp (Pavel and DeJong, 1993b). In this study of peach, the endocarp and the seed were considered as one entity; however, in apricot, the growth of the endocarp and different parts of the seed were each evaluated: thus making it possible to compare the growth rates of the different parts of the fruit (mesocarp + epicarp and endocarp) and seed (integuments and embryo + endosperm) (Baldicchi et al., 2015). In apricot, the endocarp had a higher RGR than the mesocarp + epicarp during stage I of growth; however, the RGR of the mesocarp + epicarp was also high (Baldicchi et al., 2015). During stage II (when stone hardening takes place) the embryo + endosperm had the highest RGR. This suggested that at this time, the strongest sink is the seed, which is the organ with the greatest ability to import assimilates (Baldicchi et al., 2015). During stage III of growth the RGR of both the epicarp + mesocarp and the embryo + endosperm had similar high values while the RGR of the endocarp was low (Baldicchi et al., 2015). During stages II and III, the high RGR of the seed (in terms of DW) was due to the import of assimilates used in the synthesis of storage compounds. Further, the daily growth rate of the different parts of the fruit and seed (which also depends on their size) were compared in order to calculate the amount of assimilates imported into each of them per day (Baldicchi et al., 2015). On per fruit basis, the amount of assimilates imported into the mesocarp + epicarp was high during stage I and very high during stage III; while in the endocarp, import was highest during stage II (Baldicchi et al., 2015). The lower values of seed daily growth rate, despite a higher RGR than the fruit during stage II, was due to its smaller size. However, in marked contrast to the epicarp + mesocarp, fat accounts for a large percentage of the DW of stone

fruit seeds (Femenia et al., 1995). To synthesize this fat from imported sugars, a large amount of ATP and reductant is required, and this is produced from the metabolism of imported sugars. Hence, the daily growth rate of the seed would be higher if the amount of sugars used to produce this energy and reductant is taken into account. Similar considerations apply to the endocarp because this consists largely of lignin; again, a compound which requires large amounts of ATP and reductant for its synthesis.

Changes in Cell Structure During Flesh Development and Dilution Effects

There are marked changes in the structure of the flesh of stone fruits during development (Scorza et al., 1991; Zanchin et al., 1994) and, in order to understand their metabolism, these must be taken into account. The increase in the size of the flesh results from both an increase in the number of parenchyma cells (brought about by cell division) and an increase in their size (arising from cell expansion), along with an increase in intercellular spaces (Masia et al., 1992). The contribution of each of these processes is dependent upon the stage of development. Stage I begins at full bloom and in peach it can be divided into stages Ia and Ib: in the flesh, the cell number increases 11.3 times and cell area 3.5 times during stage Ia, whereas cell number increases 1.1 times and cell area 9.2 times during stage Ib (Scorza et al., 1991; Masia et al., 1992). Similarly, in cherry, the bulk of cell division occurs during the first half of Stage I, and the greatest increase in cell size occurs during the second half of stage I (Tukey and Young, 1939).

In peach, fruit size is determined, at least in part, by the characteristics of the flower and particularly the ovarian tissues. Large-fruited peach cultivars produce ovaries and fruits with a higher number of cells than small-fruited cultivars (Scorza et al., 1991). The same is true for olive, which is also a drupe, where ovary size and cell number correlate with fruit size at maturity and the correlations hold even at the individual tissue (i.e., endocarp and mesocarp) level (Rosati et al., 2009; Rosati et al., 2012). By contrast, this is not true in cherry (*Prunus avium* L.). While fruit size in cherry is also associated with cell number and not with cell size, it is not correlated with the ovary size. Instead, differences in fruit size at maturity arise from a longer post-bloom cell division phase and are not related to ovary size and cell number at anthesis (Olmstead et al., 2007). Also in plum, differences in the number of cell divisions after anthesis are largely responsible for the genetic differences in fruit size among different species/varieties (Cerri et al., 2019). For both plant breeders and growers, it is important to know the mechanisms that influence fruit size; because in species in which fruit size is determined primarily by post-bloom processes, the possibility of altering fruit size (e.g., through irrigation or fertilization) is greater than in species where differences in fruit size are already determined at anthesis (Rosati et al., 2009; Cerri et al., 2019).

Although the number of parenchyma cells of the flesh is the major factor that determines the difference in potential fruit size among cultivars of both peach and cherry, environmental factors and cultural practices also affect the final size of the fruits of a given cultivar, largely as a result of an alteration in the size of the parenchyma cells of the flesh (Scorza et al., 1991; Zanchin et al., 1994; Olmstead et al., 2007). In peach flesh, the volume of individual cells increased nearly 200-fold during stage I, and from the end of stage I to ripeness about 20-fold (Zanchin et al., 1994); altough in absolute terms the increase in volume is greater during stage III. Clearly, this increase in cell size results in a large decrease in the cell wall area per g FW of flesh (g^{-1} FW). In peach, the expansion of the parenchyma cells during stage I was associated with an increase in intercellular spaces, and this was associated with the digestion of the middle lamella (Masia et al., 1992). Nevertheless, these cells remained connected by plasmodesmata, and the latter were grouped together to form pit fields (Zanchin et al., 1994). During stage I, there is a large increase in the proportion of total cell volume occupied by the vacuole (Famiani et al., 2012; Génard et al., 2014). The concentration of protein in these vacuoles is much less than in the cytoplasm and this results in a large decrease in the total soluble protein content of both the flesh and skin, as it is observed in both cherry and plum (Donen, 1939; Walker et al., 2011b; Famiani et al., 2012; Génard et al., 2014). In the parenchyma cells of tomato flesh, the change in the proportions of cell volume occupied by the vacuole and cytoplasm during development has been investigated in more detail: the large increase in the proportion of cell volume occupied by the vacuole occurs largely during the cell division stage of development, but also continues into the cell expansion stage (Beauvoit et al., 2014). This is consistent with the decline in total polypeptide abundance on SDS-PAGE gels of stone fruits flesh that is evident during development when gels are loaded with extracts of flesh (Walker et al., 2011b; Famiani et al., 2012). This change in the ratio of the cytoplasm to the vacuole can be a major factor that contributes to the changes in the abundance of individual enzymes per g FW of flesh during development. Thus, it is essential to take into account how enzyme abundance is expressed; because, if the content is expressed on a per g FW basis, different patterns of changes during development will be obtained than if expressed on per g total protein. Similarly, expansion of the flesh can lead to a decrease in the concentration of specific metabolites (g^{-1} FW) that is not brought about by a net catabolism of the metabolite but by a dilution effect (Famiani et al., 2015; Famiani et al., 2016; Moscatello et al., 2019). Similarly, when content is expressed on per g DW basis, a decrease during development could be a result of a dilution effect arising from the accumulation of large amounts of other material (for example sugars during ripening). Expressing content on a per fruit basis discriminates between these two possibilities (Famiani et al., 2015; Famiani et al., 2016; Moscatello et al., 2019). During the development of both peach and Japanese plum, the amount of CO_2 released per unit time and per g of FW or DW (g^{-1} FW or g^{-1} DW) decreases greatly during stage I (Pavel and DeJong, 1993a; Zuzunaga et al., 2001), and a large part of this decrease could result from an increase in the proportion of cell volume occupied by the vacuole (Famiani et al., 2016). Indeed, the 3- to 5-fold decrease in CO_2 release per unit time and g^{-1} FW or DW that occurs during stage I in stone fruit (Pavel and DeJong, 1993a; Zuzunaga et al., 2001) and the 4- to 5-fold increase in the proportion of cell volume occupied by the vacuole in tomato fruit during the equivalent stage of growth (Beauvoit et al., 2014) are consistent with this. The large decrease during development in the amount of cell wall area g^{-1} FW (Scorza et al., 1991) has also an effect on the abundance g^{-1} FW flesh

of enzymes that are located in the cell wall. A further complicating factor is that the flesh and other tissues of fruit and seeds are composed of a number of distinct tissues, and many proteins and metabolites are not uniformly distributed between these tissues or between the different cell types that make up each of these tissues (Walker et al., 1999; Famiani et al., 2000; Walker et al., 2001; Famiani et al., 2016; Zanon et al., 2015).

IMPORT OF NITROGENOUS COMPOUNDS INTO THE FRUIT AND SEED AND RELATED ASPECTS

Import of Nitrogenous Compounds Into the Fruit and Seed

Almost all the nitrogenous compounds required by fruits are imported *via* the xylem and phloem of their vasculature (Layzell and LaRue, 1982; Peoples et al., 1985). During the growth of cowpea fruits 72% of nitrogenous materials are imported in the phloem, and the xylem provides the remainder (Peoples et al., 1985). In sweet cherry, the amide/amino acid content of the xylem was about 35–60 mM about 2 weeks after bud burst and declined to about 2-5 mM 2 months later (Grassi et al., 2002; Millard et al., 2006). In the xylem of terminal shoots of peach and Japanese plum (both growing in Florida, USA in July/August), this concentrations was 1–10 mM (Andersen et al., 1995a; Andersen et al., 1995b). Given the content of nitrogenous material in the ripe flesh of stone fruits (see below), and taking into account the amounts of liquid entering through the xylem and phloem (Morandi et al., 2007; Brüggenwirth et al., 2016), it would appear that the xylem can potentially supply a considerable proportion of the required nitrogenous compounds. However, this may not necessarily be the case because, in both soybean and wheat, there is a considerable transfer of nitrogenous compounds from the xylem to the phloem during their transport through the plant, and the input of nitrogenous compounds into the fruit is largely *via* the phloem (Layzell and LaRue, 1982: Simpson et al., 1983). In the xylem of both peach and sweet cherry, asparagine and glutamine account for a large proportion of the nitrogenous material (Andersen et al., 1995b; Grassi et al., 2002). Similarly, in the phloem of the shoot apex of peach, asparagine, glutamine, and glutamate accounted for the bulk of the amide/amino acid content (Moing et al., 1997). Both asparagine and glutamine are also abundant in the phloem and xylem of almond (Youssefi et al., 2000). Thus, in stone fruits, it appears likely that a large proportion of the nitrogenous compounds imported into the fruits and seeds consists of glutamine and asparagine.

Changes in the Ratio of Contents of Non-Nitrogenous to Nitrogenous Compounds During Fruit and Seed Development

The mature flesh and mature seed of stone fruits contain a very different ratio of contents of non-nitrogenous to nitrogenous compounds, and for the flesh a ratio of 40–80:1 is typical, whereas for the seed the ratio is about 5:1 (Donen, 1939; Hawker and Buttrose, 1980; Famiani et al., 2012). Further, in the seeds of stone fruits, there is a considerable accumulation of lipids after the accumulation of nitrogenous compounds is almost complete (Hawker and Buttrose, 1980; Bassi and Ryugo, 1990; Walker et al., 2011a; Famiani et al., 2012). Thus, the ratio of sugars (also used in lipid synthesis) to nitrogenous compounds required by the seed changes during development. Fruits and seeds of other plants also require different ratios of these compounds, and potentially this can be achieved either by export of material or by adjusting the solute contents of the xylem and/or phloem saps, the latter being possible by the transfer of solutes between the xylem and phloem (Layzell and LaRue, 1982; Peoples et al., 1985). In stone fruits, export of nitrogenous material from the endocarp occurs. In both cherry and plum, vegetative storage proteins are accumulated at earlier stages of endocarp development and then decline to almost undetectable amounts (Walker et al., 2011a; Famiani et al., 2012).

Source of the Nitrogenous Compounds That Are Imported Into the Fruit and Seed During Their Early Growth

Trees of stone fruits that flower before the leaves are developed are thought to utilize a considerable proportion of carbohydrates stored in the roots and crown for the early growth of their fruits (Keller and Loescher, 1989; Loescher et al., 1990; Flore and Layne, 1999). During dormancy, these reserves consist largely of starch and sucrose, but sorbitol becomes the most abundant soluble sugar when bud burst approaches (Keller and Loescher, 1989; Jordan and Habib, 1996; Flore and Layne, 1999). Similarly, during the early growth of both peach and cherry fruits, a large proportion of the imported nitrogenous compounds arises from mobilization of nitrogen compounds that are stored in the roots and crown, and not from uptake from the soil (Taylor and May, 1967; Rufat and DeJong, 2001; Grassi et al., 2002; Policarpo et al., 2002; Millard et al., 2006). In peach, the largest proportion of these reserves consists of soluble nitrogenous compounds, and arginine accounts for the bulk of these. The remainder of these reserves consists of proteins that include vegetative storage proteins (Taylor, 1967; Taylor and May, 1967; Gomez and Faurobert, 2002; Wisniewski et al., 2004). As in the case of carbohydrate reserves (Loescher et al., 1990; Flore and Layne, 1999), the amount of stored nitrogenous compounds in the crown and roots follows an annual cycle. Nitrogenous material is accumulated after the end of shoot and fruit growth, and export of nitrogenous compounds from senescing leaves makes an important contribution to this accumulation. In spring, the nitrogenous reserves are mobilized, and the amounts of stored nitrogenous material decrease both before and after bud burst (Taylor and May, 1967; Gomez and Faurobert, 2002). When both nitrogenous and carbohydrate reserves are mobilized, the soluble products of these reserves are transferred to the xylem, and then move along with water in the transpiration stream (Van Bel, 1990; Aubry et al., 2019). For example, in the case of carbohydrates in the xylem sap of cherry, their contents drop from 15 to 2–3 mg ml^{-1} after bud break (Loescher et al., 1990), and given the amount of water imported into cherry during this period (Brüggenwirth et al., 2016), this content is insufficient to

meet the demands of the fruit. This implies that during this period of early growth of the fruits there must be a considerable transfer of sugars from the xylem to the phloem in order to increase the concentration of sugars entering the fruit, and this is also known to occur in some other fruits (Layzell and LaRue, 1982; Peoples et al., 1985: Simpson et al., 1983; Van Bel, 1990; Aubry et al., 2019). A similar mechanism could also be used for nitrogenous compounds. Unlike in some other plants (Aubry et al., 2019), in stone fruits, virtually nothing is known regarding either the sites of these transfers between xylem and phloem or the underlying molecular physiology.

IMPORT OF ORGANIC ACIDS INTO THE FRUIT AND SEED

It is generally thought that, in fleshy fruits, the bulk of the organic acid content of the flesh is synthesized from sugars (Walker and Famiani, 2018). The following shows that in the pericarp of stone fruits, this is almost certainly the case. Organic acids are the compounds that give rise to the bulk of the protons that determines the titratable acidity of fruits. Malic, citric, and quinic acids account for the main part of the organic acid content of the flesh of all stone fruits (Walker and Famiani, 2018). In both ripe cherry flesh and peach flesh, titratable acidity throughout development was close to the amount of protons that can be calculated to arise from the organic acids that are present (Girard and Kopp, 1998; Moing et al., 1998). This shows that the bulk of these must either be synthesized in the flesh or imported in the undissociated form. Organic acids dissociate in solution to give the organic acid anion and a proton(s). The degree of dissociation is dependent on the pKa of the proton donating group(s) of the acid in question. The pKa is the pH at which the proton donating group is 50% dissociated, and at one pH unit higher than this it is about 90% dissociated (malic acid $pKa_1 = 3.4$, $pKa_2 = 5.3$; citric acid $pKa_1 = 3.1$, $pKa_2 = 4.8$, $pKa_3 = 6.4$; quinic acid pKa = 3.4). At the high pH of the phloem sap, these organic acids will be almost totally dissociated; hence, it is unlikely that a large proportion of the organic acid content is imported in the phloem. In the xylem of trees, the malate content is generally lower than 12 mM (Schell, 1997), and in both terminal shoots of peach and Japanese plum (growing in Florida in July/August), the organic acid content of the xylem was less than 3.5 mM, with malate accounting for most of it (Andersen et al., 1995a; Andersen et al., 1995b). Comparing the inflows of liquid from the xylem (about 0.28 g fluid g^{-1} FW day^{-1} for stage I in peach; Morandi et al., 2007), a potential malate content of 3.5 mM in the xylem and the content of malate in peach flesh (about 50 mM in peach around the end of stage I; Famiani et al., 2016), it is clear that a large proportion of the malate content could potentially be imported. However, the pH of the xylem is often in the range 4.5–5.5, and at this pH a substantial proportion of the organic acid content would be dissociated. Therefore, it would appear that, although a small proportion of malic acid could be imported from the xylem, the bulk of the content is synthesized within the fruit.

CONTENTS OF NITROGENOUS COMPOUNDS IN THE FLESH

In this review, only abundant nitrogenous compounds are considered. Donen (1939) reported the contents of nitrogenous compounds in the flesh, endocarp, and seed of Japanese plum during development. In the flesh, protein content decreased from about 10 mg g^{-1} FW during stage 1 to about 3 mg g^{-1} FW in ripe fruits. Similarly, protein abundance g^{-1} FW in the skin and flesh of cherry and plum and in peach flesh decreased greatly during stage I (Lombardo et al., 2011; Walker et al., 2011b; Famiani et al., 2012). Subsequently, from stage II to ripeness the change in protein content of peach flesh was much less (Lombardo et al., 2011; Famiani et al., 2016), and during ripening, total protein content decreased from 1.10 to 0.77 mg g^{-1} FW (Prinsi et al., 2011). The content of total protein in the flesh of common apricot decreased from 0.42–0.38 to 0.31 mg g^{-1} FW during ripening (D'Ambrosio et al., 2013; Zhang et al., 2017). In cherry flesh, but not in Japanese plum or peach, the total protein content decreased during ripening (Krishnan and Pueppke, 1990; Walker et al., 2011b; Famiani et al., 2012; Famiani et al., 2016). In cherry flesh, there was a large accumulation of a thaumatin-like protein during ripening which accounted for around 42% of the total soluble protein content (Fils-Lycaon et al., 1996). Similarly, large amounts of both a thaumatin-like protein and lipid-transfer proteins are present in ripe peach flesh, and these can produce an allergic reaction in susceptible individuals (Palacin et al., 2010). These abundant proteins serve as a store of nitrogen and could potentially have other roles such as in plant defence and responses to stresses (Wisniewski et al., 2004; Tian et al., 2007; Dagar et al., 2010). The content of amides and amino acids can be increased greatly in the flesh of stone fruits by feeding the trees with nitrogenous fertilizer (Taylor, 1967; Jia et al., 2000). It is likely that the content of protein (and especially those that act as a store of nitrogen) will also be increased, as occurs in other fruits (Delgado-Alvarado et al., 2007: Famiani et al., 2012).

Asparagine accounts for a large proportion of amide/amino acid content of the flesh of plums, apricots, cherries, and peaches, and also in the endocarp and seeds of stone fruits species in which it has been studied (Kakiuchi et al., 1985; Famiani et al., 2012; Rodriguez et al., 2019). The amount of non-protein nitrogen present in Japanese plum flesh (Donen, 1939), would be equivalent to about 7.5 mg g^{-1} FW (stage I) and 3.5 mg g^{-1} FW (ripe flesh) of asparagine being present. In Japanese plum, flesh ammonium content was highest toward the end of stage I (0.2–0.3 mg g^{-1} FW), which accounted for 10–15% of the total N content of the flesh, and then decreased (Donen, 1939). Similarly, in the flesh of one cultivar of Japanese apricot, large amounts of ammonium accumulated up to ripening and then declined; this accumulation of ammonium was not observed in another cultivar (Otoguro and Kaneko, 1994). During the ripening of peach flesh, total amide/amino acid content increased from 1 to 1.3 mg g^{-1} FW (Prinsi et al., 2011).

In ripe peach flesh, the total amide/amino acid content of fruits from trees fed low amounts of nitrogenous fertilizer was 1.3 mg g^{-1} FW (0.8 mg of this was asparagine), and from trees fed larger amounts of nitrogenous fertilizer it was 4.6 mg g^{-1}FW (2.6 mg was

asparagine) (Jia et al., 2000). Moing et al. (1998) found that asparagine accounted for the bulk of the amino acid/amide content of peach flesh throughout development. In ripe peach flesh from three cultivars, the content of asparagine was between 3.2 and 4.7 mg g^{-1} FW and this accounted for over 85% of the amide/amino acid content (Moing et al., 2003). In the ripe flesh of *Prunus davidiana* (a related species to peach), asparagine content was about 9.5 mg g^{-1}FW and accounted for over 90% of the amide/amino acid content (Moing et al., 2003). In Japanese apricot, asparagine also accounted for about 90% of the soluble nitrogenous compounds of the flesh throughout development. Asparagine (mg g^{-1} FW) content changed during development and was 4.5 in stage I, 7.0 in stage II, and 3.2 in stage III (ripeness) in 1982 season, and 3.0 in stage I, 5.6 in stage II, and 2.5 in stage III (ripeness) in 1983 season (Kakiuchi et al., 1985). Comparable amounts and patterns of changes in asparagine content were also found in two cultivars of small Japanese apricot (Otoguro and Kaneko, 1994). In cherry flesh, amide/amino acid content decreased from roughly 9.7 to 4.4 mg g^{-1} FW during ripening (Prinsi et al., 2016).

CONTENTS OF ORGANIC ACIDS IN THE FLESH

In this review, only the quantitatively most important organic acids are considered. For previous reviews dealing with organic acids in stone fruits and additional details, refer to Famiani et al. (2015) and Walker and Famiani (2018). In stone fruits, the most abundant organic acids are usually malic, citric, and quinic (Walker and Famiani, 2018). The contents of these acids per g FW differ between the species and cultivar of stone fruits and is also dependent on the tissue and stage of development (**Table 1**; Walker and Famiani, 2018). In the flesh of apricots and peaches both malic and citric acids can be abundant (Moing et al. 1998; Baldicchi et al. 2015; Famiani et al. 2016), whereas in the flesh of cherries and plums, malic acid is much more abundant (Walker et al., 2011b; Famiani et al., 2012; Moscatello et al., 2019). In most stone fruits the contents of malic/citric acid per g FW (i.e., concentration: mg g^{-1} FW) of both skin and flesh, are usually higher before stage III and then decrease during stage III. However, this decrease is at least in part due to a dilution effect which arises from cell expansion (fruit growth), and in some cases there is no net dissimilation of the acids (Famiani et al., 2015; Walker and Famiani, 2018). In order to establish if net dissimilation of the acids occurs, it is necessary to express their contents at different stages of development on a per fruit basis (i.e., mg $fruit^{-1}$). Doing this, it was observed that in cherry flesh, the decrease in the content of malic acid per g FW during ripening, and up to the time of commercial harvesting, was due to a dilution effect; because its content per fruit increased for the whole period. Thus, there was net synthesis and not dissimilation of malic acid (Walker et al., 2011b). In apricot flesh, during the first part of ripening the decrease in both malic and citric acids per g FW was due to the growth of the fruit, because their contents per fruit

TABLE 1 | The concentration of organic acids (mg g^{-1} FW) in the flesh of unripe and ripe stone fruits.

	Unripe (stage II)			
	Malic acid	**Citric acid**	**Total soluble sugars**	
Apricot (common)	33	1.5	6.6	Baldicchi et al., 2015
Apricot (Japanese)	34	13	6.6	Otoguro and Kaneko, 1994
Cherry (sweet)	8	low	41	Walker et al., 2011b
Peach Hakuto			33	Moriguchi et al., 1990
Peach Adriatica	6.6	4.8	43	Famiani et al., 2016
Plum (Japanese)	17	0.1	35	Donen, 1939 Famiani et al., 2012
	Ripe (end of stage III)			
	Malic acid	**Citric acid**	**Total soluble sugars**	
Apricot (common)	8	10	92	Baldicchi et al., 2015
Apricot (Japanese)	21	40	12	Otoguro and Kaneko, 1994
Cherry (sweet)	6.7	low	126	Ballistreri et al., 2013 Walker et al., 2011b
	9.4	0,2	165	Winkler and Knoche, 2018
Cherry (sour)	21.6	0.3	115	Winkler and Knoche, 2018
Cherry (Morello)				
Line 16	35	low	171	Proietti et al., 2019
Line 37	37	low	55	
Peach Hakuto			72	Moriguchi et al., 1990
Peach Adriatica	4.8	0.9	68	Famiani et al., 2016
Peach Pamirskij	3	3	141	Moing et al., 2003
Nectarine Summergrand	4.3	3.7	113	Moing et al., 2003
Plum (Japanese)	11	0.1	104	Donen, 1939 Famiani et al., 2012
Prunus davidiana	15	0.3	17.5	Moing et al., 2003

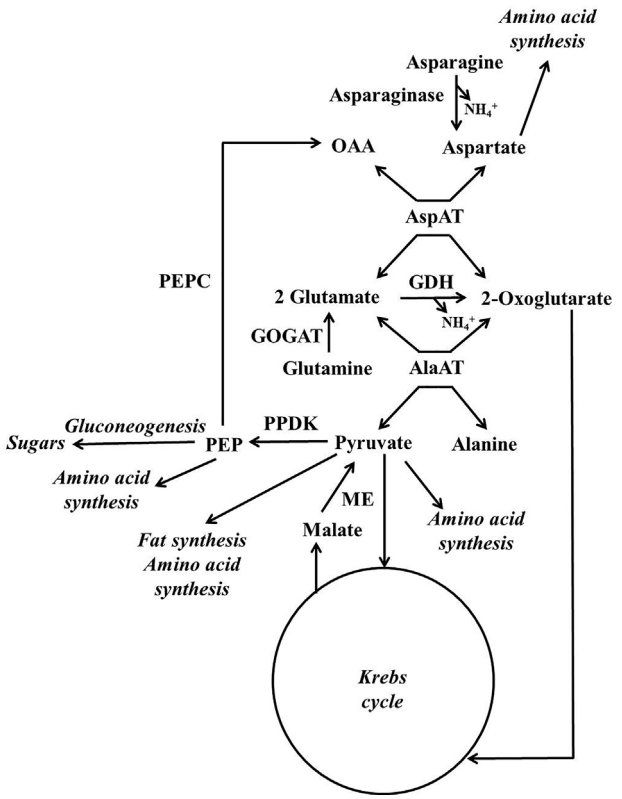

FIGURE 3 | Simplified scheme outlining the major pathways involved in the metabolism of nitrogenous compounds and malic and citric acids in fruits and seeds of stone fruits (based on Walker et al., 2018). AlaAT = alanine aminotransferare; AspAT = aspartate aminotransferare; GDH = glutamate dehydrogenase; GOGAT = glutamate synthase also known as glutamine oxoglutarate aminotransferase; ME = malic enzyme; OAA = oxalacetate; PEP = phosphoenolpyruvate; PEPC = phosphoenolpyruvate carboxylase; PPDK = pyruvate orthophosphate dikinase.

increased, and a net dissimilation of these compounds only occurred in the last part of ripening (Baldicchi et al. 2015). A similar behavior was observed in the fruits of the plum cultivar Ozark premier (*P. salicina*) (Famiani et al., 2012). Up to commercial harvest the decrease in the concentration of malic acid in the flesh of the plum species/cultivars President (*P. domestica*), Shiro (*P. salicina*) and Mirabolano (*P. cerasifera*) was due to a dilution effect, and the amounts per fruit increased; after this time a net dissimilation of malic acid occurred in the over-ripe fruits (Moscatello et al., 2019). In peach flesh, during ripening, there was a net dissimilation of citric acid, whereas there was a synthesis of malic acid (Famiani et al. 2016). Therefore, during ripening there can be either a net dissimilation or synthesis of stored Krebs cycle acids, and which occurs is dependent on the species/cultivar and on the stage of ripening. The metabolism and functions of quinic acid in fruits (including stone fruits) has been recently reviewed by Walker and Famiani (2018), and hence, this topic is omitted in the present review.

ENZYMES OF NITROGEN AND KREBS CYCLE ACID METABOLISM IN THE FLESH

A scheme illustrating the position in metabolism of the enzymes mentioned in this section is given in **Figures 3**, **4**. Evidence has been provided that in both stone fruits and various other tissues of plants, malate and citrate, which are stored in the vacuole, can be released at certain situations, and then serve as a substrate for nitrogen metabolism (Walker et al., 2015; Famiani et al., 2016; Bräutigam et al., 2017; Walker et al., 2018; Walker and Famiani, 2018). To more fully understand this area of metabolism, it is essential to take this into account (Walker et al., 2018). The schemes depicted in **Figure 4A** show events occurring when malate and/or citrate are accumulated in the vacuole, and **Figure 4B** depicts events when they are released. When the amount of malate and/or citrate released from the vacuole is in excess of demands of processes other than gluconeogenesis, the latter occurs.

For simplicity in this review, we refer to malate and citrate as Krebs cycle acids, and this is because they are associated with the Krebs cycle. The metabolism of malate and citrate is intimately linked with that of amino acids and amides (**Figures 3**, **4**), and one reason for this is that the carbon skeletons of many nitrogenous compounds are synthesized from these organic acids (Walker and Famiani, 2018). Asparagine and glutamine appear to account for the bulk of the nitrogenous compounds imported into the fruits and seeds of stone fruits, however, little glutamine is present in the flesh, whereas large amounts of asparagine are present. Thus, it appears that there is a preferential utilization of glutamine in stone fruits, as also seen in some other plant tissues (Walker et al., 2011a; Famiani et al., 2012). An inspection of the data given in the section "contents of nitrogenous compounds in the flesh", reveals that asparagine often accounts for over 50% of the protein and non-protein nitrogenous compounds present in the ripe flesh of stone fruits. Nevertheless, imported glutamine and asparagine must be converted to the spectrum of amides and amino acids required by the fruit and seed (Walker et al., 2011a; Famiani et al., 2012). The potassium-dependent asparaginase is the principle route by which asparagine is further metabolized in sink tissues (**Figures 3**, **4**; Lea et al., 2007). The enzyme glutamate synthase also known as glutamine oxoglutarate aminotransferase (GOGAT) converts glutamine to glutamate and is the main pathway used in the breakdown of glutamine (**Figures 3**, **4**; Lea, 1993). *In vivo*, glutamate dehydrogenase (GDH) catalyzes the deamidation of glutamate to 2-oxoglutarate (Lea, 1993). The enzymes glutamine synthetase (GS) and GOGAT acting in concert are responsible for the incorporation of ammonium into organic forms of nitrogen. The enzymes aspartate aminotransferase (AspAT) and alanine aminotransferase (AlaAT) catalyze reversible reactions which occupy a central role in nitrogen metabolism (**Figures 3**, **4**).

Phosphoenolpyruvate carboxylase (PEPC) is a key enzyme of both Krebs cycle acid and nitrogen metabolism, and it is a component of the predominant pathway used in the synthesis of malate in most plant tissues. PEPC catalyzes the carboxylation of

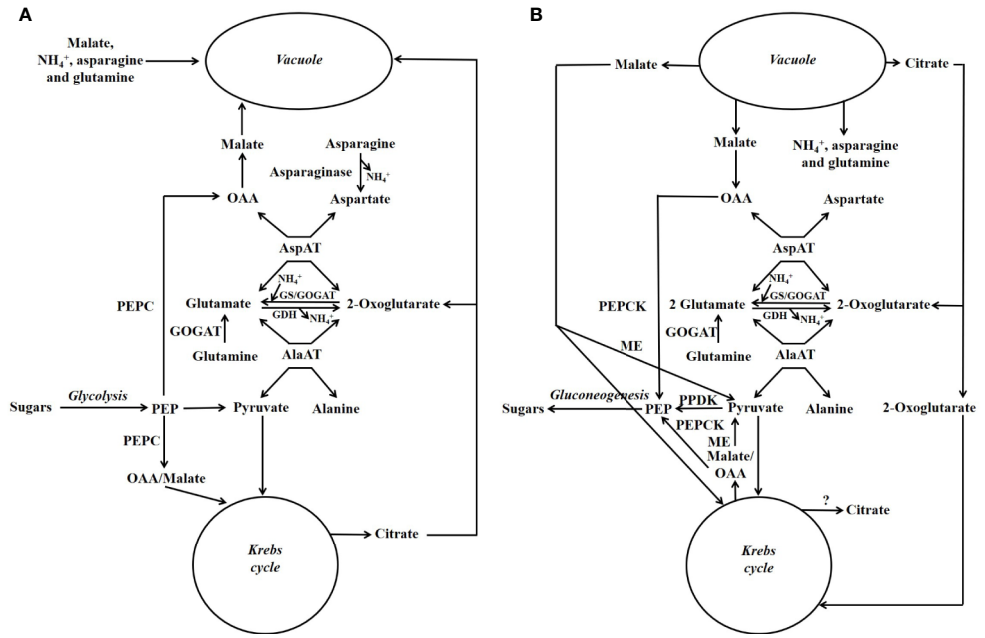

FIGURE 4 | Simplified scheme outlining the major pathways involved in the metabolism of nitrogenous compounds and malic and citric acids in fruits and seeds of stone fruits (based on Walker et al., 2018). Unlike in **Figure 3**, the temporal organization of metabolism is taken into account. **(A)** Storage phase. **(B)** Utilization phase. GDH = glutamate dehydrogenase; GOGAT = glutamate synthase also known as glutamine oxoglutarate aminotransferase; GS = glutamine synthetase; ME = malic enzyme; OAA = oxalacetate; PEP = phosphoenolpyruvate; PEPC = phosphoenolpyruvate carboxylase; PEPCK = phosphoenolpyruvate carboxykinase; PPDK = pyruvate orthophosphate dikinase.

phosphoenolpyruvate to oxaloacetate, whereas phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the reverse reaction (Leegood and Walker, 2003). There is evidence that in sweet cherry phosphorylation of both these enzymes contributes to the coordinate regulation of PEPC and PEPCK and hence flux between PEP and OAA (Walker et al., 2016). Similarly, in peach flesh, it is possible that phosphorylation contributes to the regulation of PEPC activity (Moing et al., 2000). In addition, in peach flesh (unlike both cherry and plum flesh) PEPC is present as a polypeptide doublet, and this raises the possibility that PEPC ubiquitination also contributes to its regulation (Famiani et al., 2016).

In plants, two pathways can be used in the conversion of Krebs cycle acids and the carbon skeletons of many amino acids/amides to sugars, and one utilizes PEPCK, whereas the other utilizes pyruvate orthophosphate dikinase (PPDK) (Leegood and Walker, 2003). In the flesh of peach, both PEPCK and PPDK are present (Walker and Chen, 2002; Borsani et al., 2009; Lara et al., 2009; Lara et al., 2010; Famiani et al., 2012; Famiani et al., 2016). However, it would appear that PEPCK is many times more abundant than PPDK in both peach flesh and that of other stone fruits, and this implies that the bulk of any gluconeogenic flux utilizes the PEPCK pathway (Famiani et al., 2016). NADP-malic enzyme, which converts malate to pyruvate, is also present in the flesh of peach, plum, and cherry (Borsani et al., 2009; Walker et al., 2011b; Famiani et al., 2012; Famiani et al., 2016). PPDK, which converts pyruvate to PEP, is required to convert pyruvate produced by NADP-ME to sugars by gluconeogenesis. However, the low abundance of PPDK in the flesh of stone fruits implies that the bulk of pyruvate produced by NADP-ME is used by processes other than gluconeogenesis such as the Krebs cycle (Famiani et al., 2016).

In peach, a recent study found that both metabolism and storage in the vacuole contributed to the amount of malate that was accumulated, while metabolism was crucial for citrate accumulation. In particular, low-acid cultivars showed higher citrate degradation and less transport of malate into the vacuole, and this was due to up- and down-regulation of a GABA (γ-aminobutyric acid) pathway gene and a malate transporter gene, respectively (Zheng et al., 2021).

For a detailed description of pathways regarding malate and citrate synthesis and breakdown refer to Famiani et al. (2015) and Walker and Famiani (2018). In the flesh of sweet cherry, Japanese plum and peach, cytosolic GS, cytosolic AspAT, GDH, asparaginase, PEPC and PEPCK are present throughout development. Similarly, at the time when there is an extensive metabolism of nitrogenous compounds, these enzymes are also abundant in the developing seed and endocarp of sweet cherry and Japanese plum (Moing et al., 2000; Lombardo et al., 2011; Walker et al., 2011a; Famiani et al., 2012; Rodriguez et al., 2019).

FUNCTIONS OF KREBS CYCLE ACIDS

In addition to a direct role in metabolism, the Krebs cycle acids also have other functions. In the flesh of stone fruits, the ratio of

organic acids to sugars is much higher in unripe as compared to ripe fruits (**Table 1**). This high ratio is thought to contribute to making the flesh less palatable, and this protects the developing fruits and seeds from animals (Walker and Famiani, 2018). The Krebs cycle acids are also important in turgor regulation. In tomato flesh, malate is an important osmoticum and is thought to play a key role in generating turgor pressure to drive cell expansion (Guillet et al., 2002; Beauvoit et al., 2014). At certain stages of the development of the flesh of some stone fruit species such as apricot, malic and citric acids can be more abundant than sugars (Baldicchi et al., 2015), and clearly, these acids are a major osmoticum. The sugar content of Japanese apricot flesh is quite low throughout development and citric acid is more abundant (Otoguro and Kaneko, 1994); hence, citric acid can be a predominant osmoticum even in ripe fruit. In carrot tap root, the relative contributions of organic acids and sugars for generating cell turgor pressure vary according to the position in the root (Korolev et al., 2000) and, similarly, both sugars and malate can contribute to turgor pressure changes in stomata that bring about stomatal movement (Talbott and Zeiger, 1996). Thus, it is clear that either organic acids or sugars can be the major osmoticum used to generate cell turgor, and which is used depends on a number of factors that include species, cell type, and stage of development.

ENDOCARP METABOLISM

During stage I of development, the endocarp increases greatly in size, and then, during stage II, the endocarp hardens to form the stone. There are marked changes in the structure of the parenchyma cells of the endocarp during development (Masia et al., 1992). At both 1 and 4 weeks after full bloom, these cells contained vacuolar inclusions of a phenolic nature. These inclusions were absent in the mesocarp, and it was hypothesized that they might contain precursors used in lignin synthesis. These inclusions disappeared by the middle of stage II (8 weeks after full bloom), and at this time, the cell walls were lignified and contained numerous simple pits containing cytoplasmic channels (Masia et al., 1992).

The endocarp of Japanese plum during stage I contained about 7 mg g^{-1} FW each of sucrose, glucose, and fructose. During stage II, the contents of glucose and fructose decreased and during stage III, the values of these sugars, together with that of sucrose, increased to 3–10 mg g^{-1} FW (Famiani et al., 2012). The sucrose content of cherry endocarp is low throughout development, and the contents of glucose and fructose are in the range 2–14 mg g^{-1} FW and show a similar pattern of changes during development to that observed in Japanese plum endocarp (Walker et al., 2011a; Famiani et al., 2012). In both Japanese plum and sweet cherry, the decrease in soluble sugar contents during stage II occurred at the same time as the massive rate of increase in endocarp DW, and the increase in soluble sugar contents during stage III occurred when this rate of DW increase slowed, altough some of this latter increase could also result from contamination of endocarp by the flesh/juice which, at this time, contain large amounts of soluble sugars (Walker et al., 2011a; Famiani et al., 2012). In peach endocarp during stage I, sucrose, sorbitol, and starch contents were <1.3 mg g^{-1} FW and glucose/fructose contents were 7–18 mg g^{-1} FW (Lo Bianco and Rieger, 2002). In peach endocarp during stage I, the activity (μmol g^{-1} FW h^{-1}) of sucrose synthase (SuSy) was 1.0, acid invertase was 6.5 and alkaline/neutral invertase was 0.5 (Lo Bianco and Rieger, 2002). Either invertase or Susy is required for the breakdown of sucrose in most plant tissues (Kingston-Smith et al., 1999). Hu et al. (2011) reported that a number of enzymes, including several involved in the glycolytic pathway, decreased in peach endocarp after 28 DAB; however, this decrease could be misleading because results were expressed on a per DW basis, and there is a massive increase in endocarp DW arising from lignification.

There is a massive and transient accumulation of vegetative storage proteins in the endocarp of both sweet cherry and Japanese plum. Enzymes involved in the metabolism of nitrogenous compounds (e.g., GS, cytosolic AspAT, and PEPC) are abundant at the time when these storage proteins are accumulated and subsequently mobilized. The accumulation of these storage proteins is likely associated with the storage of excess imported nitrogenous compounds; because the endocarp requires a very large amount of sugars and not nitrogenous compounds to fuel lignin synthesis (Walker et al., 2011a; Famiani et al., 2012). In stone fruits, export of nitrogenous material from the endocarp occurs; thus, in both cherry and plum, vegetative storage proteins are accumulated at early stages of endocarp development, and subsequently decline to almost undetectable amounts (Walker et al., 2011a; Famiani et al., 2012). This decline coincided with storage protein accumulation in the seed (Walker et al., 2011a; Famiani et al., 2012). In addition, the total N content of the endocarp of plum declines greatly as the fruit matures, and it can be calculated that this decline in the content of nitrogenous material is equivalent to about 35% of the nitrogenous compounds accumulated in the seed (Donen, 1939; Famiani et al., 2012). In Japanese apricot, asparagine accounted for about 60–80% of the soluble nitrogenous compounds of the endocarp throughout development. Asparagine (mg g^{-1} FW) content changed during development: 5.0 in stage I, 3.3 in stage II, and 1.3 in stage III (ripeness) in 1982 season, and 3.2 in stage I, 1.7 in stage II, and 0.8 in stage III (ripeness) in 1983 season (Kakiuchi et al., 1985). In the endocarp, nitrogen metabolism is also associated with lignin synthesis. This is because massive amounts of ammonium are released during this process by the action of phenylalanine ammonium lyase (Lea, 1993). The presence of enzymes involved in nitrogen metabolism in endocarp is also likely related to the reassimilation of this ammonium. The occurrence of PEPCK in the developing endocarp (Walker et al., 2011a; Famiani et al., 2012; Walker et al., 2018), raises the possibility that (as described in **Figure 4**) ammonium could accumulate in the vacuole and then, sometime later, could be released and re-assimilated.

SEED METABOLISM

The principal tissues of the seeds of stone fruits are the seed coat (integuments), nucellus, endosperm, and embryo. The whole seed, integuments, and nucellus reach their final size during stage I of development. During the early part of stage II, the endosperm grows and largely replaces the nucellus, while later in stage II, the embryo grows and replaces much of the endosperm (Marshall, 1954). The growth of the different parts of the seed follows a sigmoidal pattern; however, the growth of the whole seed is double-sigmoidal, and this is because the growth of the different parts is asynchronous (Chalmers and van den Ende, 1975; Bassi and Ryugo, 1990). During development, the nucellus and endosperm serve as temporary stores for small amounts of imported assimilates (Hawker and Buttrose, 1980; Bassi and Ryugo, 1990). Most of the storage reserves in the mature seeds are located in the embryo, and the bulk of these are only deposited after the embryo approaches its maximum size (Lilleland and Newsome, 1934; Tukey and Lee, 1937; Hawker and Buttrose, 1980; Walker et al., 2011a; Baldicchi et al., 2015). The storage reserves in the mature embryo consist largely of lipids (c300-420 mg g^{-1} FW), storage proteins (c120 mg g^{-1} FW) and smaller amounts of soluble sugars (20–70 mg g^{-1} FW). Very low amounts of starch are present in the mature seed (Tukey and Lee, 1937; Hawker and Buttrose, 1980; Bassi and Ryugo, 1990; Lo Bianco and Rieger, 2002; Walker et al., 2011a; Famiani et al., 2012).

The seed coat of stone fruits possesses a well-developed vasculature, and it is likely that imported material is distributed throughout the seed coat in the phloem and then unloaded and transported to the developing storage tissues (Hawker and Buttrose, 1980). Similarly, in some other seeds, the seed coat plays a key role in the distribution of imported materials (Walker et al., 1999; Patrick and Offler, 2001). In these seeds, it appears that water and assimilates exit the phloem and enter the parenchyma cells of the seed coat by bulk flow through plasmodesmata, which is driven by the high hydrostatic pressure of the phloem (Patrick and Offler, 2001). However, recent studies have indicated that the hydrostatic pressure in the phloem in unloading zones could be much lower than previously thought (Ross-Elliott et al., 2017; Milne et al., 2018). In the seed coat of grape, cell wall invertase is abundant in the palisade layer, a tissue which is thought to transport imported nutrients to the developing storage tissue, and whose cells are connected by numerous plasmodesmata (Walker et al., 1999; Famiani et al., 2000). In this tissue, cell wall invertase could contribute to the regulation of the turgor pressure of the parenchyma cells by increasing the solute concentration in the apoplast, and thus may function in contributing to maintaining a decreasing turgor pressure gradient between the phloem and sink cells that allows a symplastic flow. The seeds of stone fruits approach their maximum FW and size before the bulk of their storage materials are accumulated (Lilleland and Newsome, 1934; Tukey and Lee, 1937; Hawker and Buttrose, 1980; Walker et al., 2011a; Famiani et al., 2012; Baldicchi et al., 2015). Thus, water and large amounts of assimilates must be imported into the seed after it approaches its maximum size. It is possible to estimate the amount of water required for the import of the assimilates necessary for the synthesis of these storage materials. This can be done by comparing the amount of dry matter accumulated in the seed with the dry matter content of the phloem. Thus, in the case of one almond seed about 1,200-mg dry matter are accumulated (Hawker and Buttrose, 1980). For peach and sweet cherry the dry matter content of the phloem sap is about 160–180 mg dry matter ml^{-1} (Morandi et al., 2007; Brüggenwirth et al., 2016). This means that about 8 ml of water is needed to import this material, and this excess water must be removed from the seed. In the case of one apricot seed about 600-mg dry matter are accumulated (Baldicchi et al., 2015) and, using the same figure for the concentration of assimilates in the phloem, this would require about 4 ml of water. In apricot, the FW of seed coat about midway through the period of storage deposition in the embryo is about 0.1 g per seed, and the bulk of the storage reserves are deposited over a period of 50 days. Thus, if 4 ml of water pass through the seed coat during these 50 days, then on average at least 0.08 ml of water passes through the coat each day, and this is similar to the FW of the coat (Baldicchi et al., 2015). The most likely fate of this excess water is that, as in seeds in which it has been studied, it is exported in the xylem (Pate et al., 1985; Patrick and Offler, 2001; Milne et al., 2018). Thus, water enters the apoplast of the seed coat (this is not in apoplastic contact with the developing storage tissues) *via* aquaporin water channels located in the plasma membrane of the parenchyma cells and then enters the xylem which exports the water out of the seed (Patrick and Offler, 2001). Various transporters are involved in the transport of assimilates across the plasma membrane of the parenchyma cells of the seed coat into the apoplast that is in contact with the developing storage tissues. Assimilates are then taken up into the storage tissues and various transporters are involved (Milne et al., 2018). Almost nothing is known about the occurrence and localization of these different transporters and channels in the seeds of stone fruits.

The seed coats of both cherry and pea play a role in the interconversion of imported nitrogenous compounds, and one function of these interconversions could be to provide nitrogenous compounds whose subsequent metabolism in the storage tissues produces lower amounts of CO_2, consumes less O_2, and does not lead to pH perturbations (Delgado-Alvarado et al., 2007; Walker et al., 2011a). Storage proteins are also accumulated in the seed coat of cherry (Walker et al., 2011a). Enzymes involved in nitrogen metabolism (e.g., PEPCK, PEPC, cytosolic GS, and cytosolic AspAT) are present in cherry seed coat and their abundance g^{-1} FW is highest when both storage proteins are accumulating in the seed coat and a large transfer of nitrogenous compounds to the developing storage tissues is occurring (Walker et al., 2011a). Thus, in the seed coats of stone fruits, it is likely that an extensive metabolism of imported nitrogenous materials occurs.

In the whole seed of various stone fruits, storage proteins are accumulated rapidly during stage II and the beginning of stage III, and a small number of abundant polypeptides account for the bulk of these (Hawker and Buttrose, 1980; Bassi and Ryugo, 1990; Walker et al., 2011a; Famiani et al., 2012). These proteins

were accumulated at the same time that proteins were mobilised from the endocarp (Walker et al., 2011a; Famiani et al., 2012). This raises the possibility that proteins stored in the endocarp are translocated to the seed during the hardening of the stone and beginning of stage III, when it accumulates storage proteins (Walker et al., 2011a; Famiani et al., 2012). In black cherry (*Prunus serotina*) seeds, several of these proteins appear to be enzymes (such as amygdalin hydrolase) that have been recruited into the role of seed storage proteins (Zheng and Poulton, 1995). In Japanese apricot, asparagine accounted for about 60–80% of the soluble nitrogenous compounds of the whole seed during stage I, and this percentage decreased to around 35% in ripe fruit. Asparagine (mg g^{-1} FW) content of whole seeds was 6.9 in stage I, 7.5 in stage II, and 1.3 in stage III (ripeness) in 1982 season, and 8.1 in stage I, 4.4 in stage II, and 0.8 in stage III (ripeness) in 1983 season (Kakiuchi et al., 1985). In both the embryo of cherry and whole seed of plum, there is also a large increase in the abundance g^{-1} FW of enzymes involved in nitrogen metabolism (e.g., PEPCK, PEPC, cytosolic GS, and cytosolic AspAT) when storage proteins are being deposited, and this abundance decreases once this accumulation nears completion (Walker et al., 2011a; Famiani et al., 2012). In peach embryos, the bulk of the lipid content is also accumulated during stage III, however, this accumulation continues after that of protein is essentially complete (Bassi and Ryugo, 1990; Walker et al., 2011a; Famiani et al., 2012). Clearly, there must be a massive glycolytic flux from imported sugars to provide pyruvate for this fatty acid synthesis. The seeds of developing stone fruit are white in color, and at least in the case of cherry and plum, do not contain chlorophyll (Walker et al., 2011a). Nevertheless, Rubisco is present and the pattern of changes in its abundance during development mirrors that of enzymes such as GS and PEPC and also that of storage protein accumulation (Walker et al., 2011a; Famiani et al., 2012). Further, it was estimated that the potential amount of activity of Rubisco could be similar to that of some other enzymes involved in storage material deposition (Walker et al., 2011a; Famiani et al., 2012). It has been suggested that Rubisco might function in removing CO_2 from the tissue and hence reducing its potentially toxic effects (Walker et al., 2011a; Famiani et al., 2012).

In whole seeds of cherry, the contents of malate, glucose, and fructose were similar and around 25 μmol g^{-1} FW when seed FW was rapidly increasing. When seed FW approached its maximum value, the malate content declined to about half this value, whereas, the contents of glucose and fructose increased to 120–160 μmol g^{-1} FW (Walker et al., 2011a). In whole plum seed, each of the content of malate, glucose, and fructose was about 40 μmol g^{-1} FW when seed FW was increasing; and malate content declined when seed FW attained its maximum value (Famiani et al., 2012). In both cherry and plum seeds. sucrose content is much lower than that of glucose and fructose when the seed FW is increasing. These results are consistent with malate, glucose, and fructose contributing to the osmotic potential of the cells in the seeds in order to drive their expansion.

In whole mature seeds of both Japanese plum and sweet cherry the amount of soluble sugars g^{-1} FW is approximately 30% of the amount g^{-1} FW present in the ripe flesh of these fruits (Walker et al., 2011a; Walker et al., 2011b; Famiani et al., 2012). In whole mature cherry seeds, both glucose and fructose are much more abundant than sucrose, whereas, in both mature whole peach and plum seeds sucrose is more abundant than glucose and fructose (Lo Bianco and Rieger, 2002; Walker et al., 2011a; Famiani et al., 2012). In both cherry and Japanese plum seeds, the content of total soluble sugars increases greatly during stage III of development. In cherry seed, sucrose content is low throughout development, whereas in Japanese plum seed, glucose and fructose contents are higher than that of sucrose early in development and sucrose became the most abundant sugar as the seed matures (no data were presented regarding sorbitol contents) (Walker et al., 2011a; Famiani et al., 2012). There are differences in the soluble sugar contents of the embryos of different cultivars of peach. In one cultivar there was a large accumulation of sucrose as the seed matured and its final content was about 12 mg g^{-1} FW, and the contents of sorbitol and hexoses remained low. By contrast, in the embryo of a second cultivar sucrose, glucose and sorbitol were accumulated to final values of 7–15 mg g^{-1} FW (Bassi and Ryugo, 1990).

In whole peach seeds, SuSy activity for stages I, II and III was 1.5, 15.2 and 9.9 μmol g^{-1} FW h^{-1}, respectively (Lo Bianco and Rieger, 2002). The values for soluble acid invertase activity at these stages of development were 0.8, 5.2, and 2.7 μmol g^{-1} FW h^{-1}, respectively (Lo Bianco and Rieger, 2002). The values for alkaline/neutral invertase activity at these stages of development were 0, 0.8, and 0.5 μmol g^{-1} FW h^{-1}, respectively (Lo Bianco and Rieger, 2002). In whole peach seeds at stage III, Morandi et al. (2008) reported an activity of 2 μmol g^{-1} FW h^{-1} for acid invertase, a similar amount of alkaline/neutral invertase activity and an activity of 23-31 μmol g^{-1} FW h^{-1} for SuSY. In the developing seeds and some other storage tissues of some of other plants, it has been suggested that under conditions of low O_2 status, SuSy is often utilized in catabolizing sucrose in order to provide precursors that are used in the synthesis of storage products (Sturm and Tang, 1999; Morandi et al., 2008; Falchi et al., 2013; Stein and Granot, 2019). In whole peach seeds, the values for NAD-SDH activity at stages I-III were 0.2, 11.2, and 6.5 μmol g^{-1} FW h^{-1}, respectively, and the activities of sorbitol oxidase (SOX) at the corresponding stages were 0, 0, and 0.8 μmol g^{-1} FW h^{-1} (Lo Bianco and Rieger, 2002). Very different values were reported in whole peach seeds at stage III by Morandi et al. (2008), where SOX activity was 28–40 μmol g^{-1} FW $h^{-1}$and sorbitol dehydrogenase (SDH) 0.18–0.23 μmol g^{-1} FW h^{-1}. Clearly, to understand further the functions of enzymes involved in sugar metabolism in stone fruit seeds, their abundance and locations in the different tissues of the seeds needs to be determined.

CONCLUSIONS AND FUTURE PERSPECTIVES

Our understanding of the structure, growth and nitrogen and organic acid metabolism of stone fruits is far from rudimentary, and this has allowed an outline of these subjects to be presented in this review. Nevertheless, there are gaps in our detailed

understanding of these topics that should be addressed. For example, there is little information available regarding the regulation and localization of enzymes in different tissues and in their different cell types. Further, there is little or no information available regarding certain aspects of the functioning of the vasculature. For instance, how is it possible to supply the different parts of the fruit/seed with a different ratio of sugars to nitrogenous compounds? How are nitrogenous compounds exported from endocarp, and how are these then distributed to other parts of the fruit and seed? Finally, there is the need to further understand the potential process of storage and subsequent release of malate/citrate from the vacuole and its interaction with nitrogen metabolism.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version. However, FF and RW had a major role in the design and writing of the article.

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REVIEW
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Non-structural Carbohydrate Metabolism in the Flesh of Stone Fruits of the Genus *Prunus* (Rosaceae) – A Review

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Non-structural carbohydrates are abundant constituents of the ripe flesh of all stone fruits. The bulk of their content comprises sucrose, glucose, fructose and sorbitol. However, the abundance of each of these carbohydrates in the flesh differs between species, and also with its stage of development. In this article the import, subcellular compartmentation, contents, metabolism and functions of non-structural carbohydrates in the flesh of commercially cultivated stone fruits of the family *Rosaceae* are reviewed.

Keywords: cell turgor regulation, fructans, invertases, primary metabolism, subcellular compartmentation, sugars, SPS, SuSy

INTRODUCTION

The term stone fruits refers to a number of species that are trees belonging to the genus *Prunus* of the rose family (*Rosaceae)*, that are characterized by fruits which possess a lignified endocarp called the stone or pit. These fruits are classified as drupes and are composed of a thin epicarp (skin), a fleshy mesocarp (flesh) and a woody/lignified endocarp which encloses the seed (Romani and Jennings, 1971). The commercially most important stone fruits are: plums (several species), among which the most important are the European plums (*P. domestica*) and the Asian or Japanese plums (*P. salicina*), sweet cherry (*P. avium*) and sour cherry (*P. cerasus*) (and hybrids between them), peach and nectarine (*P. persica*), apricot (*P. armeniaca*) and almond (*P. dulcis*). The rose family also includes other commercially important fruit trees and the main group is the pome fruits (subfamily *Pomoideae*), that have not been specifically considered in the present review. The term pome fruits refers to the fruit derived by the fusion of the ovary and receptacle (from which derives the flesh), and thus this is botanically a false fruit. Pome fruits include apple (*Malus domestica*), pear (*Pyrus communis*), quince (*Cydonia oblonga*), loquat (*Eriobotrya japonica*), medlar (*Mespilus germanica*), rowan (*Sorbus* spp.), and some other minor species.

The growth pattern, of either the whole fruit or the flesh of stone fruits, can usually be described as a double-sigmoidal curve, where three phases of growth are generally recognized: a first period of rapid growth defined as stage I, a second period with reduced growth depicted as stage II and a third period, characterized by a further rapid growth, defined as stage III (Lilleland, 1933; Pavel and DeJong, 1993b; Zuzunaga et al., 2001). The only exception is almond in which the flesh does not expand during stage III (Hawker and Buttrose, 1980). During stage I, all the components of the pericarp (epicarp, mesocarp and endocarp) increase greatly in size, and both the endocarp and the seed approach their maximum size. During stage II, the increase in the size of the flesh slows down and the endocarp, whose cells develop into sclerenchyma, hardens to form the stone. During stage III, there is a large increase in both the weight and volume of the fruit, arising from the expansion of the parenchyma cells of the flesh and skin, and it ripens. During ripening, the edible parts soften, change color and accumulate soluble sugars (Brady, 1993; Zuzunaga et al., 2001). In peach, some studies have subdivided stage I into stages Ia and Ib, and similarly stage III has been subdivided into stages III and IV (Chalmers and van den Ende, 1975; Scorza et al., 1991; Zanchin et al., 1994; Tonutti et al., 1997). However, because all stone fruit species are considered in the present review, we have used the traditional division into three stages.

A large number of different carbohydrates can be present in the flesh of stone fruits (Cirilli et al., 2016); however, in this article only abundant non-structural carbohydrates are considered, and their contents, metabolism and functions are reviewed. The bulk of the non-structural carbohydrates present in the flesh of all stone fruits at all stages of their development consists of one or more of the soluble sugars sucrose, glucose and fructose, the sugar alcohol sorbitol (henceforth referred to as a soluble sugar) and very small amounts of starch. The metabolism of these non-structural carbohydrates is linked by the sucrose cycle which, together with proteins that transport sugars across membranes, plays a pivotal role in determining the contents of these sugars in the different compartments of the cell. This cycle allows sugar utilization and accumulation to be coordinated and also plays a key role in maintaining the osmotic potential and turgor of different subcellular compartments (Li et al., 2012, 2016, 2018). A simplified scheme depicting the enzymes involved in the sucrose cycle and allied reactions is shown in **Figure 1**. Soluble sugars usually account for 70–90% of the dry weight of the ripe flesh and skin of commercially cultivated stone fruits (Moing et al., 1998; Baldicchi et al., 2015). The soluble solids content (SSC or °Brix) of the flesh and skin generally accounts for 9-22% of the fresh weight (Marshall, 1954; Moing et al., 1998; Famiani et al., 2012; Zanon et al., 2015; Baldicchi et al., 2015; Cirilli et al., 2016). A large percentage of the SSC usually consists of sugars, and in the ripe flesh of both peach (*P. persica*) and sweet cherry (*P. avium*) the percentage is typically 65-85% (Brady, 1993; Winkler and Knoche, 2018). However, there are exceptions, and in Japanese apricot (*P. mume*) this percentage is only about 16% (Otoguro and Kaneko, 1994). For most stone fruits, the content of sugars is a major determinant of the taste of the flesh and skin, and a high content is a major factor in determining the quality of the crop (Desnoues et al., 2014; Cirilli et al., 2016).

SORBITOL AND SUCROSE ACCOUNT FOR THE BULK OF THE SUGARS IMPORTED INTO STONE FRUIT FLESH

The bulk of the sugars required for stone fruit growth and development are imported into them, and only a very small amount is produced by the fruits' own photosynthesis. These sugars largely arise from photosynthesis in the leaves during the same growing season that the fruit develops, although, during early fruit growth, some sugars arise from carbohydrates stored in the roots and crown during the previous season (Loescher et al., 1990; Pavel and DeJong, 1993a). Sucrose and sorbitol account for the bulk of sugars synthesized in peach leaves, and although sorbitol content is usually higher, the ratio of the contents of these two sugars is dependent on factors such as cultivar, rate of photosynthesis and age of the leaf (Escobar-Gutiérrez and Gaudillére, 1994; Moing et al., 1997; Hartman et al., 2017). Both sucrose and sorbitol are synthesized in the cytosol of mesophyll cells from either triose phosphate or the products of starch degradation (i.e., glucose and maltose) that are exported from the chloroplast. The triose phosphate and starch are produced by photosynthesis within the chloroplast (Cho et al., 2011). In the cytosol of mesophyll cells, a large proportion of triose phosphate, maltose and glucose are then converted to glucose-6-phosphate and fructose-6-phosphate and then used in both sucrose and sorbitol synthesis. Glucose-6-phosphate is used as a precursor for sorbitol synthesis (**Figure 1**). Aldose-6-phosphate reductase (also known as sorbitol-6-phosphate dehydrogenase) is a key regulatory enzyme utilized in sorbitol synthesis in leaves of Rosaceous plants (**Figure 1**; Hirai, 1981; Hartman et al., 2017; Shen C. et al., 2018). Sucrose synthesis in leaves predominantly utilizes the sucrose phosphate synthase (SPS) and not sucrose synthase (SuSy) pathway, and the SPS pathway uses as precursors both glucose-6-phosphate and fructose-6-phosphate (**Figure 1**). Sorbitol and sucrose then move to the phloem and enter it (phloem loading). It is a matter of debate as to the relative contributions of apoplastic (sugars enter apoplast before entering the phloem) and symplastic (sugars do not enter apoplast before entering the phloem) phloem loading in Rosaceous fruit trees such as peach and apple (Moing et al., 1997; Watari et al., 2004; Nadwodnik and Lohaus, 2008). Apoplastic loading in Rosaceous fruit trees, as in plants in which the process it has been studied in more detail, is likely to utilize Sugars Will Eventually be Exported Transporter (SWEETs: sucrose and hexose facilitator transporters) for the movement of at least sucrose into the apoplast, and sucrose transporters (SUC's/SUTs: sucrose/H+ symporters) and sorbitol transporters for their uptake into the phloem (Watari et al., 2004; Jeena et al., 2019). Sorbitol transporters are encoded by a number of genes in Rosaceous fruit trees and the expression of each of these genes is often dependent on the tissue, stage of development and other factors

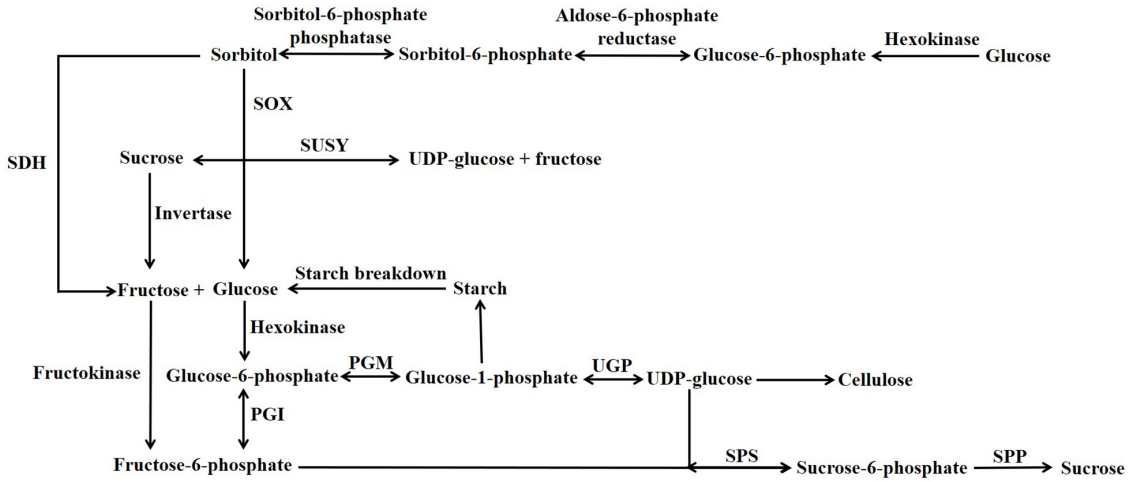

FIGURE 1 | Simplified scheme illustrating sucrose and sorbitol degradation and synthesis. PGI, Phosphoglucose Isomerase; PGM, Phosphoglucomutase; SDH, Sorbitol Dehydrogenase; SOX, Sorbitol Oxidase; SPP, Sucrose phosphate phosphatase; SPS, Sucrose Phosphate Synthase; SUSY, Sucrose Synthase; UGP, UDP-Glucose Pyrophosphorylase.

(Gao et al., 2003; Li et al., 2015, 2018; Shen C. et al., 2018). Compared to sucrose transporters the role of these sorbitol transporters in phloem loading is less understood (Li et al., 2015; Shen C. et al., 2018). Whatever, the loading mechanism used, sugars then move to sink tissues by bulk flow through the phloem, and this is driven by a pressure gradient that is largely produced by an inflow of water into the phloem of source tissues by osmosis (Voitsekhovskaja et al., 2006). In the fruit sugars can then travel from the phloem to sink cells by either an intracellular (symplastic) route via plasmodesmata, an extracellular route (apoplastic) or a combination of both routes (Grappadelli et al., 2019). Both sucrose and sorbitol are transported in the phloem of stone fruits. The aphid stylet technique revealed the ratio of sorbitol to sucrose to be 2.8–4.5 in the phloem of both the leaf and shoot apex of peach (Moing et al., 1997; Nadwodnik and Lohaus, 2008). In total extracts of different organs of peach the ratio of sorbitol to sucrose differs, and this ratio is highest in the leaf lamina (about 2–3), medium in the fruit stalk (pedicel) (about 1.3–2) and lowest in the ripe fruit (about 0.03–0.2 in the flesh) (**Table 1**; Nii et al., 1994; Lo Bianco, 2009). In ripe peach flesh, more than 70% of soluble sugars is usually sucrose, and sorbitol content is commonly less than 15% (Moing et al., 1998). Why then is the sorbitol content of ripe peach flesh much less than that of sucrose? One potential explanation is that sucrose is the main sugar accumulated to make the fruit palatable/edible during ripening. Another explanation, not in contrast and potentially consequent to the first one, is that sorbitol and not sucrose is the main substrate utilized by metabolism (Desnoues et al., 2014). However, even if equal amounts of sorbitol and sucrose were imported into the flesh, the former would exceed the metabolic demands of the tissue. Thus, metabolic substrate in ripening peach flesh is required for both the synthesis of various components of the flesh and as a source for respiration (Famiani et al., 2016). However, most of the increase in the dry weight of peach flesh during stage III of development is the result of the increase in soluble sugar content, and in the flesh of fruits such as grape during the stage of development equivalent to stage III only about 9–14% of sugars present in the flesh are used in respiration (Famiani et al., 2014). An alternative explanation to why sorbitol content is low is that it is in part converted to sucrose, and there is evidence that this occurs. Firstly, feeding radiolabelled sorbitol to detached prune fruits (a European plum cultivar, i.e., *P. domestica*) via the pedicel resulted in 25–50% of the radiolabel in the flesh being present in sucrose. By contrast, when radiolabelled sucrose was fed to the fruits only 1.6% of the radiolabel was present in sorbitol (Hansen and Ryugo, 1979). Of course, in this latter study this does not preclude that there is a conversion of sorbitol to sucrose during its transfer from the pedicel to the parenchyma cells of the flesh. Secondly, key enzymes required for the synthesis of sucrose from sorbitol such as SPS, SuSy and/or sorbitol dehydrogenase are present in ripening peach flesh (Yamaki, 2010). This raises the question of which factors are important in determining the sorbitol content of the flesh. In contrast to peach flesh, the sorbitol content of cherry flesh can be quite high (**Table 1**), and it has been suggested that this is because imported sorbitol is little metabolized (Gao et al., 2003). In addition, in the flesh of some fruits such as pear and loquat, there is evidence that sorbitol can be re-synthesized; however, Desnoues et al. (2018) reported that this may not be the case in peach. Nevertheless, the potential re-synthesis of sorbitol in the flesh of stone fruits requires detailed investigation. In general, it seems that sorbitol is low in stone fruits which accumulate sucrose and tends to be higher in those that accumulate glucose/fructose (**Table 1**). On balance, it is not implausible that both imported sucrose and sorbitol are both quantitatively important metabolic substrates for stone fruit flesh, and that their relative contributions could depend on several factors such as species and developmental stage.

TABLE 1 | Typical contents of soluble sugars (mg g^{-1} FW) in the flesh of unripe and ripe stone fruits.

	Sorbitol	Sucrose	Fructose	Glucose	
Stage II – Unripe fruits					
Apricot (common)	0.6	2	1	3	Baldicchi et al., 2015
Apricot (Japanese)	2.3	1.3	1.1	2.0	Otoguro and Kaneko, 1994
Cherry (sweet)		<1	9	23	Walker et al., 2011
Peach	3	3	13	14	Moriguchi et al., 1990
Plum (Japanese)	3	7	11	14	Famiani et al., 2012
					Donen, 1939
Stage III – Ripe fruits					
Apricot (common)	2.8	65	6	18	Baldicchi et al., 2015
Apricot (Japanese)	1.7	9.0	0.9	0.5	Otoguro and Kaneko, 1994
Cherry (sweet)	40	<1	65	75	Walker et al., 2011
					Ballistreri et al., 2013
	14	1.5	71	78	Winkler and Knoche, 2018
Cherry (sour)	16	4.2	43	52	Winkler and Knoche, 2018
Cherry (Morello) Line 16	43	low	60	68	Proietti et al., 2019
Cherry (Morello) Line 37	14	low	17	24	Proietti et al., 2019
Peach Hakuto	8	48	9	7	Moriguchi et al., 1990
Peach Pamirskii	9.3	115	11	6	Moing et al., 2003
Nectarine Summergrand	2.7	82	16	11	Moing et al., 2003
Plum (Japanese)	25	92	21	27	Famiani et al., 2012
					Donen, 1939
Prunus davidiana	0.3	10	3.8	2.7	Moing et al., 2003

CONTENT OF NON-STRUCTURAL CARBOHYDRATES IN THE FLESH OF STONE FRUITS DURING DEVELOPMENT

In the ripe flesh and skin of all stone fruits differing amounts of sucrose, glucose, fructose and sorbitol account for the bulk of the sugar content, and little or no starch is present (**Table 1**; Pavel and DeJong, 1993b; Moing et al., 1998; Desnoues et al., 2014, 2018; Baldicchi et al., 2015; Cirilli et al., 2016). However, the absolute amounts and relative proportions of each of these carbohydrates depends on the species of stone fruit and the cultivar, stage of development and growth conditions (Nonis et al., 2007; Morandi et al., 2008; Famiani et al., 2012; Desnoues et al., 2014; Baldicchi et al., 2015; Moscatello et al., 2019). In the ripe flesh of most, if not all, peaches the bulk of the soluble sugar content consists of sucrose, although smaller amounts of glucose, fructose and sorbitol are present. Further, there are differences among cultivars in the absolute amounts and relative proportions of these sugars (Moriguchi et al., 1990; Chapman and Horvat, 1990; Byrne et al., 1991; Chapman et al., 1991; Pavel and DeJong, 1993b; Vizzotto et al., 1996; Moing et al., 1998; Bae et al., 2014; Desnoues et al., 2014, 2018; Famiani et al., 2016; Cirilli et al., 2016). The amounts and proportions of sucrose, sorbitol, glucose and fructose present in the ripe flesh of most cultivars of common apricot (*P. armeniaca*) are similar to ripe peach flesh, however, differences have been detected between cultivars and tissues (i.e., peel and flesh) (Akin et al., 2008; Bae et al., 2014; Baldicchi et al., 2015; Xi et al., 2016). Nevertheless, peaches generally (but not always) contain more fructose than glucose, and the opposite has been observed in common apricot (**Table 1**; Cantín et al., 2009; Zanon et al., 2015; Baldicchi et al., 2015; Xi et al., 2016). The ripe flesh of both peaches and common apricots contains little starch (Pavel and DeJong, 1993b; Baldicchi et al., 2015). The soluble sugar content of the ripe flesh of Japanese apricots (*P. mume*) is low, and sucrose mostly accounts for this, although smaller amounts of glucose, fructose and sorbitol are present (**Table 1**; Otoguro and Kaneko, 1994). Glucose, fructose and sorbitol account for the bulk of the sugar content of the ripe flesh of all cherries studied; however, there is a variation in the proportions of these sugars between both species and cultivars (**Table 1**; Gao et al., 2003; Walker et al., 2011; Ballistreri et al., 2013; Winkler and Knoche, 2018; Proietti et al., 2019). The sucrose content of ripe flesh of different cherry species and their hybrids is usually very low, and generally less than 1% of the soluble sugar content (Walker et al., 2011; Ballistreri et al., 2013; Alrgei et al., 2016). However, certain genotypes can contain more, and one genotype contained around 4 mg g^{-1} FW (Alrgei et al., 2016). No starch was detected in the ripe flesh of either sweet cherry or sour cherry (*P. cerasus*) (Widdowson and McCance, 1935; Gao et al., 2003). Ripe plum flesh contains quite large amounts of sorbitol, sucrose, glucose and fructose (**Table 1**; Winkler and Knoche, 2018). However, there can be marked differences in the relative proportions of these between plum species and cultivars, and contents in the flesh and skin can be different (Donen, 1939; Forni et al., 1992; Nergiz and Yildiz, 1997; Singh et al., 2009; Famiani et al., 2012; Bae et al., 2014; Moscatello et al., 2019). No starch or very small amounts were detected in the ripe flesh of different plum species and several cultivars (*P. salicina*) (Widdowson and McCance, 1935; Donen, 1939;

Moscatello et al., 2019). In the flesh of young fruits of different species of plum a low amount of starch (< 5 mg^{-1} DW) was present (Moscatello et al., 2019).

The absolute and relative abundance of the different sugars in the flesh of all stone fruit changes during development (**Table 1**; Gao et al., 2003; Walker et al., 2011; Famiani et al., 2012; Bae et al., 2014; Cirilli et al., 2016; Lombardo et al., 2011). In general, the content of glucose plus fructose in the flesh of peaches, plums and apricots is much higher than sucrose content during stages I and II. Then during stage III large amounts of sucrose accumulate and this becomes the most abundant soluble carbohydrate (**Table 1**). In common apricot it has been noted that sucrose imported into the flesh before stage III is mostly metabolized to other compounds and during stage III this is not the case (Zhang et al., 2017), and the data of Baldicchi et al. (2015) are consistent with this. This is not the situation in Japanese apricot flesh because low amounts of sucrose and other soluble sugars are present during stage III, and there is a large accumulation of citric acid (Otoguro and Kaneko, 1994). In peach, during stage III large amounts of sucrose accumulate, and glucose and fructose, expressed as g^{-1} FW, usually decrease, but the content per fruit increases (Moriguchi et al., 1990; Pavel and DeJong, 1993b; Vizzotto et al., 1996; Lo Bianco et al., 1999; Famiani et al., 2016). Both sorbitol and starch contents (in g^{-1} FW) are low throughout development (Moriguchi et al., 1990; Pavel and DeJong, 1993b; Vizzotto et al., 1996). Sorbitol content g^{-1} FW has been found to either increase or decrease during stage III (Moriguchi et al., 1990; Vizzotto et al., 1996; Lo Bianco et al., 1999; Lombardo et al., 2011). In the flesh and skin of peach starch is localized in chloroplasts (Zanchin et al., 1994). Starch content increases to a maximum of about 3 mg g^{-1} FW during mid stage I and then decreases to about 0–0.3 mg g^{-1} FW in ripening flesh (Lo Bianco et al., 1999; Lo Bianco and Rieger, 2002; Rodriguez et al., 2019). This is consistent with the development of the plastids in peach parenchyma cells; four weeks after full bloom the plastids of the parenchyma cells contain few thylakoids and no starch granules, however, one week later numerous starch grains are present and are localized in the plastids of the peripheral parts of the flesh (Masia et al., 1992; Zanchin et al., 1994). The appearance of starch occurs before the full development of the thylakoids, and therefore some starch could be synthesized from imported sugars (Zanchin et al., 1994). By the end of stage II, the plastids of the peripheral part of the flesh (unlike the inner part) possess well developed thylakoids and numerous starch grains. Thirteen weeks after full bloom (middle of stage III), these starch grains are absent, chloroplasts had been converted to chromoplasts and large intercellular spaces were present (Masia et al., 1992). Similar results were obtained in 'Dixiland' peach fruit during development (Rodriguez et al., 2019). The pattern of changes in carbohydrates content in apricot flesh during development is broadly similar to those described for peach (Baldicchi et al., 2015; Xi et al., 2016). In both sweet cherry and sour cherry, glucose and fructose are much more abundant than sucrose throughout development. During stage III large amounts of glucose and fructose are accumulated, and in many cultivars sorbitol is also accumulated (**Table 1**) (Gao et al., 2003; Walker et al., 2011; Ballistreri et al., 2013). In sour cherry flesh, starch was not detected at any stage of development (Gao et al., 2003). In the flesh of the Japanese plum Ozark Premier glucose and fructose are more abundant than sucrose before stage III, then the content g^{-1} FW of each of these sugars increases and sucrose becomes the most abundant (**Table 1**; Famiani et al., 2012). In the flesh of the Japanese plum Kelsey, somewhat similar observations were made, and changes in sorbitol content were generally similar to those of sucrose (Donen, 1939). Also in the flesh of Mirabolano (*P. cerasifera*), President (*P. domestica*) and Shiro (*P. salicina*), fructose and, especially, glucose are more abundant than sucrose during stages I and II, then, during stage III, sucrose becomes the most abundant (Moscatello et al., 2019). In the skin of Ozark Premier plum glucose and fructose were more abundant than sucrose before stage III, then the contents g^{-1} FW of these sugars increased and glucose and fructose remained more abundant than sucrose (Famiani et al., 2012). In plums, the content of sorbitol and its changes in content during development are dependent on the species/cultivar. In the flesh of Mirabolano, sorbitol was low during stage I and II of fruit development, then during stage III it increased greatly, and its abundance was similar to sucrose (Moscatello et al., 2019). In Shiro flesh sorbitol content was low and changed slightly during development, whereas in the flesh of President sorbitol content was low in young fruits and then increased to become the second most abundant sugar during stage III (Moscatello et al., 2019). As far as the hexoses are concerned, in the ripe flesh of six European plum cultivars, glucose was generally more abundant than fructose (Dugalic et al., 2014), and this was the case throughout the development of both the flesh and skin of 'Ozark Premier' plum (Famiani et al., 2012). This was also the case in the ripe flesh of Mirabolano, President and Shiro (Moscatello et al., 2019). However, in some varieties of Japanese plum fructose is more abundant than glucose (Singh et al., 2009). In the flesh of plums of Mirabolano, President and Shiro, glucose was the most abundant sugars throughout stages I and II, with amounts strongly dependent on the species: it was very high in Mirabolano, followed by President and Shiro (Moscatello et al., 2019).

SUBCELLULAR COMPARTMENTATION OF NON-STRUCTURAL SOLUBLE CARBOHYDRATES

In order to understand certain aspects of sugar metabolism in stone fruit flesh it is important to know the intracellular compartmentation of sucrose, glucose, fructose and sorbitol. In addition, it is clearly important to know what proportion of each of these sugars is located in the apoplast. However, determining these distributions is extremely difficult to do experimentally, and has not been established with certainty for any stone fruit (Desnoues et al., 2018). A modeling approach applied to peach flesh predicted an almost equal concentration of glucose, fructose and sorbitol in the cytosol and vacuole and a much higher concentration of sucrose in the vacuole than in the cytosol. Nevertheless, the bulk of the content of each of these sugars would be located in the vacuole because it occupies a very large fraction of cell volume (Desnoues et al., 2018). These results are

at variance with the experimentally (the 'wash out' technique was used) derived results of Jiang et al. (2013), who found that a considerable proportion of the sugar content was in the cytoplasm. The results of Jiang et al. (2013) imply a very high cytoplasmic sugar concentration (because the cytoplasm only occupies a small proportion of the total cell volume) (Desnoues et al., 2018). Using the non-aqueous fractionation technique, Nadwodnik and Lohaus (2008) found that in mature peach leaves the bulk of the contents of sucrose, glucose, fructose and sorbitol were located in the vacuole. However, because of the large proportion of the cell occupied by the vacuole the concentrations of sorbitol and sucrose were higher in the cytosol, while the concentrations of glucose and fructose were much higher in the vacuole (Nadwodnik and Lohaus, 2008). Beshir et al. (2019) used the non-aqueous fractionation technique to determine the distribution of metabolites between different subcellular compartments during the development of apple flesh. The bulk of the contents of sucrose, glucose, fructose and sorbitol were located in the vacuole throughout development. These sugars were often also present in the cytosol and plastid, and this was dependent on both the sugar and stage of development. However, because of the large volume of the vacuole the actual concentration of some of these sugars could at certain stages of development be higher in the cytosol and plastid than in the vacuole (Beshir et al., 2019). It is plausible that in the flesh of stone fruits the subcellular distribution of sugars is comparable to apple flesh; however, it requires to be determined experimentally. In the flesh of stone fruit the glucose:fructose ratio usually is not one, and the reasons for this in peach have been recently considered. It was suggested that both the presence of isoforms of fructokinase with different affinities for fructose, and differences in the transport of fructose and glucose at the tonoplast could contribute to a higher content of glucose than fructose (Desnoues et al., 2014, 2018). However, the situation could be far more complicated than this because in tomato a glucose efflux transporter (a SWEET facilitator) located in the plasma membrane appears to be responsible for determining the ratio of glucose to fructose (Shammai et al., 2018). Thus, it is possible that different mechanisms are responsible for determining the ratio of glucose to fructose in the flesh of stone fruits, and which is predominant could depend on factors such as the species, cultivar and stage of development and environment.

The question arises as to what proportion of each sugar is located outside the cell. In grape berries, there is a large increase in the apoplastic concentrations of glucose and fructose just before the onset of ripening, and this higher concentration persists throughout ripening. During ripening, these hexoses are the predominant osmoticum in the apoplast with a total concentration of about 500 mM, and this is thought to be important in the process of turgor regulation associated with fruit softening (Wada et al., 2008). Similarly, in ripe sweet cherry flesh (and in the ripe flesh of sour cherry, European plum, tomato and a range of soft fruits) the most likely explanation for the low turgor pressure of the parenchyma cells is a build-up of apoplastic solutes (Knoche et al., 2014; Schumann et al., 2014). In kiwifruit, Gould et al. (2013) provided evidence that a large proportion of these apoplastic sugars arise not from apoplastic transport from the phloem, but from release from sink cells as part of a mechanism used to regulate cell turgor pressure. According to Schumann et al. (2014), in cherry the apoplastic volume is only around 10% of cell volume, and this would mean that the bulk of the sugar content was located within the cell. A physiological disorder of fruits termed watercore/glassines is associated with an increase in the content of apoplastic sorbitol (Gao et al., 2005).

METABOLISM OF NON-STRUCTURAL SOLUBLE CARBOHYDRATES IN THE FLESH

Sucrose and sorbitol account for the bulk of the sugars imported into stone fruits; however, to enter metabolism they must be transformed into other compounds. For sucrose this usually requires either invertase (which catalyzes: sucrose + $H_2O \rightarrow$ glucose + fructose) or sucrose synthase (SuSy, which catalyzes: sucrose + UDP $\leftrightarrow$ UDP-glucose + fructose) (**Figure 1**). On the basis of the pH optimum for their catalytic activity, invertases are subdivided into the acid and neutral/alkaline invertases (henceforth referred to as neutral invertase). Acid invertases are located in either the vacuole or cell wall; whereas, the neutral invertases are located in either the cytosol, nucleus, mitochondrion or plastid (Kingston-Smith et al., 1999; Sturm, 1999; Nonis et al., 2008; Vargas and Salerno, 2010). SuSy is often located in the cytosol, but it is not uncommonly found in other locations (Granot and Stein, 2019). In plants (including those stone fruits studied), acid invertase, neutral invertase and SuSy are each encoded by small gene families (Nonis et al., 2008; Zhang et al., 2015; Vimolmangkang et al., 2016; Wang et al., 2017; Shen L. B. et al., 2018; Granot and Stein, 2019). The genes for the invertases, SuSy, SPS and SDH present in the peach genome are shown in **Table 2**. Amongst these, a SuSy (Prupe.5G241700.1) is a candidate gene for controlling the SSC because it is within the interval (located between 12 and 18 Mbp of chromosome 5) of a Quantitative Trait Loci linked to SSC (Nuñez-Lillo et al., 2019; Rawandoozi et al., 2020).

In the flesh of both stone fruits and pome fruits, both sucrose and sorbitol can be broken down and then potentially resynthesized as either sucrose or sorbitol, and this turnover is referred to as the sucrose cycle (Li et al., 2012, 2016, 2018). A simplified scheme depicting the enzymes utilized in this process is shown in **Figure 1**. This cycle is central to carbohydrate metabolism in sink tissues and in conjunction with proteins that transport sugars across membranes allows sugar utilization and accumulation to be coordinated. In addition, the cycle together with sugar transporters plays a key role in maintaining the osmotic potential and turgor of different subcellular compartments (Li et al., 2012, 2016, 2018). There is a large increase in the content of soluble sugars g^{-1} DW of flesh during stage III of development in stone fruits such as peach (Pavel and DeJong, 1993b; Moing et al., 1998). This shows that after the onset of ripening a much lower proportion of imported sugars are used in the synthesis of compounds other than non-structural carbohydrates than before this time. In stone fruits the amount of CO_2 released g^{-1} FW of flesh

TABLE 2 | Genes encoding for the invertases, SuSy, SPS and SDH identified in the peach genome.

Transcript ID version 2	Transcript ID version 1	Enzyme	TAIR match	Location	References transcripts analysis
Sucrose synthase					
Prupe.1G131700.1	ppa001573m, ppa001845m	Sucrose synthase	ATSUS2	Pp01:10355300-10373098	Zhang et al., 2015; Vimolmangkang et al., 2016; Aslam et al., 2019
Prupe.1G192300.1		Sucrose synthase	n.a.	Pp01: 17838341-17839225	Aslam et al., 2019
Prupe.2G242300.1	ppa001535m	Sucrose synthase	ATSUS3	Pp02:26135523-26137574	Zhang et al., 2015; Vimolmangkang et al., 2016
Prupe.3G014100.1	ppa017606m	Sucrose synthase	ATSUS6	Pp03:1010886-1015717	Zhang et al., 2015; Vimolmangkang et al., 2016; Aslam et al., 2019
Prupe.5G241700.1	ppa001135m	Sucrose synthase	ATSUS6	Pp05:18195911-18200676	Zhang et al., 2015; Vimolmangkang et al., 2016; Aslam et al., 2019
Prupe.7G192300.1	ppa001535m	Sucrose synthase	ATSUS4	Pp07:18350215-18356360	Lombardo et al., 2011 (SS); Zhang et al., 2015; Vimolmangkang et al., 2016
Prupe.8G264300.1	ppa002723m	Sucrose synthase	ATSUS3	Pp08:22179197-22184773	Zhang et al., 2015; Vimolmangkang et al., 2016; Aslam et al., 2019
Invertase					
Prupe.1G111800.1	ppa002847m	Alkaline/neutral invertase	Plant neutral invertase family protein	Pp01:8933938-8938072	Lombardo et al., 2011 (NI1); Vimolmangkang et al., 2016; Aslam et al., 2019
Prupe.1G365400.1	ppa025225m	Alkaline/neutral invertase	Plant neutral invertase family protein	Pp01:33537122-33539832	Vimolmangkang et al., 2016
Prupe.1G556900.1		Cytosolic invertase	CINV2		Aslam et al., 2019
Prupe.2G075000.1	ppa004112m	Alkaline/neutral invertase	Plant neutral invertase family protein	Pp02:11444746-11449675	Vimolmangkang et al., 2016
Prupe.2G083900.1	ppa002625m	Alkaline/neutral invertase	alkaline/neutral invertase	Pp02:13283820-13288728	Lombardo et al., 2011 (NI2); Vimolmangkang et al., 2016
Prupe.2G191400.1	ppa019684m	Cytosolic invertase	S CINV2	Pp02:23073519-23078158	Vimolmangkang et al., 2016
Prupe.2G277900.1	ppa002732m	Vacuolar invertase	ATBETAFRUCT4, VAC-INV	Pp02:27860807-27865049	Vimolmangkang et al., 2016
Prupe.3G009500.1	ppa003412m	Cell wall invertase	ATCWINV1, ATBFRUCT1	Pp03:606163-610062	Vimolmangkang et al., 2016
Prupe.3G048300.1	ppa003470m	Cell wall invertase	ATCWINV1, ATBFRUCT1	Pp03:3405921-3409831	Vimolmangkang et al., 2016; Aslam et al., 2019
Prupe.5G075600.1	ppa002334m	Vacuolar invertase	ATBETAFRUCT4, VAC-INV	Pp05:8955357-8959521	Vimolmangkang et al., 2016; Aslam et al., 2019
Prupe.6G122600.1	ppa002614m	Alkaline/neutral invertase	INV-E, At-A/N-InvE	Pp06:9128385-9135532	Vimolmangkang et al., 2016
Prupe.6G309800.1	ppa002385m	Alkaline/neutral invertase	Plant neutral invertase family protein	Pp06:27815656-27819789	Lombardo et al., 2011 (NI3); Vimolmangkang et al., 2016; Aslam et al., 2019

(Continued)

TABLE 2 | Continued

Transcript ID version 2	Transcript ID version 1	Enzyme	TAIR match	Location	References transcripts analysis
Prupe.7G103100.1	ppa022745m	Cell wall invertase	ATCWINV4, CWINV4	Pp07:13274130-13276372	Vimolmangkang et al., 2016
Prupe.7G103200.1	ppa019728m	Cell wall invertase	ATCWINV4, CWINV4	Pp07:13285991-13289044	Vimolmangkang et al., 2016; Aslam et al., 2019
Prupe.7G103300.1	ppa003343m	Cell wall invertase	ATCWINV2, CWINV2	Pp07:13293841-13296921	Lombardo et al., 2011 (AI2); Vimolmangkang et al., 2016
Prupe.7G103400.1	ppa004218m	Cell wall invertase	ATCWINV4, CWINV4	Pp07:13299771-13304593	Vimolmangkang et al., 2016
Prupe.8G159800.1	ppa003483m	Alkaline/neutral invertase	Plant neutral invertase family protein	Pp08:16872814-16878303	Vimolmangkang et al., 2016; Aslam et al., 2019
Sucrose-phosphatase synthase					
Prupe.1G159700.1	ppa000622m	Sucrose-phosphatase synthase	ATSPS3F, SPS3F	Pp01:12702147-12709381	Vimolmangkang et al., 2016; Aslam et al., 2019
Prupe.1G483200.1	ppa000636m	Sucrose-phosphatase synthase	ATSPS1F, SPS1F	Pp01:40288494-40295210	Lombardo et al., 2011 (SPS2); Vimolmangkang et al., 2016; Aslam et al., 2019
Prupe.7G249900.1	ppa000639m	Sucrose-phosphatase synthase	ATSPS1F, SPS1F	Pp07:21151882-21157785	Lombardo et al., 2011 (SPS1); Vimolmangkang et al., 2016; Aslam et al., 2019
Prupe.8G003700.1	ppa000716m	Sucrose-phosphatase synthase	ATSPS4F	Pp08:302873-308259	Vimolmangkang et al., 2016; Aslam et al., 2019
Sorbitol dehydrogenase					
Prupe.1G057900	ppa007458m	Sorbitol dehydrogenase	GroES-like zinc-binding alcohol dehydrogenase family protein	Pp01:4111842-4114761	
Prupe.2G288800	ppa007458m	Sorbitol dehydrogenase	GroES-like zinc-binding alcohol dehydrogenase family protein	Pp02:28363713-28365855	Lombardo et al., 2011 (SDH1)
Prupe.4G240300	ppa007327m	Sorbitol dehydrogenase	GroES-like zinc-binding alcohol dehydrogenase family protein	Pp04:15817263-15819830	
Prupe.8G142900	ppa007374m	Sorbitol dehydrogenase	GroES-like zinc-binding alcohol dehydrogenase family protein	Pp08:15994437-15996971	
Prupe.8G143000	ppa007343m	Sorbitol dehydrogenase	GroES-like zinc-binding alcohol dehydrogenase family protein	Pp08:15999040-16001622	

In the table are reported the IDs assigned to each gene in the first (The International Peach Genome Initiative – Verde et al., 2013) and the second version (Verde et al., 2017) of the peach genome to make easier the identification of genes for which have been carried out the analysis of transcripts (see references in the last column). For each gene is also reported the location in the peach genome (to retrieve all the information see at Prunus persica Genome v2.0.a1: JBrowse | Genome page) and best hits against the Arabidopsis genome sequences (TAIR, https://www.arabidopsis.org/Blast/).

is much lower during stage III as compared to stage I (Pavel and DeJong, 1993a; Famiani et al., 2016). The bulk of this CO_2 arises from the action of the Krebs cycle which is associated with the respiratory processes that provide ATP and reductant. However, the reason for this lower CO_2 output during stage III likely arises from a dilution effect brought about by a large increases in the ratio of the vacuole to cytoplasm of fruit parenchyma cells (Famiani et al., 2016, 2020). In stone fruits the relative importance of enzymes of the sucrose cycle (and enzymes catalyzing allied reactions), sugar transporters and other factors in determining the increased accumulation of sugars during ripening is unknown. In apple, detailed studies have provided a useful model of sugar metabolism in the flesh at different stages of development (Li et al., 2018). The reader is referred to this work because it provides valuable insights into the situation in stone fruits.

Membrane Sugar Transporters

The proteins that transport sugars across membranes in plants can be divided into three families: sucrose transporters (SUC's/SUTs: sucrose/H^+ symporters), monosaccharide transporters (MST's: hexose/H^+ antiporters/symporters) and Sugars Will Eventually be Exported Transporter (SWEETs: sucrose and hexose facilitator transporters). At least some sorbitol transporters are members of the MST family (Cheng et al., 2018; Ma et al., 2018). In stone fruits, there have been few detailed studies of these transporters. The abundance and locations of transcripts encoding SUTs have been investigated in peach flesh, and possible roles in sucrose retrieval from the apoplast and in sucrose release from the vacuole suggested (Zanon et al., 2015). In peach flesh two SWEET genes were expressed, and their protein products might play a role in the unloading of sucrose from the phloem (Zanon et al., 2015). Earlier uptake studies in peach flesh are consistent with a proportion of apoplastic sucrose being hydrolysed by cell wall invertase, and the hexoses produced being transported into parenchyma cells by a hexose transporter(s) (Vizzotto et al., 1996). A recent study of a tonoplast sugar transporter (*PpTST1*; a MST) found that the gene for this transporter is located in a quantitative trait locus (QTL) for sucrose, and that transient silencing of *PpTST1* significantly reduced sucrose accumulation (Peng et al., 2020). The abundance of transcripts of two sorbitol/proton symporters in sour cherry flesh are consistent with a proportion of sorbitol being unloaded from the phloem into the apoplast (Gao et al., 2003).

Sucrose Synthase (SuSy) and Sucrose Phosphate Synthase (SPS)

Sucrose synthase activity is present in extracts of peach flesh throughout its development, and the amounts of activity in peach flesh and in some other fruits are shown in **Table 3**. In peach flesh, SuSy activity g^{-1} FW is highest during the earlier part of stage I, it then declines, and then, according to some studies, increases during ripening (Moriguchi et al., 1990; Hubbard et al., 1991) or, to others, does not (Vizzotto et al., 1996; Lo Bianco and Rieger, 2002). A survey of a large number of peach genotypes showed that there are considerable differences in the amounts of SuSy present in the flesh of the different genotypes (**Table 3**; Desnoues et al., 2014). Similarly, there are differences in the abundance of SuSy RNA transcripts among cultivars of peach (Vimolmangkang et al., 2016). Peach contains six SuSy genes (Verde et al., 2013) and transcripts arising from three of these are abundant in the flesh, and thus the function of SuSy in peach fruits is complex, not well understood, and could depend on both the tissue and its stage of development (Zhang et al., 2015). In apple, the situation appears to be similar (Tong et al., 2018). From studies of other plants, it is clear that SuSy could potentially play diverse roles in sucrose metabolism in stone fruits, and these include phloem/xylem metabolism, cellulose and callose synthesis, and the provision of substrate for metabolism especially when O_2-supply is low (Granot and Stein, 2019). Nevertheless, the absolute requirement for SuSy in some of these processes has been questioned (Barratt et al., 2009).

SuSy catalyzes a reversible reaction, and Moriguchi and Yamaki (1988) and Moriguchi et al. (1990) suggested that in ripening peach flesh SuSy could play a role in sucrose synthesis. In plants either SPS or SuSy are required for the synthesis of sucrose from either glucose or fructose; however, the predominant route in most tissues is via SPS (Hubbard et al., 1991). The activity of SPS (which catalyses: fructose-6-phosphate + UDP-glucose ↔ sucrose-6-phosphate + UDP – **Figure 1**) has been measured in peach flesh. Thus, Vizzotto et al. (1996) found that throughout development SPS activity was about 2.5 μmol g^{-1} FW h^{-1}, whilst Moriguchi et al. (1990) reported values of about 1 μmol g^{-1} FW h^{-1}. In contrast, Hubbard et al. (1991) found that during the ripening of peach flesh SPS activity increased from about 8 to 14 μmol g^{-1} FW h^{-1} and SuSy activity increased from 5 to 25 μmol g^{-1} FW h^{-1}, however, they were non-committal as to whether SuSy functioned in sucrose synthesis. A study carried out on different peach genotypes showed that there were considerable differences in the amounts of SPS present in their flesh; with an average value of 0.6 and a range of 0–2.7 μmol g^{-1} FW h^{-1} (Desnoues et al., 2014). Similarly, the abundance of transcripts arising from different SPS genes differs among cultivars of peach (Vimolmangkang et al., 2016). The expression of the different members of the gene families that encode SuSy and SPS (4 genes, according to Verde et al., 2013) has been investigated in peach flesh, and only certain member(s) of each family show increased expression during ripening (Lombardo et al., 2011; Zhang et al., 2015; Vimolmangkang et al., 2016). Yamaki (2010) reported that SuSy can function in both the synthesis of sucrose and its degradation in the flesh of fruits and was of the opinion that this was dependent on the species of fruit, tissue, stage of development and isoform of SuSy.

Invertases

Although the acid and neutral invertases have different pH optima these are quite broad, therefore, when an enzyme activity assay is conducted at either acidic or alkaline pH, both invertases can contribute to the total activity that is measured (Lowell et al., 1989; Burger and Schaffer, 2007). Hence, for tissues such as cherry flesh that contain high amounts of acid invertase (**Table 3**), in crude extracts it is impossible to obtain reliable measurements

TABLE 3 | Typical approximate activities of sucrose synthase and invertases (μmol g^{-1} FW h^{-1}) in the flesh of fruits at the stage of development roughly equivalent to late stage I in stone fruits (young fruits) and during ripening.

	Sucrose synthase (cleavage)	Neutral invertase	Total acid invertase	
Late stage I – Young fruits				
Cherry (sweet)			22	Krishnan and Pueppke, 1990
Peach	10–20	2–12	6–30	Moriguchi et al., 1990
				Vizzotto et al., 1996
Kiwifruit	40	3	8	Moscatello et al., 2011
Grape (hexose accumulator)	0.15–5		130–200	Takayanagi and Yokotsuka, 1997; Wu et al., 2011
Grape (sucrose containing)	0.15–2		< 4	
Tomato (hexose accumulator)	30		240	Yelle et al., 1991
Tomato (sucrose accumulator)	18		4	
Asian pear	2–10		8–33	Moriguchi et al., 1992
Strawberry	6	2.5	25	Hubbard et al., 1991
Grapefruit juice sacs	15	3.9	25	Lowell et al., 1989
Major vascular bundles	12	2.0	49	
Albedo of peel	4	1.6	91	
Stage III – Ripening				
Cherry (sweet)			180	Krishnan and Pueppke, 1990
Peach	1–14	0–1.5	2–6	Moriguchi et al., 1990
				Vizzotto et al., 1996
Peach	5–25	< 3	0	Hubbard et al., 1991
Peach (large number of genotypes)		average		Desnoues et al., 2014
	3.6	1.8	1.5	
		range		
	0–13	0–11	0–6	
Kiwifruit	4	1	4	Moscatello et al., 2011
Grape (hexose accumulator)	0.3–5		150–250	Takayanagi and Yokotsuka, 1997; Wu et al., 2011
Grape (sucrose containing)	0.3–5		< 1.5	
Tomato (hexose accumulator)	1–3		1200	Yelle et al., 1991
Tomato (sucrose accumulator	0.06–2.5		< 0.3	
Strawberry	6	10	3	Hubbard et al., 1991
Asian pear	1–6		1–7	Moriguchi et al., 1992
Grapefruit				Lowell et al., 1989
Juice sacs	0.1	0.6	0.4	
Major vascular bundles	1.0	0.3	1.3	
Albedo of peel	0.1	0.5	1.9	

of the neutral invertase activity. On the other hand, peach flesh contains much lower amounts of acid invertase, and it has been possible to measure both acid and neutral invertase in crude extracts (Vizzotto et al., 1996). In tissues, in which one form of invertase is much more abundant than the other, it is possible to rapidly separate them by using Concanavalin A chromatography (Walker et al., 1997).

A comparison between neutral invertase activity in extracts of peach flesh and in some other fruits are shown in **Table 3**. Neutral invertase is present in peach flesh throughout its development, and its activity shows two peaks: one occurring during the first part of stage I and the other during stage II (Vizzotto et al., 1996). By contrast, the amount of neutral invertase protein visualized on western blots (loaded so that each track contained an equal amount of total protein) was highest in 'Redhaven' peach flesh during stage II (Nonis et al., 2007). This difference is likely a result of the decline in total protein g^{-1} flesh FW during stage I (Lombardo et al., 2011; Famiani et al., 2012). In 'Springcrest' peach flesh, neutral invertase protein declined much more during development than in 'Redhaven' peach flesh (Nonis et al., 2007). Considerable differences in the amounts of neutral invertase were pointed out in the flesh of peach genotypes (**Table 3**; Desnoues et al., 2014). In the flesh of 'Dixiland' peach, neutral invertase activity per mg of total protein was lower during stage I, and was much higher during stages II to IV (Lombardo et al., 2011). In both peach and grape, neutral invertase is encoded by a small gene family, and in peach eight genes have been identified, six of which are expressed in the fruit (Nonis et al., 2008; Vimolmangkang et al., 2016). Indeed, in peach the abundance of transcripts arising from each of these genes is dependent on both

the stage of development and the cultivar (Vimolmangkang et al., 2016). Analysis of the neutral invertase gene family in different plant species shows that they can be divided into α (located in organelles) and β (located in the cytosol) neutral invertases. These two forms of neutral invertase are of ancient origin (Nonis et al., 2008; Shen L. B. et al., 2018). In peach flesh, as in a range of other tissues, cytosolic neutral invertase is likely to play a role in providing substrates for metabolism (Ricardo and ap Rees, 1970; Nonis et al., 2007; Barratt et al., 2009; Borsani et al., 2009; Rossouw et al., 2010). Other potential functions for the neutral invertases in plants include osmoregulation, signaling and the involvement in responses to various stresses (Nonis et al., 2007; Dahro et al., 2016).

Acid invertase activity is present in the flesh of stone fruits, and the amounts of its activity in these and some other fruits is shown in **Table 3**. A major difficulty arises in determining whether the acid invertase activity in extracts of a tissue is due to the vacuolar or cell wall enzyme. Usually, the acid invertase activity is measured in both the soluble and particulate fractions obtained after centrifugation of extracts. However, there can be considerable uncertainty as to whether during extraction cell wall invertase was solubilised or vacuolar invertase became bound to the cell wall fraction (Ricardo and ap Rees, 1970; Lowell et al., 1989). In tissues, such as the flesh of grape and cherry, in which vacuolar acid invertase is very abundant (**Table 3**), this problem regarding cell wall invertase can be particularly acute. Indeed, in grape flesh attempts to determine the relative proportions of vacuolar and cell wall invertase activity has been the subject of several studies, and these proportions remain uncertain (Davies et al., 2012). On the basis of these pieces of evidence, at the moment, it appears evident that it is not possible to determine the single contribution of the two forms (vacuolar and cell wall) to the acid invertase activity when the latter is measured in crude extracts of soluble proteins. To determine this further investigation is required. Vacuolar invertases have a low pI (isoelectric point) whereas cell wall invertases have a high pI (Sturm, 1999). The pI determination of the invertases present in soluble extracts of a tissue by chromatofocusing, as well as the use of antibodies specific for either the vacuolar or cell wall acid invertase (Simpson et al., 1991; Tang et al., 1999; Famiani et al., 2000) will give a strong indication of their subcellular location.

Soluble acid invertase activity is present in peach flesh throughout development, and its activity g^{-1} FW is highest during the early part of stage I, then it decreases, and during stage III one study found that it increased whilst another did not (Moriguchi et al., 1990; Vizzotto et al., 1996). However, there are considerable differences in the amounts of soluble acid invertase present in the flesh of different peach genotypes (**Table 3**; Desnoues et al., 2014). Soluble acid invertase activity per mg of total protein showed a different pattern of changes during development and was lowest during stage I (Lombardo et al., 2011). Insoluble acid invertase is present in peach flesh throughout development and its activity g^{-1} FW is highest in early stage I and then it declines, and, according to one study, it increases during stage II whilst another study found that this increase occurred during stage III (Moriguchi et al., 1990; Vizzotto et al., 1996). Further, it was found that acid invertase activity during stage III was largely in the insoluble fraction, and it was suggested that the enzyme was predominantly located in cell wall (Ugalde et al., 1988; Moriguchi et al., 1990, 1991). In the peach genome, two genes for vacuolar and six genes for cell wall acid invertase have been identified (Vimolmangkang et al., 2016), and this is similar to the acid invertase gene family from some other plants (Wang et al., 2017). In peach flesh, there are differences among cultivars in the abundance of transcripts arising from the different acid invertase genes, and the abundance of these transcripts also changes during development (Vimolmangkang et al., 2016). These differences among cultivars are likely related to the different amounts of acid invertase activity present in the flesh of different peach genotypes (**Table 3**; Desnoues et al., 2014). In the flesh of stone fruits, as in a range of other tissues, the acid invertases could potentially function in the provision of substrate for metabolism, osmoregulation associated with the regulation of turgor pressure, phloem unloading and the generation of hexoses used in sugar sensing (Sturm, 1999; Sturm and Tang, 1999). These roles are discussed in more detail in the section dealing with functions of sugars.

In many sink tissues (such as grape berry flesh, tomato fruit flesh, sugarcane internode, sugar beet roots and carrot tap roots) there is an inverse relationship between sucrose content and the abundance of vacuolar invertase (Ricardo and ap Rees, 1970; Sturm and Tang, 1999). In ripening cherry flesh the activity of soluble acid invertase is 30–90 times higher than in ripening peach flesh (Krishnan and Pueppke, 1990; **Table 3**). Cherry flesh unlike that of peach contains little sucrose and large amounts of glucose and fructose (**Table 1**). It is therefore likely that the abundance of vacuolar acid invertase in the flesh of stone fruits is a major factor in determining their sucrose:(glucose + fructose) ratio (Walker et al., 2011). However, in some tissues it has been found that the relationship between the ratio of sucrose:(glucose + fructose) and the abundance of soluble invertase is not linear (Zhu et al., 1997; Beauvoit et al., 2014). Thus, at lower sucrose content and higher glucose plus fructose content more acid invertase activity is required to bring about the same decrease in sucrose content than is required at high sucrose content and lower glucose plus fructose content. One explanation for this is that *in vivo* vacuolar acid invertase activity is markedly inhibited by high concentrations of these hexoses (Walker and Pollock, 1993; Walker et al., 1997; Kingston-Smith et al., 1999), and work on tomato fruit supports this explanation (Beauvoit et al., 2014). Desnoues et al. (2014) failed to find a correlation between soluble acid invertase activity and sucrose content in the flesh of a range of peach genotypes; however, the proportions of invertase activity measured that were due to the vacuolar and cell wall forms of the enzyme were not determined.

Enzymes of Sorbitol Metabolism

In order to enter metabolism imported sorbitol must be transformed into other compounds. To achieve this, potentially either NAD-sorbitol dehydrogenase (NAD-SDH) (NAD-SDH catalyzes: sorbitol + $NAD^+ \leftrightarrow$ fructose + NADH + H^+), NADP-sorbitol dehydrogenase (NADP-SDH) or sorbitol oxidase (SOX) can be used (**Figure 1**) (Loescher, 1987; Lo Bianco and Rieger, 2002; Yamaki, 2010; Wang et al., 2016). In peach fruits, the

relative contributions of NAD-SDH, NADP-SDH and SOX to the catabolism of sorbitol are dependent on both the tissue and its stage of development (Moriguchi et al., 1990; Lo Bianco et al., 1999; Lo Bianco and Rieger, 2002; Moscatello et al., 2017). Yamada et al. (2001) stated that in some earlier studies of NAD-SDH activity in peach flesh unsuitable extraction/assay conditions were used which led to inaccurate measurements of its abundance. Thus, although Moriguchi et al. (1990) detected SOX in peach flesh throughout its development, NAD-SDH and NADP-SDH were barely detectable. In the flesh of Encore peach NAD-SDH was only detected during stage III and SOX was not detected during at least stages I and II (Lo Bianco et al., 1999). However, later studies showed that in Encore peach not too dissimilar amounts of both NAD-SDH and SOX specific activity were detected in the flesh during stages I and III (Lo Bianco and Rieger, 2002). Similarly, Yamada et al. (2001) showed that both SDH protein and activity g^{-1} FW were highest early in development, then declined and subsequently increased during ripening. In sour cherry flesh, only very low activities of NAD-SDH were present throughout development and SOX was not detected (Gao et al., 2003). In Japanese plum flesh both SDH and SOX were present, and their specific activities (expressed on a FW basis) decreased during development (Kim et al., 2015; Farcuh et al., 2017). Nevertheless, some very different values for both NAD-SDH and SOX are reported for peach flesh at comparable stages of development. Thus, in peach flesh NAD-SDH activity for stages I-III were 1.3, 0.1 and 0.2 μmol g^{-1} FW h^{-1}. The values for SOX at these stages of development were 0.9, 0 and 0.3 μmol g^{-1} FW h^{-1}, respectively (Lo Bianco and Rieger, 2002). By contrast, for peach flesh during stage III, Morandi et al. (2008) reported values of SOX activity of 10–11.4 μmol g^{-1} FW h^{-1} and for NAD-SDH activity of 0.07–0.09 μmol g^{-1} FW h^{-1}. Yamada et al. (2001) reported values of NAD-SDH activity in peach flesh at stages I-III of 420, 30 and 240 μmol g^{-1} FW h^{-1}, respectively. In the ripening flesh of a large number of peach genotypes, the average value of NAD-SDH was 2.4 μmol g^{-1} FW h^{-1} and the range was 0–18 μmol g^{-1} FW h^{-1} (Desnoues et al., 2014). In the ripe flesh of 4 cultivars of peach the activity of NAD-SDH was 0.06–0.27 μmol g^{-1} FW h^{-1} (Kanayama et al., 2005). Moriguchi et al. (1990) reported values of SOX activity in peach flesh at stages I-III of 0.08, 0.03 and 0.03 μmol g^{-1} FW h^{-1}, respectively. In the ripening flesh of a large number of peach genotypes, the average value of SOX was 2.4 μmol g^{-1} FW h^{-1} and the range was 0–11 μmol g^{-1} FW h^{-1} (Desnoues et al., 2014). In peach endocarp at stage I the activities of SOX was 2.3 and NAD-SDH was 0.07 μmol g^{-1} FW h^{-1} (Lo Bianco and Rieger, 2002). Clearly, in order to further understand the function of NAD-SDH and SOX in stone fruits it is essential to determine the abundance of the different forms of these enzymes in the various tissues of the fruit and seed. In the peach genome there are seven genes that encode NAD-SDH and at least four are expressed in the fruit (Cirilli et al., 2016). Apple contains nine NAD-SDH genes and five of these are expressed in the fruit plus seed (Nosarzewski and Archbold, 2007).

Much less work has been done on the enzymes involved in the catabolism of sucrose and sorbitol in plums and apricots than in peach. The studies on plum (Kim et al., 2015; Farcuh et al., 2017) and apricot (Xi et al., 2016) were not inconsistent with the results obtained for peach and cherry in terms of which enzymes were present and the patterns of changes in their activity during development. However, in the case of Japanese plum, the amounts of enzyme activity were orders of magnitude higher (Kim et al., 2015; Farcuh et al., 2017), and the reason for this difference requires clarification.

FRUCTANS IN THE FLESH OF STONE FRUITS

Low amounts of fructan have been detected in the ripe flesh of one peach cultivar (4 mg g^{-1} FW) (Muir et al., 2007) and ripe plum flesh (c0.6 mg g^{-1} FW) (L'homme et al., 2001). Fructans are fructose oligomers/polymers, and the bulk of their content is usually located in the vacuole. In plants that accumulate large amounts of fructan they are synthesized by a range of fructosyl transferase enzymes, which have evolved from the vacuolar acid invertases (Kingston-Smith et al., 1999; Van den Ende, 2013). However, the synthesis of the small amount of fructan present in banana flesh could be a result of the inherent fructosyl transferase activity of acid invertase (Henderson et al., 1959; Cruz-Cárdenas et al., 2015). Indeed, purified acid invertases can synthesize fructans *in vitro*. This synthesis is favored by high concentrations of sucrose and low amounts of acid invertase, because at higher concentrations of invertase, sucrose and fructans are rapidly degraded by invertase (Pollock et al., 1989; Cairns and Ashton, 1991; Kingston-Smith et al., 1999). In ripe peach flesh, the content of vacuolar sucrose is likely to be high and acid invertase activity low (Moriguchi et al., 1991; Vizzotto et al., 1996; Desnoues et al., 2018), and this would favor the synthesis of fructans as a consequence of the inherent transferase activity of vacuolar acid invertase. Further support for this view in peach comes from studies of the acid invertase gene families. Fructan accumulating species contain further copies of genes that are highly homologous to vacuolar acid invertase, and these encode the fructosyl transferase enzymes used in fructan synthesis (Van den Ende, 2013). No extra copies of vacuolar acid invertase genes were detected in peach (Vimolmangkang et al., 2016), and this suggests that it does not contain genes that encode specialized fructosyl transferases. Similarly, Arabidopsis does not contain fructosyl transferase genes, and the simplest explanation for the presence of small amounts of fructan in this plant is that it they are synthesized by vacuolar acid invertase (Van den Ende, 2013).

FUNCTIONS OF NON-STRUCTURAL SOLUBLE CARBOHYDRATES IN THE FLESH

In addition to their role in enticing animals to disperse the fruits and hence their seeds, sugars play several vital roles in the metabolism of the flesh of stone fruits. These functions include providing substrate for metabolism and acting as a major osmoticum used in turgor regulation. Further, the contents

of sugars in a tissue can be sensed, and consequently bring about changes in both metabolism and development; and a striking example of this is the induction of fructan biosynthesis in the leaves of many temperate grasses by conditions that increase the sucrose content of the leaf (Pollock et al., 1989; Kingston-Smith et al., 1999).

Non-structural Soluble Carbohydrates Are the Major Metabolic Substrate Used in Stone Fruit Flesh

Imported sugars provide the bulk of the carbon skeletons used in the synthesis of the non-nitrogenous organic constituents of the flesh (Pavel and DeJong, 1993a; Famiani et al., 2016). In addition, sugars usually provide the bulk of the substrate utilized by the Krebs cycle and respiration to provide NADH and ATP (Famiani et al., 2016). During the growth of whole peach fruits, including their enclosed seed, the proportion of sugars used by respiration is about 20% (Pavel and DeJong, 1993a). For the flesh during ripening, the proportion is likely to be lower, for example, in grape flesh during ripening about 9–14% of sugars present in the flesh are used in respiration (Famiani et al., 2014). In ripe peach flesh, 70–90% of dry matter consists of soluble sugars so roughly 10–30% of the sugars not used in respiration are used in the production of compounds other than soluble sugars. So taking average values for these would mean that about 65% of the soluble sugars imported into the fruit stay as soluble sugars.

Non-structural Soluble Carbohydrates and Turgor Regulation

Large amounts of sugars are accumulated in the ripening flesh of stone fruits in which they are a predominant osmoticum. Thus, they have a pivotal influence on cell turgor pressure (Winkler and Knoche, 2018). Turgor pressure influences several processes that occur during the growth of the flesh of stone fruits, and these include cell expansion, the import of materials and fruit softening (Wächter et al., 2003; Wada et al., 2008). Sugars increase the turgor pressure of expanding cells and can facilitate cell expansion (Pritchard, 1994). A feature of expanding cells is often a high ratio of (glucose + fructose): sucrose, and these hexoses are often produced from sucrose by acid invertase (Ricardo and ap Rees, 1970; Sturm, 1999). The flesh of stone fruits until stage III of development usually contains a lower content of total sugars and a high ratio of (glucose + fructose): sucrose, and it is possible that this high ratio also facilitates cell expansion at this stage of development when total sugar content is lower and turgor pressure is high. Further, in order for fruits to soften during ripening it is necessary that the parenchyma cells of the flesh do not have a too high turgor pressure, and a suitable concentration of sugars in the apoplast is required to reach a lower turgor pressure (Wada et al., 2008). In a range of tissues including ripening fruit one mechanism that appears to be important in maintaining a suitable concentration of sugars in the apoplast is the hydrolysis of sucrose to glucose plus fructose by cell wall invertase (Wada et al., 2008). Turgor pressure also influences the import of materials via the phloem, and a high turgor pressure inhibits symplastic flow into the tissue (Andersen et al., 2002; Gould et al., 2013). It has been hypothesized for decades that cell wall invertase could play a role in apoplastic phloem unloading, and it was suggested that it could do this by increasing the sucrose concentration gradient between the sites of unloading and sink cells and hence increase the rate of diffusion (Sturm, 1999; Tang et al., 1999). One alternative explanation is that cell wall acid invertase increases the solute concentration in the apoplast by hydrolysing sucrose, which reduces the turgor pressure of the sink cells and facilitates symplastic flow from the phloem. In stone fruits, such as plum, it appears that apoplastic movement via diffusion makes only a very small contribution to apoplastic transport of sugars, and further, there is evidence that phloem unloading can rapidly switch between apoplastic and symplastic (Grappadelli et al., 2019). Thus, it is possible that in stone fruit flesh the relative actions of vacuolar and cell wall invertase can contribute to altering turgor pressure that modifies the contributions of the symplastic and apoplastic pathways to post-phloem transport. Studies on the distribution of cell wall invertase in developing grape berries and their seed are consistent with this view (Walker et al., 1999; Famiani et al., 2000). In the seed coat of developing grape seeds, a specialized tissue called the palisade layer functions to distribute imported assimilates from the phloem to the developing storage tissues. The cells of the palisade layer are connected by numerous plasmodesmata which are thought to facilitate symplastic movement of unloaded assimilates, Further, cell wall invertase is particularly abundant in the palisade layer (Walker et al., 1999; Famiani et al., 2000), Thus, cell wall invertase could potentially function in altering the turgor pressure of the palisade cells, and thus function in regulating symplastic phloem unloading. A feature of many sink tissues is the presence of enzymes that degrade sucrose (invertases and SuSy) together with enzymes involved in sucrose synthesis (SPS and potentially SuSy), and in some of these tissues a cycle of sucrose synthesis and breakdown (termed sucrose cycling) occurs. One function of this cycle could be in sucrose breakdown and re-synthesis associated with turgor regulation (Rossouw et al., 2010; Li et al., 2012, 2016, 2018), and in the flesh of stone fruits it is possible that one function of SPS (and potentially certain forms of SuSy) is in this process.

CONCLUSION

From the foregoing, it is clear that the work of a large number of people over several decades has resulted in an impressive understanding of non-structural carbohydrate metabolism in the flesh of stone fruits. What is also apparent is that it is an extremely complex subject, and that there are many intriguing aspects that require further study. These include the subcellular compartmentation of sugars, the distribution of different enzymes between the various tissues of the flesh, the potential cycle of sucrose breakdown and re-synthesis and its functions and the roles of sugars in turgor regulation together with the contribution of enzymes such as acid invertase to this process. In this regard, as stressed by Shiratake and Suzuki (2016), omic studies will, without doubt, contribute to our further understanding of these aspects and other aspects of the metabolism of the flesh of stone fruits (Shiratake and Suzuki, 2016). In this context, important insights can be obtained

by integrative omics approaches which combine genomics, transcriptomic, proteomic and metabolomic analyses, in a system biology view, as used in the identification of new candidate genes controlling peach fruit aroma volatiles (Sánchez et al., 2013); or for evaluating the impact of post-harvest treatments on the level of peach fruit polyphenols and related genes (Santin et al., 2019). Further, studies using plants that have altered amounts of either enzymes allied to the sucrose cycle or sugar transporters will provide valuable insights into sugar metabolism in stone fruits as they have done in apple (Li et al., 2018; Wang et al., 2020).

AUTHOR CONTRIBUTIONS

All authors have contributed significantly to the work and approved it for publication. However, RW and FF had a major role in the design and writing of the article.

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REVIEW
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Sugar Metabolism in Stone Fruit: Source-Sink Relationships and Environmental and Agronomical Effects

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The partitioning of assimilates in fruits, which are economically important sink organs, is ruled by different physiological processes and affected by both environmental and agronomical factors. The bulk of the water and solutes, required for growth, is imported into fruits and seeds through xylem and phloem. In the stone fruits, five vascular bundles enter the base of the fruit, then dividing to supply either the flesh or the seed. The main sugars accumulated in stone fruits include fructose, glucose, and sucrose, along with other minor saccharides. The mechanisms of phloem loading in these fruit species have not been fully elucidated yet, but the available data hint either an apoplastic or a symplastic type or possibly a combination of both, depending on the species and the sugar considered. Similarly, phloem unloading mechanisms, elucidated for a small number of species, depend on genotype and developmental stage. Remarkably, key enzymes and transporters involved in the main sugars-conversion and transport pathways have received considerable attention. In stone fruit trees, the presence of an elevated number of fruits alters the source-sink balance, with a consequent intensification of competition among them and between vegetative and reproductive growth. The main environmental factors affecting this balance and the agronomical/artificial manipulations of source-sink relationships to achieve adequate fruit production and quality are reviewed.

Keywords: assimilates partitioning, phloem loading/unloading, photosynthesis, water flow, fertilization, pruning, thinning, rootstock

INTRODUCTION

Growth and development in tree crops are combined processes in which the metabolic need of non-photosynthetic "sink" tissues, such as fruits, is balanced by the primary assimilation of photosynthetically active "source" tissues, such as mature leaves. The comprehension of source-sink relationships and the control of carbon partitioning among sinks in plants are very important to advance the knowledge about tree crops and the impact of yield-limiting

factors (Pavel and DeJong, 1993). In fact, within a tree, fruits, which are strong sinks, compete for assimilates with each other, especially in case of high crop loads, as well as with vegetative organs, such as shoots, leaves, and roots (Pavel and DeJong, 1993; Ludewig and Flügge, 2013). For this reason, balance preservation between vegetative and generative growth, also achieved by the artificial manipulation of source-sink relationships, can be essential to ensure acceptable fruit production and quality (Fischer et al., 2012). On the other hand, fruit size is affected by the ratio between source organs (leaves) that provide sugars for growth and the number of sinks, such as fruit and other non-photosynthetic organs that compete for these (Grossman and DeJong, 1994). The partitioning of assimilates in economically important sink organs, such as fruits, is ruled by several processes, including photosynthetic rate, phloem loading, translocation throughout the phloem, phloem unloading, and uptake and metabolism of carbohydrates in sink organs (Patrick, 1997; Liesche and Patrick, 2017).

ASSIMILATES PRODUCTION AND PHLOEM LOADING

Sugars are produced by fruit photosynthesis; nevertheless, over 90% of the assimilates required for peach growth are imported, and for cherries, this proportion is about 85% (Pavel and DeJong, 1993). Leaves are the most important structure for photosynthesis and assimilate production, even if a developing leaf can represent both a source and a sink (heterotrophic), by importing carbohydrates from other parts of the plant (Pavel and DeJong, 1993). Photosynthetic behavior of *Prunus* spp. has been widely described (Flore et al., 1996; Flore and Layne, 1999; Flore and Lakso, 2011), and authors found that values for the photosynthetic rate of these species range from 7 for almond to the highest 26.2 μmol m^{-2} s^{-1} of a plum variety, with cherries positioned really close to this value. However, some evidence has been reported for stone fruits indicating that leaf photosynthetic capacity varies greatly within the canopy, being affected by intrinsic factors, such as leaf age, exposition, and fruit amount (Testolin and Costa, 1991; Flore and Lakso, 2011). Moreover, Urban et al. (2003) have demonstrated that, in mango trees, the leaves in proximity to developing fruits showed increased photosynthetic capacity as compared to the others. Similar results have been reported in an earlier work carried out by Crews et al. (1975) showing higher rates of photosynthesis in peach leaves in close proximity to fruits; the same authors demonstrated that leaf photosynthesis peaked during the stage of maximum fruit growth rate. The removal of leaves (30%) in sour cherry (*Prunus cerasus*) decreased photosynthesis, but compensation, due to the rise of the photosynthesis rate in the remaining leaves, was observed when the amount of defoliation was reduced (Layne and Flore, 1992).

Little information about the leaf area index (LAI, ratio between leaf area and occupied soil area) is available for fruit tree species, although this parameter has a pivotal role in sunlight interception and therefore productivity. Peach canopies generally have a high LAI after the initial spring growth (6–8 m^2 of leaf area per m^2 of ground area; Fahey, 1992), but this value is highly variable depending on the species. Rajan et al. (2001), for example, carried out a study on 26 Indian mango varieties measuring LAI ranging from 1.18 to 4.48; authors demonstrated that the genotypes with a low LAI were better exposed to solar radiation and displayed higher-quality production.

Sugar transport, direction, and volume are determined by sink position and relative sink strength. Carbohydrates produced in leaf mesophyll are loaded into the phloem systems and unloaded in energy-demanding or storage tissues (sinks); both the mechanisms can be apoplastic (sugars cross the cell membrane) or symplastic (exclusively through the plasmodesmata-connected cells; De Schepper et al., 2013; Roch et al., 2019). Among the sugars synthesized in plants, only a small number of them, generally highly soluble and chemically inert, are transported in the phloem over a long-distance. Sucrose, which is less reactive than reducing sugars such as glucose and fructose, is the main form of carbon found in the phloem, but, in some species, polyols, such as sorbitol, are translocated in the phloem (Moing et al., 1997; Noiraud et al., 2001; Tyree and Zimmermann, 2002). Sorbitol has been detected in phloem sap of peach and apricot trees (Bieleski and Redgwell, 1985; Moing et al., 1997); stachyose and mannitol have been found in *Olea europea* L. (olive) and in coffee (Zimmermann and Ziegler, 1975; Flora and Madore, 1993).

In most of stone fruit species, the mechanisms of phloem loading have not been fully elucidated yet. The available data suggest that phloem loading can occur through an apoplastic or a symplastic path or possibly a combination of both, depending on the species and the sugar considered (Flora and Madore, 1993; Noiraud et al., 2001; Nadwodnik and Lohaus, 2008; **Figure 1**). The passive symplasmic mechanism has been identified in most fruit trees of Rosaceae family (Reidel et al., 2009; Fu et al., 2011; Roch et al., 2019). Despite the importance of sorbitol in this family, sorbitol transporters were found only in sink organs (Gao et al., 2003; Watari et al., 2004), making the knowledge on polyol phloem loading extremely limited. In plasma membrane vesicles, obtained from peach leaves, sucrose and sorbitol uptake exhibited saturated kinetics; these results, together with the observation of sucrose transporters in various plant tissues, suggest that sorbitol and sucrose loading is carrier mediated (Marquat et al., 1997; Nadwodnik and Lohaus, 2008).

There are different types of sugar transporter in plants, and these include sucrose uptake transporters (SUTs: sucrose/H+ symporters), hexose transporters (hexose/H+ symporters), and SWEETs (sucrose facilitator; Milne et al., 2018).

The functionality of a sucrose transporter (PpSUT1) has been recently demonstrated in peach. The pH-dependent activity of this transporter is consistent with the classification of other SUT1 as H+/sucrose symporters. Therefore, Zanon et al. (2015b) suggested that it mediates the phloem loading of sucrose in leaves, supporting the hypothesis of an apoplastic loading pathway in peach.

Conversely, phloem loading of oligosaccharides, such as raffinose and stachyose, is supposed to occur symplastically, and its mechanism has been described by the "polymer trap model" (Rennie and Turgeon, 2009). Briefly, sucrose produced

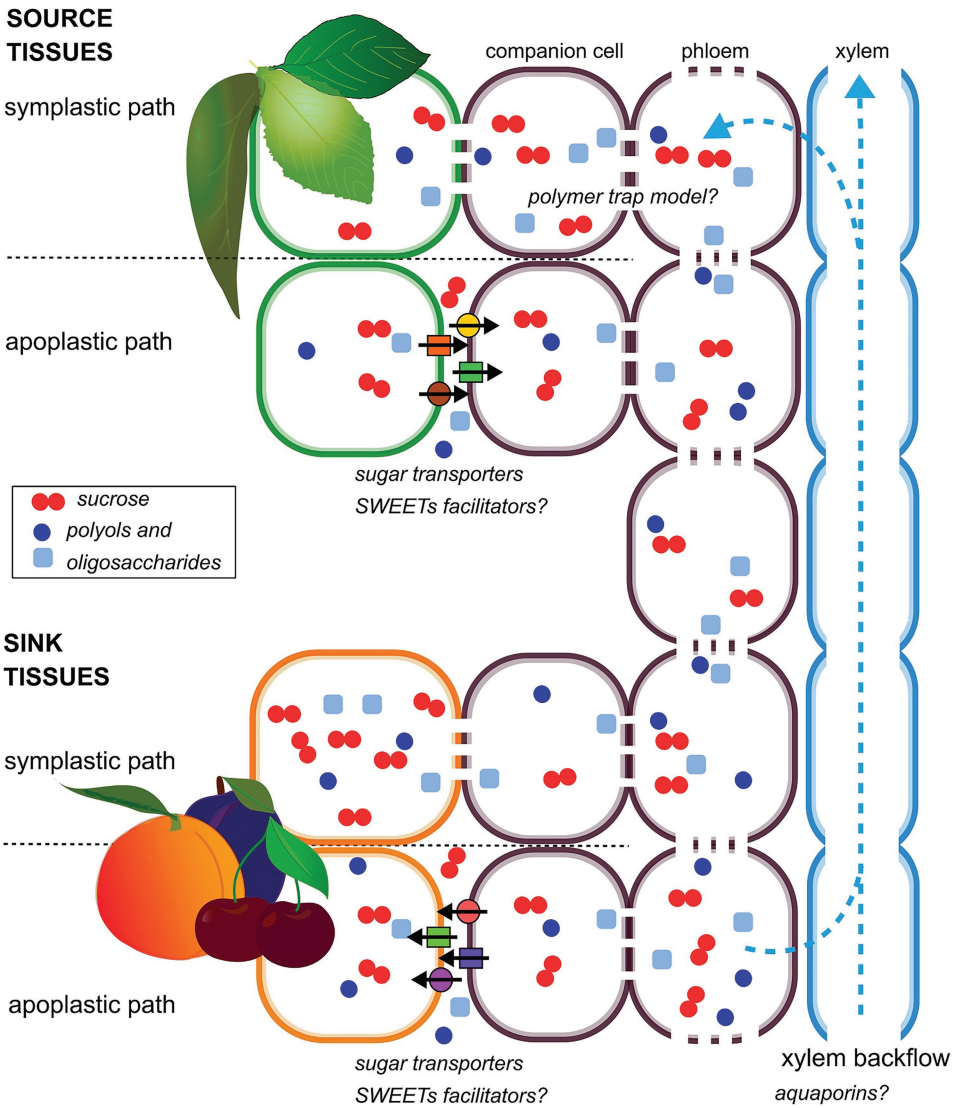

FIGURE 1 | Schematic diagram of hypothetical phloem-loading and -unloading strategies in stone fruit plants. Sucrose, polyols, and oligosaccharides are indicated as a representative of the different sugar species transported and accumulated in stone fruit plants. Both apoplastic and symplastic paths are shown, in source and sink organs, according to their coexistence, spatio-temporally regulated.

in mesophyll of source leaves diffuses to companion cells *via* plasmodesmata, and it is utilized as a substrate for oligosaccharide synthesis which flows through the sieve tubes of the phloem.

TRANSLOCATION OF WATER AND SOLUTES

The bulk of the water and solutes required for the growth of fruits and seeds is imported into them through the xylem and phloem present in their vasculature (Peoples et al., 1985; Pavel and DeJong, 1993). In the stone fruits, five vascular bundles (containing both xylem and phloem) enter the base of the fruit, then dividing to supply either the flesh or the seed. In the flesh, various branches diverge off the main bundles and ramify and anastomose throughout the flesh (Ragland, 1934; Tukey and Young, 1939; Sterling, 1953; Grimm et al., 2017). The relative contribution of xylem and phloem to fruit growth is dependent upon several factors that include the material that is being imported, the species of the stone fruit, the time of day, and both the tissue and the stage of fruit development (Matthews and Shackel, 2005; Morandi et al., 2007; Brüggenwirth and Knoche, 2016).

In sweet cherry, the input of liquid *via* the phloem is about 15% of the total imported before stage III, and it then increases to about 85–100%. Xylem flows (85% of the total liquid imported) greatly exceed phloem flows during stage II and then decrease during stage III to almost zero at harvest. Transpiration flow is

similar to xylem stream during stage II, but greater than xylem flow during stage III (Brüggenwirth and Knoche, 2016). In prunes (*Prunus domestica*), liquid input *via* the xylem accounts for a large proportion of the total liquid imported during stage II; this proportion decreases during stage III and then increases again later in stage III (Matthews and Shackel, 2005). In Japanese plum, during stage III, input can occur *via* both the xylem and phloem, and the relative contributions are dependent on the time of day (Corelli Grappadelli et al., 2019). In peach, during both stages I and III, liquid inputs to the fruit *via* phloem (30% of the total imported) or the xylem (70%) are comparable. However, in both peach and sweet cherry, the relative inputs of liquid by phloem and xylem vary differently during the diurnal cycle (Morandi et al., 2007; Brüggenwirth and Knoche, 2016).

The movement of water and solutes from the phloem into the apoplast, and their subsequent uptake into sink cells, requires their passage across plasma membranes. Albeit diffusion can potentially contribute to such membrane transport, it appears that transporters and/or channels are needed, and these greatly increase the rate of transport (Milne et al., 2018). Although few studies on stone fruit aquaporins are present in literature, in a wide range of other plant species, it is established that these channel proteins have an important role in facilitating water exchange in fruits (Milne et al., 2018). Consistently, aquaporin encoding genes appear to be modulated both in peach fruit (Sugaya et al., 2001) and in cherry (Chen et al., 2019).

In addition, once the import of phloem water into sink cells exceeds the increase in their volume, surplus of water needs to be eliminated. In several fruits and seeds, this water is recycled back to the transpiration stream, and aquaporins are likely involved also in this process, allowing water to reach sink apoplast and then xylem (Milne et al., 2018). In peach, between 55 and 65% of the total water imported into peach fruits is lost by transpiration, and the remainder is used to increase the volume of the fruit, and no backflow *via* the xylem has been detected (Morandi et al., 2007). In sweet cherry, small xylem backflows occasionally occur around midday during stage III (Brüggenwirth and Knoche, 2016). In ripening Japanese plum, some xylem backflow occurs during the morning (Corelli Grappadelli et al., 2019). The low or non-existent backflows in peach and cherry are significant because they exclude the possibility that excess water is recycled back to the axial transpiration stream, as proposed in grape (Zhang and Keller, 2017).

The concentration of sugars in the xylem of stone fruits is low after bud burst (Loescher et al., 1990; Andersen et al., 1995b). For example, their content in cherry drops from 15 to 2–3 mg ml^{-1} xylem sap after bud break (Loescher et al., 1990). Similarly, the sugar concentrations in the xylem of the terminal shoots of both peach and Japanese plum (both growing in Florida, United States in early August) were 0.1–0.4 mM (Andersen et al., 1995a,b). By contrast, the concentration of sugars in the phloem is much higher; in peach, the aphid stylet technique allowed to detect values of 800 mM in leaf phloem (Nadwodnik and Lohaus, 2008) and 700 mM in phloem of the shoot apex (Moing et al., 1997). These concentrations of sugar in the phloem sap are consistent with values of 160 (peach) and 180 (sweet cherry) mg dry matter ml^{-1} phloem sap that were estimated by comparing the phloem flow into the fruit with the rate of dry matter accumulation by the fruit (Morandi et al., 2007; Brüggenwirth and Knoche, 2016). Further, the content of dry matter in the phloem of both peach and sweet cherry fruits changes little during fruit development (Morandi et al., 2007; Brüggenwirth and Knoche, 2016). The bulk of the sugar contents of the phloem of both the leaf and shoot apex of peach consists of sorbitol and sucrose, with a ratio of sorbitol to sucrose of 2.8–4.5 (Moing et al., 1997; Nadwodnik and Lohaus, 2008).

PHLOEM UNLOADING

Water and solutes that are unloaded from the phloem can potentially move through the flesh either *via* the extracellular space (apoplastic route), directly from cell to cell by plasmodesmata (symplastic route) or possibly both (Nie et al., 2010; Zhang et al., 2014; Zanon et al., 2015b). In stone fruits, phloem unloading mechanisms have been clarified for a small number of species, showing that both symplastic and apoplastic mechanisms can occur, depending on developmental stage and genotype (**Figure 1**). In cherry fruit, an apoplastic step in sucrose and sorbitol unloading, involving sugar transporters has been shown (Gao et al., 2003). A similar behavior has been observed in jujube fruit (*Zizyphus jujuba*), but in this case, the predominantly apoplastic mechanism is interrupted by a transient symplastic unloading in the central stage of fruit growth (Nie et al., 2010).

In peach flesh, a fluorescent symplastic tracer [6(5)-carboxyfluorescein diacetate, CFDA] remained restricted to phloem strands during either stage I or at the beginning of stage III when the flesh was rapidly expanding, indicating a symplastic discontinuity between veins and parenchyma cells and suggesting that the apoplastic route predominates at these times (Zanon et al., 2015b).

A recent work in the ripening fruits of Japanese plum has shown that rapid switches between the apoplastic and symplastic routes can occur over the course of each day (Corelli Grappadelli et al., 2019). The high hydrostatic pressure of the phloem is thought to contribute to the symplastic movement of materials unloaded from the phloem (Morandi et al., 2010; Patrick et al., 2013); nevertheless, it has been recently shown that the hydrostatic pressure of the phloem from which unloading occurs could potentially be low (Milne et al., 2018). In peach flesh, xylem liquid, together with solutes and water that are unloaded from the phloem into the apoplast, appear to be largely transported through the flesh by the bulk flow of water (Morandi et al., 2007, 2010).

Subcellular compartmentation is crucial for sugar accumulation, and it can significantly affect metabolite concentrations (Patrick et al., 2013; Génard et al., 2014). Many attempts to elucidate the mechanism of carbohydrates uptake and sugar transporters have been performed in peach fruits. A carrier-mediated transport has been firstly hypothesized for mesocarp of young

peaches (Vizzotto et al., 1996). More recently, the functional characterization of three peach sucrose transporters allowed proposing different roles for these proteins in sucrose distribution. In detail, PpSUT4, localized at the tonoplast in mesocarp tissue, should act in regulating sucrose efflux from the vacuole compartment, although a different localization for this transporter cannot be excluded (Zanon et al., 2015a). In peach flesh, two SWEETs are expressed, and it can be hypothesized that they might play a role in sucrose unloading from the phloem (Zanon et al., 2015b). However, consistently with the importance of sorbitol in peach fruit, several transcripts have been annotated as *Prunus persica* sorbitol transporters, and they are more numerous than their orthologues (Verde et al., 2013).

Key enzymes involved in the main sugar conversion pathways, such as sucrose synthases and invertases, have gained attention for their involvement in determining sink strength during fruit development, and therefore, supporting the process of unloading, by accentuating the sucrose gradient between phloem and the parenchyma cells (Koch, 2004; Nonis et al., 2007; Morandi et al., 2008). Hubbard et al. (1991) indicated that activities of the sucrose metabolizing enzymes, including sucrose phosphate synthase, in fruit such as peach and mango, are pivotal in defining the content of soluble sugar in fruits. However, the relationships between enzyme activities and their products are often non-linear and are hard to evaluate; for this reason, a new kinetic model of sugar metabolism has been proposed in peach. The model suggests a different utilization of sucrose and sorbitol from sap: almost all sucrose, which is not hydrolyzed into the apoplasm, would be stored in the vacuoles; on the other hand, sorbitol would be massively catabolized in the cytosol, representing the main driver for the respiration and the synthesis of structural compounds (Desnoues et al., 2018). However, this model has recently been questioned and it has been suggested that sucrose could provide an appreciable proportion of the substrate utilized by the metabolism of peach flesh (Walker et al., 2020). Carbon translocation pathway, from leaves to fruits, has been widely studied in *Prunus* spp. (Kappes and Flore, 1986; Flore and Layne, 1999). In cherry and peach, during stage II of fruit growth, all leaves supply new shoots and fruit growth at the same time; during stages I and II, the leaves closest to fruit export basipetally, and fruits attract assimilates more strongly (Kappes and Flore, 1986). Interestingly, in sweet cherry, in which fruit matures even faster than peach and apricot, carbon labeling experiments displayed that fruit adjacent to the carbon source were very strong sinks if compared with shoots (Ayala and Lang, 2008).

The main sugars accumulated in stone fruits include fructose, glucose, and sucrose, along with other minor saccharide containing stachyose, sorbitol, raffinose, rhamnose, arabinose, galactose, and xylose (Gross and Sams, 1984; Serrano et al., 2005; Cantín et al., 2009). Both sorbitol and sucrose are imported in peaches and other stone fruits (Hansen and Ryugo, 1979; Gao et al., 2003; Lo Bianco, 2009). However, in peach, some authors have hypothesized a preferential consumption of sorbitol in vegetative sink tissues, such as young leaves and cambium, close to the source (Moing et al., 1992; Lo Bianco et al., 1999), and, conversely, a key role of sucrose in fruit growth (Vizzotto et al., 1996; Zanon et al., 2015a,b).

COMPETITION AMONG SINKS

In plants, several sinks compete for the available photoassimilates. This generates a priority system among them, in which, during the early developmental stages, roots and young leaves are the main sinks, whereas, during the reproductive stages, fruit and seeds become priorities (Lemoine et al., 2013). Partitioning of carbohydrates within a tree is not a genetically programmed process, but it is the result of a combination of competing organs and their relative aptitudes to compete for assimilates (Lakso and Flore, 2003). Fruits attract photosynthates and thus increase the assimilate production of leaves; on the other hand, the deficiency of fruits in the canopy cause accumulation of carbohydrates in leaves (Fischer et al., 2012). However, a study on sour cherry demonstrated that the sink effect on photosynthesis is not obvious under all circumstances, and this can be ascribed to the masking effect of competing sinks, of source-limiting conditions, or to the environmental conditions which favor assimilates production (Flore and Layne, 1999).

The potential capacity of a fruit to accumulate assimilates (sink strength) depends on its size, its location, and distance from the source (Pavel and DeJong, 1993). Notwithstanding, in stone fruit trees, the presence of an elevated number of fruits alters the source-sink balance, by increasing assimilate accumulation into the fruits and/or by inducing an intensification of competition among them and between vegetative and reproductive growth (Morandi et al., 2008; Costa et al., 2018). This is also the case in olive (Rosati et al., 2017, 2018b,c). As a rule, high crop loads cause a decrease in tree growth (Intrigliolo and Castel, 2009), mainly during the last phases of fruit development (Intrigliolo et al., 2014). In addition, high crop load induces a condition of constrained carbohydrates supply, which amplifies competition among fruits and causes low fruit quality at harvest (Quilot and Génard, 2008; Abrisqueta et al., 2017). In olive, which is a drupe producing species, fruit production proportionally reduced shoot length, but the leaf biomass-to-shoot wood biomass ratio increased and this may be viewed as a plant strategy to better support fruit growth in high fruit load years, also given the greater and earlier ability of short shoots to export carbon (Rosati et al., 2018a). In other words, when there is a high fruit load, the vegetative growth is reduced, but there is a higher proportion of leaf biomass with respect to the wood biomass in new short shoots.

Shoot apices can also represent competitors for a growing fruit (Génard et al., 1998a). This observation can provide an explanation for the poor quality of fruits located in the terminal section of axes. Particularly, during the earlier phases of fruit growth, when cell division primarily occurs, shoot apex has been demonstrated to be the strongest sink both in peach and Japanese apricot (Corelli Grappadelli et al., 1996; Tsuchida et al., 2011). A recent research, carried out in different plum varieties, has pointed out the important role played by the mesocarp cell number in establishing fruit sink strength

(Cerri et al., 2019) and confirmed observations obtained in other species such as peach and Japanese apricot (Yamaguchi et al., 2002, 2004). In early ripening peach cultivars, the phase of cell division in fruit overlaps with that of intensive shoot growth, thus reducing fruit fresh weight (DeJong et al., 1987). On the contrary, in sweet cherry, fruits located near source leaf appear to have stronger sinks as compared with growing shoots, the effect being also modulated by leaf:fruit ratio (Ayala and Lang, 2008). As a rule, competition between vegetative and reproductive activity may be favorably handled in cultivars characterized by a clear separation between the two stages, as observed in different peach varieties (Ruiz-Sanchez et al., 2010).

ENVIRONMENTAL FACTORS AND MANAGEMENT PRACTICES INFLUENCING THE PRODUCTION OF STONE FRUIT TREES

Agronomical Factors

Thinning

A number of manipulations have been proposed to modulate the competition inside trees and to maximize commercial yield. In fact, even if fruit trees show fruit load self-regulatory systems, linked to the partitioning of assimilates to different organs (Dennis et al., 1983), these are frequently insufficient to obtain good fruit marketable size and quality at harvest. The balance among different competing organs is performed by fruit or flower thinning and is annually carried out on peach and nectarine, while occasionally on apricot, plum, and, in recent years, also on cherry (Costa et al., 2018). In different cultivars of sweet cherry, thinning induced an increase in fruit fresh weight through a positive effect on cell length (Olmstead et al., 2007); while, in other species, the response to this agronomical technique is controversial.

Thinning could affect competition for assimilates at a different level; in fact, reducing crop load leads to the increase of the sink strength of remaining fruits and affecting endogenous factors, such as hormonal and carbon balance, regulates the transition of buds to reproductive phase, thus determining the potential production of the following season (Costa et al., 2018).

Fruit thinning is primarily adopted for peach, and the most promising approach seems to be the "multiple application strategy," which allows monitoring the crop load at different phenological stages and implicates the application of chemicals and/or agronomical techniques to reduce fruit load (Greene and Costa, 2013). However, thinner response is affected by several internal and external parameters (i.e., genotype, environmental conditions; Costa et al., 2006). Therefore, thinning strategies may be fine-tuned, modulating thinning intensity in different part of the canopy and reducing the presence of fruits in the distal portion of 1-year old shoot and in the internal part of the tree. An early thinning, after an adequate fruit set, could regulate fruit fresh weight in apricot, possibly affecting the mechanism of assimilate transport between sources and sinks (Stanley, 2016).

Several studies investigating changes in fruit growth, total soluble solids (TSSs), pulp:stone ratio, fruit quality, and fruit diameter, along with the effects of leaf:fruit ratio were carried out. Thinning on mango "Lirfa" displayed the best results (highest fresh weight) when the leaf/fruit ratio was approximately 100 (Léchaudel et al., 2004). Other authors observed that thinning (optimal 40–50 leaves/fruit) positively affected sugar content and pulp:stone ratio in the "Rubidoux" peach (Fischer et al., 2012). On the other hand, a study on the effects of source-sink balance on quality parameters of two nectarine cultivars with different harvest time demonstrated that thinning induces a transient accumulation of soluble sugar in leaves, resulting in reduced photosynthesis and stomatal closure with no significant impact on final fruit size. Authors ascribe these controversial results to the influence of crop load on fruit-water relationships, possibly improved by the frequent irrigation in unthinned plants, allowing the fruit to reach its maximum potential (Andrade et al., 2019).

Webster and Spencer (2000) reviewed the strategies for crop load reduction to improve fruit quality in plum and apricot trees; they suggest that manual thinning is still the most precise and reliable method for these species. Authors also concluded that a better understanding of the climatic site, tree, and management factors influencing seasonal variations in fruit set and abscission is needed. This knowledge could be helpful to predict fruit set and abscission, which, in turn, would aid the grower in decisions on crop load adjustments.

An accurate trial recently demonstrated that fruit quality of the European plums is almost unaffected by either mechanical or chemical thinning, probably due to the utilization of an ethylene releasing compound. On the other hand, mechanical blossom thinning has been proposed to overcome or avoid alternate/biennial bearing (Seehuber et al., 2011). Interestingly, the same authors observed that stone fruit, as compared to pome fruit, mature within a shorter time, and a greater number of leaves are required for the same final fruit size. In addition, a lower number of fruits need to be removed to obtain a faster growth and a sugar increase in stone fruit, and an upper saturation threshold is rapidly reached without further effects.

Cultivar and Rootstock

Breeding programs have given rise to many stone fruit cultivars, aiming to meet several objectives, such as fruit quality, resistance to biotic and abiotic stresses, and the extension of the harvest season with early and late varieties. Source-sink relationships differ among early-, mid-, and late-ripening varieties, as evidenced, for example, by the diverse nutrient content of peach fruits. One of the main reasons is related to the shorter period of competition between leaves and fruits in earlier ripening cultivars. In fact, during the postharvest phase, trees are allowed to store nutrients in the permanent structures to sustain the first stages of growth in the following season (Zhou and Melgar, 2019).

In several stone fruit trees, rootstocks have been proposed to play a regulative role in the interaction with environmental conditions, and also in determining fruit quality (Caruso et al., 1996; Albás et al., 2004; Sitarek et al., 2005; Daza et al., 2008; Ruiz and Egea, 2008; Iglesias et al., 2019), through a regulation of photosynthesis, water relations, tree vigor, and diverse

reproductive traits (Zarrouk et al., 2005; Goncalves et al., 2006; Basile et al., 2007). Interacting with temperature and soil, rootstocks of different stone fruit trees regulate yield, fruit weight, and nutrient partitioning to the fruits (Layne, 1994; Rato et al., 2008). The effect on tree vigor appears to influence fruit quality both directly, modifying the competition between vegetative and reproductive activity, and indirectly, determining light interception/shading by the canopy (George et al., 2005; Font i Forcada et al., 2012; Gullo et al., 2014). Commonly, there is a tight relationship between bearing capacity and canopy size (Westwood, 1978). Rootstocks have also a role in the exchange of endogenous signals (such as hormones) with scion, and among the different plant organs, modifying the source-sink relationships (Tombesi et al., 2010; Minas et al., 2018).

In cherry, the choice of the right rootstock continues to be a topic of great interest due to its importance for productivity (Correia et al., 2017). Rootstocks have noteworthy aptitudes of adaptation to different growing conditions, but a significant effect of the rootstock on fruit quality has been demonstrated by different authors for both sweet cherry and peach (Giorgi et al., 2005; Usenik et al., 2010; Orazem et al., 2011). Several studies have investigated the influence of the scion/rootstock combination on cherry fruit quality (Whiting et al., 2005; Goncalves et al., 2006; Correia et al., 2017; Morandi et al., 2019), showing that water relations and photosynthesis of cherry trees are largely influenced by the rootstock genotype. Recently, the significant effect of different size-controlling rootstocks has been remarked; a semi-dwarfing (Gisela™6) rootstock, for example, allowed a higher productivity and fruit sugar content, thanks to the fruit increased accumulation of osmotic compounds and competitiveness toward shoots (Morandi et al., 2019). Moreover, fruit firmness varies according to scion and rootstock combination; "Burlat" cherries, for instance, decrease their firmness when grafted on CAB 11E (semi-vigorous rootstock) but they are firmer when grafted on Gisela 5 (dwarfing rootstock; Goncalves et al., 2006).

On the other hand, in peach, rootstocks of similar vigor can produce fruit of different quality, indicating that vigor is not the only parameter that affects fruit production, but the different genetic origin can play an crucial role in determining yield quality (Giorgi et al., 2005; Usenik et al., 2010; Orazem et al., 2011).

Orazem et al. (2011) investigated the effect of seven rootstocks grafted with Royal Glory and Redhaven peach cultivars on tree vigor, yield, and fruit quality. Authors showed that the degree to which rootstocks affect fruit weight, sugars, phenolics, and organic acids content levels varies from cultivar to cultivar.

These outcomes point out that the effect of rootstock is significantly intricate and cannot be measured by vigor alone. Therefore, field performance of the rootstock is still the main parameter for its choice, and its impact on fruit quality should not be disregarded.

Pruning

Winter and summer pruning are largely applied techniques in all fruit trees, including stone fruit trees, providing a valuable method for size control.

Several studies about the effect of summer pruning on carbohydrate content in peach and cherry have been carried out and report similar findings (Clair-Maczulajtys et al., 1994; Ikinci, 2014). Ikinci (2014) suggested summer pruning as a standard cultural technique in the management of peach trees. The study pointed out that this practice reduced shoot length, stimulated shoot diameter enlargement, decreased fruit yield, and increased fruit weight; in addition, this technique, if applied each year, increased significantly fruit soluble solids content (SSC). The increase in fruit size and quality has been attributed to higher photosynthate availability in the fruit of summer-pruned trees due both to the increment in photosynthetic photon flux density (PPFD) and to the elimination of competing sinks, i.e., watersprouts. The improved light exposure, in turn, may increase fruit sink activity, thus positively affecting fruit size, as shown for nectarines (Day et al., 1989). On the other hand, competition between vegetative and reproductive growth, that influences fruit abscission, is well-known for deciduous fruit trees. McFadyen et al. (2011) suggested that pruning in macadamia increases fruit drop and reduces yield, as the result of the combined negative effects of leaf removal and competition on carbohydrate availability from new shoots growth. Authors demonstrated that pruning determined an increase in fruit abscission and this effect was local and related to the aforementioned competition for carbohydrates.

It is also noteworthy that, in apricot, the position along the shoot, fruit number, and fruiting node leaf area have great importance on fruit quality; the higher FW and SSC are measured in fruits present on distal zones of 2-year-old wood and the lower in fruit from distal zones of 1-year-old wood. These observations suggest that the source-sink ratio should not be considered at the tree level, as diverse positions into the canopy can differentially regulate growth, for example, interacting with light or type of bearing structure. On the other side, results allow to develop more targeted orchard practices such as pruning or training system, favoring the presence of more efficient bearing structures, depending on the species, e.g., older wood (2–3 years) in apricot (Stanley, 2016).

Pre-harvest Fruit Bagging

Pre-harvest fruit bagging is commonly used as an effective approach in Japan, Australia, and China. Bagging is a physical protection technique, applied to different fruits, aiming to improve their appearance by promoting skin coloration and reducing the incidence of fruit cracking, but can also change the microenvironment for fruit development (Sharma et al., 2014). Pre-harvest bagging on peaches and nectarines has been investigated by several authors (Li et al., 2001; Zhang et al., 2015). Bagging modified SSC content, with a different trend, depending on the type of bag and species (Sharma et al., 2014). However, this could not be ascribed to the reduced light intensity but rather to the micro-environment modification that affected the rates of transpiration and respiration in fruits (Zhang et al., 2015).

Fertilization

It is known that plant nutrition could affect different traits of fruit quality such as fruit appearance, texture, and taste. Moreover, postharvest fruit properties, and mainly storage life length, appear to be influenced by fertilization, which has a role in susceptibility

of fruits to mechanical damage, physiological disorders, and decay (Cuquel et al., 2011). Nitrogen (N) and potassium (K) are among the most important nutrients needed by plants. However, an excess of N fertilization could negatively impact the stone fruits quality by diminishing flesh firmness and sweetness, decreasing red color development, and increasing susceptibility to postharvest diseases (Crisosto et al., 1997; Rettke et al., 2006; Cuquel et al., 2011).

Most of the effects of nutrient deficiency/supply on stone fruit quality and sugar content are indirect and they depend on the nutrient impact on the canopy growth and on the source-sink balance, which is species-specific and stage-specific. In sweet cherry, nitrogen fertilization favored the carbon allocation into fruits and fine-roots while hampering the vegetative growth (Artacho and Bonomelli, 2017).

In peach, even though flower density and fruit set are generally not affected by N, overall yields are decreased by N deficiency because of reduced fruit size and less fruiting sites due to shorter shoot (Layne and Bassi, 2008). Diversely, in low chill peach cultivars, the reduction of fruit quality after high N fertilization has been related to an increase of vegetative growth and, as a consequence, of fruit shading, or to an increase of competition for carbohydrates between fruits and shoots in a critical period (Wert et al., 2009).

However, considering that N is stored and recycled each year to sustain new growth, it is important to address peach orchard fertilization based on tree N status. It has been demonstrated that the increment in N supply over the optimum level for tree maintenance does not increase yield. Yet, a recent study suggests that lower N rates may advance fruit maturation by increasing color and soluble solids (Rubio Ames et al., 2020).

There is some controversy over the effect of K on fruit quality; in detail, no correlation between leaf K content and fruit soluble solids amount has been shown in peach (Layne and Bassi, 2008). However, Tagliavini and Marangoni (2002) provided evidence that fruit size, SSC, and color can all be improved when peach trees are not deficient in K (1.35–1.6% K in leaf).

Peach is much less sensitive to boron (B) deficiency than most other plants, and this is possibly due to the easily translocated compound that is formed from B and sorbitol (or other sugar alcohols) that accounts for the high mobility of this element (Hu et al., 1997; Layne and Bassi, 2008).

In apricot (cv. Canino), different levels of N, phosphorus (P), and K fertilizers could affect biochemical markers implicated in fruit quality. The authors pointed out that the mode of N distribution could have a major role in the quality of apricot fruits, but with negligible effects on sugar content. In addition, the N-K balance was found to be the most important parameter to increase both sugars and phenolic compounds accumulation (Radi et al., 2003).

Environmental Factors

Light and Temperature

Environmental and soil conditions interact with endogenous factors and management practices to set the response of the tree and the productive performance. For instance, unsuitable winter temperature may only partially fulfill the chilling requirement, with negative consequences on regular flower formation (Petri and Leite, 2004; Blanke and Kunz, 2009) and reproductive activity. In peach, it has been demonstrated that different environmental factors (e.g., temperature, light, and precipitation) and field practices (such as irrigation, rootstocks, pruning and training system, and thinning) could affect the assimilate partitioning into the fruits, by changing both plant photosynthetic source efficacy and, therefore, carbohydrates availability and individual sink strength (Cirilli et al., 2016). A number of models were developed trying to estimate the effect of several factors on source-sink relationships and to improve efficiency in peach and other fruit crops (Quilot et al., 2004; Lescourret and Genard, 2005; Sonnewald and Fernie, 2018). Besides the number of fruits present on the tree and the fruit:shoot balance, also the relative position on the canopy of source and sink organs may influence the movement of resources (Génard et al., 1998b). In apricot, the higher SSC content of fruit was related both to light exposure and the type of bearing structure (Dichio et al., 1999; Lichou et al., 1999; Stanley et al., 2014). Longer shoots show lower fruit set as compared with spurs and maybe as a consequence, in the latter, of higher starch content in ovaries and ovules (Julian et al., 2010), earlier flower initiation during previous season (Sakayarote et al., 2005), and lower chilling requirement (Austin et al., 1992).

Light availability influences fruit development directly through primary carbon metabolism, and indirectly, favoring the translocation of assimilates from reserves accumulated in more exposed parts of the trees or by increasing fruit temperature, which enhances its sink activity (Génard and Bruchou, 1993; Cherbiy-Hoffmann et al., 2013; Reale et al., 2019).

The sunlight use efficiency has long been investigated in order to improve fruit quality at harvest. An optimum photosynthetically active radiation (PAR) interception of orchard may be achieved considering different cultural choices as orchard design, training system, and pruning that, as a whole, may also improve PAR distribution into the canopy (Jackson, 1980; Corelli Grappadelli and Lakso, 2007; Reale et al., 2019).

The interaction between light effect and canopy position has been pointed out in peach, where fruits with poor quality are usually located on older wood in the lower and central part of the tree (Luchsinger et al., 2002). This relationship was also confirmed by artificial shading that, especially if applied during the final phases of fruit growth, when assimilated accumulation is high, induces a reduction of SSC (George et al., 1996). Therefore, peach appears to be particularly sensitive to light exposure, especially in stimulating soluble sugars accumulation during the final phases of fruit growth (3 weeks) and in determining fruit quality (Marini et al., 1991; Gullo et al., 2014). Reale et al. (2019) recently demonstrated that the effect of light availability on olive fruit development is cultivar-specific, and the greater sensitivity was related to a delay in the endocarp lignification.

The exposure of fruit to sunlight also induces an increase of fruit temperature, and through an effect on respiration and transpiration rates, of sink strength (Marini et al., 1991; Pavel and DeJong, 1993; Génard and Baret, 1994). However, excessive temperature, especially during the earlier phases of growth, may induce a reduction of fruit developmental period and SSC accumulation (Lopresti et al., 2016).

Soil Conditions

Among factors that have a decisive impact on orchard production and that can be controlled are the level of macro- and micro-elements in soil. The influence of fertilizer's application, fruit species and cultivars, rootstock, and its vigor on plant nutrition has been pointed out in the previous sections. Similarly, mineral nutrient status can affect assimilate partitioning either directly by modulating phloem loading and transport or indirectly by depressing sink demand (Marschner et al., 1996). On the other hand, plants deficient in macroelements improve their ability to acquire these nutrients by altering their carbon partitioning to favor root growth (Hermans et al., 2006).

Soil conditions, such as water content, soil type and structure, organic matter content, and soil pH, affect individually the availability of nutrients, but also biological soil properties are pivotal. In general, macroelements are available to the root of fruit tree in a wider range of soil pH; in contrast, the availability of micronutrients relates closely to soil pH. The positive effect of soil organic matter content on availability and assimilation of nutrients has long been known. Naturally, organic matter or organic fertilizers, when applied to the soil in the fruit orchards, may be mineralized over time by soil microorganisms, which would increase the total organic C content and the metabolic activity of the microorganisms (Milošević and Milošević, 2020). Beside their role in mineralizing organic nutrients for fruit tree growth and development, microorganisms such as symbiont fungi (i.e., arbuscular mycorrhizal fungi) can increase plant's nutrient uptake, especially P, but also plant's tolerance to drought conditions, salinity, and biotic stresses, as reported for peach and peach-almond hybrids (Calvet et al., 2004), with indirect beneficial effects on the fruit quality (Ortas, 2018).

Water Availability

Another important environmental factor influencing physiological and metabolic activity in plants is water availability, and deficit irrigation (DI) has been adopted in several stone fruit species to regulate competition between vegetative and reproductive growth (Pérez-Pastor et al., 2007; Ruiz-Sanchez et al., 2010; Lopresti et al., 2014). In peach and nectarine, a multi-year research pointed out that DI favors carbon partitioning to fruits as compared to vegetative growth, and positively affects fruit quality, although the influence is also related to other factors such as stress intensity and period of application, crop load and thinning, and cultivar precocity (Thakur and Singh, 2013; Falagán et al., 2015; De la Rosa et al., 2016). Particularly, in extra-early nectarine trees, the postharvest phase represents a non-critical period for fruit to apply DI, even if during this stage floral differentiation occurs and assimilates are mainly accumulated to sustain the following season's early growth (Handley and Johnson, 2000). Moreover, postharvest DI reduces flower number in the subsequent year without affecting fruit quality, conceivably by a reduction of tree size (Girona et al., 2005). In early nectarines, DI treatments induce a reduction of tree size, therefore suggesting to adapt the thinning strategy to this issue (Vera et al., 2013).

In apricot, water stress imposed after harvest did not significantly affect tree yield of the following year, but improved fruit quality. However, when DI was applied during the stages II and III of fruit development, a lower production was obtained, due to a reduction of fruit size and vegetative growth, respectively (Pérez-Pastor et al., 2007, 2009). On the other hand, almond yield appears almost unaffected by moderate water stress, even when applied during kernel development, possibly as a result of intense sink strength exhibited by the fruits during this phase (Lipan et al., 2019).

Stone fruit trees with higher crop load showed an higher susceptibility to water stress, which maybe due to a reduced partitioning of carbon toward above ground organs and, therefore, to an impaired root growth (Berman and DeJong, 1996; Lopez et al., 2007; Intrigliolo et al., 2013). Therefore, DI and thinning could be efficaciously adopted to regulate source-sink relationships into the trees, allowing, in different genotypes and environmental conditions, the achievement of optimal fruit production and quality (Embree, 2007; Intrigliolo and Castel, 2010; Intrigliolo et al., 2013).

CONCLUDING REMARKS

Environmental factors exert a strong control on the timing of plant phenology, and understanding their effects is an essential step to allow predictions about plant responses, to optimize source-sink relationships in crops, and to target orchard management practices.

In the recent decades, the rate of climate changes increased dramatically, especially as regards average temperature, and this has triggered mechanisms of plant adaptation to the modifying environmental conditions. One of the main determinants of global warming is considered to be the concentration of CO_2 in atmosphere, which is rapidly increasing, and is considered to be reached in 2017, a level of 405 ppm (Dlugokencky et al., 2018). In the short term, these changes have a positive impact on plant photosynthesis, and on sucrose production into the leaves, and depending on the species, plants rearrange their source-sink relationships (Makino and Tadahiko, 1999), mainly by reducing the root:shoot ratio (Farrar and Williams, 1991). However, in the long term, the increase in leaf sucrose concentration can activate a negative feedback on photosynthesis, with a detrimental effect on the productivity (Lemoine et al., 2013). Therefore, the new scenarios have to be taken into account to elucidate the mechanisms adopted by the plants to cope with these changes and to develop strategies to improve yield and fruit quality.

AUTHOR CONTRIBUTIONS

All authors have contributed significantly to the work and approved it for publication.

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Metabolite Fruit Profile Is Altered in Response to Source–Sink Imbalance and Can Be Used as an Early Predictor of Fruit Quality in Nectarine

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Peaches and nectarines [*Prunus persica* (L.) Batsch] are among the most exported fresh fruit from Chile to the Northern Hemisphere. Fruit acceptance by final consumers is defined by quality parameters such as the size, weight, taste, aroma, color, and juiciness of the fruit. In peaches and nectarines, the balance between soluble sugars present in the mesocarp and the predominant organic acids determines the taste. Biomass production and metabolite accumulation by fruits occur during the different developmental stages and depend on photosynthesis and carbon export by source leaves. Carbon supply to fruit can be potentiated through the field practice of thinning (removal of flowers and young fruit), leading to a change in the source–sink balance favoring fruit development. Thinning leads to fruit with increased size, but it is not known how this practice could influence fruit quality in terms of individual metabolite composition. In this work, we analyzed soluble metabolite profiles of nectarine fruit cv "Magique" at different developmental stages and from trees subjected to different thinning treatments. Mesocarp metabolites were analyzed throughout fruit development until harvest during two consecutive harvest seasons. Major polar compounds such as soluble sugars, amino acids, organic acids, and some secondary metabolites were measured by quantitative ^{1}H-NMR profiling in the first season and GC-MS profiling in the second season. In addition, harvest and ripening quality parameters such as fruit weight, firmness, and acidity were determined. Our results indicated that thinning (i.e., source–sink imbalance) mainly affects fruit metabolic composition at early developmental stages. Metabolomic data revealed that sugar, organic acid, and phenylpropanoid pathway intermediates at early stages of development can be used to segregate fruits impacted

by the change in source–sink balance. In conclusion, we suggest that the metabolite profile at early stages of development could be a metabolic predictor of final fruit quality in nectarines.

Keywords: *Prunus persica*, thinning, sugars, phenylpropanoid, organic acids

INTRODUCTION

Peaches and nectarines [*Prunus persica* (L.) Batsch] are among the most important fruit crops with a world annual production of approximately 25 million tons (Food and Agriculture Organization of the United Nations (FAOSTAT), 2018). They belong to the Rosaceae family, whose species have developed a wide array of fruit types, including drupe, pome, drupetum, achene, and achenetum. *P. persica* displays a drupe-type fruit, where a fleshy juicy mesocarp encloses a lignified endocarp surrounding a seed. The presence of this lignified endocarp imposes a challenge to the proper fruit development and ripening, since the phenylpropanoid allocation must be carefully controlled for the endocarp lignification during development and the biosynthesis of flavor/aroma compounds in the ripe fruit mesocarp (Dardick and Callahan, 2014). The main difference between peaches and nectarines is the absence of trichrome in the surface of nectarines. *P. persica* is a climacteric fruit in which development and ripening are coordinated processes involving physiological, molecular, and biochemical changes (Moing et al., 1998; Lombardo et al., 2011). Fruit growth in this species follows a double sigmoidal curve with four stages clearly defined (S1–S4; Chalmers and van den Ende, 1975; Tonutti et al., 1991). The first stage (S1) corresponds to the first exponential growth due to an increase in cellular division and elongation. The second stage (S2) corresponds to endocarp lignification, which leads to an arrest in growth rate due to the high carbon and energy demand to sustain phenylpropanoid pathway (Dardick et al., 2010). In the third stage (S3), known as the second exponential growth, fruit growth is mainly due to cell enlargement because of water entrance. At the end of S3, the fruit reaches its final size and its background is green. At this moment, the fruit enters the fourth stage (S4) and can be harvested (Ognjanov et al., 1995; Lombardo et al., 2011). The S4 stage corresponds to the ripening stage. At S4 I, fruit is no longer inflated and does not release ethylene. At S4 II, fruit releases little ethylene. At S4 III, ethylene autocatalytic production increases and fruit rapidly softens (Pan et al., 2015). Peaches and nectarines as climacteric fruit show a rise in respiration and ethylene biosynthesis rates at the beginning of ripening (Carrari and Fernie, 2006; Cherian et al., 2014; Karlova et al., 2014). Ethylene induces changes in color, texture, flavor, and aroma, which all together improve the fruit nutritional value and attractiveness promoting its consumption and seed dispersal (Liu et al., 2004; Goff and Klee, 2006).

Fruit development and growth depend on photoassimilates imported from source leaves. In Rosaceae species such as peaches and nectarines, sucrose and sorbitol are the sugars translocated from source leaves to the fruit, which are non-autotrophic sink organs (Lo Bianco et al., 1999). The amount of sugars that will arrive in a fruit depends on the force attracting these "translocated sugars" known as sink strength. The competition with other sink organs (other fruits) also affects the partitioning of carbon in a tree. Once the translocated sugars are unloaded from phloem, they are metabolized into glucose and fructose in the case of sucrose, and into fructose in the case of sorbitol. These hexoses can be used to sustain aerobic respiration or they can be derived to other metabolic pathways for the synthesis of structural carbohydrates, amino acids, and other biomolecules related to growth and development (Génard et al., 2003). Fruit quality is strongly related to metabolite composition and balance (Colaric et al., 2005). Indeed, taste is mainly dependent on the balance of organic acids-to-sugar ratio, conferring acidity and sweetness, respectively (Kader, 2008; Brasil and Siddiqui, 2018). Accumulation of soluble sugars and organic acids related to organoleptic properties occurs at the late stages of fruit development and may occur through direct phloem unloading and through interconversion of metabolites by gluconeogenesis (Génard et al., 2003).

Agronomical practices like thinning consist in the removal of fruits or flowers in order to modify the source–sink balance favoring growth and advancing harvest of the fruits that remain on the tree (Grossman and DeJong, 1995; Link, 2000; Lesičar et al., 2016). Thinning is often performed in commercial orchards to increase final fruit size. The recommendation is to perform thinning in early fruit development stages to take advantage of the availability of photoassimilates (Grossman and DeJong, 1995; Costa and Vizzotto, 2000; Reighard et al., 2018; Sutton et al., 2020). Nevertheless, when thinning is performed too early, productivity may be affected by spring frosts (Byers and Marini, 1994).

Thinning practice is known to improve fruit size and in some cases also total soluble solids (TSS) content (Costa et al., 2018), but it is not clear how this practice could influence fruit quality in terms of individual metabolite composition such as major soluble sugars (Wu et al., 2011). In this work, we analyzed the primary metabolite profile of nectarine fruits cv "Magique" at different developmental stages and from trees subjected to different thinning treatments. Mesocarp polar metabolites were analyzed throughout development until harvest during two consecutive harvest seasons. In addition, harvest and ripening quality parameters such as fruit weight, firmness, acidity, and TSS content were measured. The aim of this work was to evaluate if thinning could alter the metabolic profile of fruits.

MATERIALS AND METHODS

Plant Material and Thinning Treatments

The experiments were performed using 4 year-old early harvest nectarine trees [*P. persica* (L.) Batsch var. "Magique"] from the

commercial orchard "Viveros El Tambo" located in El Tambo, O'Higgins Region of Chile (34°28′30.4″S 70°59′07.5″W) from August to December 2013 (first season), and from August to December 2014 (second season). Full bloom was on August 30, 2013, for the first season and on August 13, 2014, for the second season. For the first season, thinning was performed at 42 days after bloom (DAB), while for the second season, it was performed at 63 DAB. The thinning treatments comprised unthinned trees (UTH) that consisted in maintaining the whole fruit load resulting in a final ratio of six leaves per fruit and thinned trees (TH) that consisted in removing the fruit to the proportion 40 leaves per fruit, which is used in commercial orchard. Considering that branches of *P. persica* are autonomous concerning carbon assimilation (Volpe et al., 2008; Andrade et al., 2019), the branches were considered as biological replicates in each tree (with three trees per treatment in each year). Samples for metabolite and phenotyping measurements were harvested using four branches of each tree/thinning treatment (n = 12) every 7 days during the whole season in a randomized block design. Fruit growth was determined by measuring the equatorial diameter of 10 fruits per treatment weekly from 5 DAB until harvest at 118 DAB in the first season and from 27 DAB until harvest at 138 DAB in the second season. Once the fruits were harvested, they were stored at 20°C for 11 days until reaching the "ready to eat" stage.

Quality Parameters

For both seasons, nectarines were harvested based on fruit firmness values (40–50 N). Recently harvested fruits were transported to the laboratory to measure weight, diameter, TSS using a manual refractometer, firmness of two sides of the fruit using a penetrometer, and titratable acidity (using NaOH 0.1 N) of 30–36 nectarines per tree that belonged to different branches (**Supplementary Table S1** and **Supplementary Figure S1**). The same measurements were performed at "ready to eat" nectarines apart from the juice percentage (absorbent tissue, Infante et al., 2009). The variability was evaluated by a box plot analysis (**Supplementary Figure S1**) and fruits with outlier values were not used (values shaded in gray in **Supplementary Table S1**). All the nectarines were cut, endocarp was discarded, and mesocarp was frozen in liquid nitrogen and stored at −80°C for further analysis.

Lignin Staining

To determine the start of pit hardening, lignin staining was performed as described by Dean (1997). Fruit was sectioned in transverse and longitudinal sections and then placed immediately in phloroglucinol-HCl staining solution (5% phloroglucinol, 85% ethanol). The excess was removed and fuming HCl was added to start the reaction causing the lignin to become magenta. The samples were washed using 95% ethanol.

RT-PCR of Fruit Development Gene Markers

A piece of frozen fruit was ground using a mortar and pestle previously chilled with liquid nitrogen, then 3 g of sample was used for the RNA extraction following the protocol of Gudenschwager et al. (2012). One microgram of RNA treated with 2 U of DNase I (Invitrogen, CA, EE.UU) was used for cDNA synthesis using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen). The sequences of the primers used in the qRT-PCR assays are listed in **Supplementary Table S2**.

Extraction of Polar Metabolites and Measurement by 1D ^{1}H-NMR

Polar metabolites were extracted from mesocarp lyophilized powder (30 mg DW) using a hot ethanol/water series and determined using proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) profiling as described previously (Botton et al., 2016). pH-adjusted extracts in 200 mM deuterated phosphate buffer were analyzed using a 500 MHz Avance III spectrometer (Bruker, Wissembourg, France) with a BBI 5 mm Bruker probe. The ERETIC method was used for quantification of absolute concentration of all identified metabolites with three calibration curves (glucose and fructose: 2.5, 5, 10, 25, 50, and 100 mM; quinic acid: 1, 2.5, 5, 10, and 15 mM) using Amix Bruker v. 3.9.14 software (**Supplementary Data S6**). The glucose calibration curve was used for the quantification of all metabolites, as a function of the number of protons of selected resonances, except fructose and quinic acid that were quantified using their own calibration curve. The content of each organic acid was expressed as g of the acid form per weight unit. The metabolite concentrations in each NMR tube and the contents in fruit were calculated using AMIX (version 3.9.14, Bruker) and Excel (Microsoft, Redmond, WA, United States) software.

Extraction of Polar Metabolite and Measurement by GC-MS

The extraction of polar metabolites was carried out with the protocol described by Hatoum et al. (2014). Briefly, 20 mg of lyophilized mesocarp was placed in a tube containing 500 µl of cold methanol, and 20 µl of 2,910 ng/µl phenyl β-D-glucopyranoside was added as an internal standard. Tubes were then incubated at 70°C for 15 min using a shaking incubator (VorTemp™ 56, Labnet, Woodbridge, NJ, United States). Tubes were centrifuged for 20 min at 14,000 *g* and then the precipitate was discarded. One hundred microliters of the supernatant was dried using a stream of nitrogen gas. For derivatization, 120 µl of methoxyamine solution (Sigma-Aldrich, St. Louis, MO, United States) and pyridine 20 mg/ml (Sigma-Aldrich) was added to the dry sample and shaken for 90 min at 30°C. To each tube, 120 µl of BSTFA [*N*-*O*-Bis(trimethylsilyl)trifluoroacetamide] (Sigma-Aldrich) was added and shaken for 30 min at 37°C. The content of each tube was transferred into a vial with a micro insert.

Metabolomic analysis was performed by gas chromatography–mass spectrometry (GC-MS). Data were obtained using the protocol described by Fuentealba et al. (2017). Briefly, 1 µl of sample was injected on the GC column of an Agilent GC-MS system (GC7890 with a 5,977 single quadrupole MS with electron impact ionization source; Agilent Technologies, Palo Alto, CA, United States). Each derivatized extract was

analyzed twice; a split (1:150) method was used for the abundant compounds such as major sugars and a splitless mode for the less abundant compounds such as organic acids and amino acids. The GC column used was an HP-5-MS capillary column of 30 m length, 0.25 mm internal diameter, and 0.25 μm film thickness (Agilent Technologies). For both methods (split and splitless), the injection and interface temperatures were 220 and 280°C, respectively. Helium was used as carrier gas with a constant flow of 1 ml/min. The GC temperature program started isothermal at 50°C for 1 min (acids method) or at 120°C for 1 min (sugar method) and was then ramped at a rate of 10°C/min to 310°C where it was kept for 13 min (acid method) or to 300°C for 6 min (sugar method). The total run time was 40 min for the acid method and 25 min for the sugar method. Mass spectra in the 50–600 *m/z* range were recorded at a scanning speed of 2.66 scan cycles per second. The MS ion source and quadrupole temperatures were 230 and 150°C, respectively.

Mass Hunter Data Analysis Software (Agilent Technologies) was used to deconvolute the chromatographic peaks. Identification was performed by comparing the peak retention and mass spectra to the NIST library in the quantitative method. Raw peak area data were corrected using the actual peak area of the internal standard, the sample fresh weight, and a quality control (QC) sample representative of all samples.

Extraction of Water-Soluble Sugars and Measurement by HPAEC-PAD

The extraction of soluble sugars was performed following the protocol described by Schmitzer et al. (2011) with modifications. Three milligrams of lyophilized powder mesocarp was gently mixed with 2 ml of Milli-Q water per 2 h at room temperature. The supernatant was filtered using 0.45 μm nylon and then diluted 1:2. Sugars were measured using the HPLC Dionex DX-500 system equipped with two CarboPac PA1 (4 mm × 250 mm) analytical columns connected in series, a CarboPac PA1 (4 mm × 50 mm) guard column, and a pulse amperometric detector. Soluble sugars were separated at a flow rate of 1.5 ml min^{-1} at 40°C. The elution protocol consisted on isocratic gradients of 100 mM NaOH for 25 min. Finally, a washing step with 20 mM NaOH for 10 min was performed. Sugar content (myo-inositol, sorbitol, fructose, glucose, and sucrose) was determined by reference to a standard curve from 10 to 200 μM.

Statistical Analyses

Phenotypic data were analyzed using Student's *t*-test for mean comparisons. For metabolite data, principal component analysis (PCA), partial least square regression discriminant analysis (PLS-DA), and clustering analysis with Euclidian distance were performed on the normalized data from NMR and GC-MS data using Metaboanalyst 4.0 (Chong et al., 2019). All variables were mean-centered and reduced to unit variance before PCA, PLS-DA, and clustering analysis. For the clustering analysis for the different features, Euclidean distance similarity measure and ward.D clustering algorithm were used with the hclust function in stat package using the top 24 compounds filtered by one-way ANOVA.

RESULTS

Characterization of Fruit Development

In this study, we evaluated two harvest seasons of nectarine cv "Magique." On both seasons, trees subjected to standard commercial thinning treatments were evaluated. During both seasons, the pattern of fruit growth was the typical double-sigmoid curve in accordance with the behavior reported previously (Chalmers and van den Ende, 1975; Tonutti et al., 1991). Fruits continuously grew in an exponential rate starting at 20 DAB. This first stage of development (S1) was characterized by a first increase of fruit size, which in the first season lasted until 68 DAB, and in the second season until 70 DAB (**Figure 1A**). The end of S1 stage was determined by the beginning of lignin production in the endocarp (pink staining, **Figure 1B**). These results were consistent with the transcript expression of dehydration-responsive protein RD22 (RD22-like protein, **Figure 1C**), a marker gene that is expressed in fruit mesocarp mainly at this stage; this transcript is under control of ABA and seems to be related to stress response (Bonghi et al., 2011). Thinning treatment was performed during S1 stage in both seasons. In the first season, thinning was performed earlier than the second season: 42 DAB compared to 63 DAB.

Stage 2 (S2) of fruit development was characterized by pit hardening and little or no increase in fruit size, especially in early to mid-harvest nectarines such as "Magique" variety. End of S1 and start of S2 were determined based on the beginning of stone lignification (lignin becomes pink using phloroglucinol as stained in **Figure 1B**). During the first season, S2 lasted from 68 DAB to 88 DAB (**Figures 1A,B**), and in the second season, it lasted from 70 DAB to 100 DAB. In this stage, we observed the first differences in fruit size between thinned and unthinned trees in the first season, when fruits from thinned trees started to become significantly larger (Tukey test, $P < 0.05$) than fruits from unthinned trees. This trend was maintained until the end of fruit development.

In stage 3 (S3), a second increase of fruit size occurred, which corresponded to the period of 90 DAB until harvest at 118 DAB for the first season, and of 100 DAB until harvest at 138 DAB for the second season. The larger differences in fruit diameter between the thinning treatments were observed in S3 in both seasons (**Figure 1A**). In the first season, the rate of fruit growth during S3 was slower in unthinned trees compared to thinned trees. Stage 4 (S4) corresponded to the postharvest period, when the fruit ripens. The harvest maturity was corroborated with the expression of auxin-responsive protein IAA gene (Aux/IAA), a marker gene for S4 (Bonghi et al., 2011; **Figure 1C**).

The effect of thinning treatment on final fruit size was observed in the first season only (**Figure 1A**) when fruits from thinned trees were considerably larger than fruits from unthinned trees.

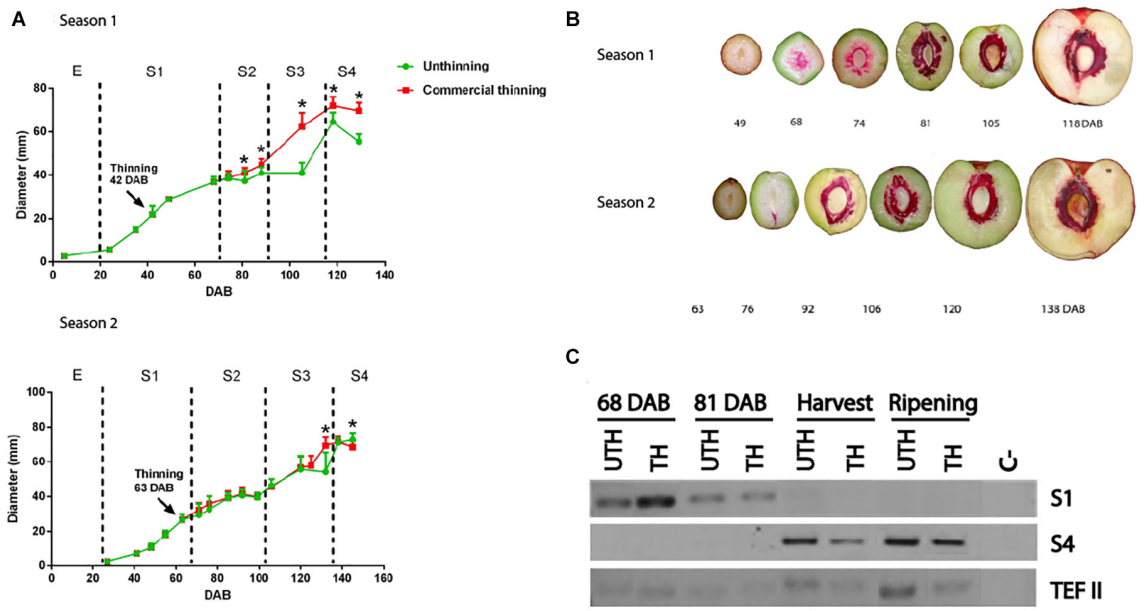

FIGURE 1 | "Magique" nectarine fruit development. **(A)** Fruit growth curve. Equatorial diameter was measured throughout development of fruits during two seasons. Thinning practice was done at 42 DAB in the first season (top) and at 63 DAB during the second season (bottom) at stage 1 (S1). Green lines represent fruits from unthinned (UTH) trees and red lines from thinned (TH) trees. Each point represents the mean of 10 fruits and the bars represent SD. Asterisks denote statistical difference of fruit diameter between UTH and TH trees (Tukey test, $P > 0.05$). **(B)** Progression of lignin deposition in developing nectarine fruit. Sectioned fruits were stained with phloroglucinol-HCl for lignin staining (pink color). **(C)** Development stage gene marker for S1 (RD22-like gene) and S4 (Aux/IAA gene) measured by RT-PCR in mesocarp of nectarines from season 1 in four stages of development using fruits from UTH and TH trees. The reference gene was Translation elongation factor 2 (TEF II).

TABLE 1 | Maturity and physiological parameters of "Magique" nectarines at harvest and ripening (shelf-life at 20°C).

	Harvest				Ripening			
	1st Season		2nd Season		1st Season		2nd Season	
	UTH	TH	UTH	TH	UTH	TH	UTH	TH
Firmness (N)	44.69 ± 2.29*	47.51 ± 1.42*	41.76 ± 9.08	44.97 ± 2.91	12.54 ± 5.02*	0.43 ± 0.72*	6.39 ± 2.65	8.89 ± 7.35
Weight (g)	135.8 ± 21.1*	185.1 ± 37.5*	186.4 ± 20.1	192.4 ± 21.6	90.14 ± 14.1*	175.9 ± 32.8*	193.8 ± 33.4	173.0 ± 26.2
TSS (° Brix)	10.81 ± 1.23*	12.31 ± 1.49*	11.77 ± 2.40	10.97 ± 1.86	10.22 ± 1.46*	12.33 ± 1.87*	12.04 ± 1.85*	8.46 ± 1.35*
Acidity (%)	–	–	–	–	0.15	1	1.36 ± 0.19	1.61 ± 0.30

*TSS, total soluble solids; UTH, unthinning; TH, thinning. *Values (means) followed by asterisks are significantly different between thinning treatments at Tukey test $P < 0.05$.*

Thinning Treatment Modified Quality Parameters of "Magique" Nectarine Differently in the Two Seasons

To evaluate quality parameters of fruits such as firmness, weight, TSS, and titratable acidity, these parameters were measured at harvest and "ready to eat" stages in the two seasons (**Table 1**). In both seasons, fruits were harvested with a firmness value of around 40–47 N. After 10 days at 20°C, firmness steeply decreased. In the first season, fruits from unthinned (UTH) trees presented a firmness of 12.5 N at the ready-to-eat stage while fruits from thinned (TH) trees soften much more rapidly reaching 0.4 N at ripe stage. In the second season, the softening of UTH and TH fruits was very similar, reaching 6.4 and 8.9 N of firmness at ripe stage, respectively (**Table 1**). On one hand, in the first season, TH fruit weight was significantly higher than UTH fruit weight in both stages (Tukey test, $P < 0.05$). On the other hand, in the second season, no differences in fruit weight were observed between treatments (**Table 1**). During the first season, TH fruits displayed significantly higher TSS than UTH fruits at harvest and ripening stages. Unfortunately, only one fruit from thinning treatment was analyzed for acidity, so in this point, no statistical analysis could be performed. However, fruits from TH trees in the second season showed no differences in TSS compared to fruits from UTH trees at harvest, but when they ripened, fruit from TH trees had significantly lower TSS than fruit from UTH trees (**Table 1**). For acidity in the second season, no differences were found between fruits from TH or UTH trees at ripe stage.

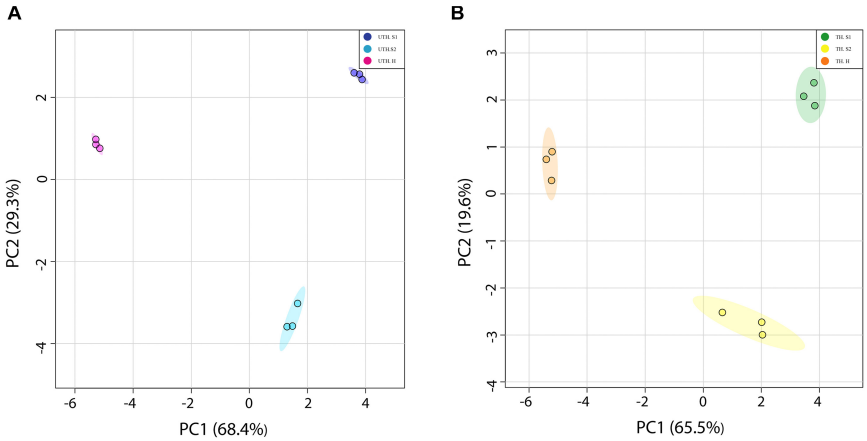

FIGURE 2 | Principal component analysis (PCA) of "Magique" nectarine metabolites detected using ^{1}H-NMR. The detected metabolites were employed as predictor variables, and the stages of development were used as response variables. The left panel **(A)** shows the score plot of S1, S2, and harvest (H) stages from unthinned (UTH) trees during the first season, where the explained variance by each principal component corresponded to 68.4 and 29.3% for PC1 and PC2, respectively. The right panel **(B)** shows the score plot of S1, S2, and harvest (H) stages from thinned (TH) trees during the first season, where the explained variance by each principal component corresponded to 69.5 and 19.6% for PC1 and PC2, respectively. Dark blue circles represent UTH S1, light blue circles represent UTH S2, and pink circles represent UTH harvest (H) fruit samples. Green circles represent TH S1, yellow circles denote TH S2, and orange circles represent TH H fruit samples.

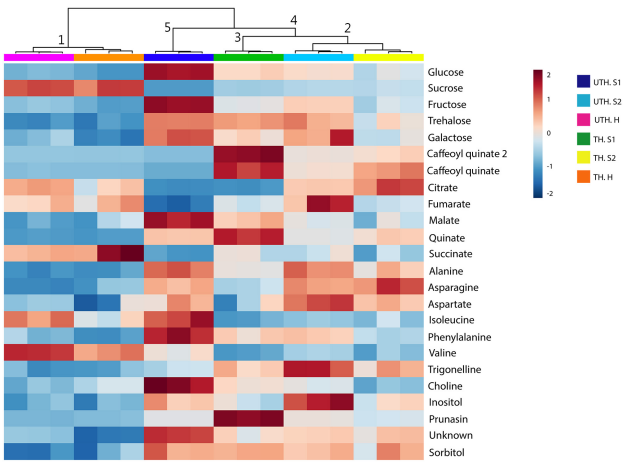

FIGURE 3 | Heatmap analysis of "Magique" nectarine metabolites detected by ^{1}H-NMR at S1, S2, and harvest (H) stages from unthinned (UTH) and thinned (TH) trees during the first season. The analysis was based on the top 24 significant metabolites revealed by ANOVA. The columns represent the biological replicates for each stage (S1, S2, and Harvest). Dark blue rectangles represent UTH S1, light blue rectangles represent UTH S2, and pink rectangles represent UTH harvest (H) fruit samples. Green rectangles represent TH S1, yellow rectangles denote TH S2, and orange rectangles represent TH H fruit samples. Cluster numbers are indicated on the upper side of the figure. The distance measure for the different features was Euclidean and the clustering algorithm corresponded to Ward.

Effect of Thinning Treatment on Metabolite Profiles of "Magique" Nectarines

Metabolite profiles (mainly primary metabolites) were determined using ^{1}H-NMR metabolomics profiling in three stages of development: S1 (68 DAB), S2 (81 DAB), and harvest (118 DAB) in the first season. A total of 24 polar metabolites were quantified in all stages of development and thinning treatment (**Supplementary Tables S3**, **S4**). The data of each thinning treatment were evaluated separately by an unsupervised multivariate statistical analysis using the stage of development as response variables and the identified metabolites as the predictor variables. PCA of nectarine metabolites that belong to UTH trees was able to explain 68.4 and a 29.3% of total variance with the first two components (**Figure 2A**). Similarly, PCA of TH was able to explain 65.5 and 19.6% for PC1 and PC2, respectively (**Figure 2B**). Interestingly, both projections clearly separate by developmental stages and the distribution of groups was similar as well. PC1 tended to separate earlier stages (S1 and S2) on the positive side from harvest (H) on the negative side. In the second season, the metabolite profiles were measured by GC-MS, and no compositional differences between UTH and TH fruits were found for the 124 compounds quantified (**Supplementary Table S5** and **Supplementary Figure S2**), in agreement with no changes in phenotypic parameters such as fruit weight.

In order to determine how thinning treatment affected metabolite profile, a clustering analysis was performed based on the top 24 metabolites shown by ANOVA in the first season and visualized with a heatmap (**Figure 3**). Harvest samples (H) of both thinning treatment grouped together in cluster 1. Cluster 2 grouped S2 of both UTH and TH, and shared node with cluster 3 that grouped only S1 TH. S1 UTH clustered separately from S1 TH. The main differences in accumulation of metabolites in nectarine samples between thinning treatments were found in S1, in a lesser extent in S2, and a few differences in H. For example, caffeoyl quinate, caffeoyl quinate 2, and prunasin were completely absent in S1 UTH, while in S1, TH were highly abundant. On the other hand, several metabolites were more accumulated in S1 UTH than S1 TH such as glucose, fructose, malate, isoleucine,

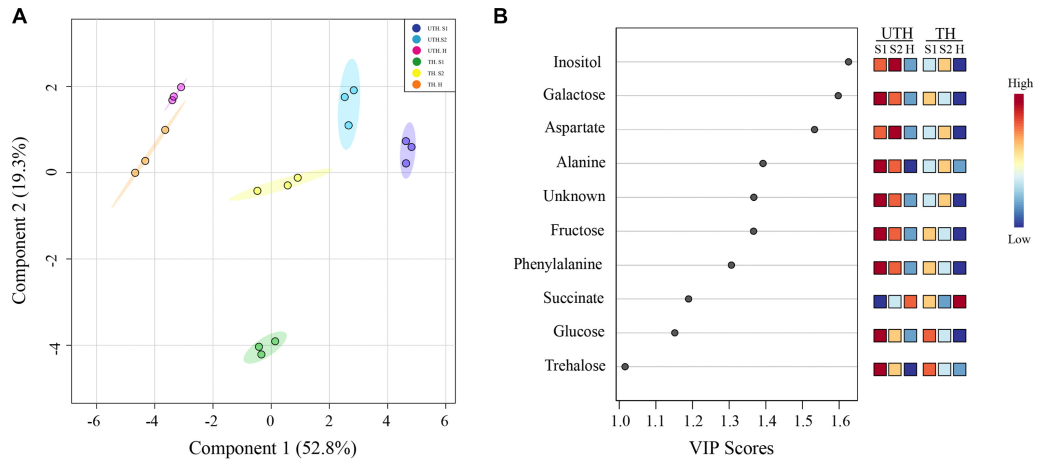

FIGURE 4 | Partial least square discriminant analysis (PLS-DA) of "Magique" nectarine metabolites detected using ^{1}H-NMR for first season. The detected metabolites were employed as predictor variables, and the stages of development and thinning treatments were used as response variables. The left panel **(A)** shows the loading plot where the explained variance by each component corresponded to 52.8 and 19.3% for C1 and C2, respectively. Dark blue circles represent UTH S1, light blue circles represent UTH S2, and pink circles represent UTH harvest (H) fruit samples. Green circles represent TH S1, yellow circles represent TH S2, and orange circles represent TH H fruit samples. The right panel **(B)** shows the top 10 variable importance in projection (VIP) identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of each metabolite at each stage of development.

phenylalanine, and choline. In S2, the main differences were found in galactose, fumarate, and inositol, which were more abundant in UTH than TH.

A multivariate statistical analysis was performed with the data obtained from both UTH and TH samples, using detected metabolites as predictor variables and stage of development and thinning treatments as response variables. PLS-DA was able to explain 52.8 and 19.3% of total variance with the two components (**Figure 4A**). In this projection, C2 tended to separate by thinning treatments, where in the positive side was UTH and in the negative side was TH, except for harvest from TH (TH H), which is in the middle tending to the positive side. C1 separated the early stages of development from harvest independently of thinning treatment. On the positive side of C1 were UTH S1 and S3, as well as TH S3. TH S1 metabolite composition was very different from all other samples. TH S1 was at the negative side of C2, isolated from all the others. At this same position, caffeoyl quinate 1, caffeoyl quinate 2, prunasin, and quinate were observed in the loadings plot (**Supplementary Figure S3**). The 10 most important metabolites involved in the discrimination between developmental stages and thinning treatment were obtained by a variable importance in projection (VIP) analysis identified by PLS-DA (**Figure 4B**). Inositol, galactose, aspartate, alanine, unknown (multiplet at 0.97 ppm) compound, fructose, phenylalanine, succinate, glucose, and trehalose were the most important metabolites identified by PLS-DA.

Figures 5, **6** show the changes of the main soluble sugars and organic acids during fruit development in the first season. As expected, sucrose was the predominant sugar at harvest in both UTH and TH fruits (**Figure 5**) and its concentration increased during fruit development. Sorbitol and trehalose remained practically constant during development, showing just a decrease in harvest stage in both thinning treatments. Hexose concentrations decreased during fruit development. The most striking differences observed between thinning treatments were that sucrose concentration was significantly higher in TH fruit, while hexose concentrations were significantly and consistently higher in UTH fruit in all development stages, with the exception of H where sucrose was similar in both treatments. Inositol, the metabolite that presented the highest VIP, had its concentration discretely increased in S2 and then diminished at harvest. However, during S2, its concentration was different between thinning treatment. Soluble sugars from mesocarp of fruits at harvest and ripening stages were also measured in parallel using HPAEC-PAD. Myo-inositol, sorbitol, glucose, fructose, and sucrose were found in similar proportions in both thinning treatments at the harvest stage (**Supplementary Figure S4**). In order to compare the results obtained by this method, the data obtained by NMR were plotted as proportions. A similar trend was found where sucrose was the most predominant sugar at the harvest stage, being more abundant in TH than UTH fruits (**Supplementary Figure S4**). Sorbitol was found in less concentration and inositol was found as just traces. At the ripening stage, sucrose proportion increased compared to the other sugars measured and reached approximately 89% of total sugar in TH reached and 75.5% in UTH (**Supplementary Figure S4**). Concerning organic acids, differences between thinning treatments were observed in the earlier stages of development (**Figure 6**). Succinate concentration was significantly higher in TH than in UTH in S1, but the differences disappeared along development, gradually increasing its concentration similar to citrate but in a different scale. Malate concentration was significantly higher in UTH than in TH S1 fruit and decreased along development. On the other hand, quinate concentration was significantly higher in TH than in UTH fruit at S1 and S2 developmental stages and

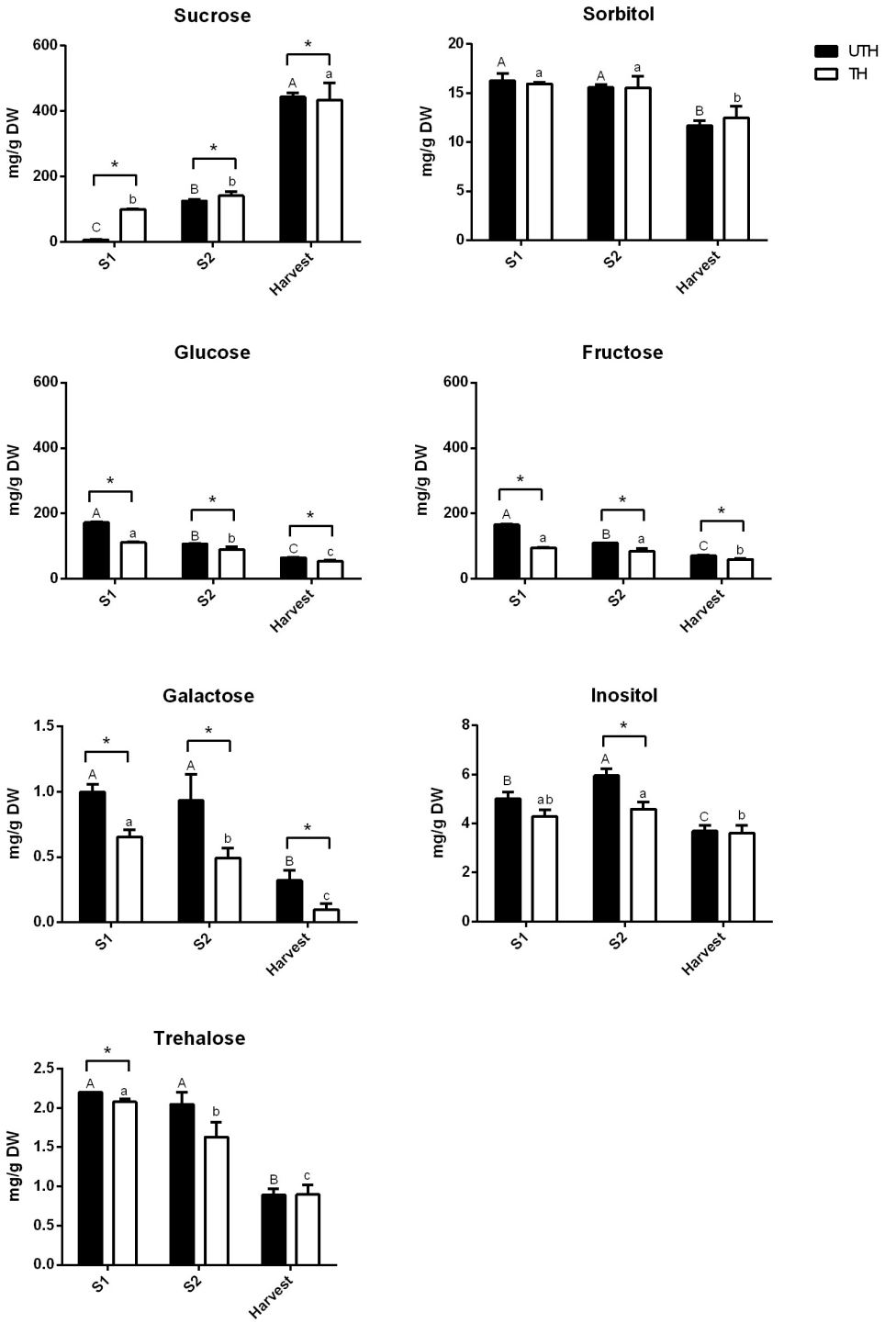

FIGURE 5 | Contents of sugars in fruits from unthinned (UTH) and thinned (TH) trees during first season development. Each column represents the average of three biological replicates (n = 3); bars are the standard deviations. Capital letters indicate comparison of each sugar concentration during UTH fruit development. Lowercase letters indicate comparison of each sugar concentration during TH fruit development. Asterisks indicate differences between thinning treatments. Statistical analysis was performed by Holm–Bonferroni method using $P < 0.05$.

also decreased until harvest. Phenylalanine content (**Figure 7**) was higher at S1 phase in both UTH and TH fruits and then decreased smoothly in S2 and harvest stages, and its content was significantly higher in UTH fruits than in TH fruits at all stages. Similarly, alanine concentration decreased along the development, but from S2 to harvest, the decrease was steep.

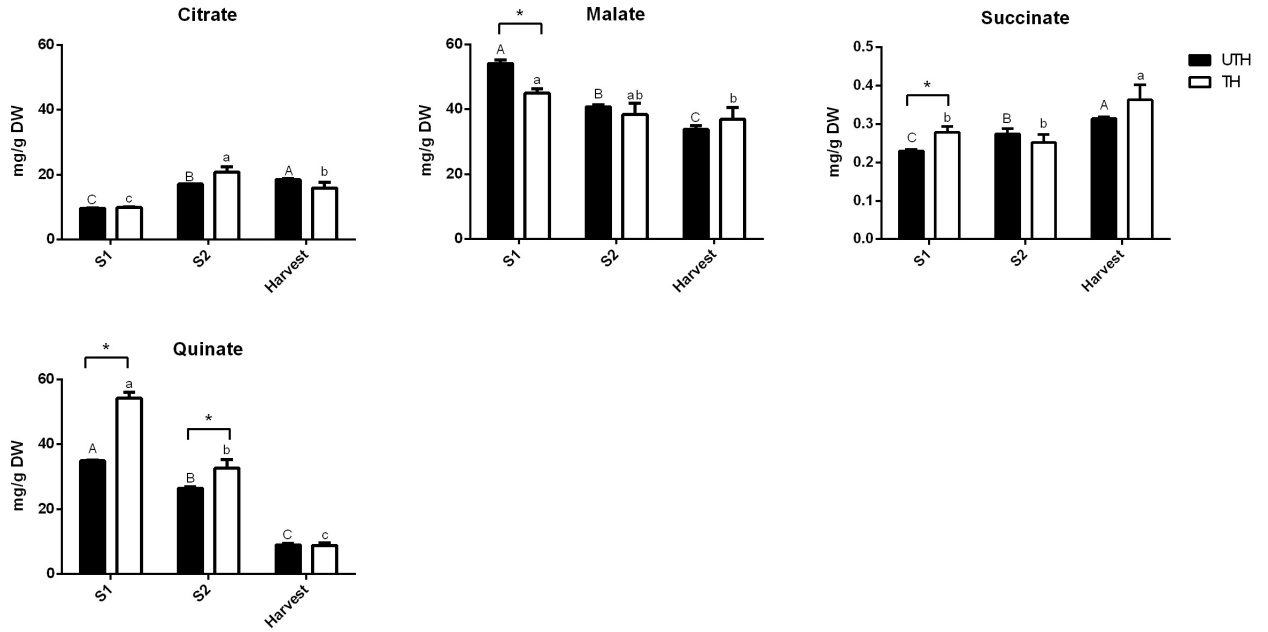

FIGURE 6 | Contents of organic acids in fruits from unthinned (UTH) and thinned (TH) trees during first season development. Each column represents the average of three biological replicates ($n = 3$); bars are the standard deviations. Capital letters indicate comparison of each organic acid concentration during UTH fruit development. Lowercase letters indicate comparison of each organic acid concentration during TH fruit development. Asterisks indicate differences between thinning treatments. Statistical analysis was performed by Holm–Bonferroni method using $P < 0.05$.

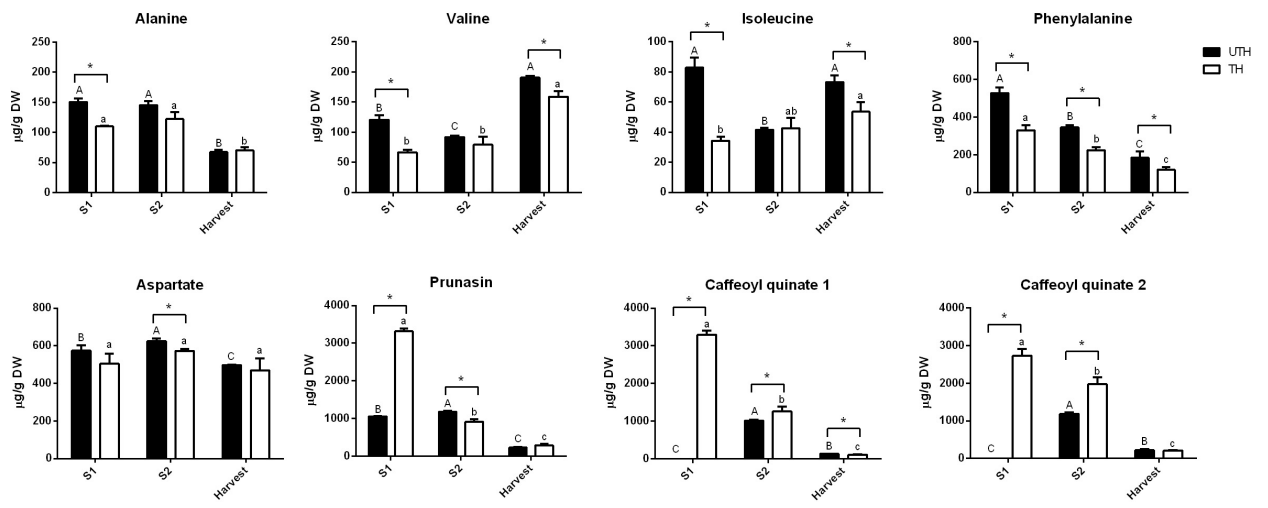

FIGURE 7 | Contents of amino acids and phenylpropanoid pathway intermediates in fruits from unthinned (UTH) and thinned (TH) trees during first season development. Each column represents the average of three biological replicates ($n = 3$); bars are the standard deviations. Capital letters indicate comparison of each metabolite concentration during UTH fruit development. Lowercase letters indicate comparison of each metabolite concentration during TH fruit development. Asterisks indicate differences between thinning treatments. Statistical analysis was performed by Holm–Bonferroni method using $P < 0.05$.

Alanine content was higher in UTH than in TH in S1, while in the other stages of development, there was no difference between thinning treatments. Isoleucine and valine showed the same pattern with a decrease from S1 until S2 and then an increase at harvest in the UTH fruit (**Figure 7**). On the other hand, these amino acids showed constant concentrations in TH fruit during development. The amount of these amino acids was higher in UTH than TH fruit. Aspartate evolution showed a different pattern. This amino acid was the most accumulated throughout all fruit development in both thinning treatments followed by phenylalanine (**Figure 7**). Aspartate concentration remained practically steady and in equivalent concentrations in both thinning treatments. The only exceptions were at S2 when UTH fruits presented more aspartate than TH. The two caffeoyl

quinates and prunasin showed the same concentration pattern in both thinning treatments throughout fruit development (**Figure 7**) with higher contents at S1 and a steep decrease until harvest. These compounds were consistently higher in TH than in UTH fruit.

DISCUSSION

Thinning is an agronomical practice used worldwide in several fruit tree species to increase fruit size (Myers et al., 2002). It is very well described in the literature that thinning affects fruit size and even development time. It is also known that thinning may impact organoleptic traits such as TSS content, without any detail of the metabolite composition changes induced by this agronomical practice (Costa et al., 2018). The effectiveness of thinning depends largely on the stage of development in which it is performed. The recommendation is to perform thinning at the beginning of fruit development before the mesocarp sucrose accumulation starts (Costa and Vizzotto, 2000). In this study, we analyzed the mesocarp metabolic profile during the development of nectarine fruits when trees were subjected to different thinning treatments. We worked with an early to mid-season variety that takes around 120 DAB to be harvested in two consecutive seasons (Ognjanov et al., 1995; **Figure 1**). We observed an increase in final fruit size and TSS in response to thinning only in the first season evaluated (**Figure 1A** and **Table 1**). No differences in diameter, TSS, or acidity were observed between fruits from thinned compared to unthinned trees during the second season (**Figure 1A** and **Table 1**). Fruits from thinned trees seemed to soften faster than unthinned trees as after 10 days at 20°C, the firmness decreased more intensively. These differences between the seasons are probably due to the different times of thinning. In the first season, thinning was performed at 42 DAB at the middle of the first exponential growth phase (S1), while in the second season, thinning was performed at 63 DAB, which corresponds to the end of S1. During S1, the first increase in fruit volume occurs mainly due to the high rate of cell proliferation and expansion process (Scorza et al., 1991; Zanchin et al., 1994), which leads to an increase in fruit metabolic rate (Moing et al., 1998). The increase in photoassimilate demand directly impacts the strength of the fruit as a sink (Grossman and DeJong, 1995). Unfortunately, we did not count the number of mesocarp cells in the fruit to verify this hypothesis. It was shown that peach trees with light to moderate crop load (thinned trees) harbor fruit with higher dry matter and water potential (Berman and DeJong, 1996). On the other hand, a strong cutinization of the epidermal layer occurs during S3 that might be related to limited respiration to avoid water loss (Masia et al., 1992). When crop load was high (unthinned trees), photoassimilates had to be distributed among many fruits, resulting in smaller fruit with lower dry matter. This limitation in the growth rate due to limited photoassimilate resources is called source-limited (Pavel and DeJong, 1993). The S1 stage is the first source-limited growth period. During the first harvest season, thinning was performed at the beginning of S1 phase diminishing the competition of resources among the remaining fruit. In the second season, thinning was performed late during S1, after the first source-limited growth period (the second was during S3 stage), and the remaining fruits were unable to take full advantage of the decreased competition for resources caused by thinning and potential yield was lost (Grossman and DeJong, 1995). In other Prunus species, such as sweet cherry (*Prunus avium*) and plum (*P. salicina, P. domestica*, and *P. cerasifera*), it was shown that genotypic differences in final fruit size are primarily a function of cell number (Olmstead and Iezzoni, 2007; Cerri et al., 2019). It is possible that the lower competition for photoassimilates in trees that were thinned at the beginning of S1 phase (first season) stimulated a higher post-bloom cell division rate compared to the unthinned condition, positively impacting the final fruit size.

The metabolic profile of peach fruit has been broadly studied at different stages of development, during postharvest and treatments to prevent chilling injury (Moing et al., 1998; Lombardo et al., 2011; Botton et al., 2016; Brizzolara et al., 2018; Nuñez et al., 2019; Lillo-Carmona et al., 2020). Here, we showed the metabolic response to a source–sink manipulation removing fruits in order to obtain an approximately 36 leaves per one fruit in a branch (thinning performed in commercial orchards in Chile). A total of 24 metabolites were quantified by ^{1}H-NMR profiling: 7 sugars, 7 organic acids, 6 amino acids, 2 amine derivatives, 1 cyanogenic glucoside, and 1 unknown compound. PCA analysis revealed that each stage of development has their unique metabolic profile since each group was separated from the others in both thinning treatments (**Figure 2**). On the other hand, the heatmap analysis showed that S1 showed a different metabolic profile depending on the thinning treatment (**Figure 3**), and along the development of the nectarines, these differences decrease. The S1 samples used for metabolite analysis were sampled 26 days after thinning, sufficient time to observe any effect of the treatment. Since the main compositional separation was observed during earlier stages, we suggest that an early change of the metabolite profile could witness metabolism rearrangements that cause changes in the final stages of development.

We had shown before that sorbitol was the most abundant soluble sugar in "Magique" leaves followed by sucrose and that thinning induced an increase in the sugar contents in leaves at S1 and S2 phases (Andrade et al., 2019). These are the main exportable sugars in *P. persica*, and they were previously reported as the major soluble sugars in the leaves of this species (Moing et al., 1992). The increased content of exportable sugars together with the increase in the expression of sugar transporters related to phloem loading/unloading in response to thinning (Nuñez et al., 2019; Aslani et al., 2020) probably sustained the high metabolic rates and carbohydrate demand to sustain cell proliferation and endocarp lignification at S1 and S2 phases in fruit from thinned trees. We did not observe accumulation of these sugars in fruits, because once they arrived in the fruit, they were converted to glucose and fructose to sustain respiration and also directed to other biosynthetic pathways such as synthesis of cell wall components, amino acids, and precursors related to lignin synthesis. The higher content of hexoses in S1 UTH fruits in comparison to TH fruits may be due to the lower metabolic

rates of UTH fruit. Inositol is a polyol that maintained low levels throughout the development with a peak at S2. This metabolite was the highest VIP, indicating its importance in the separation of the variables in PLS-DA. It also showed a strong correlation with early stages of UTH fruit development (**Supplementary Figure S3**). Inositol is synthesized from glucose 6-phosphate and can act in different plant metabolisms as cell wall biosynthesis, auxin physiology, and response to stress among others (Loewus and Murthy, 2000). At the harvest stage, inositol contents are equivalent in UTH and TH. Its accumulation in S2 UTH could suggest a delay in the development of UTH fruits.

Previous studies showed that lignin gene expression is induced at extremely high levels just before the end of S1 phase (Dardick et al., 2010). Phenylalanine is an aromatic amino acid and the main phenylpropanoid pathway precursor that leads to lignin and flavonoid biosynthesis (Singh et al., 2010; Botton et al., 2016). Our data showed that this amino acid accumulated at S1 phase in both UTH and TH fruit, followed by its content decreasing in S2 and S3 phases. Nevertheless, phenylalanine content was higher in S1 UTH fruits than TH fruits (**Figures 3**, **7**). The same pattern was observed for alanine, asparagine, isoleucine, and valine (**Figure 7**), which are also substrates for the phenylpropanoid pathway that lead to lignin and flavonoid biosynthesis (Lombardo et al., 2011). In contrast, quinate and caffeoyl quinate 1 and 2, which belong to this same pathway downstream phenylalanine (Del Cueto et al., 2018), were more abundant in S1 TH than UTH (**Figures 6**, **7**). These metabolites and prunasin showed a high correlation with S1 UTH samples (**Supplementary Figure S3**). Thinning may also advance fruit development and harvest (Nuñez et al., 2019). It seems that UTH fruits were delayed in contrast to TH fruits, because the content of phenylalanine during S2 UTH was similar to S1 TH. Therefore, phenylalanine previously accumulated in TH fruit was already used for lignin synthesis and endocarp hardening. Indeed, prunasin is a cyanogenic glucoside biosynthesized from phenylalanine and then metabolized into amygdalin, which is localized only in seeds at later developmental stages (Mizutani et al., 1991). It was detected in higher amounts in TH S1 than UTH S1 fruit, indicating that endocarp development was advanced in TH fruit. This is in agreement with the UTH fruit growth curve shown in **Figure 1A**, where S2 lasted more in UTH than TH. The pit hardening process requires high amounts of energy sustained by imported photoassimilates (Dardick et al., 2010), which were more abundant in TH than in UTH trees. This increased availability of photoassimilates in TH trees accelerated fruit development. Aspartate, the third metabolite by VIP, is involved in the biosynthesis of asparagine, lysine, threonine, isoleucine, and methionine (Azevedo et al., 2005). Besides, its concentration was almost constant throughout the development, except for S2 UTH (**Figure 7**), which tended to accumulate, suggesting that the metabolism of aspartate was slower than in TH fruit, in agreement with the hypothesis that UTH fruits were delayed in development.

All the sugars detected had an accumulation pattern characterized by higher contents at early stages and progressively declined until harvest. The only exception was sucrose that presented exactly the opposite trend, with lower levels at early stages of development and being the predominant sugar at late stages. In the fruit, high hexose concentrations observed in S1 were associated to high sucrose synthase (SS, EC 2.4.1.13) and acid invertase (AI, EC 3.2.1.26) activities (Moriguchi et al., 1990; Lo Bianco and Rieger, 2002) when the energy requirement of the dividing cells was high. Trehalose evolution during development presented exactly the opposite trend of sucrose independent of thinning treatment (**Figure 5**). Trehalose is the precursor of trehalose 6-phosphate (Tre6P), which is a critical signaling metabolite that is important for plant growth and development at all stages of the plant's life cycle (Figueroa and Lunn, 2016). In sink organs where sucrose and sorbitol in Rosaceae species are the major carbon import, it has been suggested that Tre6P promotes growth and acts as a major hub when sucrose supply is high (Schluepmann et al., 2003; Baena-González and Lunn, 2020). The decreased trehalose concentration may reflect an increase in Tre6P production signaling sucrose availability at the different stages of fruit development.

The main organic acids found in all stages of development of "Magique" nectarine were malate and quinate (**Figure 6**) whose content declined along development. On the other hand, succinate, citrate, and fumarate were found in a lesser extent, and their content rose to reach a maximum at harvest (**Figures 3**, **6**). This profile was similar to the high-acid peach cultivars (Wu et al., 2005; Zheng et al., 2021). Taste is a multifactorial aspect in which sweetness and sourness play a key role. Sugars and organic acids are involved in both characteristics, respectively (Cirilli et al., 2016). Sucrose accumulation at the end of peach development originates from phloem import and gluconeogenesis with organic acids as substrates and is directly related to the fruit sweetness (Vizzotto et al., 1996; Cirilli et al., 2016; Desnoues et al., 2016). Sucrose presented its highest content at harvest. NMR was used to measure metabolites in three stages of development, from S1 until harvest while HPLC was used to measure water-soluble sugars at harvest and ripening stages. NMR and HPLC data were quite similar at the harvest stage when a higher proportion of sucrose was observed in TH than in UTH fruits (**Supplementary Figure S4**). This trend was exacerbated at the ripening stage in agreement with phenotypic analysis, where TSS of TH were higher than UTH fruits in the first season evaluated (**Table 1**). This pattern of sugar accumulation throughout fruit development in "Magique" is quite similar to that observed in other peach and nectarine varieties (Moing et al., 1998; Lombardo et al., 2011; Zhang et al., 2013; Bae et al., 2014; Desnoues et al., 2014; Roch et al., 2020). Nuñez et al. (2019) reported that this steep increase of sucrose at the end of development was in parallel to an increase in the sucrose transporter gene *PpeSUT1* expression mainly in thinned trees probably supporting apoplasmic sucrose unloading at harvest. We also observed higher sucrose content at ripening in TH fruits compared to UTH (**Supplementary Figure S4**), which sustains this hypothesis. The organic acid accumulation during early stages of fruit development is directly related to the supply of substrates for respiration process maintenance during fruit development (Seymour et al., 2013) while its decrease at later stages is related to their consumption for sucrose synthesis.

CONCLUSION

Our results indicated that fruit thinning in peach (i.e., source–sink imbalance) should be performed at the beginning of fruit development to be effective in increasing final fruit size. We showed that when performed at the right moment, thinning affects the metabolite composition of fruit mainly in earlier stages of development and driving resources to sustain cell division that will impact final fruit size. Metabolite data revealed that sugar, organic acid, and phenylpropanoid pathways at early stages of development (S1 and S2 stages) can be used to segregate fruits impacted by the change in source–sink balance. In conclusion, we suggest that the profile of these metabolites in early developmental stages could be a metabolite predictor of final fruit quality in nectarines.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AMA and MLV designed the research. MPC, LM, GB, and DA conducted field work, fruit phenotyping, and sampling. MPC, MM, CD, and AM performed proton NMR metabolite analyses. VL-C, CF, and RP performed GC-MS analyses and data analysis using Metaboanalyst 4.0. MPC, LM, GB, and MLV performed HPAEC-PAD analyses. MC and AMA wrote the original manuscript. MPC, VL-C, AM, RP, and AMA edited and reviewed the original manuscript. All authors read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.604133/full#supplementary-material

Supplementary Figure 1 | Phenotypic analysis of "Magique" nectarines harvested from unthined (UTH) and thinned (TH) trees in both seasons. A boxplot analysis was performed to select the most similar fruits. In harvest, firmness was evaluated and in ripening firmness and juiciness were analyzed.

Supplementary Figure 2 | Principal component analysis (PCA) of the 124 metabolites detected by GC-MS at S1, S3 and harvest (H) stage from unthinned (UTH) and thinned (TH) trees during the second season. The detected metabolites were employed as predictor variables, and the stages of development as a response variable. The panel shows the score plot where the variance explained of each component corresponded to 21.1% for PC1 and 13.7% for PC2. Blue circles represent S1 fruit samples from unthinned trees and green circles from thinned trees. Light blue circles represent S3 fruit samples from unthinned trees and yellow circles from unthinned trees. Pink circles represent harvested fruit samples from unthinned trees and orange circles from thinned trees.

Supplementary Figure 3 | Loadings plot between the selected PCs of Partial Least Square Analysis Discriminant Analysis (PLS-DA) of "Magique" nectarine metabolites detected using ^{1}H-NMR for first season.

Supplementary Figure 4 | Sugar proportions in the mesocarp of "Magique" nectarine fruit during the first season measured by HPAEC-PAD (harvest and ripening stages) and ^{1}H-NMR (harvest stage). The total concentration of myo-inositol, sorbitol, glucose, fructose and sucrose together was considered as 100% and the proportion of each sugar was plotted.

Supplementary Table 1 | Phenotypic data obtained from fruit harvested in both seasons evaluated.

Supplementary Table 2 | Primers used for RT PCR analysis.

Supplementary Table 3 | Metabolites quantified by ^{1}NMR in the mesocarp of nectarines at different stages of development in the first season.

Supplementary Table 4 | ^{1}H chemical shifts (δ_H in ppm) of caffeoyl quinic derivatives.

Supplementary Table 5 | Metabolites quantified by GC-MS in the mesocarp of nectarines at different stages of development in the first season.

Supplementary Data 6 | NMR calibration curves.

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REVIEW
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Biosynthesis and Cellular Functions of Tartaric Acid in Grapevines

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Tartaric acid (TA) is an obscure end point to the catabolism of ascorbic acid (Asc). Here, it is proposed as a "specialized primary metabolite", originating from carbohydrate metabolism but with restricted distribution within the plant kingdom and lack of known function in primary metabolic pathways. Grapes fall into the list of high TA-accumulators, with biosynthesis occurring in both leaf and berry. Very little is known of the TA biosynthetic pathway enzymes in any plant species, although recently some progress has been made in this space. New technologies in grapevine research such as the development of global co-expression network analysis tools and genome-wide association studies, should enable more rapid progress. There is also a lack of information regarding roles for this organic acid in plant metabolism. Therefore this review aims to briefly summarize current knowledge about the key intermediates and enzymes of TA biosynthesis in grapes and the regulation of its precursor, ascorbate, followed by speculative discussion around the potential roles of TA based on current knowledge of Asc metabolism, TA biosynthetic enzymes and other aspects of fruit metabolism.

Keywords: grape, fruit, tartaric acid, metabolism, gene, enzyme, antioxidant

INTRODUCTION

L-Tartaric acid, (2,3-Dihydroxybutanedioic acid, L-threaric acid, TA) accumulates in comparatively few plant species despite its close structural similarity to many other C_4-dicarboxylates. A product of the oxidation of sugars, in many cases via L-ascorbate (Asc) breakdown, it appears to have no known physiological or biochemical function. In cultivated crops the accumulation of TA is largely non-responsive to stresses or environmental or cultural management practices. A five or six-step synthesis pathway was proposed almost 40 years ago, and whilst candidate enzymes have been characterized for two steps, little is understood about how, where or indeed why this relatively simple compound accumulates in the tissues of just a few plant species (DeBolt et al., 2007).

Scientific interest in TA would likely have been minimal were it not for the happy circumstance that it is the principal acid found in berries of the cultivated grapevine *Vitis vinifera* (Kliewer, 1966). At harvest, there are 4 to 8 grams of TA per L of grape juice, contributing in large part to a pH of 2.9 to 3.8 (Amerine et al., 1965). TA is thereby responsible for much of the 'vitality' of wine, balancing out the inherent sweetness of alcohol on the palate and contributing to low pH conditions needed to enable the wine to age over an appropriate period whilst minimizing spoilage from microbial and oxidative processes (Plane et al., 1980; Zoecklein et al., 1995; Liu et al., 2007).

Notwithstanding its economic and cultural value, important questions remain unanswered about TA. The most widely accepted TA synthesis pathway begins with Asc, although precisely how Asc is converted to 2-keto L-gulonate, the first intermediate in the pathway, remains unknown. Some suggest that the oxidized form of Asc, dehydroascorbate (DHA; a product of ROS scavenging), serves as the precursor (Hancock and Viola, 2005; Melino et al., 2009a). Either way, it would seem an unusual fate for Asc, more commonly referred to as vitamin C, which is better known for its role in regulating the cellular redox state, among other metabolic and signaling processes (Gilbert et al., 2009; Smirnoff, 2011; Foyer et al., 2020). None of the intermediate compounds in the pathway are commonly found in plants, nor do they have roles in alternative pathways. It is therefore to be wondered why five or six enzymes have been retained in grapevine for the sole function of producing a compound with no known role, from a compound with several enormously important roles.

It is of course likely that we are yet to elucidate its function, a fate which for many plant compounds has previously seen them allocated to the category of 'secondary metabolite'. More recent definitions have revised this classification to 'plant specialized metabolites', with the defining characteristics of such compounds being that they are (i) not directly involved in growth or development, (ii) restricted to a narrow set of species, (iii) not necessary for survival and (iv) support stress responses. Additionally, specialized metabolites are classified as either phenolics, alkaloids or terpenes. TA does not fit this latter requirement and may therefore be proposed as a 'specialized primary metabolite', being a product of essentially primary (i.e., carbohydrate) metabolism, but with none of the energy conservation, biosynthetic or regulatory functions normally associated with these biomolecules.

Trending decreases in titratable acidity and increases in pH of grape juice have been observed over time and in response to increased temperatures or other climatic effects (Spayd et al., 2002; de Orduña, 2010; Sadras et al., 2013; Godden et al., 2015). These can lead to the need for acid additions to restore appropriate pH and TA levels if wine quality is to be preserved. This is often a significant expense to winemakers, particularly during warmer growing seasons. In the broader food industry, TA can be used as an additive for its acidic and antioxidant properties (Silva and Lidon, 2016; EFSA Panel on Food Additives and Flavourings et al., 2020). Unlike TA, malic acid (MA), which also accumulates in grapes during early development, is susceptible to enzymatic catabolism during ripening, particularly at high temperatures (Buttrose et al., 1971; Kliewer, 1971; Ruffner et al., 1976). Potassium levels in grape berries can increase with temperature due to increased water uptake, thus exacerbating the effect on pH that arises from the exchange of protons from the vacuole during cation transport (Boulton, 1980; Coombe, 1987). With increased pH of juice and must (the mixture of juice, skins and seeds that is fermented to make red wines), larger SO_2 additions may be required to effectively curb the growth of undesirable microbes. This can lead to wines exceeding legal SO_2 thresholds, also affecting wine flavor and aroma (de Orduña, 2010). Several decades of work has explored acidity in winegrapes, including the variation of acidity measured among different *Vitis* species (Stafford, 1959; Kliewer et al., 1967; Shiraishi et al., 2010; reviewed by Dai et al., 2011), *V. vinifera* cultivars (Shiraishi, 1995; Liu et al., 2007) and within populations (Liang et al., 2011; Viana et al., 2013; Duchêne et al., 2014; Chen et al., 2015; Houel et al., 2015; Ban, 2016), as well as the metabolic effects of temperature on organic acids in grapes (Kliewer, 1973; Ruffner et al., 1976; Sweetman et al., 2014).

The aim of this review is to summarize new developments in the area of TA accumulation and biosynthesis in grape berries, although some parallels are drawn with other fruits. Furthermore, potential roles of TA in fruit metabolism are proposed, drawing on what is now known of the biosynthetic enzymes and precursors.

TARTARIC ACID ACCUMULATION IN GRAPE BERRIES (CONCENTRATION AND DISTRIBUTION)

In fruit, TA was once thought to be uniquely accumulated in grapes, but it has since been shown to occur at significant levels in a range of other fruits, including avocado (Pedreschi et al., 2019), lychee (Wang et al., 2006), sweet cherry (Mahmood et al., 2012), blueberry (Li et al., 2015), tamarind (Van den Bilcke et al., 2014), some citrus fruits (Nour et al., 2010) and in one report, banana (Wyman and Palmer, 1964). A tabulated summary is available in a recent review on fruit organic acids (Walker and Famiani, 2018). TA has also been measured in leaves of bean (Saito and Loewus, 1989), tamarind (Lewis and Neelakanthan, 1959), geranium and grapevine (Williams and Loewus, 1978). Recent technological advances have enabled very accurate and high-throughput quantification of organic acids in individual grapes (Melino et al., 2009b; Higginson et al., 2016), revealing large variation in TA concentrations between bunches within a vine and between berries of a single bunch, likely due to asynchronous development of the individual fruit.

Net TA accumulation is exclusive to the first stage of berry development, associated with rapid cell division (Kliewer and Nassar, 1966). Asc shares an almost identical developmental accumulation pattern although at approximately one-fiftieth the concentration (Melino et al., 2009a; Cholet et al., 2016). TA biosynthesis reactions are thought to occur in the cytosol of plant cells (Pignocchi et al., 2003), however the proposed involvement of transketolase and succinate semialdehyde dehydrogenase in late steps (Salusjärvi et al., 2004; DeBolt et al., 2006), as well as a

protein localization study of the L-idonate dehydrogenase (Wen et al., 2010), discussed in later sections, suggest that additional cellular compartments and even the apoplast may be involved in grape TA biosynthesis. Once synthesized, TA resides in the vacuole (DeBolt et al., 2004).

Accumulation of TA occurs in all *V. vinifera* cultivars and across the Vitaceae family (Stafford, 1959). TA levels in *V. vinifera* fruit are largely unaffected by environmental conditions other than in a small handful of studies that report responses to light (Melino et al., 2011; Reshef et al., 2017), water deficit (Grimplet et al., 2009; Savoi et al., 2017), fertilization with silicon and calcium chloride (Gomes et al., 2020), grafting onto different rootstocks (Zhang et al., 2020b) and seasonal variability due to water status and light exposure (Cholet et al., 2016). Notwithstanding these reports, there is no evidence that any one or combination of cultural practices can be used to modulate levels of TA in berries at harvest.

The search for QTLs relating to grape berry acidity has seen a lot of activity in the last decade, with some promising breakthroughs for total or titratable acidity (Liang et al., 2011; Duchêne et al., 2014; Ban, 2016), pH (Viana et al., 2013; Chen et al., 2015), MA (Chen et al., 2015; Duchêne et al., 2020) and various acid ratios in young berries (Houel et al., 2015), however in most cases there was no satisfactory marker for TA level (Liang et al., 2011; Viana et al., 2013; Chen et al., 2015; Bayo-Canha et al., 2019). This may be due to the involvement of several biochemical steps and potential sites of regulation, as well as other factors that affect berry acid levels such as potassium accumulation (Duchêne et al., 2020). Despite these challenges, two major QTLs for TA concentration on linkage groups LG7 and LG4 were stably detected using a Picovine x Ugni Blanc flb population under different environmental conditions (Houel et al., 2015). Most of the above QTL analyses were based on mapping within bi-parental populations, which may be restricted by the limited genetic diversity between the specific parental varieties or species. Therefore, the selection of appropriate crossing parents may be critical for the success of TA QTL studies. In addition, genome-wide association studies (GWAS) that exploit the great genetic and phenotypic diversities of large grapevine germplasm collections deserve more attention for the study of complex biological traits such as TA accumulation. This approach is becoming increasingly feasible for woody species like grapevine (c.f. cereal crops), due to the lower costs of high-throughput sequencing and phenotyping techniques. Recently, GWAS analyses of 472 *Vitis* accessions (Liang et al., 2019) and 279 *V. vinifera* L. cultivars (Flutre et al., 2020), were employed to identify loci associated with traits including TA accumulation, detailed further in Section 9.

BIOSYNTHETIC ENZYMES OF TARTARIC ACID

Three pathways of TA biosynthesis occur in higher plants, each identified by the precursor and cleavage site, namely "Asc C4/C5," "Asc C2/C3," and "D-gluconic acid C4/C5," (Loewus, 1999), the former being the primary pathway in grape and described in detail by Ford (2012). Briefly, in the Asc C4/C5 pathway (depicted in **Figure 1**), Asc is converted by one or more uncharacterized steps to 2-keto L-gulonate and thence via reduction to L-idonate and oxidation to form 5-keto D-gluconate. This six-carbon intermediate is cleaved by an unknown enzyme to yield a four-carbon intermediate, possible tartaric acid semialdehyde that is finally oxidized to produce TA. In the Asc C2/C3 pathway, Asc is cleaved between carbons two and three: the two-carbon fragment forms oxalic acid and the four-carbon fragment forms L-threonic acid which is subsequently oxidized to form TA. The D-gluconic acid C4/C5 pathway, which has been identified in leguminous species, has a direct conversion of D-gluconic acid to 5-keto-D-gluconic acid via an unidentified oxidation step, whereafter the formation of TA is thought to occur by the same steps as proposed for the Asc C4/C5 pathway. The sequence of redox-associated steps in the confirmed reactions of TA synthesis, which may feature also in the conversion of Asc to 2-keto L-gulonate and in the steps leading from 5-keto D-gluconate, has been suggested to reflect a common strategy in the generation of phytochemical diversity (Horn, 2021).

By feeding labeled Asc to immature berries of a number of species from the Vitaceae family it was determined that [1-^{14}C]Asc and [4-^{14}C]Asc corresponded to the C1 and C4 carbons respectively of the TA skeleton (Williams and Loewus, 1978). Earlier studies that identified the key intermediates and organization of the C4/C5 pathway (Saito and Kasai, 1969; Wagner and Loewus, 1974; Wagner et al., 1975) were complemented by later work identifying the final intermediates (Saito and Kasai, 1982, 1984). Accumulation of radiolabeled L-idonate in grapevine leaves also fed with labeled Asc confirmed that the oxidation of L-idonate to 5-keto-gluconic acid is a rate-limiting step of TA synthesis (Malipiero et al., 1987).

Non-targeted metabolomics studies in grape berries provide opportunities to look for accumulation of other intermediates under a range of conditions, however our own search of published metabolomics datasets for the purpose of this review, turned up little evidence. One report showed developmental and temperature regulation of ketogluconate levels (Gouot et al., 2019), but it is unclear which isomer(s) were present in the analysis. Although the steps of the "Asc C4/C5" pathway were identified decades ago, further work on the biosynthesis of TA in grape berries and identification of the enzymes responsible has been limited (Ford, 2012), but some recent findings warrant summarizing.

L-Idonate Dehydrogenase

L-idonate dehydrogenase (L-IDH) catalyzes the above-mentioned "rate-limiting" step of L-idonate to 5-keto-gulonic acid (**Figure 1**) and was the first enzyme of the TA biosynthetic pathway to be identified and characterized (DeBolt et al., 2006). In *V. vinifera* there are three L-IDH isoforms, which are co-located on chromosome 16 but only one (*VvLIDH3*; Q1PSI9; VIT_16s0100g00290) has been shown definitively to oxidize L-idonate (DeBolt et al., 2006; Sweetman et al., 2012; Higginson et al., 2016). *VvLIDH1* (ABA01327) has a very high sequence identity to *VvLIDH3* with only three like-for-like (e.g., hydrophobic-for-hydrophobic or neutral) amino

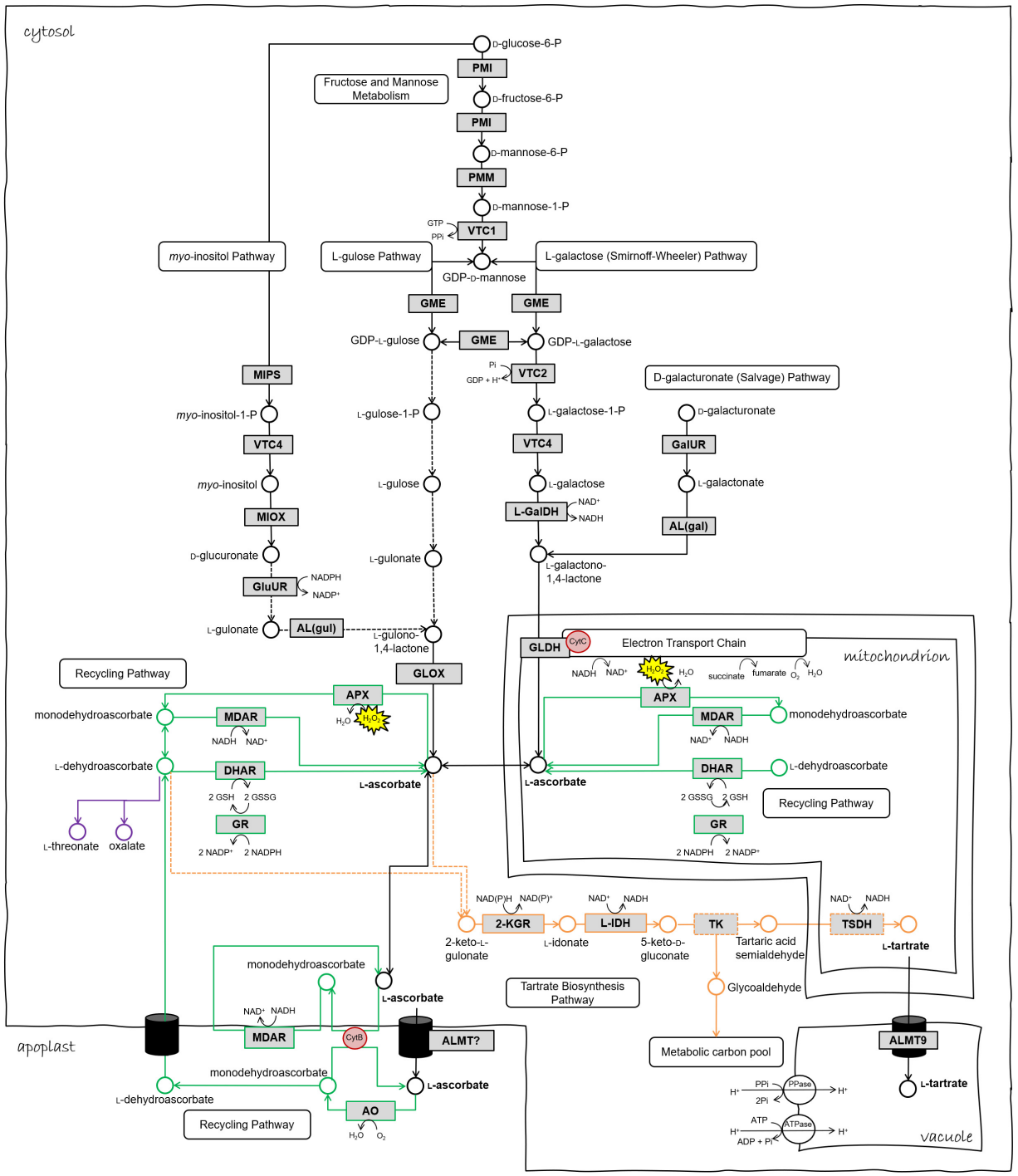

FIGURE 1 | Biosynthetic and redox pathways of ascorbic acid and tartaric acid in the plant cell. Chloroplastic ascorbate redox reactions are omitted from this schematic. Black arrows/metabolites/enzymes represent ascorbate biosynthesis steps. Green represents ascorbate recycling pathways in various cell compartments. Orange represents tartrate biosynthesis pathway. Dashed arrows or boxes indicate hypothetical reactions or enzymes. Enzyme abbreviations as follows. PMI phosphomannose isomerase, PMM phosphomannomutase, VTC1 GDP-D-mannose pyrophosphorylase, GME GDP-D-mannose epimerase, VTC2 GDP-galactose phosphorylase, VTC4 L-galactose-1-phosphate phosphatase, L-GalDH L-galactose dehydrogenase, GLDH L-galactonolactone dehydrogenase, MIPS *myo*-inositol-3-phosphate synthase, MIOX *myo*-inositol oxidase, GluUR D-glucuronic acid reductase, AL(gul) aldono-lactonase for L-gulonate, GLOX L-gulonolactone oxidase, GalUR D-galacturonic acid reductase, AL(gal) aldono-lactonase for L-galactonate, MDAR monodehydroascorbate reductase, DHAR dehydroascorbate peroxidase, APX ascorbate peroxidase, GR glutathione reductase, AO ascorbate oxidase, 2KGR 2-ketogulonic acid reductase, L-IDH L-idonate dehydrogenase, TSDH tartaric acid semialdehyde dehydrogenase, TK transketolase, ALMT aluminum-activated malate transporter, cytB Cytochrome B561. Figure adapted from Melino et al. (2009a), Burbidge (2011), and Podgórska et al. (2017).

acid substitutions at non-critical sites (Higginson, 2015; Jia, 2015). *VvLidh1* and *VvLidh3* transcripts both show preferential expression in young fruit (Deluc et al., 2007; Melino et al., 2009a; Sweetman et al., 2012; Rienth et al., 2014; Massonnet et al., 2017), thus aligning with the timing of TA accumulation. This may be seasonally dependent in some cultivars such as Trincadeira (Fortes et al., 2011), although it is unclear whether the microarray used in this study could discriminate between the three *VvLidh* isoforms. Data from another study using microarray data and potted vines in climate chambers suggest that *VvLidh3* may be up-regulated at night in ripening berries (Rienth et al., 2014).

Development of an antibody to L-IDH (*VvLIDH3* peptide was used as a target but the antibody was not checked for cross-reactivity against the other isoforms) and an enzyme assay compatible with berry extracts enabled the first observations that the L-IDH protein and its activity peak during early berry development, similar to transcript levels of both *VvLidh1* and *VvLidh3*, but linger during ripening (Wen et al., 2010, 2014; Cholet et al., 2016). Given the persistence of the protein beyond the period of TA accumulation, post-translational mechanisms such as protein degradation or sequestration of the enzyme by vacuolar autophagy (Wen et al., 2010), feedback inhibition (Cholet et al., 2016), or the availability of precursors (Melino et al., 2009a, 2011) may explain the cessation of TA accumulation at veraison. Wen et al. (2010) suggested that L-IDH continues to function in the berry vacuole during ripening: if so, it must occur at a low rate, with optimal pH of the recombinant enzyme between pH 8 and 9 and a five-fold drop in specific activity at pH 7 (DeBolt, 2006; Jia, 2015).

Berries of the wild Chinese grapevine *Ampelopsis aconitifolia* lack *Lidh* transcripts and TA (DeBolt et al., 2006), suggesting that L-IDH is essential for TA biosynthesis. Wen et al. (2014) showed that the transcript levels of *Lidh* (in this case a combination of *VvLidh3* and *VvLidh1* orthologs, as it is difficult to design unique primer sets) did not correlate with berry TA levels across *Vitis* species but instead, a high TA accumulator had a slower decline of the L-IDH protein throughout ripening as compared to a lower TA-accumulator. However, it is important to factor in berry size, which can differ significantly across species and cultivars, exerting a dilution effect on the TA content of berries. This warrants further analysis of the data by Wen et al. (2014). In one of the first successful CRISPR/Cas9 mutagenesis studies in grapevine, disruption of *VvLidh3* led to a decrease, but not a total loss of TA in Chardonnay cell cultures (Ren et al., 2016). The authors suggested that residual TA synthesis could be symptomatic of the type of mutation (i.e., no frameshift) or due to a combination of wild type and transgenic cells present within the cell mass. Complementary activity from the *VvLIDH1* isoform might also explain this, unless the guide RNA could not discriminate between both copies and thereby both isoforms were edited within the same cell.

L-IDH enzymes have recently been classified as "Class II" plant sorbitol dehydrogenases (SDH), with a set of key amino acid residues (His42, Gly112, and Ser113) likely responsible for the evolutionary diversification from sorbitol to L-idonate substrate binding and oxidation (Jia et al., 2015). These key residues are conserved in *VvLIDH1* and *VvLIDH3* but not *VvLIDH2*, which is instead designated as a "Class I" SDH and likely involved in sorbitol metabolism rather than TA biosynthesis. Residues predicted to be important for L-idonate selectivity have also been highlighted in the L-IDH ortholog of the TA-accumulating geranium (Narnoliya et al., 2018). The expression patterns of the Class I and Class II SDHs of grapevine also differ, with the former increasing throughout development and the latter aligning more closely with the timing of TA biosynthesis, i.e., decreasing throughout development (Sweetman et al., 2012; Jia et al., 2015; Cholet et al., 2016). *VvLidh1* and *VvLidh3* are genetically linked at 10 kb apart, in the same orientation on chromosome 16, whereas *VvLidh2* is also situated nearby but in the opposite orientation (Higginson, 2015; Jia et al., 2015), suggesting regulation by different promoters.

In apple and sweet orange, the presence of putative L-IDH orthologs did not equate to TA accumulation, leading to some doubt as to the importance of L-IDH in TA biosynthesis (Shangguan et al., 2015). Upon closer inspection of the apple genome, 11 of the 12 potential orthologs had low sequence similarity to the confirmed *VvLIDH3* and importantly, they lacked the key residues identified by Jia et al. (2015) and Narnoliya et al. (2018). That is, only one copy of a Class II plant SDH is present in apple and sweet orange (Jia et al., 2015) and the Class I SDHs may not catalyze the L-idonate oxidation reaction. The only "Class I" SDH to be tested for L-IDH activity, COC280 (Uniprot: A0A061FZU3) from *Theobroma cacao* did not display any convincing oxidation of L-idonate despite its close homology to *VvLIDH3* (Jia, 2015). Therefore, the enzymatic function of these L-IDH orthologs remains undetermined. With respect to the lack of TA in apple and sweet orange, low expression of proteins responsible for upstream steps of the pathway could also conceivably deprive these fruits of L-idonate precursors, an avenue yet to be explored. Therefore L-IDH remains the best-characterized enzyme of the grape berry TA biosynthesis pathway.

2-Keto-L-Gulonic Acid Reductase

Efforts to identify enzymes responsible for other steps of the TA biosynthesis pathway have proven challenging, however some progress has been made toward the 2-keto-L-gulonic reductase (2-KGR; VIT_09s0002g04300), which precedes the L-IDH step (**Figure 1**). A grape gene with homology to the *Escherichia coli* 2-KGR from Yum et al. (1998) showed the same developmental expression pattern as *VvLidh3* (Burbidge, 2011). The recombinant enzyme had 2-KGR activity, however substrate affinity kinetics favored the enzyme as a glyoxylate or hydroxypyruvate reductase that may have additional activity as a 2-KGR (Burbidge, 2011; Jia et al., 2019). Indeed, in plants the grape *Vv2KGR* has the highest sequence similarity with the cytosolic *Arabidopsis thaliana* hydroxypyruvate reductase isoform 2 (*AtHPR2*), which acts as a compensatory bypass for hydroxypyruvate and glyoxylate reduction (Timm et al., 2008). However, recombinantly expressed *Vv2KGR* was also active, albeit far less efficiently, with L-idonate (i.e., the reverse reaction) as well as Asc, formate, sorbitol, D-glucose, 6-phosphogluconate, 5-keto-D-gluconate and D-gluconate (Burbidge, 2011). Subsequently, the protein structure of *Vv2KGR*

was resolved using x-ray crystallography, and molecular docking studies revealed 2-keto-L-gulonate as the optimal substrate, while GC-MS confirmed the product of this reaction as L-idonate (Jia et al., 2019). Based on *in vitro* studies this enzyme may be considered a 2-KGR that has been commandeered from another metabolic pathway. Both *Vv2KGR* and *VvLIDH3* retain significant "original" enzyme activities, i.e., HP/glyoxylate reduction and sorbitol oxidation, respectively (Jia, 2015; Jia et al., 2019). This is a common observation for gene evolution via duplication, in which the newly duplicated genes evolve novel functions while still retaining their original functions (Flagel and Wendel, 2009).

Intracellular Transporters

Once synthesized, TA is translocated to the vacuole for storage. An aluminum-activated malate transporter (ALMT) protein, encoded by *VvAlmt9* (XM_002275959), is likely to shuttle both TA and MA into the vacuole (Patel, 2008; Rongala, 2008; De Angeli et al., 2013). It is expressed throughout berry development but highest in ripe fruit according to transcriptomic data from Shiraz berries (Sweetman et al., 2012) and qPCR data from Aragonez berries (De Angeli et al., 2013), therefore its activity is likely to remain important long after net accumulation of MA and TA.

At present there are no candidates for TA transport at any other membrane of the plant cell but there are some candidates for transport of the precursor, Asc. There are twelve candidate genes for nucleobase ascorbate transporters (NAT) in Arabidopsis, three of which (*AtNat7*, *8* and *12*) are localized to the plasma membrane and at least one in the thylakoid membrane. However, it is unknown if these transport Asc or nucleobases (Maurino et al., 2006; reviewed by Foyer et al., 2020). A chloroplast envelope transporter of Asc (*AtPht4:4*) has also been identified (Miyaji et al., 2015), and chloroplast uptake is dependent on membrane potential and cytosolic Asc concentration. There is little understanding of the mechanisms by which Asc travels from its site of synthesis in the mitochondrion throughout the berry cell.

TA CATABOLISM

Endogenous TA in the grape berry is not catabolized at any appreciable rate (Ruffner, 1982). Instead, it is widely accepted that the acid forms a stable salt of potassium bitartrate, which is sequestered in the vacuole away from potential catabolizing enzymes and thereby essentially untouched throughout ripening (Takimoto et al., 1976; Moskowitz and Hrazdina, 1981; Ruffner, 1982; DeBolt et al., 2004). However, degradation of exogenously applied radiolabeled TA could be recovered as CO_2 from excised grape berries, suggesting that TA-catabolizing enzymes do exist in *V. vinifera* (Hrazdina et al., 1984). *E. coli* and *Pseudomonas* spp. can use D-tartrate as a carbon source via oxidation to oxaloacetate or glycerate and eventually pyruvate (Vaughn et al., 1946; Dagley and Trudgill, 1963; Kohn and Jakoby, 1968). L-tartrate, the isomer present in grapes, can be used for carbon fixation by *Agrobacterium vitis*; a bacterium that harbors genes for TA-metabolizing enzymes and is hosted by *V. vinifera* (Otten et al., 1995). L-tartrate from grape must can also be catabolized by *Botrytis cinerea*, resulting in several different organic acids including malate, pyruvate, acetate, oxalate and oxaloacetate (Shimazu et al., 1984). Therefore, while the berry is intact, TA remains sheltered from catabolism; however, upon its release from the vacuole via mechanical damage (i.e., during harvest and crush), or upon pathogen invasion, TA likely becomes susceptible to catabolic enzymes from such microorganisms.

ASCORBATE AS A PRECURSOR FOR TA BIOSYNTHESIS

In plants, Asc is amongst the major vital antioxidants and fulfils a plethora of functions in different cellular components (Smirnoff, 2011; Foyer et al., 2020). For example, Asc acts as an enzyme cofactor and modulator of enzyme activity in the thylakoid membrane (Müller-Moulé et al., 2004), a reducing agent in the chloroplast (Krieger-Liszkay et al., 2008), a substrate for ethylene biosynthesis (Mirica and Klinman, 2008), and has roles in regulation of cell expansion, fruit-ripening and softening in the apoplast (Green and Fry, 2005; Gilbert et al., 2009). Physiological roles of Asc in plants also include defense (Conklin et al., 1996), growth and development (Dowdle et al., 2007), hormone and pathogen responses (Pastori et al., 2003) and programmed cell death (de Pinto et al., 2006). The redox couple Asc to DHA can influence the cellular redox state, which may be an important component in ROS signaling (Foyer and Noctor, 2005; Noctor, 2006).

Asc does not accumulate to high quantities in grapes compared to some other fruits, for example, ripe Shiraz grapes contain approximately 0.7 $\mu mol.g^{-1}$ FW (Melino et al., 2009b) which is significantly lower than ripe strawberries (3.37 $\mu mol.g^{-1}$ FW) and kiwifruits (3.41 $\mu mol.g^{-1}$ FW) (Davey et al., 2000). It is not known for certain whether low Asc accumulators have a lower rate of Asc biosynthesis or an increased turn-over capacity, although *A. aconitifolia* berries, which do not accumulate TA, contain 3-fold more ascorbate compared to *V. vinifera* cv. Cabernet Sauvignon (DeBolt et al., 2006). Therefore, TA may primarily function to catabolize excess Asc, as recently proposed by Cholet et al. (2016) and blocking the TA biosynthetic pathway may increase vitamin C content in *V. vinifera* berries. Considering that there is no sudden accumulation of Asc at the conclusion of net TA biosynthesis in grapes, but there is reduced transcription of Asc biosynthetic genes in the Smirnoff-Wheeler pathway, a halt in the biosynthesis of Asc is a likely contributor to the plateau in TA levels from veraison (Melino et al., 2009a).

DEHYDROASCORBATE AS A PRECURSOR FOR TA BIOSYNTHESIS

Ascorbate peroxidase (APX) catalyzes the two-electron oxidation of Asc, reducing hydrogen peroxide (a potent ROS that can inactivate CO_2-fixation enzymes) to water and forming DHA (via monodehydroascorbate, MDHA) in numerous cellular

compartments. Asada (1992) described the value of regenerating reduced Asc as two-fold: to maintain capacity to donate electrons for the reduction of hydrogen peroxide and to protect against inactivation of APX.

In very young grape berries (10 days after flowering), over 70% of ^{14}C from exogenously applied Asc was recovered within TA (Saito and Kasai, 1969). Generally, the reduced form of Asc is considered the fundamental precursor of TA in the cytosol due to its vast predominance over oxidized forms in the cell (Smirnoff, 2018). However, the cellular location of TA biosynthesis has not yet been definitively shown. Radiolabelling studies may largely represent apoplastic events due to the methods of radiolabel application (Smirnoff, 2018) and in the apoplast the Asc pool is rapidly oxidized to DHA (Pignocchi et al., 2003). Hancock and Viola (2005) proposed DHA as an important intermediate of TA biosynthesis; also labeled a "branch-point" for Asc catabolism by Parsons et al. (2011). When fed to grapevine leaf apices, radiolabeled DHA led to 2KGA, L-idonate and ultimately TA in a similar manner to radiolabeled Asc (Saito and Kasai, 1984). This suggests DHA as an intermediate in the conversion of L-Asc to 2KGA, a reaction that at the present time remains to be characterized. DHA catabolism to oxalic acid (OA) and other products in the culture media of *Rosa* cells suggest that such Asc catabolism can occur via DHA in the apoplast (Green and Fry, 2005). In grapes, OA and TA both accumulate within the same cells and demonstrate strikingly similar developmental accumulation profiles that show highest biosynthesis at a time when ascorbate is predominantly in the oxidized form (DeBolt et al., 2004; Melino et al., 2009a). During ripening, when net TA accumulation no longer occurs, there is a gradual shift to the reduced form (i.e., increased Asc to DHA ratio), likely due to decreased transcription of key genes responsible for Asc biosynthesis and increased transcription of genes responsible for recycling Asc from its oxidized forms (Melino et al., 2009a). There is also a substantial increase in the concentration and reduction state of glutathione (Adams and Liyanage, 1993; Okuda and Yokotsuka, 1999), which may redirect DHA toward Asc recycling and away from catabolic processes. Further correlative support arises through different grapevine tissues: roots have a small and highly reduced ascorbate pool (Asc to DHA ratio of 13.4) and accumulate little or no TA as compared to berries, rachis and leaves which have an Asc to DHA ratio of 1.1, 1.9, and 2.6 respectively (Kliewer, 1966; Melino et al., 2009b).

POTENTIAL ROLES OF TA IN GRAPE BERRIES BASED ON PREDECESSORS OF TA BIOSYNTHETIC ENZYMES

An Incidental Route to TA Accumulation?

As new enzymes of the TA biosynthesis pathway are uncovered, it will be interesting to see if these too are adapted from, or closely related to orthologs from other metabolic pathways, as proposed for L-IDH and 2-KGR. If so, TA accumulation could be a consequence of unrelated biochemical phenomena: high expression of enzyme isoforms that primarily catalyze other reactions but have adapted to recognize TA precursors as substrates, and where low affinity can be overcome by high precursor concentration (Melino et al., 2009a; Jia et al., 2015, 2019). That is, enzymes from other metabolic pathways could become "hijacked" when DHA or Asc levels are high, provided a particular (as yet unidentified) set of primary metabolic pathways are operating. This "incidental" route of TA biosynthesis might explain the narrow distribution of TA in the plant kingdom and the specific developmental pattern of accumulation in grape berries. Even the ALMT responsible for TA uptake into the vacuole (Terrier et al., 1998; De Angeli et al., 2013) could have been adapted from an exclusively MA transport function. Such evolution of a metabolic pathway is not unheard of in plants and may not mean that the pathway is unimportant: consider the glycolate pathway, adopted by modification of probably existing enzymes, now occurring in at least three organelles and cytosol, and essential in dealing with oxygenic photosynthesis (reviewed by Fernie and Bauwe, 2020).

From what we have garnered of grape berry TA biosynthetic enzymes so far, one enzyme (*Vv2KGR*) probably diverged from, or shares functionality with, a hydroxypyruvate or glyoxylate reductase (Jia et al., 2019) while another (*VvLIDH3*) diverged from a sorbitol dehydrogenase (Jia et al., 2015). The functions of these precursor enzymes will be explored below, to speculate on potential roles or reasons for TA biosynthesis in grape berries. Importantly, TA is also synthesized in grapevine leaves, likely by the same C4/C5 cleavage pathway found in grape berries (Williams and Loewus, 1978). Therefore, the roles of these divergent or promiscuous enzymes (outside of TA biosynthesis) are likely to be common between young berries and leaves. A role in photosynthesis or photorespiration seems most likely.

A Sink for Ascorbate – The Hydroxypyruvate/Glyoxylate Reductase Alternative

Hydroxypyruvate reductase catalyzes the reversible conversion between hydroxypyruvate and glycerate in the peroxisome accompanied by the oxidation of NADH to NAD^+. This reaction is essential during carbon recovery in photorespiration (Fernie and Bauwe, 2020). Glyoxylate reductase converts glyoxylate to glycolate in the cytosol, oxidizing NADPH to $NADP^+$, and probably participates in the removal of toxic glyoxylate leaked from peroxisomes (Zhang et al., 2020a). Interestingly, these two activities arise from the same enzyme, along with 2-KGR activity. Considering the proposed dual roles of 2-KGR in reactions related to photorespiration, early reports of increased TA levels in light-exposed grape berries (Saito and Kasai, 1969; DeBolt et al., 2006) would seem to support a function for this enzyme in its synthesis. However, further investigation of berries grown for extended periods in light-excluding boxes suggested that the regulation by light applies also to the biosynthesis of Asc and that the transcription of *VvLidh3* was unaffected by light (Melino et al., 2011). These observations complicate the story, the light-mediated effects on ascorbate probably act to provide antioxidant buffering when ROS-generating photosynthetic activities are high. Only immature grape berries are capable of photosynthesis

(Ollat and Gaudillère, 2000), coinciding with the timing of TA accumulation, which may therefore act as a sink for excess Asc as recently proposed elsewhere (Cholet et al., 2016). However, while the over-exposure of ripening berries to light caused depletion of Asc and glutathione levels, likely due to oxidation (DHA was not measured), there was no effect on TA levels (Rustioni et al., 2020).

An Osmolyte – The Sorbitol Dehydrogenase Alternative

SDHs oxidize sorbitol and other sugar alcohols such as xylitol and ribitol to their corresponding monosaccharides (i.e., fructose, xylulose, and ribulose), reducing $NAD(P)^+$ to NAD(P)H in the cytosol (Aguayo et al., 2013). Sorbitol is the main form of translocated sugar in some plants (mostly Rosaceae), though *V. vinifera* transports predominantly sucrose instead, and does not accumulate sorbitol to a high level (Swanson and El-Shishiny, 1958). In water-deficit conditions, grape berry SDH activity is down regulated, allowing sorbitol levels to increase (Conde et al., 2015). Knockdown of SDH in Arabidopsis led to improved tolerance to drought stress, likely due to osmo-protection from the higher levels of sorbitol (Aguayo et al., 2013). TA has also been suggested to enhance osmotic potential, especially in pre-veraison berries (Bigard et al., 2018) where it can be responsible for over 50% of total berry osmolarity, followed by MA at approximately 25% (Diakou et al., 1997). In the fruits of *Citrus reticulata*, *C. sinensis* and *C. paradisi*, treatment with proline (a stress-induced amino acid and osmo-protectant) led to decreased levels of TA, H_2O_2, and MDA (a lipid peroxidation product indicative of oxidative stress), as well as decreased lipoxygenase activity and increased APX activity (Mohammadrezakhani et al., 2019). In this case, excess proline may have reduced the need for other osmoprotective or ROS-protective mechanisms such as TA biosynthesis. Support for an osmo-protectant role of TA in grape and other fruits is merely correlative but warrants a targeted investigation.

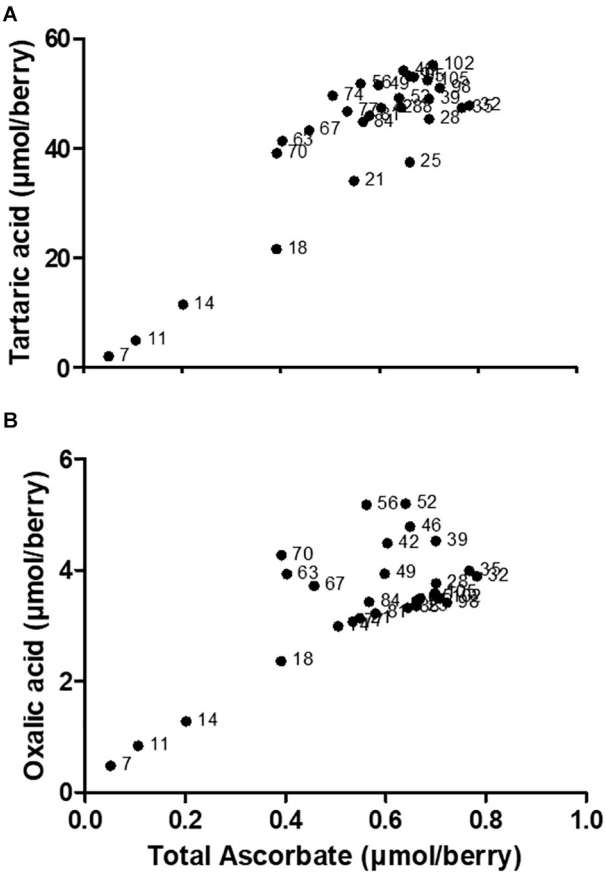

FIGURE 2 | Correlation between **(A)** tartaric acid and **(B)** oxalic acid, with total ascorbate across *V. vinifera* c.v. Shiraz berry developmental series. Data points are labeled with physiological stage of berry (days after flowering). Correlation analysis was conducted using GraphPad Prism 5.01. The spearman correlation coefficients (rs) are 0.6486 and 0.4466, respectively. Adapted from Melino (2009).

POTENTIAL ROLES OF TA IN GRAPE BERRIES BASED ON PRECURSORS OF THE TA BIOSYNTHETIC PATHWAY

An Incidental or Dedicated Route to TA Accumulation?

In young grape berries a significant proportion of Asc is present as DHA (i.e., a low Asc to DHA ratio), which is engaged in the biosynthesis of OA and TA (Melino et al., 2009a). There is a strong positive correlation between the accumulation of total ascorbate (the sum of reduced and oxidized forms), and TA or OA in young berries (**Figure 2**). A regulatory link between total ascorbate levels and *VvLidh3* transcription has been proposed, and it follows that TA biosynthesis could be a storage mechanism for excess Asc or DHA under certain conditions (Cholet et al., 2016), generating an osmolyte that is sequestered in the vacuole during times of high Asc biosynthesis. Such a role is also consistent with the substantial accumulation of TA in grapevine leaves (Ruffner, 1982). This may support the hypothesized "incidental" route of TA biosynthesis, whereby the availability of precursors (i.e., Asc or DHA) is the limiting factor.

Apoplastic Redox State, ROS Signaling and Cell Wall Softening

Additional roles for TA in grape berries may be found in the apoplast where it co-exists with other organic acids including Asc, OA and MA, as well as sugars and cations such as potassium (Wada et al., 2008; Keller and Shrestha, 2014; Podgórska et al., 2017). In leaves, the apoplast can contain up to 10% of the total cellular Asc content but this is highly oxidized due to the activity of ascorbate oxidase (AO), localized to the cell wall (Pignocchi et al., 2003; Karpinska et al., 2018). Although AO activity is enhanced by photosynthetic O_2 (De Tullio et al., 2007), excessive AO activity can impair photosynthesis (Karpinska et al., 2018). In tobacco, high AO activity also led to the accumulation of apoplastic threonate, a DHA degradation product (Karpinska et al., 2018). The genome of *V. vinifera* (cv Pinot Noir) contains an unusually large

number of genes encoding AO (9 copies) and APX (12 copies) compared to Arabidopsis (3 and 6 copies, respectively) and sweet orange (2 and 5 copies, respectively). Transcript levels of at least three AO genes (*VvAO1*, VIT_06s0009g01320; *VvAO3*, VIT_07s0031g01010; and *VvAO4*, VIT_07s0031g01040) followed a consistent developmental down-regulation in the grape berry (Fasoli et al., 2012; Sweetman et al., 2012; Savoi et al., 2017), analogous to the pattern of TA accumulation. In young *V. vinifera* berries with their multitude of AO genes and highly oxidized ascorbate pool, the accumulation of TA (instead of, or in addition to threonate) in the presence of light and photosynthetic O_2, could provide an avenue for DHA degradation, in an attempt to balance the redox state of the apoplastic Asc pool.

The redox state of the apoplastic Asc pool is involved in ROS signaling (reviewed by Podgórska et al., 2017; Karpinska et al., 2018; Smirnoff, 2018). Signal transmission from the environment requires that some of the apoplastic Asc is kept in an 'active' (reduced) state (Pignocchi and Foyer, 2003), while an oxidized apoplastic Asc pool (i.e., high DHA) can inhibit photosynthesis and cell division but promote cell elongation and cell wall loosening (Pignocchi and Foyer, 2003; Pignocchi et al., 2003; Sanmartin et al., 2003; Karpinska et al., 2018). In tomato fruit, at the beginning of ripening Asc is excreted from the cell to the apoplast where it can generate hydroxyl radicals by reacting with H_2O_2 in the presence of ascorbate peroxidase, facilitating non-enzymatic degradation of polysaccharides in the cell wall and thus cell expansion (Dumville and Fry, 2003; Green and Fry, 2005). On the other hand, upon entry into the cell as a signal from the apoplast, DHA can slow cell cycle and proliferation in tobacco cell cultures (de Pinto et al., 1999; Potters et al., 2000). Reduction of DHA to Asc can apparently only occur in the cytosol, likely due to the lack of NADH and NADPH in the apoplast (Pignocchi and Foyer, 2003). Therefore, a specific transporter that can exchange apoplastic DHA for cytosolic Asc (Horemans et al., 2008), could simultaneously facilitate inhibition of cell division and enhancement of cell expansion – hallmarks of fruit ripening. It follows that TA biosynthesis could postpone cell wall loosening by catabolizing Asc (or DHA) in the apoplast. Based on transcriptomic datasets, higher transcription of *VvLidh3* may be associated with the firmer berries of Red Globe compared to soft berries of Muscat Hamburg (Ma et al., 2020). This transcript was also upregulated in berries afflicted with shrivel compared to non-shriveled berries co-existing on the same Zweigelt vines (Savoi et al., 2019). From veraison, phloem unloading into the berry switches from a symplastic route to apoplastic (Zhang et al., 2006), flooding the apoplast with high concentrations of hexoses and other solutes, potentially interrupting TA biosynthesis at the cell wall. Changes in apoplastic solutes affect cell turgor and berry firmness associated with veraison and necessary for ripening (Wada et al., 2008; Keller and Shrestha, 2014; Rogiers et al., 2017), as also seen in other fruits (Canton et al., 2020).

Although TA synthesis could take place in either or both cytosol and apoplast, further work is needed to confirm the apoplast as a site of synthesis. If a significant proportion of TA is synthesized in the apoplast there must be a transport mechanism for entry into the cell, to enable accumulation in the vacuole. Identification and characterization of this, and other transporters of the grape plasmalemma will help to determine DHA import kinetics and a mechanism for TA import, if it exists.

Antioxidant Metabolism and the Oxidative Burst

ROS, antioxidants and their interactions are key to regulating fruit developmental stages and ripening (Muñoz and Munné-Bosch, 2018; Decros et al., 2019). Despite being classified as a non-climacteric fruit, grapes exhibit an oxidative burst in the skin cells as the berries begin to soften but prior to color change (Pilati et al., 2007). The ROS in this oxidative burst accumulate as superoxide in the chloroplast and hydrogen peroxide in the cytosol (Pilati et al., 2014). Hydrogen peroxide, when applied to bunches of young berries can also advance ripening (Guo et al., 2019). The mechanism by which hydrogen peroxide accumulates in the cytosol is unknown but a loss of catalase activity is unlikely to explain it (Pilati et al., 2014). Most likely it originates from another organelle and is transported to the cytosol via aquaporins (Bienert et al., 2006; Smirnoff and Arnaud, 2019).

Hydrogen peroxide accumulation may be a side-effect from the removal of superoxide from the chloroplast or from the loss of photosynthetic functionality. Alternatively, hydrogen peroxide could be released from the peroxisomes/glyoxysomes or mitochondria for example during photorespiration or oxidation of excess reducing equivalents that have exited the chloroplast (Smirnoff and Arnaud, 2019). Pilati et al. (2014) also speculate that it may signal a temporary switch to fermentative respiration. In any case it likely relies on the activity of a superoxide dismutase (SOD), of which isoforms are present in almost all organelles (Alscher et al., 2002). Up-regulation of a SOD transcript (VIT_214s0030g00950) has been reported at veraison in berries of both Kyoho and its early ripening mutant Fengzao (Guo et al., 2016). Despite considerable differences in ripening time between the two cultivars, the SOD transcript began to accumulate in skins of both cultivars at the initiation of berry softening and peaked at the initiation of berry coloring. It was unclear what type of SOD isoform this gene encoded, although a follow-up study found transcriptional up-regulation of a putative cytosolic Cu/Zn-SOD (Vitvi14g02607) and a putative mitochondrial MnSOD (Vitvi13g00177) in veraison berries of both cultivars (Guo et al., 2020). This work would benefit from more biological replication and expansion to other cultivars, and it would be of great interest to know which organelle generates the hydrogen peroxide that is involved in regulating berry ripening.

The accumulation of ROS is followed by a surge in the appearance of ROS-detoxification transcripts and their cognate proteins including ascorbate and glutathione peroxidases, ascorbate oxidase, catalase, polyphenol oxidase, peroxiredoxins, thioredoxins, glutaredoxins, glutathione-S-transferases, metallothioneins, tocopherol cyclase and lipoxygenases (Jimenez et al., 2002; Pilati et al., 2007, 2014; Negri et al., 2008; Agudelo-Romero et al., 2013; Rienth et al., 2014). During ripening, increases in total glutathione and total ascorbate levels have also been observed as well as an increase in their reduction state (Melino et al., 2009a; Fortes et al., 2011). There is also an increase in the production of galactolipid peroxidation products, directed

by lipoxygenase activity (Pilati et al., 2014). The molecular and biochemical changes that occur with the oxidative burst at veraison are indicative of an oxidative stress or "oxidative signaling" event (Foyer and Noctor, 2005) analogous to that of the climacteric burst of tomato fruit (Jimenez et al., 2002).

A negative regulator of transcription, NOR (non-ripening) was recently discovered in tomato, which prevents the transcription of ripening-related genes. The NOR protein is susceptible to post-translational regulation via methionine sulfoxidation (i.e., oxidative damage), causing a loss of DNA-binding capacity and thus enabling the transcription of ripening-related genes (Jiang et al., 2020). Therefore, oxidative stress signals are involved in the initiation of ripening, and such a transcription factor could be explored in relation to grape berry oxidative stress and ripening. A surge in ROS levels in the chloroplasts was also observed during the chloroplast-to-chromoplast transition in the exocarp. The trigger for the oxidative burst in grape berries is currently unknown but it is tempting to speculate that the developmental degeneration of chloroplasts, or chloroplast-to-chromoplast transition (Jimenez et al., 2002; Decros et al., 2019), and diminishing capacity for photosynthesis (Palliotti and Cartechini, 2001) leads to decreased accumulation of Asc and TA at veraison, thus the ascorbate/glutathione antioxidant system becomes overwhelmed, resulting in excess ROS generated from respiration or from photons that can no longer be captured for photosynthesis. As such, it would be expected that decreased Asc (or TA) levels and increased ROS could advance ripening. However, in Micro-Tom tomato fruit lacking GDP-L-galactose phosphorylase (VTC2), low levels of Asc (30% relative to wild type fruit) attenuated the ripening-related peak in H_2O_2 and delayed ripening, which was recoverable with the addition of exogenous Asc but further delayed by H_2O_2 (Steelheart et al., 2020). Meanwhile ripening of Kyoho grape berries could be brought forward by spraying with H_2O_2 (Guo et al., 2019). This underscores the importance, and complicated interplay of Asc and H_2O_2 for fruit ripening. Non-enzymatic catabolism of Asc (via DHA) to either OA and threonate (then potentially on to TA, at least in tomato), or to 2,3-diketogulonic acid (and several downstream catabolites) can generate H_2O_2 in the apoplast (Green and Fry, 2005; Kärkönen et al., 2017). Therefore, diversion of Asc to TA via the 4,5 cleavage pathway and L-idonate, as seen in grapes, could avert this pro-oxidant characteristic of Asc. Transgenic lines of tomato overexpressing VTC2 resulted in very large increases in Asc (3 to 6-fold) but impaired fruit growth and seed production (Bulley et al., 2012). Therefore a potentially important role for TA may be prevention of Asc or DHA over-accumulation in the apoplast, thus postponing the initiation of ripening until the seed is ready for dispersal. It should be noted, however, that translational repression of VTC2 via an upstream ORF (uORF) may prevent over-accumulation of Asc via a clever negative feedback loop as seen in Arabidopsis, tomato and lettuce leaves (Laing et al., 2015; Broad et al., 2020). If this mechanism is active in fruit cells, TA is less likely to be involved in the moderation of Asc accumulation except perhaps in circumstances where very rapid Asc accumulation requires an immediate overflow valve. A link between the oxidative burst and the abrupt changes in TA biosynthesis and ascorbate/glutathione redox state is hereby tentatively proposed. A higher level of regulation is also likely to occur via crosstalk between hormones including ethylene and ABA (Chervin et al., 2004; Lijavetzky et al., 2012; Böttcher et al., 2013a; Carvalho et al., 2015; Pilati et al., 2017).

Stress Tolerance

Several studies (many reviewed by Gill and Tuteja, 2010) have used over-expression of DHAR genes from various sources, to improve tolerance of tobacco, potato, and Arabidopsis plants to stresses such as salinity, drought, cold, ozone, heavy metal, herbicide treatment, or H_2O_2 application (Chen et al., 2003; Kwon et al., 2003; Chen and Gallie, 2005, 2008; Eltayeb et al., 2006, 2011; Ushimaru et al., 2006; Yin et al., 2010; Chang et al., 2017). Generally, the transgenic leaves demonstrated increased Asc due to recycling and in some cases increased levels of total ascorbate, leading to lower ROS accumulation and improved growth metrics in response to stressors. These studies demonstrate the importance of DHA recycling to Asc for tolerating oxidative stress. A study with tomato found that overexpression of DHAR increased total ascorbate levels in fruit but not leaves (Haroldsen et al., 2011). Redirection of DHA to TA biosynthesis removes the potential for Asc recycling and necessitates continuous biogenesis of Asc in pre-veraison berries. It is unclear whether this would have an impact on the antioxidant capacity of the Asc pool and stress tolerance. Studies of transgenic grapevines or natural mutants with impaired or increased TA accumulation are necessary to determine whether the promotion of TA biosynthesis could increase or decrease stress tolerance of grapevines. An exploration of publicly available datasets (below, **Table 1**) showed that TA biosynthesis genes are largely unaffected by environmental conditions.

Transcriptional profiling of all three *VvLidh* genes and the *Vv2kgr* gene with publicly available datasets was difficult due to a lack of microarray probesets and genome accessions for *VvLidh2*, and due to the similarity between *VvLidh1* and *VvLidh3* isoforms such that they may have been indistinguishable in RNAseq experiments. For the purpose of this analysis we assumed that *VvLidh* (VIT_16s0100g00290) represents transcripts of both *VvLidh1* and *VvLidh3*. Most datasets (**Table 1**) exhibited no changes in expression of *VvLidh* nor *Vv2kgr* (VIT_09s0002g04300), including experiments featuring water limitation (Berdeja et al., 2015; Catacchio et al., 2019), cold night temperature (Sawicki et al., 2019), elevated light (du Plessis et al., 2017), diurnal regulation (Rienth et al., 2014), salt stress in leaves (Upadhyay et al., 2018; Das and Majumder, 2019), increased source-sink ratio via cluster thinning (Pastore et al., 2011), copper stress (Leng et al., 2015) and abscisic acid application (Rattanakon et al., 2016; Pilati et al., 2017). In an experiment reporting the differential terroir effect on Cabernet Sauvignon berries in Bordeaux and Reno, there was also no change, but these experimental samples were skins collected during late ripening so expression of *VvLidh* was likely low anyway (Cramer et al., 2020). In response to elevated temperature exposure there was a small down-regulation of *VvLidh* at green and veraison berry stages (Rienth et al., 2016) and a small up-regulation at

TABLE 1 | Summary of transcriptomic responses of *VvLidh* and *Vv2kgr* to environmental cues.

Treatment/Condition	Log2 FC		Source
	VvLidh	***Vv2kgr***	
Water deficit	–	–	Berdeja et al., 2015
Water deficit	–	–	Catacchio et al., 2019
Cold night temperature	–	–	Sawicki et al., 2019
Elevated light	–	–	du Plessis et al., 2017
Salt (leaves)	–	–	Upadhyay et al., 2018
Salt (leaves)	–	–	Das and Majumder, 2019
Increased source:sink	–	–	Pastore et al., 2011
Copper stress	–	–	Leng et al., 2015
Terroir	–	–	Cramer et al., 2020
Abscisic acid	–	–	Pilati et al., 2017
Abscisic acid	0.08	−0.001	Rattanakon et al., 2016
Water deficit	−0.01 to 0	−0.02 to 0.02	Dal Santo et al., 2016
Water deficit	−0.2 to 0.9	−0.4 to 0.3	Ghan et al., 2015
Water deficit	**0.4 to 1.4 (veraison or ripening berries)**	–	Savoi et al., 2017
Extended drought	**0.6 (veraison berries)**	**0.4 (veraison berries)**	Savoi et al., 2016
Elevated temperature	−0.5 to 1.0 (green or veraison berries)	–	Lecourieux et al., 2017
Elevated temperature	**1.3 (ripening berries)**	–	Lecourieux et al., 2017
Elevated temperature	**−1.4 to −2.1 (green or veraison berries)**	–	Rienth et al., 2016
Elevated temperature	**1.1 (ripening berries)**	–	Rienth et al., 2016
Day (c.f. Night)	**−1.2 (late ripening)**	–	Rienth et al., 2014
Cold, anaerobic storage	**2.8**	–	Maoz et al., 2019

Dashes indicate datasets where log2-fold data could not be retrieved, as only DEGs were presented. In these cases it is suspected, but not confirmed, that the transcript was unchanged between treatment groups, however it could also reflect limits of detection or data lost during filtering steps. Bold indicates statistically significant differences.

ripening stages (Rienth et al., 2016; Lecourieux et al., 2017). There was also a slight up-regulation of both *VvLidh* and *Vv2kgr* transcripts in response to extended drought stress at veraison and during ripening (Savoi et al., 2016), while another water deficit experiment showed up-regulation of *VvLidh* only but was accompanied by a slight increase in TA levels (Savoi et al., 2017). Cold storage of table grapes post-harvest led to an increase in *VvLidh3*, if stored under anaerobic conditions (Maoz et al., 2019). Overall the *VvLidh3* and *Vv2kgr* transcripts are largely unaffected by environmental conditions.

Flowering Time and Senescence

As reviewed by Foyer et al. (2020), Asc can regulate flowering time via the NO-mediated flowering repression pathway (Kumar et al., 2016), such that application of Asc or L-galactonolactone delays flowering, while knockout mutants of various *Vtc* genes in *A. thaliana* (i.e., less Asc) flower earlier (Kotchoni et al., 2009). Genetic modification of AO led to altered expression and diurnal regulation of catalase genes and photorespiration-related genes in tobacco, as well as altered DHAR and APX activities, MAPK activity and growth response to auxins (Pignocchi et al., 2006). A cytosolic APX knockout in Arabidopsis increased H_2O_2 content, delayed flowering, decreased photosynthesis rates, prevented stomatal closure in the dark, and decreased expression of some photorespiration-related genes (Pnueli et al., 2003). Overall, Asc metabolism can regulate many developmental switches including flowering and senescence via altered ROS (or reactive nitrogen species (Podgórska et al., 2017; Smirnoff, 2018; Foyer et al., 2020), while TA biosynthesis could indirectly regulate these by catabolizing Asc in the grape.

OTHER POTENTIAL ROLES OF TA BIOSYNTHESIS

The high levels of TA in Vitaceae may be considered an evolutionary consequence to aid in the dispersion of the mature seed of the grape berry by birds or animals, ensuring the berry is unpalatable until the seed is mature (Hardie and Obrien, 1988; Hardie, 2000; Brummell et al., 2016). Asc has also long been linked to plant defense against biotic and abiotic stressors (Smirnoff, 2000; Zechmann, 2011). Therefore, the possibility of TA being synthesized and stored in the cell as an alternative defensive mechanism, should also be considered at the whole organism level. The potential role in defense against herbivores was suggested by Böll et al. (2005), who found evidence of calcium tartrate crystals in the midgut of the phloem-sucking grape leafhoppers (*Empoasca vitis*) after feeding on grapevine leaves, with levels increasing during ripening of berries of the vine. Although TA was ineffective at preventing leafhoppers from eating the grapevine leaves, calcium tartrate crystals may have a more toxic effect on other insects that feed on grapevine. Both TA and OA have been identified in the hairs of stinging nettle (*Urtica dioica*) as eliciting a pain response in rats (Fu et al., 2006). The

human body, along with rabbits, dogs and rats cannot process TA, which instead must be expelled, or destroyed by microorganisms in the intestinal tract (Underhill et al., 1931; Finkle, 1933; Lord et al., 2005). Nevertheless, TA has been deemed non-toxic and appropriate for use as a food additive (EFSA Panel on Food Additives and Flavourings et al., 2020) although the nutritional benefits have not been fully investigated (Spiller et al., 2003).

TA may be used as a carbon source by some microorganisms. Grape berries infected with *Botrytis cinerea* contained less TA and more sorbitol than uninfected berries (Ribereau-Gayon et al., 2006; Blanco-Ulate et al., 2015), hinting at a negative relationship between these two osmolytes that warrants further investigation considering the *VvLIDH3* is a Class II SDH. Based on transcriptomic data (Blanco-Ulate et al., 2015), infection of Semillon berries with *B. cinerea* led to consistent and significant up-regulation of *VvLidh3* expression in harvest-ripe berries ($r = 0.78$ between transcript level and measures of rot). However at harvest-ripeness, net TA biosynthesis is expected to be low or negligible. Transcript levels of a Botrytis-encoded tartrate dehydrogenase were also enhanced (Blanco-Ulate et al., 2015), therefore catabolism of TA by the fungus could explain the loss of TA. There were also some changes in Asc biosynthesis genes of the grape (Blanco-Ulate et al., 2015), suggesting that this whole metabolic pathway may be reprogrammed, possibly to elicit an antioxidant response. Meanwhile, transcript levels of *Vv2kgr* were largely unaffected but there were negative correlations ($r = -0.77$ and -0.79) between measures of rot and the transcript levels of two genes putatively associated with later steps in the TA synthesis pathway, a transketolase and a succinic semialdehyde dehydrogenase. This could offer another explanation for lower TA levels in infected fruit, however these genes are yet to be confirmed as components of the TA biosynthesis pathway. Some yeast strains can also utilize L(+)-tartaric acid as a sole carbon source, including a significant number of Basidiomycetous species (Fonseca, 1992). Basidiomycetes, responsible for white rot (Fischer and García, 2015) have been shown to preferentially degrade the L-isomer over the other isomers (Fonseca, 1992). *Pseudomonas syringae* pv. *Syringae*, responsible for bacterial inflorescence rot (Whitelaw-Weckert et al., 2011), has also been shown to utilize TA (Hall, 2015).

IDENTIFYING NEW CANDIDATES FOR THE REMAINING STEPS OF TA BIOSYNTHESIS

Currently, candidate genes in TA biosynthesis have been mainly identified through a biochemistry-guided approach, i.e., based on their annotated enzymatic functions in catalyzing one of the proposed biochemical reactions in the TA biosynthetic pathway. Other than *Vv2kgr* and *VvLidh3*, no candidates have been identified for the remaining steps of TA biosynthesis. These include the first dedicated step, i.e., the conversion of either Asc or DHA to 2-keto-L-gulonate (which may occur in multiple steps), and the final two steps, i.e., 5-keto-D-gluconate to tartaric acid semialdehyde, and subsequently to TA (**Figure 1**). It was earlier proposed that a transketolase could cleave 5-keto-D-gluconate into 4C and 2C fragments; its normal role would likely be in the Calvin Cycle or the pentose phosphate pathway but perhaps under certain conditions 5-keto D-gluconate could be a substrate (Salusjärvi et al., 2004). For the final step, we suspect a "tartaric acid semialdehyde dehydrogenase", which could be a promiscuous succinic acid semialdehyde dehydrogenase or a divergent isoform with tartaric acid semialdehyde as a new preferred substrate. It may also be possible that some of these reactions occur non-enzymatically, or that one enzyme may be responsible for multiple steps, e.g., the *Vv2KGR* could conceivably convert Asc directly to L-idonate, as some activity was observed when the recombinant protein was provided Asc as a substrate (Burbidge, 2011).

To overcome the limitations faced by the biochemistry approach, alternative solutions such as QTL analyses based on bi-parental genetic mapping and GWAS have also been used to identify genetic loci linked to TA production. However, due to the complexity of TA biosynthesis and also the challenges in accurately measuring TA content, many genetic mapping studies using bi-parental crossing populations have failed to identify significant QTLs associated with TA production (Liang et al., 2011; Viana et al., 2013; Chen et al., 2015; Bayo-Canha et al., 2019). Houel et al. (2015), however reported consistent QTLs for TA on linkage groups LG4 and LG7 under multiple environmental conditions. The successful identification of genetic loci in this study may be attributed to the specific parent varieties used, likely to be a critical consideration in designing future QTL studies. In addition to bi-parental mapping studies, GWAS analyses using large germplasm collections have emerged as a promising approach in the search for candidate genes for TA biosynthesis. For example, a recent whole-genome resequencing of 472 *Vitis* accessions allowed comprehensive GWAS analyses on many grapevine traits including TA accumulation (Liang et al., 2019), which identified a putative deacetoxyvindoline 4-hydroxylase (VIT_05s0049g00420) and a cinnamoyl-CoA reductase 1-like protein (VIT_13s0064g00270) as potential candidates related to TA biosynthesis. Noteworthy, deacetoxyvindoline 4-hydroxylase belongs to the oxidoreductase superfamily and catalyzes the oxidation of 2-oxoglutarate (5-carbon) to succinate (4-carbon), thereby potentiating this enzyme in the last two steps of TA biosynthetic pathway, i.e., the conversion of 5-keto gluconate to TA. Experimental evidence is required before we can further speculate on their potential functions in TA biosynthesis. In addition, another comprehensive GWAS analysis of 279 *V. vinifera* L. cultivars also identified loci associated with TA accumulation (Flutre et al., 2020), although at this stage there is insufficient publicly available data to link this to a specific gene or set of genes.

A new approach to identifying candidates for the remaining steps takes advantage of publicly available 'omics' data banks and analysis tools. Gene co-expression networks (GCNs) are an emerging resource to study grapevine metabolism (Malacarne et al., 2016; Vannozzi et al., 2018), fruit development/ripening (Massonnet et al., 2017) and stress responses (Savoi et al., 2017; Sun et al., 2018, 2019). Central to GCN analysis is the 'guilt-by-association' principle whereby genes that share common functions or related processes are often co-ordinately

regulated across a wide range of conditions (e.g., multiple tissues, developmental stages, stress, hormones, etc.). Resources providing customized gene co-expression interrogation are available for grapevine (e.g., Moretto et al., 2016; Wong, 2020). To give an example of how such resources could be used to assist functional gene characterization within grape biosynthetic pathways, we used VTC-Agg (https://sites.google.com/view/vtc-agg) to search for genes that are highly co-expressed with *VvLidh3* and *Vv2kgr*. The data from this database consist of 33 separate experiments including over 1300 different samples across a range of tissues and developmental stages (Wong, 2020). This includes the grapevine transcriptome atlas dataset of Fasoli et al. (2012), which surveyed 54 different sample types, representing vegetative and reproductive organs at numerous developmental stages, including postharvest. The GCN data sets could be mined in multiple ways, including scanning for genes encoding specific proteins of interest such as transketolases and succinate semialdehyde dehydrogenases, or exploring the most highly co-expressed genes (i.e., the top 1% of genes) for other pathways that may occur in parallel with TA biosynthesis, or identify transcription factors or other regulators of the pathway. Surprisingly, *VvLidh3* and *Vv2kgr* were not highly co-expressed with one another based on expression patterns in >1,300 samples (**Figure 3**), despite showing strong developmental similarities (Jia et al., 2019). This suggests that other conditions, e.g., stress or hormones, affect the expression of these two genes differently. Interestingly, and encouragingly, the transcript that was most highly co-expressed with *VvLidh3*, belonged to a putative GDP-D-mannose pyrophosphorylase gene (*VvVtc1*), while the most highly co-expressed transcript

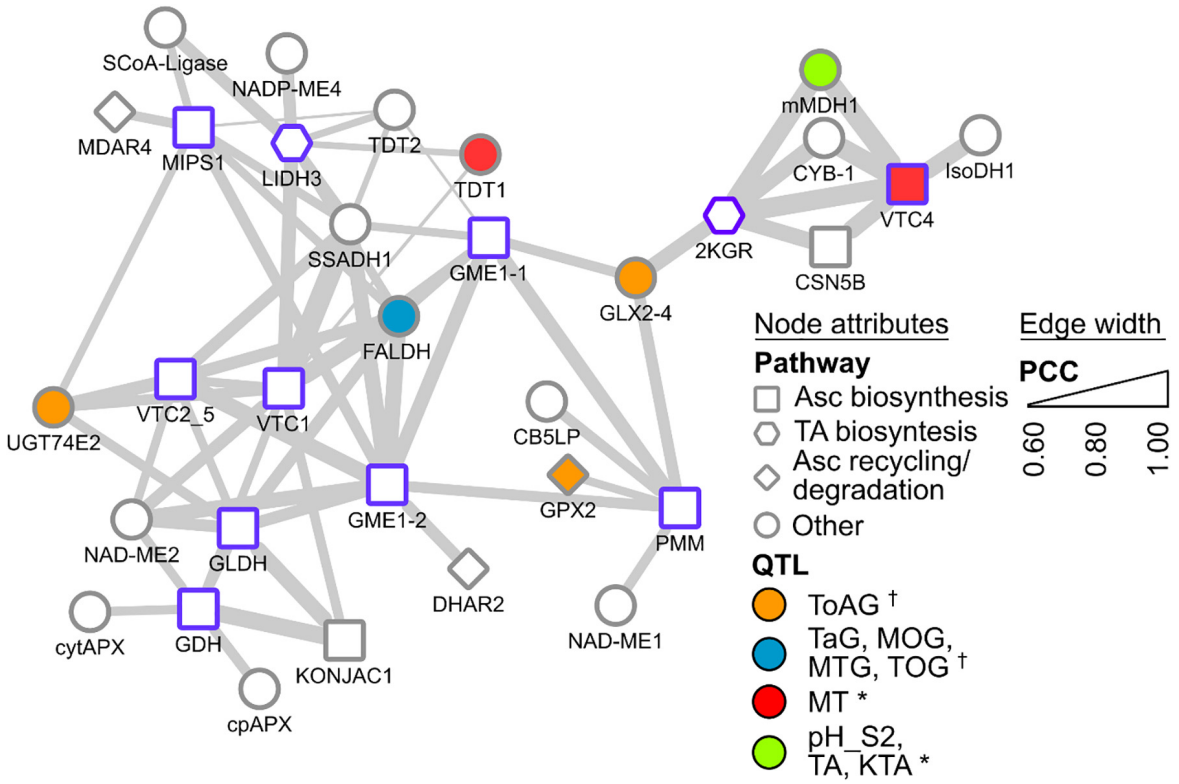

FIGURE 3 | Gene co-expression subnetwork of selected Asc and TA biosynthesis genes. Genes are depicted as nodes and edges depict Pearson correlation coefficients (PCCs) between nodes. Node borders in purple indicate query gene. Node shapes indicate their annotated functional categories. Node color indicate association with known berry acidity QTLs [e.g., †, Houel et al. (2015); *, Duchêne et al. (2020)]. Edge width indicates increasing PCC. Gene abbreviations are SCoA-Ligase succinyl CoA ligase, NADP-ME NADP-dependent malic enzyme, MDAR monodehydroascorbate reductase, MIPS *myo*-inositol-3-phosphate synthase, L-IDH L-idonate dehydrogenase, SSADH succinate semialdehyde dehydrogenase, TDT tonoplast dicarboxylate transporter, GME GDP-D-mannose epimerase, FALDH glutathione-dependent formaldehyde dehydrogenase, VTC1 GDP-D-mannose pyrophosphorylase, VTC2_5 GDP-galactose phosphorylase, NAD-ME NAD-dependent malic enzyme, GLDH L-galactono-1,4-lactone dehydrogenase, GDH L-galactose dehydrogenase, cytAPX cytosolic ascorbate peroxidase, cpAPX chloroplastic ascorbate peroxidase, DHAR dehydroascorbate reductase, UGT74E2 UDP-glucosyltransferase, KONJAC a sugar pyrophosphorylase that can stimulate VTC1 activity, GLX glyoxylase, GPX glutathione peroxidase, CB5LP a cytochrome B5, PMM phosphomannomutase, 2KGR 2-ketogulonic acid reductase, mMDH mitochondrial malate dehydrogenase, CYB Cytochrome B561, CSN5B interacts and modulates activity of VTC1, VTC4 L-galactose-1-phosphate phosphatase, IsoDH isocitrate dehydrogenase. Relevant QTLs from Houel et al. (2015) include: ToAG, total acids at green lag phase; TaG: tartrate at green lag phase; MOG: malate/total acids ratio at green lag phase; MTG, malate/tartrate ratio at green lag phase; and TOG, tartrate/total acids ratio at green lag phase. Relevant QTLs from Duchêne et al. (2020) include: MT, malate/total acids ratio in green berries (Riesling); pH_S2, pH in mid-ripening berries (Gewurztraminer); TA, tartaric acid concentration; KTA, potassium/tartaric acid ratio in berries (Gewurztraminer). For the full lists of co-expressed genes, refer to Wong (2020) supplementary data at https://sites.google.com/view/vtc-agg.

with *Vv2kgr*, was a L-galactose-1-phosphate phosphatase gene (*VvVtc4*). Both of these gene products are involved in plant Asc biosynthesis (**Figure 1**), indicating close regulation of Asc and TA biosynthesis pathways. A succinic acid semialdehyde dehydrogenase was linked to *VvLidh3* and is therefore a good candidate for the final step of the TA biosynthetic pathway. This strategy was also extended by integrating data from QTL studies aimed at berry acidity (e.g., Houel et al., 2015; Duchêne et al., 2020), to assist with candidate prioritization (**Figure 3**). Some interesting candidates that were both highly co-expressed with *VvLidh3*, and associated with berry acidity QTLs included two putative tonoplast dicarboxylate transporters (VIT_00s2188g00010 and VIT_00s0187g00130), which associated with a QTL for berry MA:TA concentration ([MA]:[TA]) on chromosome 10 (Houel et al., 2015) and two glutathione-dependent formaldehyde dehydrogenases, or GSNORs (VIT_07s0005g04600, VIT_07s0005g04610) associated with a major QTL for titratable acidity on chromosome 4 (Duchêne et al., 2020). For *Vv2kgr*, candidates included a L-galactose-1-phosphate phosphatase gene, *VvVtc4* (VIT_10s0405g00030) and mitochondrial malate dehydrogenase (VIT_10s0003g01000), which have been implicated with QTLs for [MA]:[TA] and combined pH, TA concentration and potassium:TA concentration ([K+]:[TA]), respectively on chromosome 10 (Duchêne et al., 2020) as well as a predicted glyoxalase (VIT_05s0102g01180), which aligns with a QTL for total acids at green lag phase (Houel et al., 2015). Therefore this analysis has identified several promising candidates for the regulation of TA accumulation in grapevine.

Co-expression analyses were also conducted with known Asc pathway genes (from enzymatic steps summarized in **Figure 1**), such as phosphomannomutase (*VvPmm*, VIT_15s0046g03520), GDP-D-mannose pyrophosphorylase (*VvVtc1*, VIT_13s0019g02330), GDP-D-mannose epimerase 1 and 2 (*VvGme1-1*, VIT_05s0020g04510; *VvGme1-2*, VIT_14s0030g02180), GDP-galactose phosphorylase (*VvVtc2_5*, VIT_19s0090g01000) and L-galactose-1-phosphate phosphatase (*VvVtc4*, VIT_10s0405g00030). L-Galactose dehydrogenase (*VvGdh*, VIT_03s0088g01250), L-galactono-1,4-lactone dehydrogenase (*VvGldh*, VIT_08s0007g05710), and *myo*-inositol-3-phosphate synthase (*VvMips1*, VIT_07s0031g00920) demonstrated strong and extensive co-expression among various Asc biosynthetic pathway genes, as well as some co-expression with Asc degradation and recycling pathways. For example, co-expression of (i) *VvGdh* with chloroplastic and cytosolic APX (*VvCpapx* and *VvCytapx*), (ii) *VvGme1-2* with DHA reductase 2 (*VvDhar2*), and (iii) *Vv2kgr* and *VvVtc4* with Cytochrome B561. The latter is very interesting, as it links a TA biosynthesis candidate with an Asc biosynthesis candidate and an Asc-dependent oxidoreductase of the plasma membrane involved in electron transport, supporting the idea of TA biosynthesis in the apoplast. Together with the coordinated expression of *VvLidh3* with *VvVtc1*, and *Vv2kgr* with *VvVtc4*, as well as similar metabolite accumulation profiles, there is likely to be coordinated regulation of Asc and TA biosynthetic pathways, potentiating an efficient flux to the endpoint metabolite, as seen for other metabolic pathways in plants (Winkel, 2004; Obata, 2019).

The tight co-regulation of Asc and TA metabolism pathways may involve regulatory control by hormones. A sizable proportion (ca. 10%) of co-expressed genes (of both Asc and TA genes) in the berry were differentially expressed in response to auxin (Dal Santo et al., 2020). A smaller proportion (ca. 5%) of co-expressed genes were modulated by abscisic acid (Pilati et al., 2017). Interestingly, *VvLidh3* was also significantly upregulated in berries by naphthalene acetic acid three hours post-treatment compared to the non-treated control (Dal Santo et al., 2020). These findings indicate a key role of auxin in the regulation of TA metabolism and co-expressed pathways both directly or indirectly. However, we cannot discount the involvement of other hormones given the lack of genome-wide transcriptome studies pertaining to hormonal regulation (e.g., ethylene, jasmonic acid, salicylic acid). Together, this demonstrates how Asc pathway genes can be effectively leveraged to identify more TA candidate genes (e.g., biosynthetic, regulatory, transporters) compared to the restricted lists when using just *VvLidh3* and *Vv2kgr* as guides. We predict that Asc pathway co-expressed genes that also associate with TA concentration or other acidity-related QTLs will likely be relevant in this endeavor.

FUTURE DIRECTIONS FOR TARTARIC ACID RESEARCH

Despite being first proposed over 50 years ago (Saito and Kasai, 1969), our understanding of the biochemical and genetic pathways to TA in grapes remains significantly under-developed. Two enzyme candidates have been characterized but definitive (i.e., genetic) evidence of their roles remains elusive. Thus far there are no known genetic nor biochemical regulators of the pathway. While 'conventional' molecular and biochemical approaches may in time yield additional candidates, research on TA biochemistry in grapes should be conducted with a mind to alternative possibilities. For example, some parts of TA biosynthesis may occur without the assistance of an enzyme, or a single enzyme may be responsible for multiple proposed steps. Meanwhile, the search for novel proteins contributing to a specialized pathway of TA biosynthesis could be directed instead to enzymes of common metabolic pathways with potential sub-functions, or "moon-lighting" functions. For example, genes that are transcribed in a pattern that is inconsistent with their sequence-predicted functions and more consistent with the transcriptional patterns of *VvLidh3* and *Vv2kgr*, and the timing and localization of TA biosynthesis. As TA accumulates to large quantities very quickly [up to 30% of assimilated CO_2 in very young berries (Saito and Kasai, 1968; 1969)], candidate proteins are likely to be highly abundant, especially during early development.

An intriguing aspect of TA is the existence of multiple biosynthetic pathways, which have arisen separately in different organisms, despite the limited distribution of this metabolite in plants. In the leaves of Geraniaceae plants Asc is cleaved between C2 and C3, with the C3-C6 fragment giving rise to L-threonate and subsequently converted to TA, and C1-C2 becoming OA (Williams and Loewus, 1978). In grape

berries L-threonate and OA also occur via 2,3 cleavage of Asc. However, TA arises from a separate pathway whereby Asc is first converted to 5-keto-D-gulonate before being cleaved between C4 and C5, with C1-C4 going on to become TA (reviewed by Ford, 2012). It is unknown why grapevines enlist an entirely new set of enzymes when the 2,3-cleavage activity for Asc is already active. Perhaps the enzyme(s) responsible for converting L-threonate to TA in Geraniaceae are absent or inactive in Vitaceae. OA accumulation typically, but not always, follows the same developmental pattern of accumulation as TA (Melino et al., 2009a; Simson and Debolt, 2012) and the two coexist in the same cell types, thus the pathways likely compete for Asc or DHA (DeBolt et al., 2004). The different biochemical routes to TA observed between species may also reflect contrasting roles of the acid. Updated radiolabelling experiments utilizing ^{13}C-Asc and tandem mass spectrometry could be used to determine the proportion of TA biosynthesis that occurs via the 2,3 and 4,5 cleavage pathways in grapes. Measurement of TA precursors as part of wider ranging metabolomic studies may also assist in confirming the existence of a particular route(s) of TA biosynthesis without the need for radiolabeled precursors, and could identify environmental conditions that are favorable for the biosynthesis of TA precursors. Comprehensive studies taking into account environmental and vintage effects are also essential to understanding and anticipating future physiological consequences for grapevines (Cholet et al., 2016). Above all, transgenic grapevine studies with altered expression of *Vv2Kgr*, *VvLidh3* and any other candidates will be necessary for confirmation of the TA biosynthetic pathway genes. Further investigation into the potential of DHA as the precursor to TA should also be explored via transgenic work in TA-accumulating species and tissues.

The classification of *VvLIDH1* and *VvLIDH3* as "Class II" plant SDHs and *VvLIDH2* as a "Class I" SDH provides an opportunity to more deeply understand these enzymes in grapevine and in other plants. Class I SDHs should be clearly differentiated from Class II SDHs when analyzing their potential function in TA biosynthesis because the latter may be the only "genuine" L-IDH. Special attention should also be given to the clear transcriptional divergence between Class I and Class II SDHs, which may shed light on their different biological functions. While the transcript profiles of *VvLidh1* and *VvLidh3* strictly match the developmental pattern of TA accumulation in the berries, a characteristic that was key to the original functional characterization of *VvLidh3* (DeBolt et al., 2006), the Class I SDH (*VvLidh2*; VIT_16s0100g00300) transcripts actually increase from veraison (Sweetman et al., 2012; Conde et al., 2015). Therefore, the Class II SDH genes have not only diverged with respect to their substrate preference, but also expression pattern. The regulators of confirmed L-IDHs may activate transcription in response to photosynthetic, redox or ROS signals and should be investigated for such responsive elements.

Insights could also be gained from other species such as rose-scented geranium, where secondary structures have been predicted for Asc and TA biosynthesis enzymes, including substrate binding sites of L-IDH (Narnoliya et al., 2018). Some important residues included Cys47, His72, Glu73, Glu158, consistent with those identified in *VvLIDH3* for substrate binding Cys36, His61, Glu62, Glu147 (Jia et al., 2015). Predictions of isoelectric point and other functional properties were also carried out in geranium (Narnoliya et al., 2018), followed by the identification of non-coding RNAs that may regulate expression of the gene (Narnoliya et al., 2019). Such information could assist functional analysis, mutagenesis and regulation studies of *V. vinifera* L-IDH. In addition to grapevine and geranium, although at a less significant level, TA production has also been reported in potato (Galdón et al., 2010), citrus fruits (Nour et al., 2010), and pear (Hudina and Stampar, 2000; Sha et al., 2011). These three species have been shown to contain a single copy L-IDH or "Class II" SDH (Jia et al., 2015). Of particular interest was potato, which as an annual herb plant may have an advantage over other species to be exploited as a model for the identification of other TA pathway genes. On another interesting note, TA has also been identified as the main acid in avocado (Pedreschi et al., 2019) and tamarind (Van den Bilcke et al., 2014) fruits. In contrast to most fruits including grape, avocado contains very low levels of citric and malic acids (Pedreschi et al., 2019). These species would be useful models to investigate the genetic and metabolic basis of distinct organic acid profiles and to elucidate the metabolic function, if any, of TA in these plants.

The elaborate regulation of Asc metabolism via biosynthesis, recycling and transport pathways, and its fundamental importance to the ascorbate/glutathione antioxidant pathway (Foyer et al., 2020) clashes with the idea that the irreversible catabolism of Asc (or DHA) to TA should occur by chance or as an "incidental" overflow valve. We propose that the catabolism of Asc or DHA to TA is a regulated process. The hypotheses that TA biosynthesis contributes to antioxidant metabolism, ROS avoidance or as a sink for excess ascorbate in pre-veraison berries, and the potential link to oxidative burst at veraison require further investigation. Gene editing experiments with grapevines, or other TA-accumulating models mentioned above, would be highly suited to this purpose, targeting *Vv2kgr* and *VvLidh3* in the first instance but also new candidates that arise from *in silico* and QTL analyses. Measurement of H_2O_2 evolution and TA localization in berries of cultivars that harbor different oxygen distribution patterns, such as those reported by Xiao et al. (2018), could be explored, as could the use of plant growth regulators that alter the timing of ripening (Böttcher et al., 2011, 2012, 2013b), or mutants with altered ripening times (Wei et al., 2020), to test the relationship between the oxidative burst and TA/Asc metabolism at veraison. A fleshless grape berry mutant, where TA accumulates to normal levels but the lack of mesophyll results in lower MA content (Fernandez et al., 2006), could also be a useful tool for investigating the dynamics of Asc metabolism and TA biosynthesis in grapes, and to determine whether metabolism in the flesh is required to support the oxidative burst observed in the skins. Eventually, once the entire TA biosynthetic pathway and the genes responsible have been established, integration of these genes into a non-TA-accumulating plant would be a valuable approach to investigate the hypothesized roles of TA in plant metabolism.

As a final thought, the pathway to TA synthesis in grape berries may only persist due to centuries of grapevine cultivation via

clonal propagation (therefore a lack of meiotic recombination) and selection for traits beneficial to winemaking processes and wine style rather than those essential for the competitive survival of the species. In this way, human activity has cemented the relevant biochemical activities and molecular regulators into the genetic lineage of *V. vinifera*. It is another example of artificial selection of a trait considered favorable by humans but otherwise biologically useless to the host, such as early flowering time, larger seed size and determinate habit in grain crops (Izawa, 2007; Shomura et al., 2008; Tian et al., 2010), increased milk production in dairy cattle (Flori et al., 2009) or appearance and temperament characteristics in dog breeds (Akey et al., 2010). In these examples, analysis of SNPs between breeds, cultivars, progeny and progenitors has shed light on genetic components of these domesticated traits. Such an approach may also be possible for TA accumulation in grapevines, considering the absence of TA in at least one species (DeBolt et al., 2006). In any case, this unusual metabolic endpoint with its favorable acid property likely influenced the initial adoption of grapes for winemaking, as early as 5000 BC (McGovern et al., 1996) and remains a significant player in grape and wine biochemistry today. Now the technology is available to begin manipulating TA levels in grapes and wine, with an aim to improve the quality of products for both industry effectiveness and consumer preference.

AUTHOR CONTRIBUTIONS

CS and CAB formulated the fundamental structure and content of the manuscript. CF, VM, DW, YJ, CJ, and KS contributed significant ideas, text, and corrections. SC, PD, MR, CB, RW, and FF provided further useful discussions, comments, and corrections. All authors contributed to the article and approved the submitted version.

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Frontiers Media SA, Lausanne, Switzerland

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Grape Berry Secondary Metabolites and Their Modulation by Abiotic Factors in a Climate Change Scenario–A Review

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Temperature, water, solar radiation, and atmospheric CO_2 concentration are the main abiotic factors that are changing in the course of global warming. These abiotic factors govern the synthesis and degradation of primary (sugars, amino acids, organic acids, etc.) and secondary (phenolic and volatile flavor compounds and their precursors) metabolites directly, via the regulation of their biosynthetic pathways, or indirectly, via their effects on vine physiology and phenology. Several hundred secondary metabolites have been identified in the grape berry. Their biosynthesis and degradation have been characterized and have been shown to occur during different developmental stages of the berry. The understanding of how the different abiotic factors modulate secondary metabolism and thus berry quality is of crucial importance for breeders and growers to develop plant material and viticultural practices to maintain high-quality fruit and wine production in the context of global warming. Here, we review the main secondary metabolites of the grape berry, their biosynthesis, and how their accumulation and degradation is influenced by abiotic factors. The first part of the review provides an update on structure, biosynthesis, and degradation of phenolic compounds (flavonoids and non-flavonoids) and major aroma compounds (terpenes, thiols, methoxypyrazines, and C13 norisoprenoids). The second part gives an update on the influence of abiotic factors, such as water availability, temperature, radiation, and CO_2 concentration, on berry secondary metabolism. At the end of the paper, we raise some critical questions regarding intracluster berry heterogeneity and dilution effects and how the sampling strategy can impact the outcome of studies on the grapevine berry response to abiotic factors.

Keywords: grapevine berry, climate change, abiotic stress, secondary metabolism, phenolic compounds, aroma compounds, *Vitis vinifera*

GRAPE BERRY PHENOLICS

Phenolic compounds constitute a large group of secondary metabolites, which are produced via different branches of the phenylpropanoid pathway. They are widely distributed throughout the plant kingdom and function as pigments, antioxidants, signaling molecules, structural elements, and components of defense mechanisms (Rienth et al., 2019; Santos-Sánchez et al., 2019).

They are composed of a phenyl ring backbone with a hydroxyl group or other substitutes and are generally classed into non-flavonoids and flavonoids. Non-flavonoids consist of simple C6 backbone phenolics such as hydroxybenzoic acids, hydroxycinnamic acids, and volatile phenols, and C6-C2-C6 backbone compounds such as stilbenes. Flavonoid compounds that occur in grapes comprise flavones, flavonols, flavanones, flavan-3-ols, and anthocyanins (**Figure 1**).

Phenolic compounds are synthesized via the general phenylpropanoid pathway (GPP) and its downstream reactions. After the generation of intermediates in the GPP, the carbon flow is directed into specific branch pathways to produce flavonoids, stilbenes, and phenolic acids (Deng and Lu, 2017). The starting point of the GPP is the aromatic amino acid phenylalanine, a product of the shikimate pathway, which constitutes a major link between primary and secondary metabolism in vascular plants (Santos-Sánchez et al., 2019).

The GPP leads to the production of p-coumaroyl-CoA from phenylalanine, via reactions catalyzed by phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H), and 4-coumarate-CoA ligase (4CL). Further downstream p-coumaroyl-CoA and malonyl-CoA are catalyzed by chalcone synthase (CHS) and stilbene synthase (STS) in the first steps of the flavonoid and stilbenoid pathway, respectively.

Recently, two transcription factors (TFs), *VviMYB4a* and *VviMYB4b*, have been characterized as negative regulators of phenylpropanoid and hydrocinnamic acid synthesis (Cavallini et al., 2015) and can repress upstream genes of the phenylpropanoid biosynthesis pathway in berry skins (Muñoz et al., 2019).

Non-flavonoid Phenolics

The concentrations of non-flavonoid phenolic compounds present in grapes and wines are relatively low (25–60 mg L^{-1}), with the exception of hydroxycinnamic acids (150–200 mg L^{-1}) (Kennedy et al., 2006). They are principally located in the berry pulp and are the major phenolic compounds in berries of white cultivars, although they reach similar magnitudes in reds (Kennedy et al., 2006).

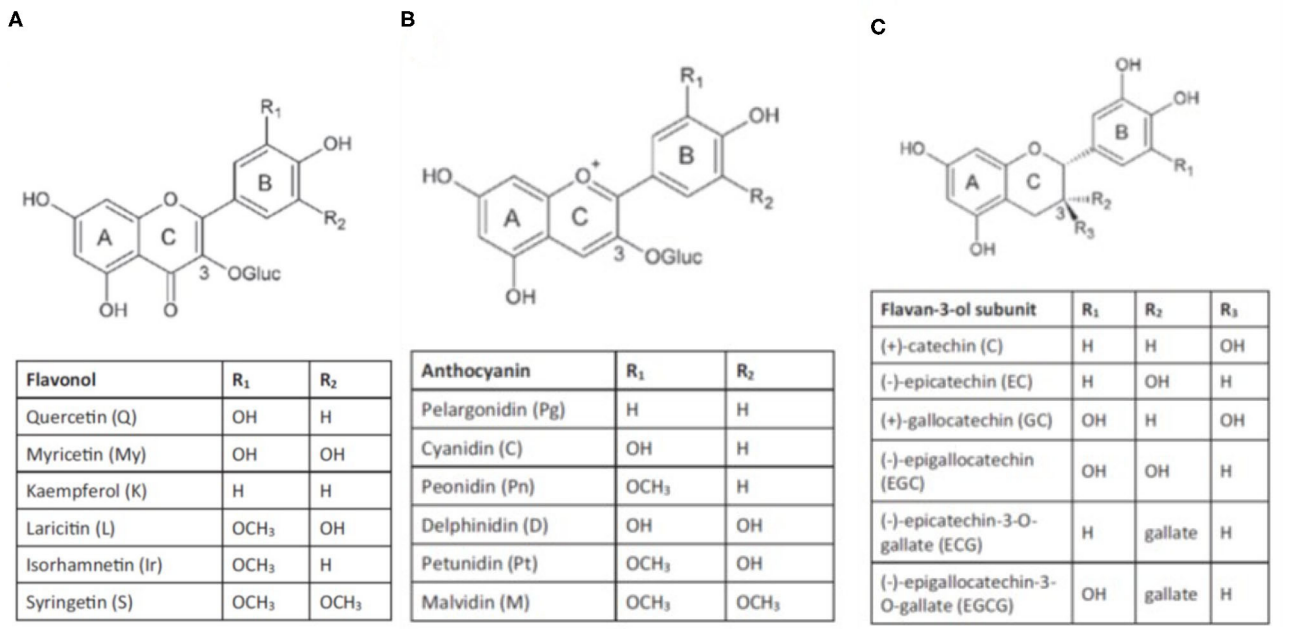
The major non-flavonoid compounds present in grapes are the hydroxycinnamic acids: p-coumaric acid, caffeic acid, ferulic acid, and their esterified forms, coutaric, caftaric, and fertaric acid (Zhang et al., 2013). Their biosynthesis occurs during the first phase of berry growth until the lag phase (herbaceous plateau) and is catalyzed by caffeic acid 3-O-metyltransferase (COMT) and caffeoyl-CoA 3-O-methyltransferase (CCoAOMT) downstream of the GPP. Although the accumulation occurs predominantly in the flesh, they are present in all berry tissues (Cadot et al., 2006; Braidot et al., 2008). In hypodermal, mesocarp, and placental cells of the pulp, hydroxycinnamates may be conjugated with anthocyanins (Cadot et al., 2006; Conde et al., 2007; Castellarin et al., 2012; Kuhn et al., 2013). Minor non-flavonoid compounds comprise hydroxybenzoic acids that are present in grapes in much lower concentrations than hydroxycinnamic acids, and include gentisic acid, salicylic acid, gallic acid, and p-hydroxybenzoic acid (Vanhoenacker et al., 2001; Pozo-Bayon et al., 2003; Ali et al., 2010). The most represented hydroxybenzoic acid is gallic acid, which is present in both free form or, in the seed, as an acyl substituent of flavan-3-ols (described below) (Adams, 2006).

Grape stilbenoids include *cis*- and *trans*-resveratrol, piceatannol, *cis*- and *trans*-piceid, astringin, pallidol and α-, β-, γ-, δ-, ε-viniferin. Although stilbenoids are present in trace quantities in wine, they have been drawing increasing attention due to their potential health benefits (Akinwumi et al., 2018). They have been shown to protect the plant against various pathogens and have been detected in significant concentrations in leaves and berries of highly stressed vines and disease-tolerant *Vitis* species such *V. amurensis* (Kiselev et al., 2017). Therefore, they represent important biomarkers and a molecular target for breeding strategies aiming to develop disease-resistant cultivars (Gindro et al., 2012; Viret et al., 2018). Recent research seeking alternatives to pesticides highlighted the potential of stilbenes extracted from wooden grapevine tissue as natural fungitoxic substances (Schnee et al., 2008; Biais et al., 2017).

Stilbenes are mostly accumulated in berries after the onset of ripening (*véraison*) (Gatto et al., 2008), and the regulation of their biosynthesis is strongly modulated by both biotic and abiotic factors (Vannozzi et al., 2012; Savoi et al., 2017). The concentration of stilbenes in grapes is variety-dependent; generally, red grapes have higher stilbene levels than white grapes (Bavaresco et al., 2016). Reported levels in grapes range from 0.2 to 1.8 mg kg^{-1} fresh weight of healthy grapes for cultivars that were classified as low stilbene producers (e.g., Nebbiolo or Aglianico) but can reach levels up to 33.4 mg kg-1 in high stilbene producers ($>$ 2.3 mg kg^{-1}) such as Pinot Noir (Gatto et al., 2008) or Syrah (Favre et al., 2020).

Stilbenes can be glycosylated or methylated. The stilbene with the simplest molecular structure is resveratrol, which exists as a *trans* or *cis* isomer (Kiselev et al., 2017). Resveratrol has been associated with the so called "French paradox," where the rather low risk of cardiovascular disease within the French population despite of a high intake of saturated fat was attributed to relatively high resveratrol intake through wine consumption (Renaud and de Lorgeril, 1992). In subsequent studies, resveratrol was designated the main substance amongst other phenolic compounds accountable for such protective effects (Pastor et al., 2019). Its anticancer, anti-inflammatory, anticarcinogenic, cardioprotective, vasorelaxant, phytoestrogenic, and neuroprotective properties have been reported in numerous studies (Guerrero et al., 2009; Chalons et al., 2018; Ramírez-Garza et al., 2018; Salehi et al., 2018; Wurz, 2019).

Resveratrol is the precursor for other stilbenoids such as piceids, *trans*- and *cis*-resveratrol-3-O-β-D-glucopyranosidade,



Flavonol	R_1	R_2
Quercetin (Q)	OH	H
Myricetin (My)	OH	OH
Kaempferol (K)	H	H
Laricitin (L)	OCH_3	OH
Isorhamnetin (Ir)	OCH_3	H
Syringetin (S)	OCH_3	OCH_3

Anthocyanin	R_1	R_2
Pelargonidin (Pg)	H	H
Cyanidin (C)	OH	H
Peonidin (Pn)	OCH_3	H
Delphinidin (D)	OH	OH
Petunidin (Pt)	OCH_3	OH
Malvidin (M)	OCH_3	OCH_3

Flavan-3-ol subunit	R_1	R_2	R_3
(+)-catechin (C)	H	H	OH
(-)-epicatechin (EC)	H	OH	H
(+)-gallocatechin (GC)	OH	H	OH
(-)-epigallocatechin (EGC)	OH	OH	H
(-)-epicatechin-3-O-gallate (ECG)	H	gallate	H
(-)-epigallocatechin-3-O-gallate (EGCG)	OH	gallate	H

FIGURE 1 | Structure of flavonols **(A)**, anthocyanins **(B)** and flavan-3-ols **(C)** (from Gouot et al., 2018).

and astringin. Pterostilbene (3,5-dimethoxy-4′-hydroxystilbene), which exhibits an enhanced antifungal activity compared to the non-methylated stilbenoid forms, is formed by the addition of two methyl groups to resveratrol (Chong et al., 2009). The oxidation of resveratrol produces oligomers called viniferins. The most important viniferins are α-, β-, γ-, δ-, and ε-viniferin, consisting essentially of cyclic oligomers of resveratrol (Castellarin et al., 2012).

In the grapevine genome, forty-five stilbene synthases genes (*STS*), the key enzyme of resveratrol biosynthesis, have been described, of which at least 33 encode full-length proteins. Multiple tandem and segmental duplication events led to the rise of this gene family (Vannozzi et al., 2012). Many of those *VviSTSs* exhibit changing expression pattern during fruit development and ripening as shown by transcriptomic analysis (Massonnet et al., 2017). In red berry cultivars, the expression of *VviSTSs* is increased during the late stages of ripening, consistently with the expression of two *R2R3 MYB* TFs, *VviMYB14* and *VviMYB15* (Holl et al., 2013), which are known to regulate stilbene biosynthesis. Amongst the many TFs proposed to regulate this pathway (Wong et al., 2016; Vannozzi et al., 2018) the two WRKY TFs, *VviWRKY24* and *VviWRKY03*, contribute at different levels to *VviSTS* regulation. This occurs by a direct activation of *VviSTSs* and synergistic action with MYB TFs. Recently, Jiang et al. (2019) indicated that *VvWRKY8* represses *VvSTS15/21* expression and thus stilbene biosynthesis through the interaction with *VvMYB14*.

Flavonoid Phenolics

Flavonoids make up a significant proportion of phenolic compounds in red grapes (1,000–1,800 mg L^{-1}) and can be considered as the most important quality-determining compounds in red wines as they contribute to color, flavor, texture, and astringency. Flavonoids are C6–C3–C6 polyphenolic compounds, where the two hydroxylated benzene rings are joined by a three-carbon chain. According to the oxidation state of the C3 ring, these compounds are divided into flavonols, flavan-3-ols (which include simple flavan-3-ols and their polymeric forms proanthocyanidins, also known as tannins), and anthocyanins (Castellarin et al., 2012). Flavonoids are mainly localized in both the peripheral layers of the berry pericarp (skin) and in the seed coat (Teixeira et al., 2013).

The flavonoid pathway has been well-characterized in grapevine. Most of the genes encoding structural elements of the flavonoid pathway are present in low copy numbers except genes coding *flavonoid-3′,5′-hydroxylases* (*F3′5′H*′s). p-Coumaroyl-CoA and malonyl-CoA are the substrates of *chalcone synthase (CHS),* which catalyzes the first step of flavonoid synthesis. Subsequently, naringenin chalcone is transformed to naringenin flavanone by *chalcone isomerase (CHI)* and to dihydrokaempferol by *flavonoid-3-hydrolase (F3H)*. The pathway is then divided into two major branches catalyzed by two flavonoid hydroxylases, *flavonoid-3′-hydroxylase (F3′H)* and *flavonoid-3′5′-hydroxylase (F3′5′H)*, which generate di-hydroxylated and tri-hydroxylated flavonoids, respectively (Azuma, 2018). In the grapevine genome a proliferation of the *F3′5′H*s has occurred, giving rise to 15 paralogs that are predominantly expressed in grapes (Falginella et al., 2010, 2012).

Flavonols, such as kaempferol, quercetin, myricetin, isorhamnetin, laricitrin, and syringetin are synthesized by *flavonol synthases (FLSs)* (Downey et al., 2004), regulated by a light-induced TF (*VviMYBF1/VviMYB12*) (Czemmel et al.,

2009). Two studies showed that three additional bZIP TFs, *VviHYH, VviHY5,* and *VvibZIPC22*, contribute to the regulation of flavonol synthases and the accumulation of flavonol in berries (Malacarne et al., 2015; Loyola et al., 2016). The TF *VviMYBF1* is part of a regulatory cascade of *VviHY5/HYH* that potentially involves positive feedback (Loyola et al., 2016; Czemmel et al., 2017). Flavonols are glycosylated by *flavonol-3-O-glycosyltransferases (VviGT3-5-6)* and *flavonol-3-O-rhamnosyltransferase (VviRhaT1)* and are present in the berry as galactosides, rhamnosides, rutinosides, and glucuronides (Ono et al., 2010; Czemmel et al., 2017).

Flavan-3-ols are synthesized from anthesis until the lag phase via *leucoanthocyanidin reductases (LAR1-2)* or an *anthocyanidin reductase (ANR)* (Bogs et al., 2005), which are regulated by TFs of the MYB family. In particular, *VviLAR1* and *VviANR* are under the control of *VviMYBPA1* and *VviMYBPA2* (Bogs et al., 2007; Terrier et al., 2009), whereas *VviLAR2* is regulated by *VviMYBPAR* (Koyama et al., 2014). In grapes, the main monomeric flavan-3-ols are (+)-catechin, (-)-epicatechin, (-)-epicatechin-3-O-gallate, (+)-gallocatechin and (-)-epigallocatechin (Mattivi et al., 2009), and are the building blocks for pro-anthocyanidins (condensed tannins). The mechanisms involved in tannin polymerization, galloylation, and transport into the vacuoles are not well-elucidated (Zhao et al., 2010). Three different glycosyltransferases (*VviGT1-3*) are ostensibly involved in the synthesis of hydroxycinnamic esters and in the galloylation of proanthocyanidins (Khater et al., 2012), and two specific transporters of proanthocyanidin have been identified (*VviPAMATE1-2)* (Perez-Diaz et al., 2014).

Anthocyanins are the pigments that are responsible for the color of red grapes and wines. They are synthesized in the epidermal and hypodermal cells of the berry skin and stored in the cell vacuoles from the onset of ripening (*véraison*). Few red cultivars, commonly called by the French word "Teinturier", accumulate anthocyanins also in the flesh (Ageorges et al., 2006; Castellarin et al., 2011; Falginella et al., 2012).

In *Vitis vinifera,* anthocyanins are glycosylated at the 3′ position. They can be substituted with two (di-oxygenated: cyanidin- and peonidin-3-O-glucosides) or three (tri-oxygenated: delphinidin-, petunidin-, and malvidin-3-O-glucosides) hydroxyl (-OH) and/or methoxyl (-OCH3) groups in the side-ring (B) of the flavonoid structure. Glycosylation occurs via the activity of the enzyme UDP-glucose, flavonoid-3-O-glucosyltransferase (UFGT) (Boss et al., 1996a,b). Anthocyanin-O-methyl transferases (*VviAOMT1-3*) methylate cyanidin-3-O-glucoside and delphinidin-3-O-glucoside into peonidin-3-O-glucoside, petunidin-3-O-glucoside, and malvidin-3-O-glucoside (Fournier-Level et al., 2011). Most anthocyanins can be acylated at the 6′ position of the glucose mediated by an anthocyanin-3-O-glucoside-6′-O-acyltransferase (*Vvi3AT*) to produce 3-O-6′-acetyl-, 3-O-6′-coumaroyl- and 3-O-6′-caffeoyl-monoglucosides (Rinaldo et al., 2015). Anthocyanins composition (the relative portion of individual anthocyanins, the ratio of di-oxygenated vs. tri-oxygenated side-ring forms, the ratio of acylated vs. non-acylated derivatives, etc.) is variable among grapevine cultivars (Monagas et al., 2003; Theodorou et al., 2019) and can be modified by abiotic factors as discussed in later sections.

The two TFs *VviMYBA1* and *A2* are the pivotal genetic determinants of berry anthocyanin synthesis (Kobayashi et al., 2004; Walker et al., 2007). Recently it has been shown that *VviMYBA6* and *VviMYBA7*, additional MYBA family members, regulate anthocyanin biosynthesis in vegetative organs (Matus et al., 2017) and that both also possess the capacity to alter fruit anthocyanin pigmentation and composition under extreme abiotic conditions (i.e., UV-B) during ripening (Czemmel et al., 2017). Recently, Costantini et al. (2017) identified a set of new candidate genes responsible for anthocyanin variation among cultivars.

The translocation of anthocyanin-acylglucosides into the vacuole is mediated by MATE-type transporters localized in the tonoplast (*VviAnthoMATE1-3*) (Gomez et al., 2009), whereas translocation of glycosylated anthocyanins occurs via a glutathione- dependent, ATP-binding cassette (ABC) protein (*VviABCC1*) (Francisco et al., 2013). Furthermore, glutathione S-transferases (*VviGST*s) have been associated with the transport of anthocyanins (Conn et al., 2008). Notably, *VviGST1* and *VviGST4* have been shown to be involved in anthocyanin accumulation in the vacuole (Pérez-Díaz et al., 2016).

The synthesis of hydroxycinnamic acids, stilbenes, flavonols, flavan-3-ols, and anthocyanins is spatially and temporally separated during grape berry development and tightly regulated by a vast transcriptional gene network. In addition to the already described TFs, two MYB (*VviMYB5a-b*) have been shown to be general regulators of the flavonoid pathway and, in particular, modulate the expression profile of several flavonoid genes such as *VviCHI, VviF3′5′H, VviLDOX, VviLAR,* and *VviANR* during berry development (Deluc et al., 2006; Cavallini et al., 2015). Recently, several R2R3-MYBs (*VviMyb4a, VviMyb4b, VviMybC2-L1, VviMybC2-L2, VviMybC2-L3,* and *VviMyb4-like*) were characterized as repressors of both proanthocyanidin and anthocyanin biosynthesis (Huang et al., 2014; Cavallini et al., 2015; Muñoz et al., 2019). Moreover, a bHLH (*VviMYC1*) interacts with *VviMYB5a-b, VviMYBPA1,* and *VviMYBA1-A2* in the transcriptional regulation of proanthocyanidin and anthocyanins biosynthesis in grapevine (Hichri et al., 2011).

GRAPE BERRY AROMA COMPOUNDS

In this section, a brief overview of aroma compound formation in grape berries is given to lay the basis for subsequent parts on abiotic interaction with their synthesis. For more detailed information we refer the reader to the recently published review of Lin et al. (2019).

The major groups of grape and wine aroma compounds are mono- and sesquiterpenes, methoxypyrazines, furan derivatives, lipoxygenase pathway products, phenylpropanoid pathway products, norisoprenoids, and volatile sulfur compounds such as thiols. They are mainly found as non-volatile precursors in the berry and require further modifications to be perceived (Dunlevy et al., 2013; Parker et al., 2018; Lin et al., 2019).

Terpenes

Terpenes play roles as phytohormones, protein modification reagents, anti-oxidants, repellents of herbivores and attractants of predators and parasitoids of herbivores (Pichersky and Raguso, 2018). The grape terpenoids of major aromatic importance in grapes can be divided according to their chemical structure into monoterpenes (C10) and sesquiterpenes (C15). Moreover, carotenoids (C40) can be included in this list as aroma precursors (Dunlevy et al., 2013; Ilc et al., 2016).

Around 50 monoterpenes (including *cis* and *trans* forms) have been identified in grapes and wine with a significant proportion of them being linalool derivatives (Strauss et al., 1986; Black et al., 2015; Ilc et al., 2016). The main monoterpenes present in grapes are linalool, geraniol, nerol, citronellol, *(E)*-hotrienol, α-terpineol, and rose oxides (**Figure 2**) (Matarese et al., 2014), which confer flowery and fruity notes to wines (Siebert et al., 2018). Generally, in aromatic cultivars, terpene concentrations peak early during green berry development, decline until *véraison*, and strongly increase during ripening (De Billerbeck et al., 2003; Matarese et al., 2013; Costantini et al., 2017). Inversely, Poitou et al. (2017b) found 1,8-cineole concentration to decrease during ripening. 1,8-cineole, commonly known as eucalyptol, is the major aroma compound present in the leaves of many *Eucalyptus* species. In Australian wines, the presence of this compound has been attributed to the proximity of vineyards to eucalyptus trees and the incorporation of *Eucalyptus* material into harvested grapes (Capone et al., 2012). 1,8-cineole was also identified as a contributor to the varietal aroma reminiscent of menthol and overall green perception of unripe grapes of the cultivars from the Carmenet family, and partially linked to the proximity of the invasive plant *Artemisia verlotiorum* (Poitou et al., 2017b).

Grape cultivars can be grouped roughly into three classes based on their total free monoterpene concentration and their monoterpene profile in their wines: cultivars named "neutral" (e.g., Chardonnay and Chasselas) have low terpene concentrations, cultivars more "aromatic" (e.g., Gewürztraminer, Albariño, Scheurebe, Auxerrois, and Riesling) have moderate (1–4 mg L^{-1}) terpene concentrations, and "Muscat type" cultivars (e.g., Muscat de Frontignan, Muscat of Alexandria, Muscat d'Ottonel, Muscat de Hambourg, White Muscat, etc.) have high (as much as 6 mg L^{-1}) terpene concentrations (Darriet et al., 2012).

Sesquiterpenes contribute little to grape and wine aroma, as their concentrations are often below the olfactory threshold. The most studied sesquiterpene is (-)-rotundone, which confers a peppery character to some red and white cultivars, such as Syrah, Duras, Durif, and Viognier (Wood et al., 2008; Geffroy et al., 2014, 2018, 2019).

Terpenes are produced via two independent pathways: (1) the plastidial 2C-methyl-D-erythritol-4-phosphate (MEP) pathway, which is the predominant pathway for monoterpenes (C10) and diterpenes (C20), and (2) the cytosolic mevalonate (MVA) pathway, which is the primary pathway for sesquiterpenes (Bohlmann and Keeling, 2008).

The key enzyme of the MEP pathway in grapevine has been shown to be 1-deoxy-D-xylulose 5-phosphate synthase (*VviDXS*) (Battilana et al., 2009, 2011; Duchene et al., 2009a). Further downstream terpene synthases (TPSs) regulate the monoterpene or sesquiterpene production (Martin et al., 2010; Matarese et al., 2013, 2014). The genome of *Vitis vinifera* contains 69 putative TPSs, of which 39 were functionally characterized (Martin et al., 2010) and can be divided into seven clades: TPS-a, -b, -c, -d, -e/f, -g, and -h (Chen et al., 2011). The TPS-a clade (30 genes) is mainly composed of sesquiterpene and possibly diterpene synthases, whereas the TPS-b clade (19 genes) and TPS-g clade (17 genes) is composed principally of monoterpene synthases and the TPS-c (2 genes) and TPS-e/f (1 gene) clades is composed of genes related to plant hormone metabolism and are typically present in a single copy in plant genomes. No full-length TPS-d and TPS-h are known yet in grapevine (Martin et al., 2010). Several genes, such as nudix hydroxylase, vesicle-associated proteins, ABCG transporters, glutathione S-transferases, and amino acid permeases are potential candidate genes for monoterpene biosynthesis regulation and accumulation in the berry (Costantini et al., 2017). Cramer et al. (2014) hypothesized that ethylene signaling could play a role in the biosynthesis of terpenes which has been point out by a positive correlation between aroma production and ERF TFs. The hormones jasmonic acid and methyljasmonate are putatively involved as major regulators of terpene biosynthesis in grapes (Savoi et al., 2016; D'Onofrio et al., 2018). The key genes involved in the synthesis of the sesquiterpene (-)-rotundone have been identified as *VviGuaS*, *VviTPS24*, and *VviSTO2* (Takase et al., 2015; Drew et al., 2016).

Most monoterpenes and sesquiterpenes are present in berries as non-volatile terpene glycosides. So far, only three monoterpenol glycosyltransferases have been characterized in grapevine, *VviGT7-14-15* (Li et al., 2017). Also, the cytochrome P450 *CYP76F14* is implicated in several enzymatic transformations of monoterpenes including hydroxylation (mono- and poly- hydroxylations), cyclization, and oxidation through successive reactions including dehydration of alcohol functions in acidic media. These phenomena can lead to an increase in the diversity of monoterpenes in grapes and wines (Schwab and Wüst, 2015). As an example, cytochrome P450 catalyzes the conversion of linalool to *(E)*-8-carboxylinalool, which, during wine fermentation, generates a wine-lactone, a key odorant of Gewürztraminer wines (Ilc et al., 2016).

Norisoprenoids

Norisoprenoids are a diverse group of widespread compounds derived from the oxidative breakdown of carotenoids (**Figure 3**) – pigments that contribute to light harvesting and to the protection of the photosynthetic apparatus from photooxidation (Rodríguez-Concepción et al., 2001). Carotenoids are synthesized from isopentenyl diphosphate (IPP) and its double-bond isomer dimethylallyl diphosphate (DMAPP) via the MEP pathway with DXS as the rate-limiting enzyme.

In grapevine, carotenoids are cleaved via carotenoid cleavage dioxygenases (CDD) to form norisoprenoids with C13 carbons

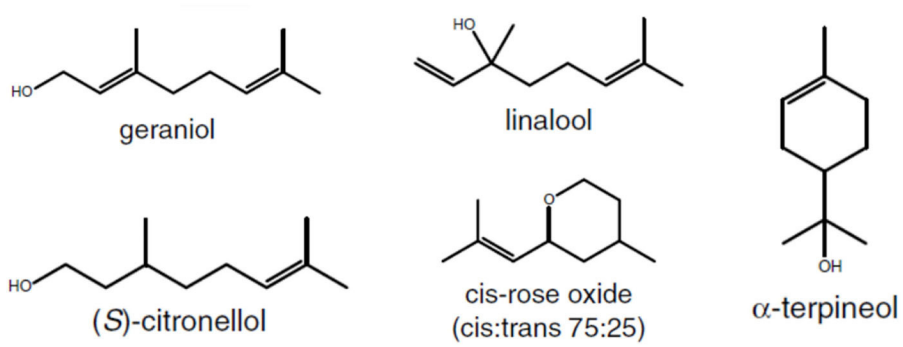


FIGURE 2 | Structure of main monoterpenes found in grapes.

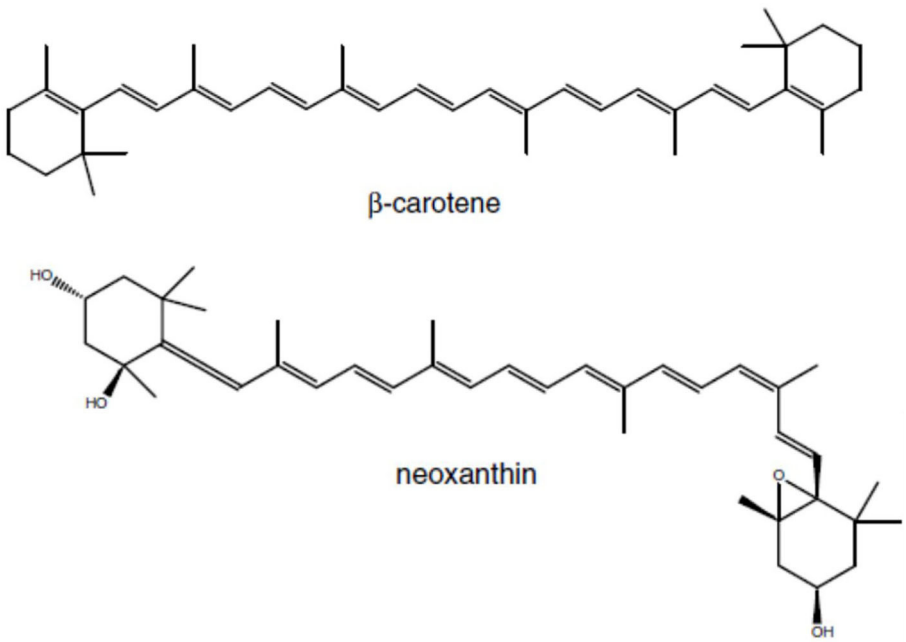


FIGURE 3 | Examples of carotenoid precurors for C13 Norisprenoids in grapes: β-carotene is the precureur of β-ionone, and neoxanthin the precurseur of β-damascenone.

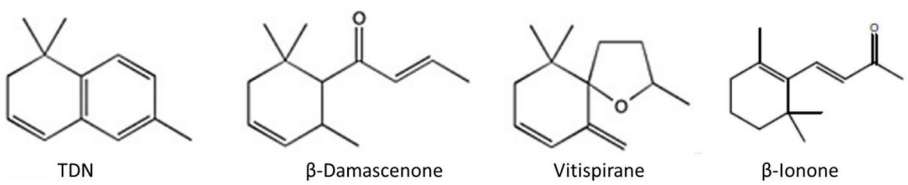


FIGURE 4 | Main C13 norisporenoids in grapes.

(C13-norisoprenoids) such as β-ionone, β-damascenone, vitispirane, actinidol, 4-(2,3,6-trimethylphenyl) buta-1,3-diene (TPB), 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), and 2,2,6-trimethylcyclohexanone (TCH) (**Figure 4**). Odorous C10 norisoprenoids such as safranal and β-cyclocytral have also been found in several grape cultivars (Poitou et al., 2017a).

The cleavage of carotenoids occurs after *véraison*, during berry ripening, where a simultaneous increase in C13-norisoprenoid

content correlates with a decrease in carotenoid content (Razungles et al., 1993; Yuan and Qian, 2016) and is followed by enzymatic reduction and glycosylation (Mathieu et al., 2005; Leng et al., 2017).

At the end of maturation, and during vinification and wine aging, C13-norisoprenoid compounds are formed in acidic media, through chemical reactions from several volatile and non-volatile precursors (Darriet et al., 2012).

β-ionone (violet-like flavor) and β-damascenone (apple sauce – rose like flavor) are the most ubiquitous C13-norisporenoids, whereas significant quantities of TDN are only found in few cultivars such as Riesling and Ugni blanc, whose wines can have a TDN concentration as high as 200 $\mu g\,L^{-1}$ and have a characteristic kerosene/petroleum flavor (Schüttler et al., 2015).

Nine CDD have been identified in Arabidospis, but only *VviCCD1* and *VviCCD4* have been shown to cleave carotenoids at the 9,10 and 9',10' double bonds (Huang et al., 2009; Ahrazem et al., 2010; Lashbrooke et al., 2013). Fifty four genes putatively involved in carotenoid metabolism in *V. vinifera* were identified by Young et al. (2012) and Leng et al. (2017) and seven CCDs have been annotated in grapevine (*VviCCD1.1*, *VviCCD1.2, VviCCD4a, VviCCD4b, VviCCD4c, VviCCD7,* and *VviCCD8*) (Young et al., 2012; Lashbrooke et al., 2013).

Fatty Acid Derivates

The unsaturated C_{18} fatty acids linoleic acid and linolenic acid are the precursors of other volatile organic compounds such as C_6-aldehydes and alcohols like hexanal and hexanol (Kalua and Boss, 2009). Their synthesis occurs mainly before *véraison* in the green berry (Kalua and Boss, 2009) and they have green-grassy aromas even though, considering their detection threshold, they rarely contribute to the herbaceous character of wines. They are formed by the activity of lipoxygenases (*VviLOX*) (Podolyan et al., 2010), *hydroperoxide lyase (VviHPL1 and VviHPL2)* (C_6-aldehydes and alcohols-*2)* (Zhu et al., 2012), *enal isomerase (3Z)-(2E)*, and *alcohol dehydrogenase (VviADH)* (Kalua and Boss, 2009). The levels of these compounds in wines are mainly modulated by winemaking processes; the more odorous C6 aldehydes (hexanal, hexenals) are reduced to less odorous C6 alcohols by *Saccharomyces cerevisiae* during alcoholic fermentation (Ferreira et al., 1995).

Methoxypyrazines

Methoxypyrazines, nitrogen heterocycle compounds belonging to the pyrazine group, are present in both animals and plants (Maga, 1992). Among the various methoxypyrazines, some alkylated methoxypyrazines, such as 2-methoxy-3-isobutylpyrazine (IBMP), 3-sec-butyl-2-l methoxypyrazine (SBMP), and 2-methoxy-3-isopropylpyrazine (IPMP) (**Figure 5**) are extremely odorous, with very low odor thresholds (nanogram per liter range in water) (Sidhu et al., 2015). In cultivars of the Carmenet family, such as Sauvignon blanc, Cabernet Sauvignon, Cabernet Franc, Merlot, and Fer, the vegetable-like aromas are reminiscent of pea pods and green peppers, and depending on the concentration they can contribute to earthy nuances (Geffroy et al., 2020). In the grape cluster, the stems contain the largest proportion of IBMP (79.2%), and in the berries, most of the IBMP is located in the skin (72%), followed by seeds (23.8%) and pulp (4.2%) (Roujou-de-Boubée and Botella, 2003).

In grape berries, methoxypyrazines rapidly accumulate between fruit set and the lag phase, peak at 2 to 3 weeks before *véraison*, then decrease continuously during ripening until harvest (Ryona et al., 2008, 2010; Gregan and Jordan, 2016). The extent to which they are synthesized is still not fully elucidated, whether *in situ* in the berries (Roujou de Boubee et al., 2000; Koch et al., 2010) or in the leaves with subsequent transport via the phloem to the berries where they are degraded (Lei et al., 2018).

Their biosynthesis begins with dicarbonyl addition to the amino acid valine or leucine for IPMP and IBMP and is followed by a methoxylation to form the final methoxypyrazines. In grape, four O-methyltransferases *(VviOMT1-4)* have been characterized, with *VviOMT3* playing a major role in IBMP production (Dunlevy et al., 2013; Guillaumie et al., 2013).

Thiols

Thiols are volatile sulfur compounds and crucial components of the "varietal character" of several cultivars. 4-methyl-4-sulfanylylpentan-2-one (4MSP), 4-merthyl-4-sulfanylpentan-2-ol (4MSPOH) and the 3- sulfanylhexan-1-ol (3SH) (**Figure 6**) are the most important thiols present as precursors in the berry of white wine cultivars such as Sauvignon Blanc, Semillon, Petite Arvine, Riesling, Chenin Blanc, Muscat blanc, Colombard, and Alvarino (Tominaga et al., 2000; Fretz et al., 2005), but were found also to contribute to perceived fruitiness in red wines made from Cabernet Sauvignon and Merlot (Bouchilloux et al., 1998). Many of the cultivars known for their high thiol content share parent-offspring or sibling relationships (Duchene et al., 2009b). Thiols are generally linked to flavors reminiscent of passionfruit, box tree, black currant, garlic, and asparagus and are also important volatile components of meat, mushrooms, and many other plants. The first discovered thiol was 4MSP, responsible for aroma of box tree (*Buxus*) (Darriet et al., 1995), followed by the characterization of 3 SH and its ester 3SHA (3-sulfanylhexyl acetate) (Tominaga et al., 1996), often associated with aroma of grapefruit and passionfruit (Tominaga et al., 1998).

Thiols are accumulated during ripening in their non-volatile form, bound to S-cysteine or S-glutathione. The biosynthesis of thiol precursors is linked to glutathione (GSH) metabolism but the biosynthetic pathway of these thiol precursor is not fully elucidated yet. It is related to the conjugation of GHS and α,β-unsaturated carbonyl compounds by S-glutathione transferase as a part of the plant's endogenous metabolism. From this, S-glutathione glutamic acid and glycine is removed resulting in the S-cysteine conjugate. Kobayashi et al. (2011) showed that two genes, *VviGST3* and *VviGST4*, are involved in the production of thiol precursors in grapes.

The localization of different thiol precursors inside the berry is also unclear, and studies often report contradictory results. For example, des Gachons et al. (2005) found that in Sauvignon Blanc grapes at harvest, 4MSP-Cys and 4MSPOH-Cys were localized in the flesh (80%), while 3SH-Cys was equally distributed (50%) between the flesh and the skin. On the contrary, Roland et al.

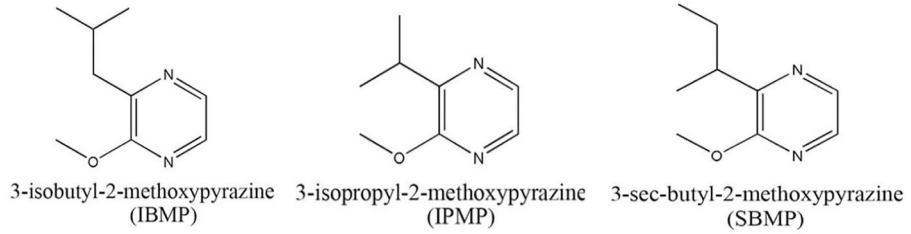


FIGURE 5 | Chemical structures main MPs in grapes.

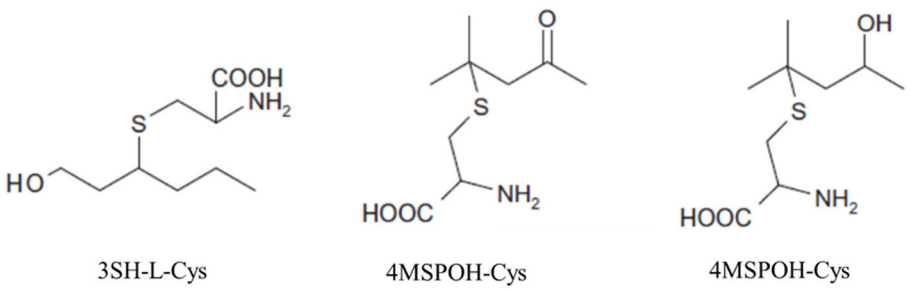


FIGURE 6 | Cysteinylated precursors of 3SH-L-Cys, 4MSPOH-Cys, 4MSP-Cys.

(2011) found that 3SH-Cys and 4MSPOH-Glu were mainly located in the skin (78 and 81%).

The yeast enzyme β-lyase, which is encoded by the gene *IRC7* (Howell et al., 2005; Roncoroni et al., 2011; Santiago and Gardner, 2015), cleaves thiol precursors during alcoholic fermentation. Several other factors, such as active transportation of S-cysteine and S-glutathione conjugates through the plasma membrane, influence thiol release during fermentation and contribute to the varietal expression of thiol-containing cultivars, which can sometimes be considered as too intense and as off-flavors, depending on the concentration.

BERRY METABOLISM MEDIATED BY ABIOTIC FACTORS

Temperature, water, light, and CO_2 concentration are among the most important abiotic factors interacting with vine and fruit development in a genotypic-dependent manner (Keller, 2010). These factors are expected to be largely modulated by global climate change. The atmospheric concentration of the greenhouse gas (GHG) CO_2, the main driver of global warming, has been continuously rising since the beginning of the industrial revolution (around 1,750) due to anthropogenic use of fossil fuel. Concentration increased in the twentieth century from 300 ppm in the early 1960's, when systematic direct atmospheric measurements started (Keeling, 1960), to 400ppm at present time and will, depending on future CO_2 emission scenarios, reach between 600 (moderate emission scenario) and 1,000 ppm (worst case scenario) by the end of the twenty first century (IPCC, 2013). This entails a rise of temperature, which is expected to be in a range of 2–4.8°C as a function of anthropogenic CO_2 emissions (IPCC, 2013, 2018). Besides its direct impact on plant physiology, higher temperatures lead to higher evapotranspiration rates and to increased water requirements during the growing season. Together with predicted alterations in precipitation patterns, more severe drought periods can be expected and have already been observed in most viticulture regions (Schultz, 2016). Regarding solar radiation of different wavelengths reaching the earth's surface, predictions of climate change models are ambiguous. Due to its high energy and impact on all living organisms, ultraviolet (UV) radiation (wavelength from 100 to 400 nm) plays a crucial role in the physiology of different plants, mammals and human beings, and terrestrial ecosystems (Ballaré et al., 2011). The UV-B radiation reaching the earth's surface is mostly impacted by the gas composition of the atmosphere. The ozone layer in the upper stratosphere absorbs most of the UV radiation and has been highly degraded by ozone-depleting substances (ODSs) such as chlorofluorocarbons (CFCs) during the last 40 years, which caused a significant increase in UV-B radiation reaching earth's surface. Since the reduction of ODS emissions following the ratification of the Montreal protocol in 1989, the depletion of the ozone layer in the stratosphere was slowed; it remains unclear how UV-B radiation will develop during different climate change scenarios. Certainly the continuous rise of CO_2 is altering the physical structure of the atmosphere (Chipperfield et al., 2017). Measured concentrations of ozone show an increase of 2–4% per decade, at mid-latitudes and the tropics, in the upper stratosphere (altitude 35–45 km) since about 2000. This increase is consistent with the recovery of stratospheric ozone as a consequence of decreasing concentrations of ODSs and increases in GHGs. In fact, GHGs in the troposphere will isolate upper layers of the atmosphere and a cooler stratosphere which slows down catalytic ozone breakdown (Williamson et al., 2014; United Nations

Environment Programme, Environmental Effects Assessment Panel, 2017; Bais et al., 2018, 2019). An additional factor to consider in respect to UV, is its variability due to cloud cover, which often exceeds the variability due to the ozone column depth and is expected to vary in the future due to global climate change as well and remains hard to predict (Enriquez-Alonso et al., 2016).

Altogether the alteration of the different aforementioned abiotic factors will continue to impact viticulture worldwide in different ways. This has triggered discussions among scientists regarding the sustainability of traditional viticulture regions (Fraga et al., 2012, 2013). Different authors predict that the land suitable for viticulture will decrease in main growing regions between 25 and 73% in the future (Hannah et al., 2013), which has however been questioned by other groups as there was no consideration of the capability of cultivars to adapt to changing conditions (van Leeuwen et al., 2013). Recently, more integrated modeling approaches predict that under the most optimistic climate change scenario (2°C increase) and in the most pessimistic scenario (4°C increase) 56 and 85% of current wine growing regions will become climatically unsuitable for viticulture by the end of the twenty first century, respectively (Morales-Castilla et al., 2020). In general, predicted and already observed consequences for wine quality are wines with higher alcohol content, lower acidity, and altered aroma profiles (Schultz, 2000, 2016; Mira de Orduna, 2010; Pons et al., 2017b; van Leeuwen and Destrac-Irvine, 2017) which altogether leads to a loss of typicity and terroir expression (van Leeuwen and Destrac-Irvine, 2017; Van Leeuwen et al., 2018; van Leeuwen et al., 2020).

In the subsequent sections, the most recent and pertinent literature dealing with the impact of the main abiotic factors modified by global climate change on berry metabolism is reviewed.

TEMPERATURE

Temperature is the main driver of vine phenology and its increase advances the vegetative and reproductive cycle of the grapevine and consequently shifts the berry developmental stages toward warmer months of the growing season (van Leeuwen and Destrac-Irvine, 2017). This has been widely confirmed in studies analyzing historical records of harvest dates worldwide which show overall advances of 1–2 weeks over the last several decades (Jones and Davis, 2000; Chuine et al., 2004; Duchêne and Schneider, 2005; Jones et al., 2005; Petrie and Sadras, 2008; Webb et al., 2012; Cook and Wolkovich, 2016). However, crop-yield reductions and evolving management practices may have also contributed to the advancement of ripening (Webb et al., 2013).

The combined effects of advanced phenology and increased temperatures during ripening lead to grapes with higher sugar and less organic acid concentration as well as altered composition in secondary metabolites, such as phenolic and aroma compounds (van Leeuwen and Destrac-Irvine, 2017). In the recent decades, myriad temperature studies have been conducted with a multitude of experimental approaches (Bonada and Sadras, 2015; Gouot et al., 2018). Results are often ambiguous, which makes it difficult to draw general conclusions. Published studies vary from whole plant to bunch level stress applications with variations in the duration of stress application from a couple of hours to several months. A wide range of temperature intensities have been tested from moderate increases (up to 35°C) to severe heat stress (up to 45°C) during day and/or night and at different berry developmental stages. The cultivar and the type of plant material used, as well as the experimental constraints for such studies, affect the responses of berry metabolism to temperature regimes. Control climate chamber experiments are mostly conducted with small model plants such as fruiting cuttings (Pillet et al., 2012; Carbonell-Bejerano et al., 2013; Lecourieux et al., 2017) or mutant microvines (Rienth et al., 2012, 2014a,b, 2016, 2017; Houel et al., 2015; Luchaire et al., 2017; Torregrosa et al., 2017, 2019; Pellegrino et al., 2019).

The dissociation of temperature and radiation effects is difficult to achieve in field trials as compared to climate chamber experiments where heat is applied by convection. However, some early and methodically groundbreaking field experiments succeeded to control clusters' temperature in an radiation-independent way by using different row orientation (Bergqvist et al., 2001), bunch cooling (Spayd et al., 2002) or *in situ* convection systems (Tarara et al., 2008). These studies concluded that some fruit components (in particular anthocyanins) are especially sensitive to temperature (Poni et al., 2018), which to some extent have been subsequently confirmed by molecular studies.

Temperature Impact on General Fruit Metabolism

Probably the earliest controlled study of the temperature effect on berry metabolism is Ravaz 1912, which inserted single clusters of a vine into a heated glass chamber. Many temperature studies focusing mainly on sugar accumulation, primary metabolisms, and berry growth dynamics have been conducted from the 1960's to the 90's (Kliewer, 1964, 1971, 1977a,b; Kliewer and Lider, 1968, 1970; Buttrose et al., 1971; Kliewer and Torres, 1972; Hale and Buttrose, 1974; Lakso and Kliewer, 1975; Matsui et al., 1986; Sepulveda and Kliewer, 1986) and are summarized in the very first review on the effect of elevated temperature on berry metabolism by Coombe (1987). In summary, moderate temperature increases during ripening hasten berry development and sugar accumulation as observed already in early studies from Buttrose et al. (1971), whichnoted an increase in total soluble solids (TSS) in Cabernet Sauvignon berries when exposed to higher temperatures (30°C). Kliewer and Torres (1972) observed a similar sugar accumulation increase, correlated with increasing temperature until around 35°C, in Cardinal, Pinot noir, and Tokay and Sangiovese even until 41.7°C (Pastore et al., 2017). During early green stages, moderate temperature increases accelerate berry growth, malic and tartaric acid accumulation (Rienth et al., 2016; Arrizabalaga et al., 2018).

When heat stress becomes severe (>35°C), a decrease or arrest of sugar accumulation as well as an inhibition or cessation of

berry growth and a delay of ripening is observed (Matsui et al., 1986; Sepulveda and Kliewer, 1986; Greer and Weston, 2010; Greer and Weedon, 2013; Lecourieux et al., 2017; Gouot et al., 2019). This slackening of berry development has been attributed to a lack of carbon supply by photosynthesis, which is impeded and slows down the rate of cell division, which may limit berry size (Keller, 2010). Furthermore, a decrease in ABA biosynthesis as indicated by the repression of *VviNCED2* and *VviNCED4* transcripts and the upregulation of *VviABI3* a B3-domain TF that is a part of the core ABA signaling network were associated with slower berry growth (Rienth et al., 2014b; Lecourieux et al., 2017). In addition, the role of auxins biosynthesis and regulation, which inhibits berry growth and sugar and anthocyanin accumulation (Davies et al., 1997; Bottcher et al., 2010) could putatively contribute to the delay of berry development and onset of ripening under severe heat stress as evidenced by the reduction of IAA-amido synthetase (*VviGH3*) and an increase in the expression of transcripts encoding IAA-amino acid hydrolases (Lecourieux et al., 2017).

The slackening of sugar accumulation rates under heat stress has been correlated with a down-regulation of sugar transporters (STPs) and invertase-encoding genes (*GIN2*) putatively involved in the import and accumulation of hexoses into vacuoles. The latter genes were found to be repressed in fruiting cuttings of Cabernet Sauvignon (Lecourieux et al., 2017), Muscat Hamburg (Carbonell-Bejerano et al., 2013) and microvines (Rienth et al., 2014b) upon exposure to high temperature. Curiously, a recent field study using an open top heating system found an altered glucose / fructose ratio due to lower glucose concentration in Riesling berries under high temperature (+10°C) (Brandt et al., 2019). Similar observations have been reported in historical studies and attributed the altered glucose/fructose ratio to over-ripeness of grapes (Kliewer, 1965, 1967; Sepulveda and Kliewer, 1986). This has, however, not been observed or closely investigated in other heat stress studies (Carbonell-Bejerano et al., 2013; Sweetman et al., 2014; Lecourieux et al., 2017).

Drawing upon the results described above, it seems that the most sensitive berry stage to biotic and abiotic stress is just around or during the *véraison* phase as evidenced by a couple of studies with precise single berry sampling protocols (Rienth et al., 2014b, 2016; Torregrosa et al., 2019; Ghaffari et al., 2020).

Concerning malic and tartaric acid, the two main organic acids in grape berries, it appears that moderately increased temperature during early berry development from anthesis to lag phase accelerates their accumulation (Sweetman et al., 2014; Rienth et al., 2016; Lecourieux et al., 2017). However, when heat stress becomes too severe, their synthesis is inhibited, as reported, for example, by Gouot et al. (2019), where temperatures reached up to 45°C during early green growth.

It has been shown in myriad historic and recent studies that high temperatures are correlated with a decrease in malic acid concentration in the ripening berry (Ruffner et al., 1976; Ruffner and Hawker, 1977; Possner et al., 1983; Sweetman et al., 2009, 2014; Carbonell-Bejerano et al., 2013; Etienne et al., 2013; Rienth et al., 2016; Lecourieux et al., 2017; Brandt et al., 2019). This degradation is highly cultivar-dependent and can even vary among clones, as shown for example on different Tempranillo clones exposed to high temperatures (Torres et al., 2017). However, the underlying regulation of malic acid degradation remains to be elucidated. Sweetman et al. (2014) found that high temperatures accelerated the NAD dependent malic enzyme activity and decreased phosphoenolpyruvate carboxylase and pyruvate kinase activities, accompanied by the accumulation of various amino acids and γ-aminobutyric acid, suggesting an enhanced anaplerotic capacity of the TCA cycle and a need to deal with decreased cytosolic pH in heated berries. This study also found differences depending on the diurnal temperature range: that is, the loss of malic acid content in response to elevated day temperature was lessened if night temperature was also increased. It was also proposed that malate concentration is mainly depends on the thermodynamics of its tonoplastic transport (Lobit et al., 2006).

It is generally reported that tartaric acid (TA) is not metabolized by the ripening berry and thus its content is not impacted by temperature and represents an important trait for breeding programs seeking new cultivars better adapted to future climate conditions (Duchêne, 2016). Observed variation of TA in the scientific literature (Cholet et al., 2016) is a likely consequence of dilution effects or precipitation during sample processing (Rösti et al., 2018).

Temperature Impact on Phenolic Compounds

The direct and indirect effects of temperature on flavonoid composition are far from being completely unambiguous. In this section we summarize and update recent findings.

Temperature effects on flavonol and flavan-3-ol composition are not always consistent among studies (Gouot et al., 2018). However, there is unambiguous scientific evidence that shows deleterious effects of high temperature on anthocyanin levels in the grape berry. This was reported in early studies (Buttrose et al., 1971; Kliewer and Torres, 1972; Spayd et al., 2002) and more recently confirmed by physiological and molecular studies (Mori et al., 2005, 2007; Yamane et al., 2006; Azuma et al., 2012; Carbonell-Bejerano et al., 2013; Rienth et al., 2014b; Lecourieux et al., 2017; Pastore et al., 2017; Torres et al., 2017; Yan et al., 2020). Heat stress has been shown to repress major anthocyanin biosynthesis regulators such as *VviMYBA1* and downstream genes such as *VviUFGT*, *VviCHI*, *VviF3H2*, *VviDFR*, and *VviLDOX*. However, not all of these studies showed unequivocal repression nor a correlation with lower anthocyanin accumulation. This is possibly due to the use of different plant materials (normal vines, different cultivars), different stages of berry development and intensities of treatment, and sampling strategy. Furthermore, the temperature effect on anthocyanin synthesis varies highly between genotypes. For example, when the temperature maximum during ripening exceeded 35°C, inhibition of color formation was much more pronounced in Grenache than in Carignan (Fernandes de Oliveira et al., 2015). In earlier studies, low temperature during ripening, in particular at night was associated with enhanced coloration of grapes (Kliewer and Torres, 1972), which was confirmed in a recent molecular experiment where low night temperatures enhanced

anthocyanin accumulation and expression of *VviCHS3*, *VviF3H1*, *VviUFGT*, and *VviMYBA1*, in particular when applied around *véraison* on Corvina grapes (Gaiotti et al., 2018). In Kyoho grapes, temperature increases from 27 to 30°C during ripening induced a strong decrease of the transcript levels of anthocyanin genes, leading to less berry color (Shinomiya et al., 2015). In Merlot, an increase of day temperature of 5°C during ripening, from 20 to 25°C, resulted in a anthocyanin decrease of 37% (Yan et al., 2020). Besides the repression of anthocyanin related genes, high temperature may promote anthocyanin degradation, possibly via the increased activity of peroxidases (Mori et al., 2007). This is evidenced by the upregulation of a gene coding for a peroxidase, *VviPrx31*, in berries exposed to high temperature (Movahed et al., 2016), and a similar effect occurs in other plant species, such as *Brunfelsia* flower petals (Vaknin et al., 2005), litchi (Zhang et al., 2005), and strawberry fruits (Chisari et al., 2007).

An increased proportion of acylated and tri-hydroxylated anthocyanins under higher temperature has been observed in experiments with Merlot (Tarara et al., 2008; Yan et al., 2020), Cabernet Sauvignon (Lecourieux et al., 2017), Sangiovese fruiting cuttings (Pastore et al., 2017), and Malbec (de Rosas et al., 2017), concomitantly with the overexpression of the acyltransferase gene *Vvi3AT* (de Rosas et al., 2017; Yan et al., 2020). Similarly for anthocyanins, high temperature impeded flavanol accumulation strongly and increased methoxylated (isorhamnetin and syringetin) and 3′, 4′, 5′-substituted (myricetin and syringetin) flavonols in Merlot (Yan et al., 2020).

Interestingly, high temperature can cause an uncoupling of sugar accumulation and anthocyanin synthesis leading to a lower anthocyanin / sugar ratio, possibly because of a delay in the onset of anthocyanin synthesis (Sadras and Moran, 2012; Sadras et al., 2013; Yan et al., 2020) or a reduced anthocyanin accumulation during ripening (Yan et al., 2020). The magnitude of this thermal decoupling is cultivar dependent as shown for Grenache and Carignan (Fernandes de Oliveira et al., 2015) and can vary between clones of the same cultivar as shown in Tempranillo (Arrizabalaga et al., 2018).

The effect of temperature on tannins is still not well-understood. The synthesis of the tannin monomers, flavan-3-ols, was increased under elevated temperature (Cohen et al., 2012a,b), although in both latter studies, differences were no longer significant at *véraison*. Tannins were not affected by heat stress in Sangiovese (Pastore et al., 2017), as well as in the study of Gouot et al. (2019) who reported an absence of effect on flavan-3-ol or tannin levels, but a significantly higher percent of galloylation of flavan-3-ols, consistently with that reported by Cohen et al. (2012a) and indicated bythe overexpression of *UDP glucose-gallic acid-glucosyltransferase* under high temperature as reported by Rienth et al. (2016).

With regards to stilbene synthesis, heat stress inhibited the expression of members of the STS biosynthetic pathway (Rienth et al., 2014b), while grape exposure to low temperature upregulated STS transcripts indicating a higher stilbene biosynthesis at low temperature (Pastore et al., 2017).

Temperature Impact on Aroma Compounds

In plants, high temperature causes an increase in aroma compound production and emission as shown particularly for terpenes up to a certain threshold, generally around 40°C (Guenther et al., 1993; Copolovici and Niinemets, 2016). In grape berries, the temperature impact on aroma compound accumulation is ambiguous (Selmar and Kleinwächter, 2013; Lecourieux et al., 2017). From recent studies, it appears that the aroma levels are reduced when heat stress is applied both at the cluster scale (Lecourieux et al., 2017) and at the whole plant scale if stress is applied during berry ripening (Rienth et al., 2014b). In Sangiovese, Pastore et al. (2017) reported an increase in TPS expression under higher temperature before ripening and a repression of linalool synthase, delta-cadinene synthase, vetispiradiene synthase, and a germacrene enzyme activity during ripening. In Sauvignon blanc exposed to higher berry temperature through leaf removal, the concentration of thiol precursors in berries at harvest was not significantly modified (Sivilotti et al., 2017). However, Wu et al. (2019) found a lower concentration of the aldehydic glutathionylated precursor of 3SH (Glut-3SH-Al) in grapes from Cabernet Sauvignon and Sauvignon Blanc under a 1.5°C temperature increase.

Temperature studies on carotenoids, the precursors of C13 norisoprenoids (see above), are also inconsistent, most likely because of the difficulty in separating temperature and radiation effects in field trials. Higher carotenoid levels were found in Riesling and Chenin Blanc grown in warmer than cooler regions (Marais et al., 1991). In the Duoro Valley in Portugal, cooler temperature at higher altitudes possibly contributed to the lower berry carotenoids concentration in Portuguese autochthonous cultivars (Oliveira et al., 2004). Fernandes de Oliveira et al. (2015) found consistently higher carotenoid content in shaded grapes compared to grapes exposed to direct sunlight. However, leaf removal in the fruiting zone, which generally increases temperature around berries, did not alter total carotenoids in Riesling (Kwasniewski et al., 2010).

The concentration of the C13 norisoprenoid 1,1,6-trimethyl-1,2-dihydronaphtalene (TDN), which determines the kerosene-like notes of aged Riesling, is generally higher in warm climates (Marais et al., 1992; Schüttler et al., 2015). Carotenoid and C13 norisoprenoid related transcripts, such as phytoene synthase (*VviPSY*), which catalyzes the first step of carotenoid biosynthesis, and downstream genes coding the phytoene dehydrogenase, carotenes desaturase, carotenoid isomerase, lycopene cyclase, and carotene hydroxylase were concomitantly downregulated by vine exposure to high temperature (Rienth et al., 2014b; Lecourieux et al., 2017).

Considering the cultivars that produce MPs, wines produced in cooler regions have higher MP concentrations and display a more vegetative odor, as shown for Cabernet Sauvignon and Sauvignon Blanc (Allen et al., 1991; Falcão et al., 2007). However, it is still not clear to what extent temperature contributes to the decrease of MPs before and during ripening, and to what extent the decrease is due to the radiation (Darriet et al., 2012; Lei et al., 2018). The limited molecular data indicates a repression of IBMP synthesis already in

the green berry exposed to high temperature as highlighted by the repression of the key gene *VviOMT3* during heat stress experiments with fruiting cuttings (Lecourieux et al., 2017).

Recent research has characterized other aroma components contributing to overripe aromatic nuances in grapes and wines of red cultivars (i.e., nuances of jammy fruit, and prune in Merlot and Cabernet Sauvignon wines) (Pons et al., 2017a; Allamy et al., 2018). Such grapes and wines presented a greater concentration of chemical compounds belonging to the ketones, furanones and lactones family. Moreover, the analysis of wines of different vintages from a Pomerol estate contained higher concentrations of 5,6-dihydro-6-pentyl-2-(2H)-pyranone (called massoia lactone or 2-decen-5-olide) and gamma-non-alactone during vintages with higher average temperatures. This leads to the hypothesis that increased temperatures due to climate change will increase the perception of overripe fruity notes, as occurring now in hot vintages (Pons et al., 2017a).

RADIATION

As described above, changes in the gas composition of the atmosphere are forecasted for the future. These changes may affect the amount and composition of solar radiation reaching the earth's surface. The exposure of grapes to sunlight is normally associated with higher berry quality due to higher levels of total soluble solids, anthocyanins, and phenolics in general, and lower levels of acids and juice pH as well as lower incidence of disease due to better microclimates (Dokoozlian and Kliewer, 1996; Bergqvist et al., 2001; Abeysinghe et al., 2019).

Impact of Radiation on Phenolic Compounds

The exposure of grapes to sunlight normally increases the level of phenolic compounds as shown, for example, in Riesling (Brandt et al., 2019), Pinot Noir (Song et al., 2015), Summer Black (Xi et al., 2016), and Cabernet Sauvignon (Blancquaert et al., 2019). In parallel, sunlight exposure enhances the expression of structural and regulatory phenylpropanoid genes (Chorti et al., 2010; Matsuyama et al., 2014; Wu et al., 2014; Friedel et al., 2015; Sun et al., 2017). Among phenolics, the most light-responsive ones are flavonoids and in particular flavonol glucosides, whose levels increase dramatically with increasing sunlight exposure, consistently with their UV radiation-screening activity and their capacity to reduce light-induced oxidative damage (Downey et al., 2004; Matus et al., 2009; Agati et al., 2013; Martínez-Lüscher et al., 2014; Reshef et al., 2017, 2018). In a recent comprehensive study that used leaf thinning and shoot removal in Cabernet Sauvignon and Petit Verdot to improve sun exposure, the flavonols kaempferol, quercetin, and myricetin significantly increased, whereas no changes of other flavonoid compounds occurred (Torres et al., 2020a). Alike, Sun et al. (2017) found higher hydroxycinnamic acids and flavonol levels in Cabernet Sauvignon when sun exposure was increasd.

Several transcriptomic studies indicate that, in the berry skin, flavonol genes – for example *VviFLS1, VviGT5* and *VviGT6* in Tempranillo berries – are more induced than those of other phenylpropanoids upon UV radiation (Koyama et al., 2012; Loyola et al., 2016). Reciprocally, Matus et al. (2009) found reduced expression of *VviFLS4* and its putative transcriptional regulator *MYB12* when berries were shaded.

It is not fully understood to what extent visible and/or UV light contribute to the stimulation of the synthesis of phenolic compounds (Keller and Torres-Martinez, 2004; Schreiner et al., 2012; Teixeira et al., 2013). Drawing upon recent studies, it seems that particularly the UV-B fraction of solar radiation is responsible for the enhanced expression of key flavonoid genes (Koyama et al., 2012; Teixeira et al., 2013; Carbonell-Bejerano et al., 2014; Martínez-Lüscher et al., 2014; Liu et al., 2015; Loyola et al., 2016). Recently, two bZIP TFs elongated hypocotyl 5 protein (HY5) orthologs, *VviHY5* and *VviHYH*, were characterized as constituents of the UV-B response pathway in grapevine and mediated flavonol accumulation in response to high radiation exposure (Loyola et al., 2016; Matus et al., 2017).

The exposure of grape clusters to light significantly increases anthocyanin accumulation, whereas shading reduces it (Spayd et al., 2002; Downey et al., 2004; Matus et al., 2009; Song et al., 2015; Guan et al., 2016). In a comprehensive *in vitro* study that considered the effect of berry exposure to light and temperature treatments, Azuma et al. (2012) reported higher anthocyanin levels in grapes exposed to higher light levels. These higher levels correlated with the upregulation of most genes of the anthocyanin biosynthesis pathways. Several other studies confirm the induction of major anthocyanin genes such as the TF *VviMYBAa* together with *VviUFGT* under elevated sun exposure (Koyama et al., 2012; Shinomiya et al., 2015). Interestingly, UV-B radiation induces the expression of *VviMYBA1* and significantly delays the down-regulation of *VviMYBA6* and *VviMYBA7* at the latter stages of berry development (Matus et al., 2017).

Low light conditions modulate the proportion of di- to tri-hydroxylated anthocyanins more toward tri-hydroxylated anthocyanins as evidenced by the downregulation of *VviF3′5′Hs* (Azuma et al., 2012; Koyama et al., 2012; Guan et al., 2016). Similar trends, but inconstent amongst years, have been recently reported in warm climates with Cabernet Sauvignon (Sun et al., 2017) and Petite Verdot (Torres et al., 2020a,b). It seems that low light conditions increase the concentration of non-acylated anthocyanins (Downey et al., 2006; Matus et al., 2009) but this remains to be confirmed in future studies.

Recently, a role of miRNA on the anthocyanin response to UV-B radiation has been suggested. Sunitha et al. (2019) hypothesize that the UV-B induced upregulation of miR3627/4376 facilitates anthocyanin accumulation by antagonizing a calcium effector, and showed that miR395 and miR399, which are induced by micronutrient deficiencies also known to trigger anthocyanin accumulation, respond positively to UV-B radiation. Finally, increases in the abundance of the MYB-bHLH-WD40 complex, which regulates anthocyanin production, are mediated by UV-B-induced changes in miR156/miR535. The same changes could contribute to the observed up-regulation of miR828. In turn, miR828 would regulate the AtMYB113-ortologues *MYBA5, A6* and *A7* (and thereby anthocyanins) via a widely conserved and previously

validated auto-regulatory loop involving miR828 and phasi TAS4abc RNAs.

Impact of Radiation on Aroma Compounds

It is generally known that light exposure increases the concentration of most aroma compounds in grape berries, but that excessive sunlight as well as its total exclusion inhibits the accumulation of most aroma compounds (Bureau et al., 2000; Zhang et al., 2014; Young et al., 2016).

Because carotenoids are pigments with photoprotective function, their biosynthesis is generally enhanced under high radiation as are their degradation products. The levels of C13 norisoprenoids are as well highly correlated with extended sun exposure (Kwasniewski et al., 2010; Schüttler et al., 2015; Young et al., 2016). Sunlight exposure increased the concentration of β-ionone, but the increase was not statistically significant for UV treatment (Song et al., 2015), and TDN is typically found in high amounts in sun-exposed Riesling berries, potentially resulting in a higher petrol aroma of wines (Mendes-Pinto, 2009).

Young et al. (2016) reported that the cytosolic CCD1-encoding genes were up-regulated in exposed clusters in the earlier stages of berry development. Conversely, the chloroplastic CCD4-encoding genes were down-regulated in exposed clusters during ripening. Therefore, Young et al. (2016) suggested that the increased levels of norisoprenoids observed in exposed berries during ripening were not due to increased gene expression (of the CCD4-encoding genes) but rather due to increased substrate (carotenoid) availability.

Monoterpenes, and particularly linalool, are highly sensitive to sunlight, as shown, for example, in Sauvignon Blanc and Riesling (Sasaki et al., 2016), where the expression of *VviDXS* and of linalool synthases was reduced by low sun exposure and by UV-B exclusion, resulting in lower levels of linalool content. Similarly, Zhang et al. (2017) showed that, in the Muscat variety Jingxiangyu, linalool was the most sensitive compound to sunlight followed by ocimene and glycosylated geraniol. The reduction in the levels of these terpenes by sunlight exclusion correlated with the reduced expression of *VviPNLinNer1*, *VviCSbOci*, *VviGT7*, and *VviGT14* genes. Similarly, Carbonell-Bejerano et al. (2014) reported an upregulation of genes involved in monoterpenoid biosynthesis, such as 1,8-cineole/eucalyptol synthase and two linalool synthases, in Tempranillo berries exposed to high UV-B radiation. In general, monoterpene levels were induced by exposing clusters to sunlight (Song et al., 2015; Feng et al., 2017).

Young et al. (2016) showed that increased photosynthetically active radiation (PAR) (+52%) led to higher levels of volatile terpenoids in the exposed Sauvignon Blanc berries; however, there were clear differences in the responses based on the developmental stage considered.

Similarly, Šuklje et al. (2014) found increased concentration of thiols in clusters exposed to sunlight, and it is generally known that light exposure during ripening reduces MP content in berries (Roujou de Boubee et al., 2000; Sala et al., 2004; Stummer et al., 2005; Darriet et al., 2012; Sidhu et al., 2015; Martin et al., 2016; Cassandra et al., 2019; Torres et al., 2020a). Interestingly, according to Koch et al. (2012), higher light intensity before and not during ripening has a greater impact of methoxypyrazine concentration at harvest. Dunlevy et al. (2013) showed that both the precursor of 3-isobutyl-2-hydroxypyrazine (IBHP) and the expression of *VviOMT3*, a gene that controls the final step of methoxypyrazine biosynthesis, were drastically reduced in exposed clusters(Koch et al., 2012).

Allamy et al. (2018) considered separately the specific impact of light exposure on berry aroma compounds during post-harvest storage. Under conditions of light exposure, significant increases in furaneol, homofuraneol and γ-nonalactone concentrations were noticed in both grape juice and wine (Allamy et al., 2018).

WATER

Impact of Vine Water Status on Phenolic Compounds

Studies investigating the effect of water availability on berry physiology and quality have been recently reviewed by Scholasch and Rienth (2019), Rienth and Scholasch (2019), and Gambetta et al. (2020). Impact of water deficit on berry development depends on the intensity and duration of the stress as well as the developmental stage. Water deficit stress during the first growth phase had the highest impact on final berry volume and consequently yield. Water deficit reduces cell expansion without impacting the rate of cell division (Ojeda et al., 2001), contrary to frequent speculations. During the ripening phase, water deficit has a smaller impact on berry size than during the first growth phase, probably because of impaired hydraulic connections with the parental plant.

It is generally known that a moderate water deficit (predawn leaf water potential between−0.3 to −0.5 MPa) is beneficial for final wine quality which is in particularly true for red cultivars (Van Leeuwen et al., 2009; Zufferey et al., 2017). Because water deficit reduced berry volume, positive effects can partly be attributed to higher concentration of quality determining compounds synthesized in the skin cells (the surface of the berry). However, an enhanced accumulation of secondary metabolites independently of berry volume changes has been already highlighted by Ojeda et al. (2002) and confirmed by several molecular studies that observe an upregulation of key enzymes of the phenylpropanoid and flavonoid pathways (Castellarin et al., 2007a,b; Cramer et al., 2007; Deluc et al., 2009, 2011; Teixeira et al., 2013; Savoi et al., 2016, 2017; Zarrouk et al., 2016a,b).

The most beneficial effects are observed when water deficit occurs throughout ripening. Aside from an overall increase in the accumulation of phenylpropanoids and flavonoids (Chorti et al., 2016; Koundouras, 2018), several studies showed a modification in composition of anthocyanins toward a relative increase of tri-hydroxylated anthocyanins (3′,4′,5′-hydroxylated: delphinidin, petunidin, malvidin) (Castellarin et al., 2007a; Ollé et al., 2011; Cook et al., 2015). However,

the changes in the anthocyanin profile in response to water deficit appear to be highly varietal dependent (Niculcea et al., 2014; Theodorou et al., 2019). Some reports showed an increase proanthocyanidin concentration and proanthocyanidin polymerization levels in grape berry skins (Kyraleou et al., 2016; Cáceres-Mella et al., 2017), and higher catechin levels (Zsófi et al., 2014). The observed increase of phenolic compounds when water deficit occurs prior to *véraison* can mainly be attributed to concentration effects (Santesteban et al., 2011; Brillante et al., 2018); however, several studies also observed an increase in anthocyanin content per berry (Ojeda et al., 2002; Castellarin et al., 2007a; Koundouras et al., 2009; Ollé et al., 2011).

Impact of Vine Water Status on Aroma Compounds

Reported effects of water availability on aroma compounds are less evident than for phenolic compounds. Most water deficit studies on grape aroma compounds show very heterogeneous results, depending on the type of aroma compounds considered, as reviewed by Alem et al. (2019). A positive relationship between increasing water deficit and the concentration of C13-norisoprenoids such as β-damascenone, β-ionone, and 1,1,6-trimethyl-1,2-dihydronaphthalene was reported. This was particularly true for red cultivars such as Cabernet Sauvignon (Bindon et al., 2007; Koundouras et al., 2009; Brillante et al., 2018), Merlot (Song et al., 2012), and Tempranillo (Talaverano et al., 2017). In of Talaverano et al. (2017), C6 compounds (hexanal, trans-2-hexenal, and 1-hexanol), phenol volatiles, ethyl esters, and lactones were also found to be increased under water deficit, as opposed to Song et al. (2012), which reported a decrease of those compounds under water deficit in Merlot.

Several studies reported increased concentrations of monoterpenes such as limonene, linalool, α -terpineol geranyl acetone, geraniol, and citronellol under light to moderate water stress (Savoi et al., 2016; Brillante et al., 2018; Wang et al., 2019; Kovalenko et al., 2021), which was associated with increased expressions of terpenoid synthases-genes in Chardonnay and Tocai Friulano (Deluc et al., 2011; Savoi et al., 2016). Some authors report higher monoterpene concentrations, even under severe water deficit (Schüttler et al., 2015). One of the few aroma compounds whose concentration in the berry is negatively correlated with moderate water deficit is the recently discovered sesquiterpene rotundone (Wood et al., 2008; Geffroy et al., 2014, 2018) associated with notes of black pepper.

The precursors of volatile thiols (S, 4MSPOH and the 3SH) present in the berry (Tominaga et al., 2000; Fretz et al., 2005) increased under mild water deficit and decreased under severe stress (predawn leaf water potential close to −1.0 MPa) (des Gachons et al., 2005). However, since nitrogen is very important for the production of volatile thiols of grapes (Helwi et al., 2015, 2016) and its absorption can be limited by water deficit (Celette and Gary, 2013), the effects observed may be indirect and largely due to a limited nitrogen absorption. Picard et al. (2017) found that the exposure of vines to water deficit positively relates to the perception of aging bouquet typicality (truffle and underwood aroma) in premium Bordeaux wines. It appears that moderate water deficit increases the concentration of C13-norisoprenoids most likely from higher sun exposure due to reduced canopy (Koundouras et al., 2009). On the other hand, the reduced canopy caused by water deficit might favor methoxypyrazine degradation (Brillante et al. (2018) and Harris et al. (2012).

CO_2 CONCENTRATION

Elevated carbon dioxide (eCO_2) concentration is generally beneficial for plants because it leads to increases in the rate of photosynthetic carbon fixation by leaves. This leads primarily to increased plant growth and biomass production and translates into increases in harvestable yield of wheat, rice and soybean, all showing 12–14% yield increases under eCO_2 in FACE (Free Air Carbon enrichment) experiments (Ainsworth and Long, 2005; Ainsworth and Rogers, 2007; Ainsworth, 2008). In fruit crops and vegetables, eCO_2 generally increased the total antioxidant capacity as well as the concentrations of fructose, glucose, total soluble sugars, total phenolics, total flavonoids, ascorbic acid, and calcium in the edible parts (Sun et al., 2017).

Most studies on grapevine dealing with eCO_2 focused on vegetative growth and photosynthesis while physiological and molecular studies on berry metabolism are relatively scarce so far. All studies report increased photosynthesis leading to a yield and biomass increase under eCO_2 (Goncalves et al., 2009; Moutinho-Pereirea et al., 2009; Kizildeniz et al., 2015; Edwards et al., 2016, 2017; Wohlfahrt et al., 2018). In climate chamber studies, Martinez-Luscher et al. (2015) highlighted the dependence of berry ripening rates on the carbon fixation process which is correlated to CO_2 concentration.

Only some grape attributes have been found to be affected by eCO_2. In particular, sugars, acids, and berry size increased under eCO_2 (Bindi et al., 2001; Kizildeniz et al., 2015). However, in a FACE study that considered Riesling and Cabernet Sauvignon vines exposed to moderate increases of atmospheric CO_2, sugar concentration was not affected even though yields were increased (Wohlfahrt et al., 2018). In wines made from the latter FACE experiment must and young wines quality and compositin was not found to be negatively influenced by an eCO_2 (Wohlfahrt et al., 2021). Anthocyanins and proanthocyanidins were not affected by eCO_2 in most studies (Goncalves et al., 2009; Salazar-Parra et al., 2012; Kizildeniz et al., 2015).

In multistress experiments with Temperanillo fruiting cuttings where future temperature (+4°C) and CO_2 (700 ppm) conditions where simulated, high CO_2 in particular when combined with high temperature hastened berry ripening, sugar accumulation, malic acid respiration and reduced the aforementioned high temperature induced anthocyanin–sugar decoupling (Arrizabalaga-Arriazu et al., 2020a).

The effect of eCO_2 on aroma compounds remains to be elucidated. Goncalves et al. (2009) found no impact on aroma compounds such as C6 alcohols, alcohols, esters, terpenols, carbonyl compounds, acids, volatile phenols, and C13 norisoprenoids under moderate eCO_2 (500 ppm). However, the same treatment induced an increased level of

ethyl 2-methylbutyrate, isoamyl acetate, ethyl hexanoate, and ethyl octanoate.

BIASES GENERATED BY BERRY HETEROGENEITY AND VOLUME VARIATION

Empirically, winegrowers and berry physiologists know that grape ripening is a very heterogenous process which can easily by visually perceived around *véraison* when, in red grape cultivars, berries of the same cluster change color asynchronously. For "fine scale" physiological studies that aim to reveal the effect of climate and abiotic factors on grape berry composition, the random time scale of berry sampling that is commonly adopted can cause important biases in chemical composition and gene expression data, which can potentially mask important physiological information. Poor correlation of phenotypic and transcriptomic data often observed in abiotic and biotic stress studies can be partially attributed to berry heterogeneity and sampling strategy.

Several molecular studies tried to address the complex issue of grape ripening on a single berry scale (Gouthu et al., 2014; Rienth et al., 2014b, 2016; Shahood et al., 2015, 2020; Carbonell-Bejerano et al., 2016; Rösti et al., 2018). For instance, Carbonell-Bejerano et al. (2016) performed berry density sorting by berry flotation in NaCl solutions and assessed the transcriptome of different berry ripening groups and showed that gene expression profiles clearly relate with ripening progression of different sorted berry groups. By contrast, when the same density series were sampled on two different dates from the same vineyard of Tempranillo, considerable differences were detected, which indicated that environmental differences between both sampling moments determined most of these expression differences. Latter findings highlight evidence on the convenience of a homogenization of the developmental stage and the sampling time condition for transcriptome comparisons by berry density sorting.

Previous heat stress studies using RNA from single berries sorted according to their biochemical characteristics led to the discovery of anthocyanin biosynthesis related transcripts (Rienth et al., 2014b) that have not been detected in other studies. In several studies (Carbonell-Bejerano et al., 2013; Lecourieux et al., 2017; Pastore et al., 2017), anthocyanin concentration was significantly decreased by heat stress, but the expression pattern of key anthocyanin regulator genes such as *VviMybA1* and *VviAOMT* did not show concomitant repressions as opposed to the heat stress study of Rienth et al. (2014a,b), where berry batches where constructed according to berry biochemical characteristics. Similar discrepancies in gene expression data were observed in molecular studies conducted on berries of virus infected vines and emphasize the heterogeneity problem, where the gene expression results changed if berries were sampled at same calendar day without sorting (Vega et al., 2011) or if individual berries were grouped according to their "internal ripening clock" (Ghaffari et al., 2020). The "highest precision" is in our opinion obtained by single berry analysis of sugar and acids and a subsequent grouping of berries according to those to parameters. Obviously, such sampling strategies are labor intensive and above all time consuming. A good compromise could be berry sorting by density, which, however, will require some time for the sorting before the berries can be frozen in liquid nitrogen, during which time the transcriptome of the abscised berry may evolve leading to biases. An alternative, less time-consuming, sampling approach is to measure sugar concentration of each berry prior to N-freezing as adopted in a recent study by Cramer et al. (2020). Researchers need to weigh the advantages and drawback of different sampling strategies for each experiment and when interpreting their results.

Another potential issue is related to how the levels of compounds are expressed. This can prevent researchers from drawing clear physiological conclusions. Commonly, metabolite (sugars, acids, phenolic compounds, etc.) levels in the grape berry are expressed as concentration, i.e., amount per volume (L) or weight (g), because the metabolite concentration in the wine is more closely related to the metabolite concentration in the berry than to the total metabolite amount in the berry. The metabolite concentration depends on the amount accumulated in the cells and the berry volume which change dramatically during development and/or due to responses to abiotic stresses (e.g., limited water availability that decreases berry size). Therefore, expressing metabolites as concentrations can lead to misinterpretations of the effects of specific treatments or stresses on the metabolic responses of the berry (Famiani et al., 2015; Moscatello et al., 2019). Thus, expressing both the concentration and the amount per berry of a given metabolite allows for a better identification of direct and indirect effects of treatments/factors on such metabolite.

CONCLUSION

A changing climate requires a profound knowledge of how abiotic factors modulate different quality-determining compounds of the grape berry in order to implement appropriate viticultural mitigation strategies (van Leeuwen and Destrac-Irvine, 2017; Rienth et al., 2020), select varieties, clones (Wolkovich et al., 2018), and rootstocks (Ollat et al., 2016), and to identify traits, genes, or QTLs for the breeding of new cultivars better adapted to future conditions (Duchêne, 2016).

The physiological and molecular knowledge of the mechanisms involved in the biosynthesis and degradation of secondary metabolites in the grape berry has significantly increased in the past two decades and is continuously advancing due to the development and improvement of omic tools. However, the impact of environmental factors, notably light and temperature, is often ambiguous (van Leeuwen et al., 2020). This can partly be attributed to the difficulty in separating light and temperature effects and the interaction between the two. A further often neglected bottleneck in physiological studies on grape berry responses to climate and abiotic factors is the effect of heterogeneity and the aforementioned berry sampling strategy, which may impair our capability to analyses complex metabolic events. Post-transcriptional and epigenetic regulation of metabolism, which have so far rarely been

addressed in physiological studies, might play important roles in the endogenous and exogenous modulation of secondary metabolism in the grape berry.

Facing global warming, viticultural practices such as cluster and shoot thinning or leaf removal, historically considered as improving quality, need to be reconsidered and adapted to changing conditions (van Leeuwen and Destrac-Irvine, 2017; Torres et al., 2020a,b). Moreover, field data on the impact of increased atmospheric CO_2 concentration on berry metabolism are scarce and need to be further investigated, in particular in combination with different abiotic stresses such as increased temperature and drought. Though difficult to conduct in field conditions and so far only carried out on fruiting cuttings (Salazar-Parra et al., 2012; Kizildeniz et al., 2015; Martinez-Luscher et al., 2015; Arrizabalaga-Arriazu et al., 2020a,b) such multi-stress experiments will improve understanding of how climate change will impact vine and berry physiology, and will help develop mitigation strategies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MR and SDC devised the main body and structure and content of the manuscript. PD, CS, CBu, CBo, NV, RW, and FF provided valuable ideas and triggered fruitful discussions via helpful comments and provided corrections. All others approved the final version of the manuscript.

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Toward Systematic Understanding of Flower Bud Induction in Apple: A Multi-Omics Approach

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The induction of flower buds in apple (*Malus* × *domestica* Borkh.) is tightly connected to biennial bearing, which is characterized by alternating years with high (ON) and low or no (OFF) crop loads. In order to study this irregular cropping behavior, spur buds from ON- and OFF-trees of the biennial-bearing cultivar 'Fuji' and the regular bearing cultivar 'Gala' were collected. First, the time of flower bud initiation was precisely determined for both cultivars by histological analysis. Moreover, for a systematic understanding of flower bud induction in apple, the physiological and molecular mechanisms within the bud tissue were evaluated over four weeks prior to flower bud initiation by employing a multi-omics approach, including RNA sequencing, proteomic and metabolic profiling. Gene and protein enrichment analysis detected physiological pathways promoting and inhibiting early flower bud development. Metabolic profiles from the cropping treatments revealed a greater abundance of thiamine, chlorogenic acid, and an adenine derivative in spur buds from OFF-trees, whereas tryptophan was more abundant in the buds collected from ON-trees. Cultivar comparison indicated that chlorogenic acid was more abundant in 'Gala' than in 'Fuji' spur buds, whereas the opposite effect was found for tryptophan. Genes controlling tryptophan biosynthesis were not affected by ON- and OFF-treatments, but genes assigned to the metabolism of tryptophan into indoleacetate were differentially expressed between cultivars and treatments. The multi-omics approach permitted analyzing complex plant metabolic processes involved in early flower bud development and more specifically presumably in flower bud induction by tracing some pathways from gene to product level.

Keywords: malus domestica, flower bud formation, RNA sequencing, proteomics, metabolomics, multi-omics

Abbreviations: ABA, abscisic acid; BS, brassinosteroids; CK, cytokinins; DAFB, days after full bloom; DAP, differentially abundant protein; DAM, differentially abundant metabolite; DEG, differentially expressed gene; GA, gibberellins; JA, jasmonic acid; KEGG, Kyoto Encyclopedia of Genes and Genomes; log2FC, log2 fold change; MS, mass spectrometry; NGS, next-generation sequencing; IAA, indole-3-acetic acid; RT, retention time; SA, salicylic acid.

INTRODUCTION

Flower induction, initiation, and differentiation are developmental stages that vegetative buds need to undergo on their way to become floral. Flower induction is commonly defined as a time point when a vegetative bud meristem perceives a signal to develop new tissue structures, so-called flower meristems. In contrast, flower initiation is characterized by distinct morphological, microscopically visible, meristematic changes in the bud (Foster et al., 2003; Hanke et al., 2007). While flower bud initiation can be determined by histological sectioning, the exact time of flower induction still remains obscure in many plant species such as apple (*Malus* × *domestica* Borkh.).

Based on mRNA expression data of flowering genes in apple shoot apices, it was assumed that the bud transition from induction to initiation could take around two weeks (Hanke et al., 2007). Early attempts to study flower initiation in apple were broadly reviewed by Jonkers (1979) and Monselise and Goldschmidt (1982). Merging the accumulated knowledge, some authors proposed hypothetical models of flower induction (Xing et al., 2015; Zuo et al., 2018); however, none of them could fully explain the genetic and physiological basis of this plant developmental stage. Understanding flower induction is of great importance for controlling crop load in perennial crops in order to establish regular annual cropping levels and to alleviate the production constraints associated with biennial bearing. The term biennial (or alternate) bearing in horticultural crops describes erratic yields when flowering density and in turn crop load in a given year strongly depends on the crop load of the previous year. It is frequently triggered by adverse environmental conditions, such as spring frost accompanied with flower damage, resulting in an OFF-year with low yield, followed by an ON-year with high yield of small-sized fruit before the repetitive cycle commences again with an OFF cropping status (Wünsche and Ferguson, 2005).

Initially, the reduced flower initiation rate on high-yielding apple trees was explained by sink–source interactions between fruit and buds concurrently developing within the growing season, with fruit being a stronger sink, thereby attracting proportionally more carbohydrate and in turn depriving the buds of the phloem-derived nutrients (Lenz, 1979). Numerous experiments indicated the importance of carbohydrate supply for flower bud development *in vitro*. Among the different sugars that have been tested in culture media, sucrose appeared to be the most effective to induce bud formation (Nitsch and Nitsch, 1967) and flower bud development (Jana and Shekhawat, 2011). Moreover, it was found that plant hormones and hormone-like acting compounds are strongly involved in the flower bud induction processes. In *Plumbago indica*, *in vitro* bud formation from callus was achieved in the presence of cytokinins and adenine and further promoted by adding indole-3-acetic acid (IAA). In the same study, flower bud formation was inhibited by application of three different gibberellins (Nitsch and Nitsch, 1967).

Recently developed omics analytical strategies have been driven largely by technological advances in mass spectrometry for proteomics and metabolomics and next-generation sequencing (NGS) assays, including RNA-Seq for transcriptomics. These high-throughput methods are cost-effective and target specific classes of biomolecules such as RNA transcripts, proteins, and primary or secondary metabolites. Despite the immense amount of data that can be obtained using each of those approaches, implementing only one of them, as for example done by Li et al. (2016, 2019) in apple and by Muñoz-Fambuena et al. (2013) in citrus, might not be sufficient to understand complex biological mechanisms such as flower induction in plants. With the appearance of gene-detection techniques, considerable research was devoted to the discovery of genes and transcription factors, which may promote or suppress flowering. Indeed, dozens of sequences were initially described as flowering regulators in Arabidopsis and later confirmed to be present as homologs in apple (Flachowsky et al., 2010, 2012; Haberman et al., 2017).

Despite these achievements in genomics, the current knowledge about the proteome and metabolome of apple is very limited and described so far only in the context of fruit development and maturation by Lin and Harnly (2013), Buts et al. (2016), and Li et al. (2016). Specifically, proteomic and metabolic data sets of apple buds in relation to biennial bearing are still missing. Many authors studied flower bud development by looking at the activity of particular genes (Zuo et al., 2018), transcription factors (Vimolmangkang et al., 2013), and proteins (Foster et al., 2007), which had already been discovered in other plants. To study the underlying biological processes involved in flower induction and to trace them from gene to product, it is necessary to combine several omics approaches in an attempt to better understand the interplay between genes, proteins, and metabolites determining the reproductive development of plants. The target and the novelty of the current work is the application of NGS in combination with two non-targeted omics approaches to develop a systematic understanding of the complex plant metabolic processes involved in flower bud induction in apple and tracing some pathways from gene to product level.

In the same experimental setup, we sampled apple buds for histological sectioning and revealed flower initiation time points for 'Fuji' and 'Gala' under field conditions in southwest Germany (Kofler et al., 2019). Considering these time points, we aimed to detect mobile signals potentially promoting or inhibiting flower bud induction such as peptides, phytohormones, phytohormone-like acting compounds, sugars, and secondary metabolites during 1–4 weeks prior to flower initiation and to link all the compounds of interest to genes and proteins involved in their biosynthesis and regulation. Specifically, the unknown mobile signals could influence or could be influenced by expression patterns of genes, determining the fate of the bud meristem. In order to test this hypothesis comprehensively on the transcript, protein, and metabolite level, we used a holistic multi-omics approach, targeting the flower induction mechanisms of the biennial-bearing apple cultivar 'Fuji' and of the regular bearing apple cultivar 'Gala'. Although we acknowledge that there is a smooth transition from induction to initiation of flower buds, the signals in the gene to product pathway we found as early as four weeks prior to flower initiation can be assertively ascribed to flower induction.

Apple spur buds, which were used for multi-omics analyses, were collected from ON- and OFF-trees over four weeks leading up to flower initiation, covering the assumed period of flower induction. RNA extracted from the buds was analyzed using next-generation RNA sequencing; proteins and metabolites were detected using electrospray ionization (ESI) mass spectrometry in order to create multi-omics profiles of apple spur buds and to reveal the differences between ON- and OFF-trees. Here we summarize the results of RNA sequencing and non-targeted proteomic and metabolic profiling.

MATERIALS AND METHODS

Plant Material and Experimental Design

The experimental apple orchard was located at the Centre of Competence for Fruit Cultivation near Ravensburg, Germany (47°46′2.89″N 9°33′21.21″E, altitude 490 m). The study was performed using 7-year-old 'Fuji' (clone "Raku-Raku") and 'Gala' (clone "Galaxy") apple trees (130 trees of each cultivar) grafted on M.9 rootstock. Trees of each cultivar were planted in two rows at 3 × 1 m spacing, respectively, trained as tall spindles of 3.5 m height and managed with standard irrigation and plant protection programs for the area. At the time of full bloom (30 April 2015), all flowers from randomly selected 65 trees per cultivar were removed by hand (OFF-trees), while the remaining trees were not thinned and maintained their natural flower density and hence high crop load (ON-trees). Subtending apple buds on 2-year-old spurs were collected weekly starting from four weeks after full bloom for 15 weeks until 2 September 2015. At each sampling week, 55 buds were collected from each of four randomly selected treatment-trees for proteomic and metabolic profiling and for RNA sequencing. After the brown bud scales were removed, the buds were placed into safe-lock tubes and snap-frozen in liquid nitrogen. The samples were stored at −80°C until used for analysis. The workflow including the plant material, treatments, sampling scheme, and analytical procedures used in this study is shown in **Figure 1**.

Sampling Time Window for the Analyses

Observing flower bud meristem development by histological sectioning of apple buds sampled in the same experiment throughout the growing season of 2015, we identified flower initiation time points for 'Fuji' (75 days after full bloom, DAFB) and for 'Gala' (97 DAFB) (Kofler et al., 2019). The findings showed not only microscopically observed flower initiation time points but also suggested the predicted (modeled) time of the onset of flower initiation. The sampling window selection for the current study was based on the microscopically identified flower initiation time points. Assuming that a signal for flower bud induction must be detectable at least two weeks prior to flower initiation, we selected four sampling weeks prior to this event in order to perform omics analyses and to capture triggers of flower induction. Consequently, the multi-omics analysis covers sampling dates 48, 55, 63, and 68 DAFB, corresponding to 17 June, 24 June, 01 July, and 07 July for 'Fuji' and 68, 75, 83, and 89 DAFB, corresponding to 07, 14, 21, and 28 July for 'Gala.'

RNA Sequencing

Three trees, serving as replicates, were randomly selected for each of the two treatments (ON and OFF) at each of four sampling weeks chosen for the analysis. Five out of 55 collected buds per replicate were randomly taken for RNA extraction. This was performed using the InviTrap Spin Plant RNA Mini Kit (Invitek Molecular GmbH, Berlin, Germany) according to the standard protocol with the following modifications: Samples were first ground to powder using a cryogenic mixer mill (CryoMill, Retsch GmbH, Haan, Germany) cooled with liquid nitrogen. The grinding was done in safe-lock tubes with two steel balls (Ø = 5 mm) in one cycle of 10 s precooling and 6 min grinding at 25 Hz. A mixture of mercaptoethanol and lysis solution RP (1:100) was used for cell membrane disruption. Total RNA of each sample was eluted in 40 µl RNase-free water. The removal of contaminating DNA from the RNA preparations was achieved with the DNA-free DNA Removal Kit (Thermo Fisher Scientific, Waltham, United States). All the RNA samples were stored at −80°C until required.

RNA sequencing was conducted with c.ATG at the University of Tübingen, Germany. All samples were sequenced on a HiSeq 2500 (Illumina machine, Illumina Inc., San Diego, United States) using a paired-end (PE) mode and producing ∼10 million reads per sample. The resulting fastq files were demultiplexed and transferred to QBiC and analyzed on an HPC cluster of the University of Tübingen in a fully automated way using a Nextflow-based RNA-Seq pipeline[1] (release 1.3). At the core of this workflow, *FASTQC* v0.11.8 (Andrews, 2010) was used to

[1]https://github.com/nf-core/rnaseq

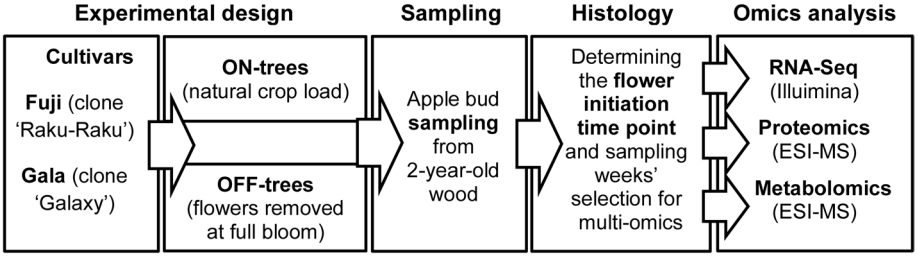

FIGURE 1 | Schematic diagram of the plant material, treatments, sampling scheme, and analytical procedures used in this study.

determine the quality of the FASTQ files. Subsequently, adapter trimming was conducted with *Trim Galore* v0.5.0 (Krueger et al., 2012). The *HISAT2* (v2.1.0) aligner was used to map the reads that passed the quality control against the GDDH13 apple genome version 1.1 from The National Institute of Agricultural Research (INRA)[2] (Daccord et al., 2017). Annotation and fasta files were downloaded from there in January 2019. Read counting of the features (e.g., genes) was done with featureCounts v1.6.4 (Liao et al., 2014). For differential expression analysis, the raw-read-count table resulting from featureCounts was fed into the R package *limma* (v. 3.32.10) and *edgeR* (v. 3.18.1). First, the raw-read-count table was filtered for genes that had no expression in any of the samples. The remaining counts were then normalized by sequencing depth and log2 transformation using the edgeR functions calcNormFactors() and cpm() in order to meet the assumptions of linear models. To identify differentially expressed genes (DEGs) at each time point between 'Fuji' ON and 'Fuji' OFF as well as between 'Gala' ON and 'Gala' OFF conditions, a simple linear model was fitted to each gene consisting of a fixed effect for a combined factor of time (levels: 48, 55, 63, 68 DAFB for 'Fuji' and 68, 75, 83, 89 DAFB for 'Gala'), genotype (levels: 'Fuji' and 'Gala'), and treatment (levels: ON and OFF). *Limma* was then also used to extract pairwise contrasts including statistics for each gene including empirical Bayes moderated *p*-values which were finally adjusted for multiple testing by controlling the false discovery rate (FDR) using the Benjamini–Hochberg procedure (Benjamini and Hochberg, 1995). As threshold, a gene was called a DEG with a multiple adjusted $p \leq 0.05\%$. No log fold change filter criterion was applied for statistical assessment to find DEGs. For exploratory analysis, counts were normalized using the *DESeq2* package (v. 1.16.1) and visualized using standard packages in R (version 3.4.0). Translation of gene IDs into gene symbols was made using Blast2GO software (Conesa et al., 2005), where both apple genomes GDDH13 apple genome version 1.1 published by INRA and MalDomGD1.0 apple genome published by The National Center for Biotechnology Information were aligned. Gene symbols corresponding to gene IDs were chosen from the best-match column. For gene enrichment analysis and gene mapping to KEGG pathways, we used gene annotation software KOBAS 3.0 (Xie et al., 2011). All enriched pathways presented in the current work were selected from the main result output according to the $p \leq 0.05\%$. A Venn diagram was created using online software InteractiVenn (Heberle et al., 2015). Raw data can be accessed on ArrayExpress with the dataset identifier E-MTAB-9644[3].

Proteomic Profiling

Three out of the four trees per treatment and sampling week were randomly selected for proteomic profiling. Each replicate consisted of 16 buds, which were randomly taken from each 55-bud sample per tree. Sample preparation and analysis were performed according to Kofler et al. (2020). In order to compare ON- and OFF-treatments, relative quantification of proteins was performed.

Reverse hits, proteins identified only by site, and potential contaminants were removed from the dataset; LFQ intensities were log(2) transformed; and rows were filtered based on a minimum of three out of six potential valid values at each sampling date and a minimum of two unique peptides per protein. In order to perform a principal component analysis (PCA), missing values were imputed by random numbers from the normal distribution and a downshift of 1.8. For other statistical tests, missing values were not imputed; however, all the proteins found only in spur buds collected from ON- or only in the buds collected from OFF-trees were taken for enrichment analysis and KEGG search. A two-sided Student T-test at each date between the treatments was calculated with S0 = 0.1 and a permutation-based FDR of 0.05. Proteins which were statistically significant according to the T-test and exhibited a log2 fold change >0.5 were considered as differentially abundant. Differentially abundant proteins (DAPs) between treatments in at least one sampling week were considered for the subsequent KEGG pathway analysis. Raw data can be accessed on ProteomeXchange Consortium via the PRIDE repository with the dataset identifier PXD021716[4].

Metabolic Profiling

For metabolic profiling, one bud from each out of four trees per treatment at each of four sampling weeks was analyzed. Each bud was randomly taken from a mixture of 55 frozen buds collected from one tree representing one out of four replicates.

Metabolites from each bud were extracted in a separate safe-lock tube. Since the buds and safe-lock tubes had a slight weight deviation, all the empty tubes (64 in total) were numbered and weighed on the analytical balance to standardize the extraction conditions by adjusting the solvent volumes according to the net sample weight. Frozen buds were then quickly placed into corresponding tubes, weighed again in order to calculate net weights of the samples, opened to put in a precooled steel ball (Ø = 5 mm), and immediately returned to liquid nitrogen. The samples were ground to powder using a CryoMill (Retsch GmbH, Haan, Germany) at a frequency of 20 Hz with 10 s precooling and 4 min grinding. Each sample was eluted with ice-cold (−20°C) solution of 80% methanol and 20% distilled water, vortexed for 10 s, and placed on ice. The samples were kept frozen until cold 80% methanol was added. Methanol volumes were adjusted for each sample by using 120 µl methanol (80%) per 1 mg bud fresh weight. Steel balls were removed from the tubes using a magnet, and extracts were left at −20°C for 24 h for incubation. Incubated samples were centrifuged at 10,000 rcf for 4 min, and the supernatant containing metabolites was transferred into new tubes, which were kept at −20°C until required.

Non-targeted metabolic profiling was carried out at the Mass-Spectrometry Core Facility Unit at the University of Hohenheim, Stuttgart, Germany, using ultra-high-performance liquid chromatography (UHPLC) coupled with electro-spray ionization mass spectrometry (ESI-MS). The UHPLC unit Agilent 1290 Infinity LC System (Agilent Technologies, Inc.,

[2]https://iris.angers.inra.fr/gddh13/

[3]https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-9644/

[4]https://www.ebi.ac.uk/pride/

Santa Clara, United States) was equipped with an Acquity CSH C18 1.7 μm, 2.1 × 150 mm column (Waters Corporation, Milford, United States). Sample components were separated under the column temperature of 40°C using 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) as solvents. From each extract, we used 7 μl for UHPLC injection with a flow rate of 400 μl min^{-1} and the following gradient: at 0 min 97% A and 3% B, from >0 to 15 min 80% A and 20% B, from >15 to 40 min 5% A and 95% B, and from >40 to 44 min 97% A and 3% B. Mass spectrometry was performed on a Thermo Fisher Scientific Q-Exactive Plus Orbitrap System (Thermo Fisher Scientific, Waltham, United States) in positive and negative ionization modes. The spray capillary voltage was set at 4.2 kV in positive mode and at 3.5 kV in negative mode, and desolvation temperature was 380°C. Mass spectra were acquired using a scan range from 140 to 1,500 m z^{-1} at a resolution of 70,000 full width half maximum (FWHM), an automatic gain control (AGC) target of 1.0 × 106, and a 100 ms maximum ion injection time. Data-dependent MS/MS spectra in a mass range of 200–2,000 m/z were generated for the five most abundant precursor ions with a resolution of 17,500 FWHM using an AGC target of 5.0 × 104 of and a 64 ms maximum ion injection time and a stepped collision energy of 15, 30, and 55 V. The measurement was started with a blank solution, after which the extracts (samples) were injected in a randomized order. Quality-control (QC) samples (a mixture of all the samples in equal proportions) were injected after every 10 measurements. Reference compounds were obtained from Merck KGaA (Darmstadt, Germany).

In order to compare ON- and OFF-treatments, relative quantification of metabolites was performed. For high-resolution accurate-mass data analysis and compound identification, we used Xcalibur 4.0.27.13 and Compound Discoverer 2.1.0.398 (Thermo Fisher Scientific, Waltham, United States) software. Fragmentation spectra and molecular masses of detected compounds were aligned to the references retrieved from the databases mzCloud, ChemSpider, Plant Metabolic Network (PMN), and PubChem. Additional statistical analyses of normalized peak areas were performed with Perseus 1.6.1.3 (Tyanova et al., 2016) and online software MetaboAnalyst 4.0 (Chong et al., 2019). PCA, including both cultivars, was not possible to perform due to analysis of samples from 'Gala' and 'Fuji' as two separate batches.

RESULTS

Gene Expression and Proteomics

Next-generation RNA sequencing of 'Fuji' and 'Gala' spur buds detected 40,916 genes (out of 46,558 total annotated genes) for which reads were successfully mapped to the double haploid 'Golden Delicious' GDDH13 genome version 1.1. In total, 6,967 genes in 'Fuji' and 3,426 genes in 'Gala' were differentially expressed between ON- and OFF-trees in any of the four selected sampling dates (**Figures 2A,B**). From those differentially expressed genes (DEGs), 1,057 were detected in both genotypes, while 5,910 DEGs were detected only in 'Fuji' and 2,369 DEGs only in 'Gala' (**Figure 3A**). The number of DEGs was not evenly distributed between the treatments and over the sampling dates. The majority of DEGs was observed 63 DAFB in 'Fuji' and 83 DAFB in 'Gala', corresponding to approximately 2 weeks prior to flower initiation in both cultivars (**Figures 2A,B**). At this developmental stage, a higher abundance of DEGs in spur buds from 'Fuji' OFF-trees was observed compared to 'Fuji' ON-trees, whereas the number of DEGs in 'Gala' was nearly the same in both treatments.

Proteomic profiling of 'Fuji' and 'Gala' spur buds resulted in a total number of 7,121 proteins detected by mass spectrometry. From them, 7,075 protein IDs could be linked to the corresponding genes identified in the transcriptomic analysis. Data quality filtering and statistical analyses of the proteomic profiles detected 316 DAPs in 'Fuji' and 512 DAPs in 'Gala' (**Figures 2C,D**). These DAPs were primarily found at 63 and 68 DAFB in 'Fuji', while in 'Gala' predominantly at 83 DAFB, a period, which corresponds to 1–2 weeks prior to flower initiation in both genotypes. From the total number of DAPs, 90 were common for both cultivars (**Figure 3B**). Comparison of transcriptomic and proteomic datasets showed that 141 DEGs in 'Fuji' and 78 DEGs in 'Gala' had corresponding DAPs (**Figures 3C,D**).

Principal component analysis (PCA) of transcriptomic (**Figure 4A**) and proteomic (**Figure 4B**) data with variances of two main components of 31.86 and 29.03, respectively, showed clear distinctions between the studied cultivars. In the transcriptomic data, we observed prominent differences between 'Fuji' ON- and OFF-trees 63 DAFB, whereas in the proteomic data there was a clear distinction between 'Gala' ON- and OFF-trees 83 DAFB. Both time points correspond to approximately two weeks prior to flower initiation in both cultivars, respectively.

DEGs and DAPs were mapped to KEGG pathways using gene list enrichment analysis with Kobas 3.0 (Xie et al., 2011). Enrichment analysis showed promoting (blue) and inhibiting (red) metabolic pathways for flower bud initiation in apple (**Figures 5A,B**), which were overrepresented in a given gene (or protein) list compared to the genome (or proteome) background information. Early flower bud development mechanisms in OFF-trees included metabolic pathways of carbon fixation, fatty acid biosynthesis, purine and pyrimidine metabolism, DNA replication, biosynthesis and metabolism of amino acids, steroid biosynthesis, amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism, flavonoid biosynthesis, and others presented in **Figure 5**.

In spur buds from ON-trees of both cultivars, plant hormone signal transduction pathway, plant-pathogen interaction pathway, and phenylpropanoid biosynthesis were overrepresented (**Figures 5A,B**). The plant hormone signal transduction pathway in 'Gala' ON included 7 DEGs regulating signaling mechanisms of auxins, cytokinins (CKs), gibberellins (GAs), abscisic acid (ABA), and jasmonic acid (JA), whereas in the same pathway in 'Fuji' ON, 46 DEGs controlling signal transduction of at least eight known phytohormone groups—auxins, CKs, JA, GAs, ABA, salicylic acid (SA), brassinosteroids (BS), and ethylene—were detected (**Supplementary Tables 1, 2**). Enriched pathways of DEGs and DAPs within OFF-trees had

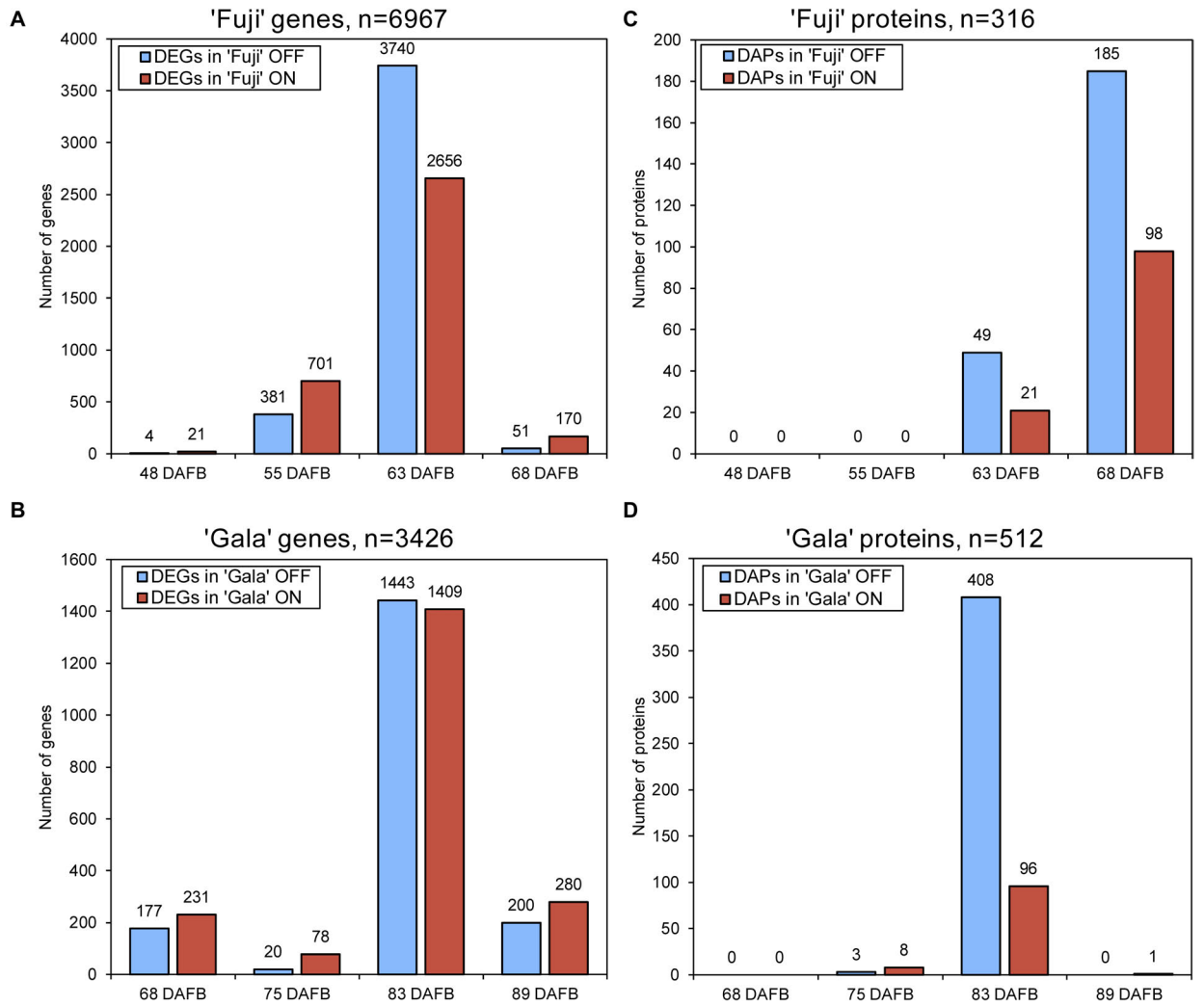

FIGURE 2 | The number of differently expressed genes (DEGs) **(A,B)** and differentially abundant proteins (DAPs) **(C,D)** in 'Fuji' **(A,C)** and 'Gala' **(B,D)** between ON- and OFF-trees over 4 weeks prior to flower initiation. Flower initiation in 'Fuji' occurred 75 DAFB and that in 'Gala' 97 DAFB. The sums of the bar plots are not equal to the numbers (*n*) shown above each plot as n indicates the total number of unique DEGs and DAPs.

partial overlaps. More distinct were enriched pathways of DEGs and DAPs in ON-trees. The cutin, suberin, and wax biosynthesis pathway was found to be enriched only in 'Fuji' ON and only in DEGs while there was no significantly enriched pathway based on DAPs in 'Fuji' ON-trees. In 'Gala', some metabolic pathways in ON-trees were detected, which were also found in OFF-trees of the same cultivar: carbon metabolism, ribosome and protein processing in endoplasmic reticulum, and pyrimidine metabolism.

Metabolic Profiling

Besides transcriptomics and proteomics analyses, we also performed metabolic profiling of apple spur buds to detect the final products of gene expression activity and analyzed the abundances of small molecules, which may serve as mobile signals to trigger or inhibit flower induction. Computation of metabolic data revealed 1,491 mass/charge signals (features) in the positive ionization mode and 796 features in the negative ionization mode. From those, 1,140 unique features had MS^1 isotope patterns and fragmentation spectra (MS^2 spectra) of sufficient quality. Based on their sum formulas and tentative assignment in ChemSpider, PubChem, and Plant Metabolic Network (PMN), databases we were able to obtain general information about the compound classes that could be detected in apple bud tissue. These included amino acids and dipeptides, plant hormone-like acting substances, polyphenols and their glucosides, vitamins, triterpenoids, fatty acids, and unknown compounds, which are not yet included in the chemical databases. By the automated analysis of accurate m/z ratios and MS^1-isotope patterns of the detected compounds, we obtained sum formulas for each of the metabolites. Based on the precise molecular mass search in the mentioned chemical databases

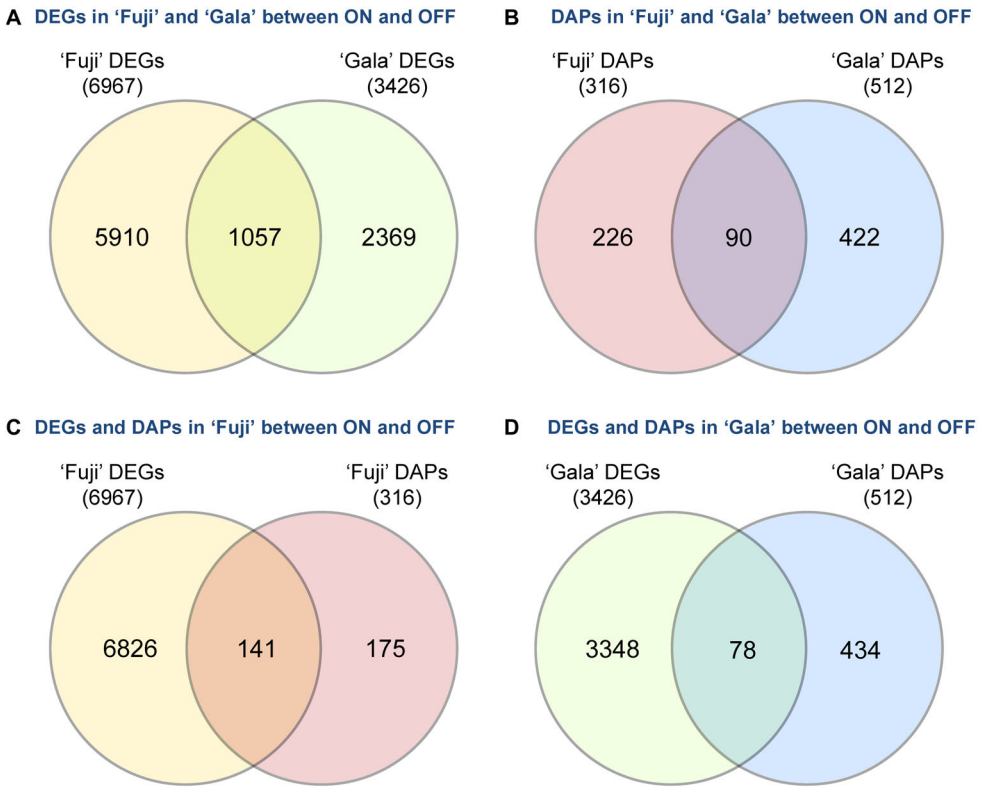

FIGURE 3 | Crop load (ON, OFF)-induced number of differently expressed genes (DEGs) and differentially abundant proteins (DAPs) between **(A,B)** and within **(C,D)** the cultivars 'Fuji' and 'Gala' during 1–4 weeks prior to flower initiation.

and comparison of fragmentation spectra with existing reference spectra in mzCloud, 159 features could be linked to potential compounds (pre-identification step). After manual inspection of MS^1 isotope patterns and fragmentation spectra in order to avoid false-positive identifications, the number of pre-identified compounds was further reduced to 111. They were characterized with robust MS^1 signals, fragmentation spectra of sufficient quality, and measurable peak areas, which were used for further downstream processing.

Statistical data analysis of pre-identified 111 compounds revealed 22 compounds, whose abundances differed significantly between the spur buds from ON- and OFF-trees of either one or both cultivars in at least one out of four sampling weeks (**Figure 6**). The metabolite identification process is considered to be completed only if the structures of all candidate substances are confirmed by reference compounds. From those 22 compounds, 6 were available for purchasing as reference substances (prolylleucine, thiamine, chlorogenic acid, arginine, tryptophan, and glutamic acid). These compounds were used for verification of the database search result by comparing MS^1 isotope patterns, retention times, and MS^2 spectra to the corresponding features obtained by metabolic profiling. As a result, five reference compounds fully confirmed the proposed structures of thiamine, chlorogenic acid, arginine, tryptophan, and glutamic acid, whereas prolylleucine was not confirmed.

Analysis of Candidates Across Omics Levels

For analysis downstream of the individual omics analysis, we focus on the fully confirmed compounds, which were already described as compounds potentially influencing (inhibiting or promoting) early flower bud development, and also genes and proteins involved in their biosynthesis and metabolism. These compounds include thiamine, chlorogenic acid, and tryptophan. Thiamine was more abundant in spur buds from OFF-trees in both apple cultivars. The compound's abundance in 'Fuji' ON and 'Gala' ON was at a similar level, whereas comparing spur buds from OFF-trees, it was slightly higher in 'Fuji'. KEGG library indicates that thiamine originates from thiamine phosphate and can be further metabolized to thiamine diphosphate and thiamine triphosphate by thiamine diphosphokinase (EC:2.7.6.2; EC—enzyme commission number) and adenylate kinase (EC:2.7.4.3), respectively. The majority of DEGs and DAPs, which are assigned to those reactions, were also higher expressed in OFF-trees at 63 DAFB ('Fuji') 83 DAFB ('Gala') corresponding to 2 weeks prior to flower initiation in both cultivars (**Figure 7**).

Chlorogenic acid is a product of coumaric acid metabolism, where enzymes belonging to shikimate O-hydroxycinnamoyltransferases (EC:2.3.1.133) catalyze multiple reactions. Among 24 genes assigned for these enzymatic reactions in KEGG, three DEGs and one DAP were identified

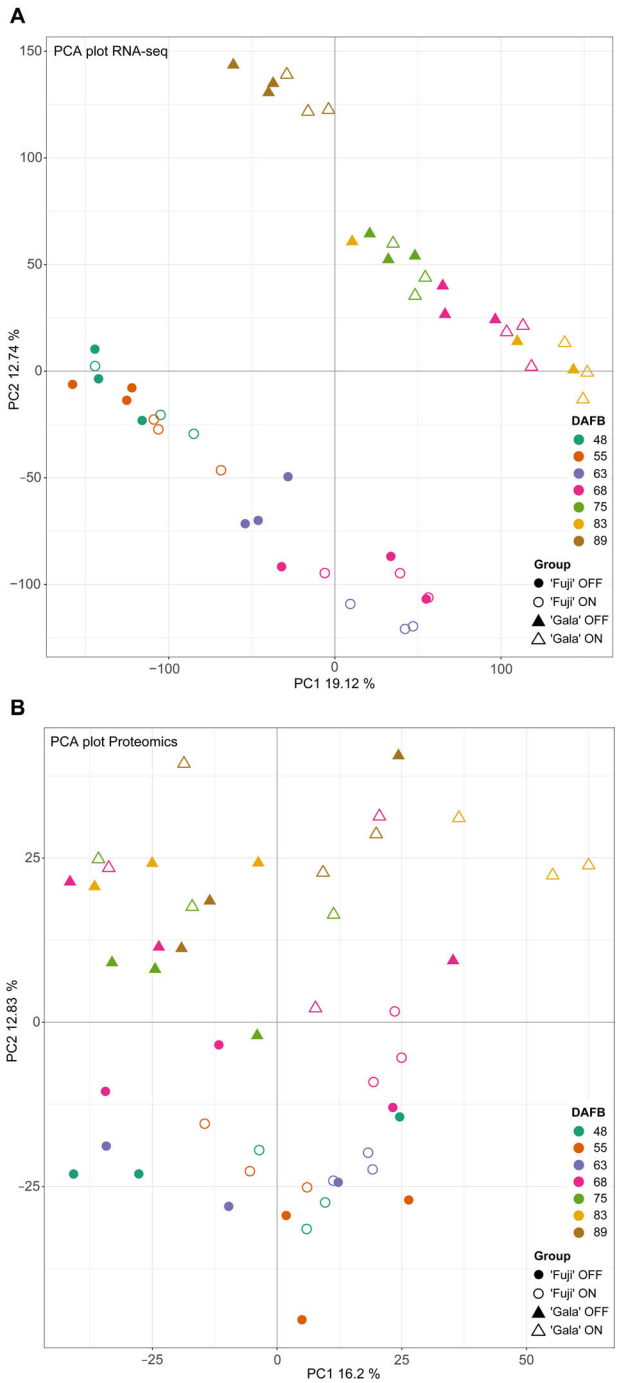

FIGURE 4 | PCA analysis of transcriptomic **(A)** and proteomic **(B)** data.

in our study (**Figure 8**). According to a gene annotation in the apple genome, all of them were classified as HXXXD-type acyl-transferase family protein and showed distinct, sometimes even inverse, expression patterns. MD13G1114800 showed up to 6.38-fold greater expression in ON-trees of both cultivars. No proteins corresponding to this gene were detected. Another gene, MD16G1108700, was more highly expressed in spur buds from OFF-trees in both apple cultivars with a significant effect in 'Fuji' OFF at 55 DAFB. Its protein product was characterized by significantly higher abundance in OFF-trees in both cultivars two weeks prior to flower initiation. Average relative abundance of chlorogenic acid was 2.0-fold higher in 'Fuji' OFF-trees and 2.7-fold higher in 'Gala' OFF-trees compared to ON-trees with prominent cultivar differences. In 'Gala' ON and OFF, this compound was 4.3- and 5.6-fold more highly abundant than in 'Fuji' ON and OFF, respectively.

Tryptophan in apple is synthesized by several reactions catalyzed by tryptophan synthase (EC:4.2.1.20), to which five genes were assigned using KEGG pathway analysis. However, none of them were differentially expressed between ON- and OFF-trees neither in 'Fuji' nor in 'Gala'. Tryptophan detected in apple spur buds was characterized by 2.3-fold higher abundance in 'Fuji' ON compared to 'Fuji' OFF and by 2.1-fold higher abundance in 'Gala' ON compared to 'Gala' OFF. Cultivar comparison demonstrated that the relative abundance of tryptophan was much higher in 'Fuji' than in 'Gala' differing within both ON and OFF treatments, by about an order of magnitude (**Figure 9**). Furthermore, tryptophan was shown to increase in concentration toward flower initiation in 'Fuji' while it was more fluctuating in 'Gala'.

To date, 66 genes are known to be involved in tryptophan metabolism in apple. Among them, 12 were found differentially expressed between ON- and OFF-trees in any cultivar, most of which were involved in indoleacetate biosynthesis. The expression patterns of those genes were not only cultivar specific but also treatment dependent. In particular, in 'Gala' OFF-trees, we detected DEGs for indoleacetate biosynthesis from indole-3-acetaldehyde (MD13G1090800) and indole pyruvate (MD00G1056000), whereas no genes showed significantly higher expression in 'Gala' ON-trees. In both 'Fuji' ON- and OFF-trees, we found DEGs coding aldehyde dehydrogenases (EC:1.2.1.3) converting indole-3-acetaldehyde into indoleacetate (MD15G1250400 in 'Fuji' OFF and MD06G1177200 in 'Fuji' ON). One gene coding indole-3-pyruvate monooxygenase (EC:1.14.13.168) that catalyzes reaction of indoleacetate biosynthesis from indole pyruvate (MD15G1184800) was more highly expressed in 'Fuji' OFF. Another gene of amidase enzyme (EC:3.5.1.4) leading to indoleacetate by catalyzing indole-3-acetamide was found more highly expressed in 'Fuji' ON (MD00G1192400). From the diversity of genes related to tryptophan metabolism, we could detect only one protein in the proteomic dataset (MD09G1128400). This protein was more highly abundant in 'Gala' OFF 83 DAFB.

DISCUSSION

Despite the fact that the apple genome has already been deciphered, even the newest high-quality *de novo* genome versions still cannot provide sufficient information about promotors and repressors of flowering and which specific metabolic pathways play a crucial regulatory role in early flower bud development. Many research groups attempted to identify genes that might induce flowering in higher plants; however,

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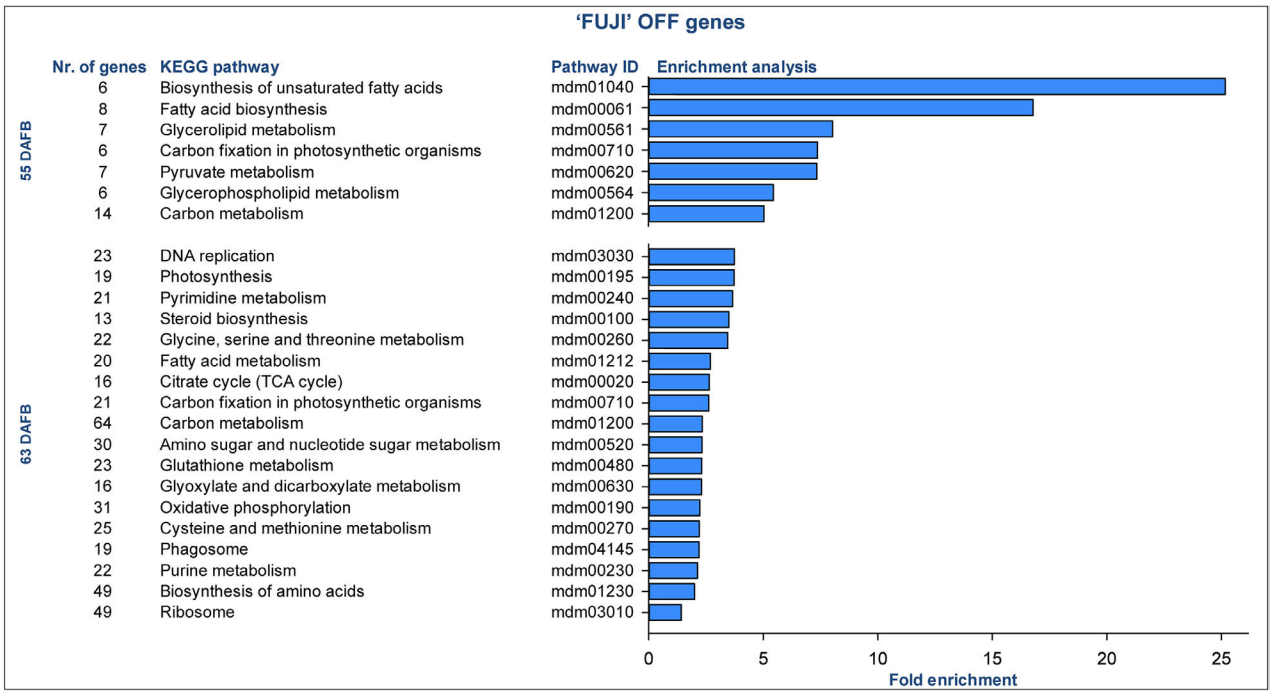

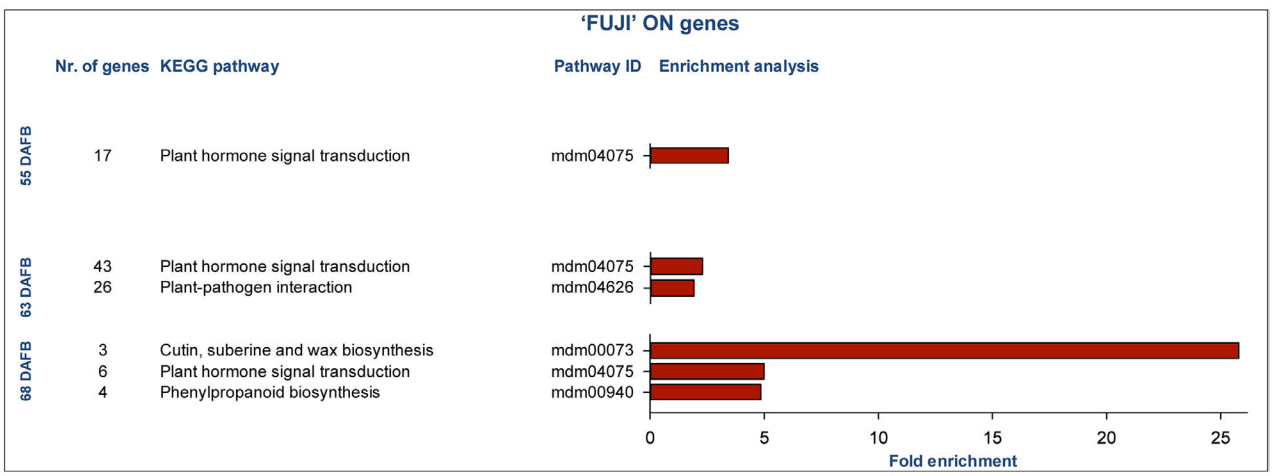

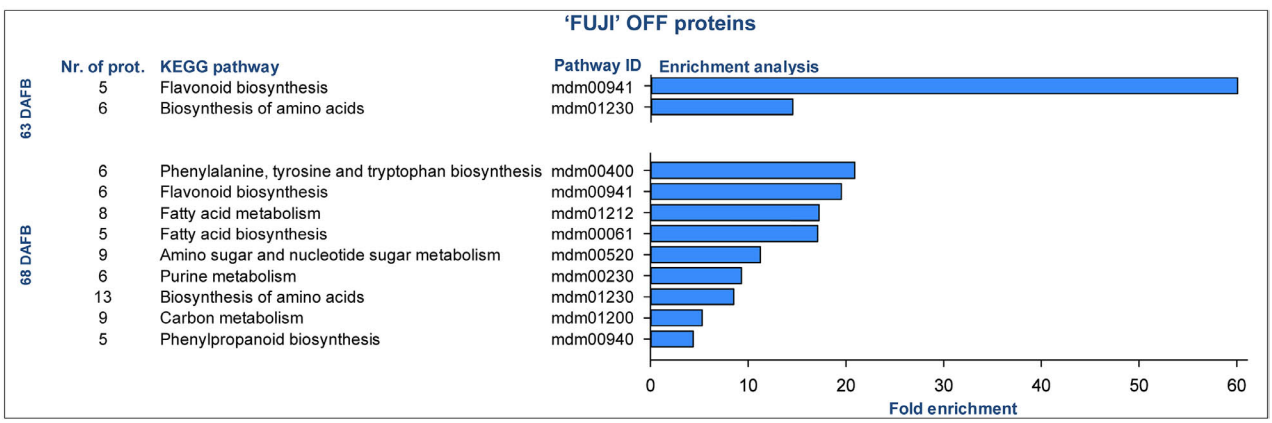

FIGURE 5 | Continued

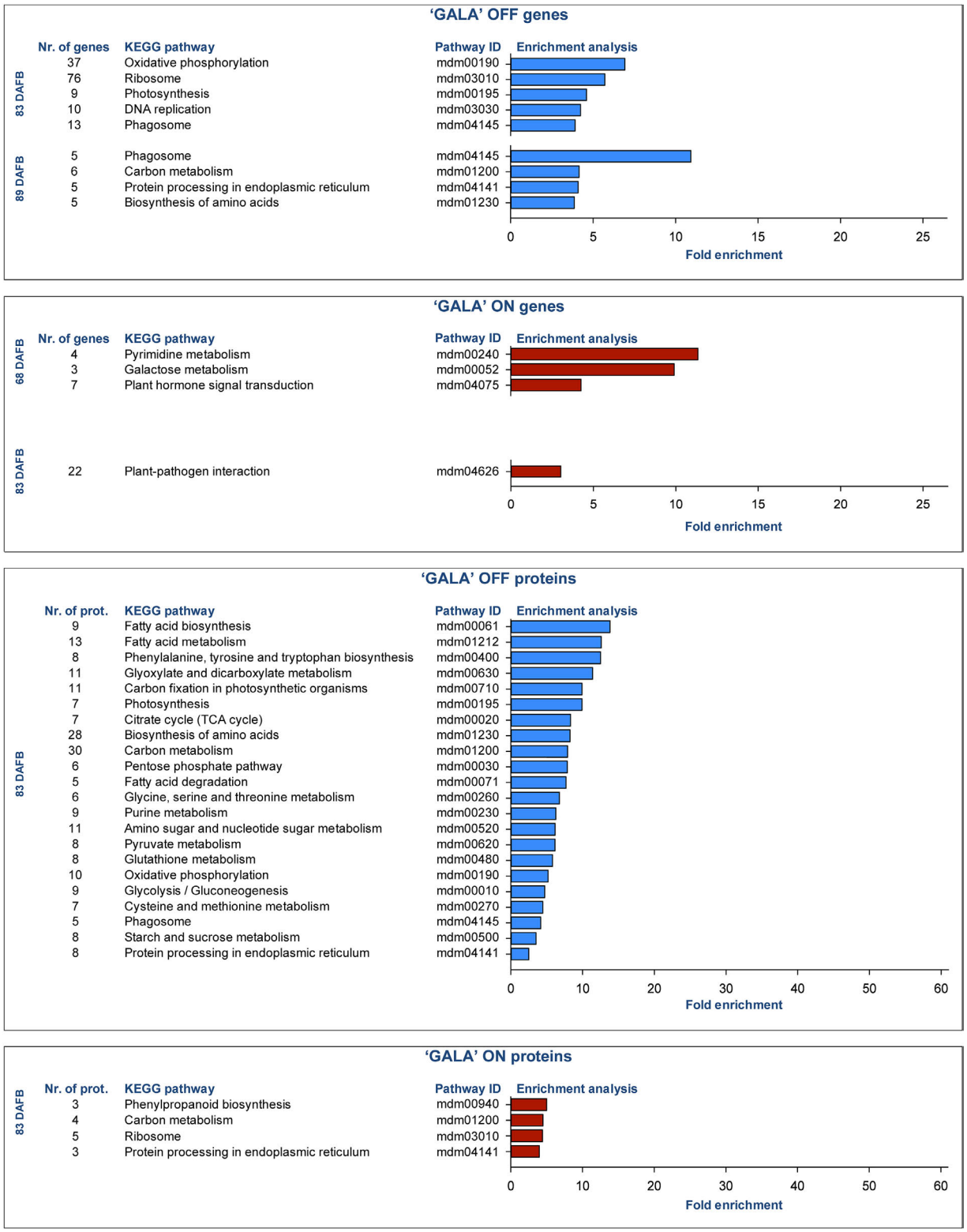

FIGURE 5 | Enrichment analysis of KEGG pathways of differently expressed genes (DEGs) and differentially abundant proteins (DAPs) between ON- and OFF-trees of 'Fuji' **(A)** and 'Gala' **(B)**. Fold enrichment was calculated by comparing the background frequency of total genes or proteins annotated to that term in *Malus* × *domestica* to the sample frequency, representing the number of genes or proteins entered that fall under the same term (Dalmer and Clugston, 2019). In 'Fuji' ON, no significantly enriched pathways of DAPs were found. Detailed information about pathway entries and statistics is provided in **Supplementary Tables 1–3**.

-4.95 0 3.55

Nr.	MS mode	Compound name (confirmed with reference compounds)	Potential candidate according to the chemical databases*	Molecular formula	Molecular weight, Da	RT, min	Compound abundance, log2FC difference between 'Fuji' ON and 'Fuji' OFF				Compound abundance, log2FC difference between 'Gala' ON and 'Gala' OFF			
							48 DAFB	55 DAFB	63 DAFB	68 DAFB	68 DAFB	75 DAFB	83 DAFB	89 DAFB
1	Neg		1-O-(4-coumaroyl)-beta-D-glucose	$C_{15}H_{18}O_8$	326.09986	8.389	2.14	2.21	3.06	3.55	1.30		2.02	1.92
2	Pos		Glucosylgalactosyl hydroxylysine	$C_{18}H_{34}N_2O_{13}$	486.20669	12.762	1.39			1.50	2.23	1.93		1.65
3	Pos	Tryptophan	Tryptophan (CONFIRMED)	$C_{11}H_{12}N_2O_2$	204.08988	3.896				1.90	1.28		1.97	
4	Pos	L-Glutamic acid	L-Glutamic acid (CONFIRMED)	$C_5H_9NO_4$	147.05274	0.934						0.62		0.66
5	Pos		Kaempferol + 5-Sugar	$C_{15}H_{10}O_6 + C_5H_{10}O_5$	418.08923	18.304			0.70					
6	Neg		Kaempferol 3-rhamnoside	$C_{21}H_{20}O_{10}$	432.10533	18.83					-0.46	-0.46	-0.49	
7	Pos		3-trans-p-Coumaroylrotundic acid	$C_{39}H_{54}O_7$	634.38552	33.049							1.13	
8	Pos		Phloridzin + 5-Sugar	$C_{21}H_{24}O_{10} + C_5H_{10}O_5$	568.17754	16.527	-0.73	-1.07		-0.65	-0.39	-0.58		
9	Pos		Astilbin	$C_{21}H_{22}O_{11}$	450.11506	15.91	-1.39			-1.68	-0.43			
10	Pos		Phloridzin + 6-Sugar	$C_{21}H_{24}O_{10} + C_6H_{12}O_6$	598.18843	15.45	-1.13	-1.36		-0.97	-0.78	-1.18		
11	Pos	DL-Arginine	DL-Arginine (CONFIRMED)	$C_6H_{14}N_4O_2$	174.11124	0.772	-1.98	-3.34						
12	Pos	Chlorogenic acid	Chlorogenic acid (CONFIRMED)	$C_{16}H_{18}O_9$	354.09412	9.31				-2.28	-1.18	-2.05	-2.14	
13	Pos		Sieboldin	$C_{21}H_{24}O_{11}$	452.13086	15.91	-1.66	-1.31		-2.31	-0.56			
14	Pos		L-1-glycero-phosphorylcholine	C8H20NO6P	257.10192	0.973	-1.55	-1.34		-1.62				
15	Pos	Thiamine	Thiamine (CONFIRMED)	$C_{12}H_{17}N_4OS$	264.10388	0.877	-2.02		-1.39				-1.31	
16	Pos		Argininosuccinic acid	$C_{10}H_{18}N_4O_6$	290.12166	0.91	-2.40	-3.86						
17	Pos		Prolylleucine (REJECTED)	$C_{11}H_{20}N_2O_3$	228.14657	1.022	-2.20		-2.55	-2.05	-1.82	-1.65	-1.85	-1.54
18	Neg		Glycerophosphoglycerol	$C_6H_{15}O_8P$	246.05001	1.904						-1.53	-1.30	-1.17
19	Pos		Glycerophosphoinositol	$C_9H_{19}O_{11}P$	334.06573	1.749	-2.36			-2.28		-1.75	-0.99	-1.41
20	Pos		N-acetyl-L-2-aminoadipic acid	$C_8H_{13}NO_5$	203.07899	1.272	-3.42				-1.50	-2.66	-2.04	
21	Pos		Allysine	$C_6H_{11}NO_3$	145.07347	0.944	-3.75		-2.82		-1.95	-1.99	-1.72	-1.65
22	Pos		1-Methyladenine	$C_6H_7N_5$	149.06976	0.833	-4.95	-3.08	-2.83	-3.73		-3.23	-2.59	

FIGURE 6 | Differentially abundant metabolites (DAMs) in spur buds from ON- and OFF-trees of 'Fuji' and 'Gala'. *Pre-identified metabolites according to mzCloud, ChemSpider, Plant Metabolic Network (PMN), and PubChem. "CONFIRMED" indicates identified organic molecules confirmed with reference compounds, "REJECTED" indicates structure and/or retention time is not confirmed using reference compounds. Negative log2FC values (blue) indicate a higher compound abundance in OFF, positive log2FC values (red) indicate a higher compound abundance in ON. Cells of the same color on the heat map indicate the same level of log2FC metabolite abundance difference between ON- and OFF-trees and do not indicate significant differences. Numbers on the heat map specify exact log2FC differences between ON- and OFF-trees and mark significant differences at multiple adjusted $p \leq 0.05$.

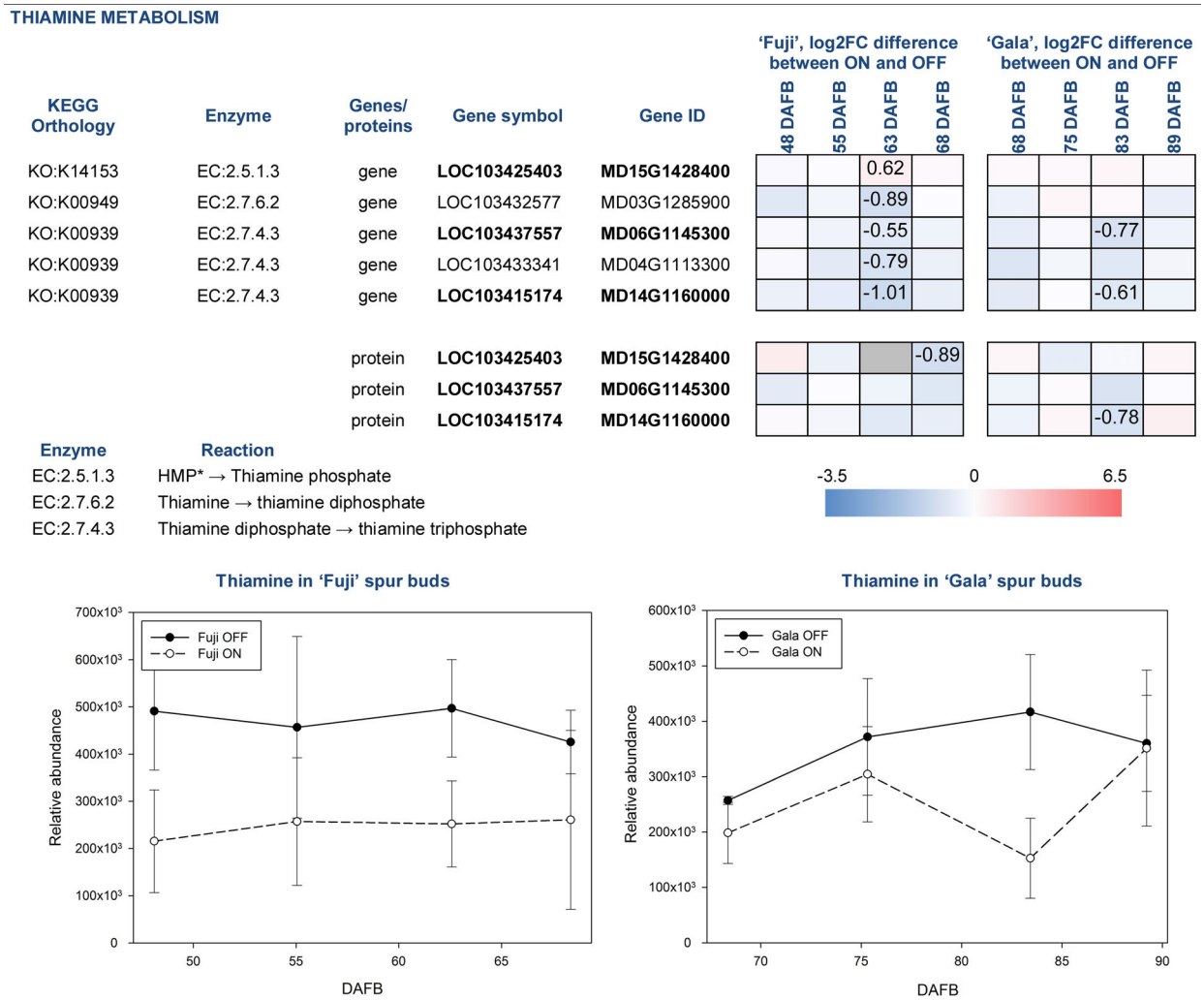

FIGURE 7 | Relative abundance of thiamine in spur buds from 'Fuji' and 'Gala' with differently expressed genes (DEGs) between ON- and OFF-trees and corresponding proteins, which are assigned to the enzymatic reactions metabolizing the compound. *HMP—4-amino-5-hydroxymethyl-2-methylpyrimidine diphosphate. Information about the reactions and genes assigned to thiamine metabolism was provided from KEGG library. Bold font of gene and protein IDs indicates a link between a gene and a corresponding protein illustrated in the figure. Negative log2FC values (blue) indicate a higher gene expression or higher protein abundance in OFF, positive log2FC values (red)—in ON. Cells of the same color on the heat map indicate the same level of log2FC gene (protein) expression difference between ON- and OFF-trees and do not indicate significant differences. Numbers on the heat map specify exact log2FC differences between ON- and OFF-trees and mark significant differences at multiple adjusted $p \leq 0.05$. Gray cells indicate no statistics possible due to "fill gap" function switched off (see Materials and Methods).

the majority of them used Arabidopsis as a model plant, whose ability to flower strongly depends on day length (Seo et al., 2011; Blümel et al., 2015). Nevertheless, there is evidence that some homologs of the flowering-regulating genes discovered in Arabidopsis also play a role in flower bud development in apple (Guitton et al., 2016). Transcriptomic analysis revealed numerous DEGs between ON- and OFF-trees two weeks prior to flower bud initiation and thereby showed that candidate genes for flower induction published in the literature is a small fraction of all transcripts differentially expressed between ON- and OFF-trees, which we could detect in this study. Gene and protein enrichment analyses illustrated metabolic pathway differences between spur buds collected from ON- and OFF-trees. In OFF-trees, we detected several enriched metabolic processes, which are assumed to lead to early flower bud development and meristem differentiation in apple. Among those were carbon fixation during cell photosynthetic activity and fatty acid biosynthesis, where enzymes from pyruvate and biotin metabolism pathways are involved (Rawsthorne, 2002). Fatty acids are then metabolized into different forms including glycerophospholipids, which are used to build cell membranes (Harwood, 2005). The metabolic profile of apple spur buds confirmed that fatty-acid-structured compounds were more abundant in OFF-trees (compounds 14, 18, and 19 in

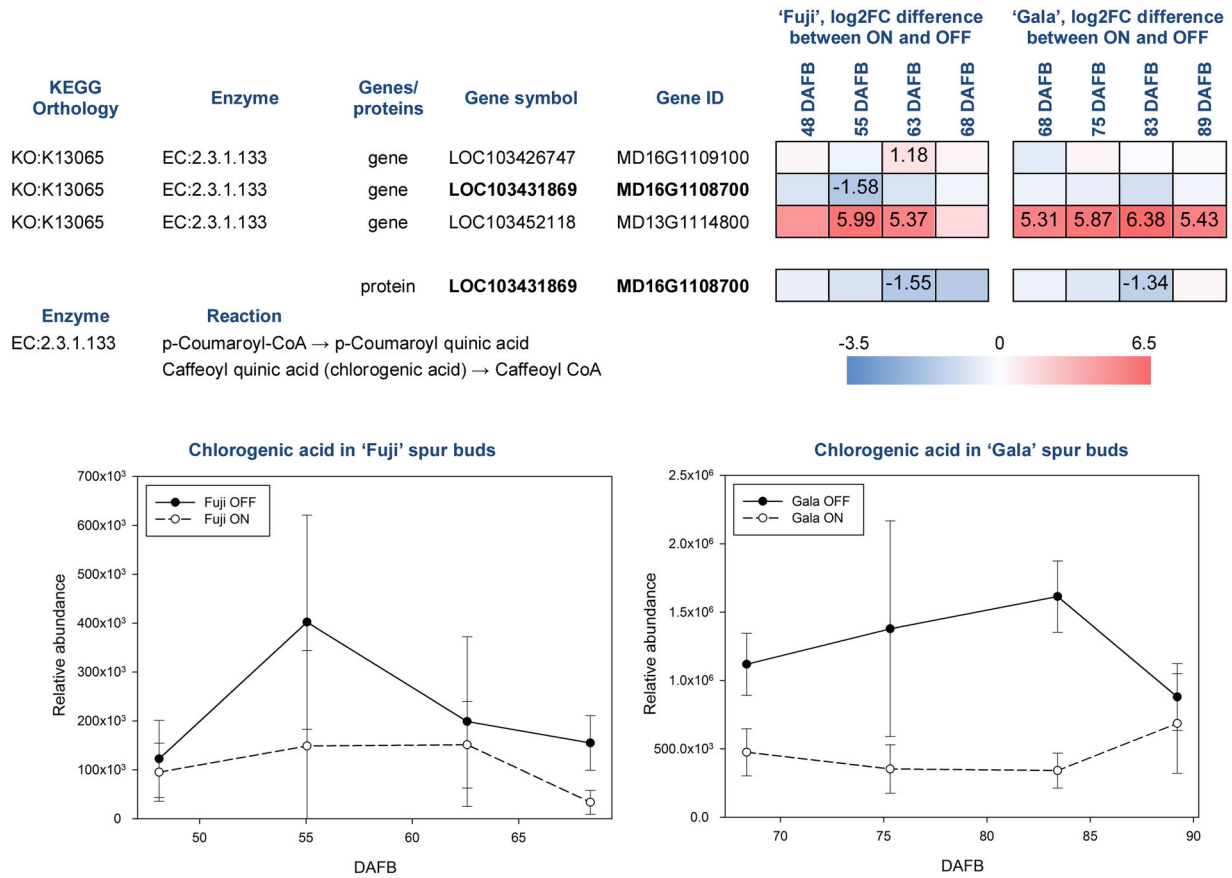

FIGURE 8 | Relative abundance of chlorogenic acid in spur buds from 'Fuji' and 'Gala' with differently expressed genes (DEGs) between ON- and OFF-trees and corresponding proteins, which are assigned to the enzymatic reactions metabolizing the compound. For detailed figure explanation see **Figure 7**.

Figure 6). Besides that, in spur buds from OFF-trees we detected physiological pathways of amino acid biosynthesis and metabolism, metabolism of purine and pyrimidine, which serve as DNA and RNA constituents, DNA replication, starch and sucrose metabolism, and flavonoid biosynthesis.

Amino acid arginine, which was identified in the metabolic profiles of both apple cultivars, was significantly higher abundant in 'Fuji' OFF at 48 and 55 DAFB in comparison to 'Fuji' ON (**Figure 6**). Besides being a protein constituent, arginine serves as a nitrogen storage in plants and enables fine-tuning of the production of nitric oxide, polyamines, and potentially proline (Winter et al., 2015). Another detected amino acid, glutamic acid, showed higher abundance in 'Gala' ON at 75 and 89 DAFB. This compound is a precursor for the biosynthesis of arginine and proline, plays an essential role in nitrogen metabolism and can be synthesized by a number of physiological pathways (Forde and Lea, 2007). Moreover, glutamic acid was shown to be involved in plant stress responses and was associated with stress-triggered signal transduction (Qiu et al., 2020). However, there is so far no evidence on the involvement of arginine or glutamic acid in flower bud development. Amino acids phenylalanine, tyrosine, and tryptophan are immediate precursors for phenylpropanoid biosynthesis pathway that includes biosynthesis of flavonoids and chlorogenic acid in particular. Metabolic profiling of 'Fuji' and 'Gala' spur buds demonstrated that chlorogenic acid showed higher abundance in spur buds collected from OFF-trees. Cultivar comparisons within the same treatments showed several-fold higher abundance of chlorogenic acid in non-biennial cultivar 'Gala'. In previous studies, chlorogenic acid was proven to inhibit IAA oxidase and therefore to protect auxin from its inactivation (Pilet, 1964). Furthermore, Lavee et al. (1986) reported that chlorogenic acid had auxin-like activity, affecting the growth of olive shoot apices cultivated *in vitro*. These findings suggest that chlorogenic acid might be involved in meristem development of a young bud. However, the mode of action of this compound in apple spur bud meristem remains unclear. The transcriptome and proteome of apple buds revealed the activity of enzymatic reaction EC:2.3.1.133 that metabolizes at least 3 derivatives of p-coumaric acid including p-coumaroyl-CoA and caffeoyl shikimic acid, which serve as chlorogenic acid precursors. The gene MD16G1108700 and the corresponding protein assigned to this reaction were detected in apple spur

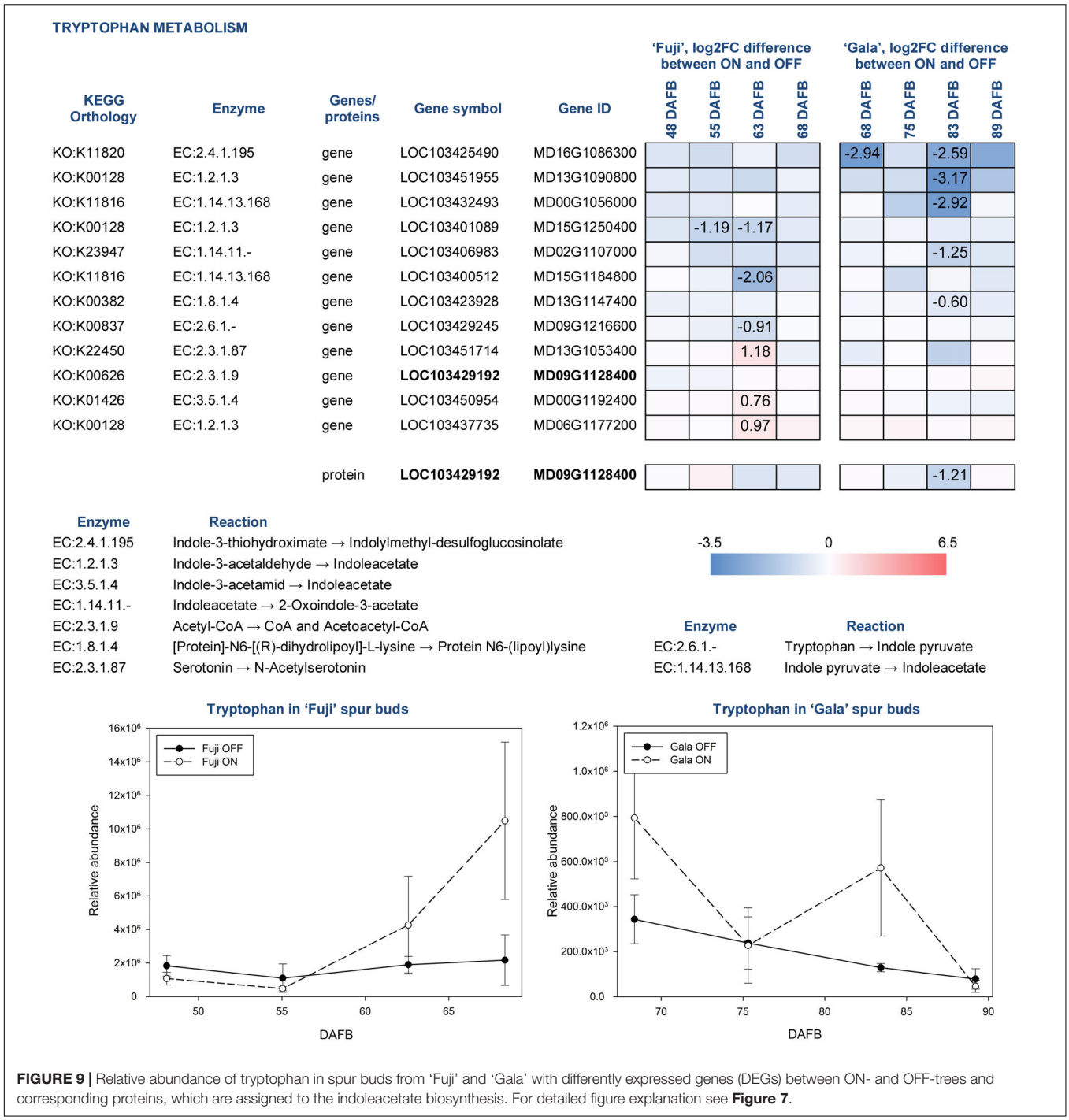

FIGURE 9 | Relative abundance of tryptophan in spur buds from 'Fuji' and 'Gala' with differently expressed genes (DEGs) between ON- and OFF-trees and corresponding proteins, which are assigned to the indoleacetate biosynthesis. For detailed figure explanation see **Figure 7**.

buds and were both more highly expressed in OFF-trees, whereas the MD13G1114800 gene showed significantly (up to 6.38-fold) higher expression in ON-trees with no detectable protein-product. Exploring the metabolic profile of 'Fuji' and 'Gala', we revealed that one compound from the metabolic dataset with m = 326.09986 and RT = 8.389 (compound 1 in **Figure 6**) showed similarity with a fragmentation spectrum of p-coumaric acid (peaks at 93.03, 119.05, and 163.04 m z^{-1}), suggesting that this compound and chlorogenic acid, having p-coumaric acid as a structural base, may belong to the same metabolic pathway. The unknown compound showed up to 3.55-fold higher abundance in spur buds from ON-trees compared to OFF-trees; however, no direct connection of the biosynthesis of this compound with a high expression of the MD13G1114800 gene could be established. None of DEGs and DAPs related to the reaction EC:1.14.14.96, which converts p-coumaroylquinic acid (a product of EC:2.3.1.133) to chlorogenic acid, were found in our datasets.

Another compound that was more highly abundant in spur buds from OFF-trees of both cultivars was thiamine. This vitamin is frequently used in plant tissue culture and is proven to positively affect cell growth and development. It was reported that in *Plumbago indica*, *in vitro* bud formation from callus could be gained by adding the mixture of glycine, *myo*-inositol, nicotinic acid, thiamine, folic acid, and biotin routinely to culture media (Nitsch and Nitsch, 1967). In the multi-omics study, the thiamine biosynthesis pathway could also be detected at all three omics-levels of 'Fuji' and 'Gala' showing a higher abundance in spur buds from OFF-trees that supports previous findings. Though it was not possible to conclude whether thiamine directly contributes the flower induction by influencing the bud meristem formation, it is known that thiamine diphosphate plays a role as an enzymatic cofactor in universal metabolic pathways including glycolysis and the pentose phosphate pathway. Moreover, it has the same function in mitochondrial and chloroplastic pyruvate dehydrogenases. The latter provides acetyl-coenzyme A and NADH for biosynthesis of fatty acids (Goyer, 2010). The KEGG library indicates that in apple, thiamine is formed by cleaving of a phosphate group from thiamine phosphate, where enzymes classified as EC:3.1.3.100 are involved. Our analyses showed no DEGs or DAPs related to this enzyme group. The gene MD15G1428400 assigned to EC:2.5.1.3, thiamine phosphate synthase, was more highly expressed in 'Fuji' ON at 63 DAFB, whereas the other 4 genes assigned to EC:2.7.6.2 and EC:2.7.4.3 converting thiamine into thiamine diphosphate and latter into thiamine triphosphate, respectively, were more highly expressed in OFF-trees.

Among the pre-identified compounds with higher abundance in OFF-trees and thus potentially involved in early flower bud development, one compound showed a fragmentation spectrum similar to 1-methyladenine (peaks at 150.08, 133.05, 94.04, 82.04, and 55.03 m z^{-1}, from which the last three are typical for adenine). It was characterized by up to 4.95-fold higher relative abundance in OFF-trees compared to ON-trees throughout all four sampling weeks in both apple cultivars. Notwithstanding all the efforts to classify the compound, we could not verify the proposed structure because of many differences in the fragmentation spectrum between the potential candidate and the reference spectrum of 1-methyladenine. It is well known that adenine serves as a base for the wide range of naturally occurring cytokinins (Kieber and Schaller, 2014). This fact raises an assumption that the unknown compound may represent a fragment, a precursor, or a product of metabolism of one of them.

The metabolic profile of 'Fuji' and 'Gala' spur buds suggests that the majority of pre-identified flavonoids were more abundant in OFF-trees (compounds 6, 8, 9, 10, 13 in **Figure 6**). This is in conformity with transcriptomic and proteomic profiles of spur buds collected from those cultivars, where the flavonoid biosynthesis pathway was significantly enriched in OFF-trees. The precise molecular masses and the fragmentation spectra of polyphenolic compounds indicated that they belong to the phloridzin and kaempferol aglycone type of flavonoids (compounds 5, 8, and 10 in **Figure 6**) with additional C5- and C6-sugar moieties attached to the aglycones. However, the precise structure of these phloridzin- and kaempferol-based flavonoids could not be determined. Scientific literature provided no evidence that flavonoids may have a direct influence on bud meristem development. However, the findings of Brown et al. (2001) and Peer et al. (2004) suggest that flavonoids play a role as negative auxin transport regulators, which would be in line with the higher abundance of flavonoids observed in OFF-trees.

In the metabolic dataset, tryptophan appeared in a cluster of small molecules, which were characterized by significantly higher abundance in spur buds from ON-trees. Moreover, cultivar comparison showed that tryptophan had 10-fold higher abundance in 'Fuji' spur buds compared to 'Gala'. These differences make this compound and the metabolic pathways, in which tryptophan is involved, interesting for further studies. Since no genes assigned to tryptophan biosynthesis differed significantly in their expression between ON- and OFF-trees, it could be assumed that the accumulation of this compound in spur buds collected from ON-trees may be the result of suspended tryptophan conversion to other primary or secondary metabolites. Transcriptomic analysis showed that the majority of DEGs from the tryptophan metabolism pathway were assigned to indoleacetate biosynthesis, an active auxin form in plants. However, none of the known auxin forms could be detected in apple buds by the applied analytical method.

Transcriptomic and proteomic analyses indicated an involvement of plant hormone signal transduction pathways in bud meristem fate. Based on this information, in our metabolic approach, we used reversed-phase (RP) chromatography which is known to be suitable to analyze hydrophobic compounds such as phytohormones (Pan et al., 2010). However, none of the known plant hormones could be successfully detected using the non-targeted metabolomics, most likely due to the low abundance of these compounds in apple bud tissue compared to other metabolites. Therefore, a further step toward a systematic understanding of flower induction in apple would be the determination of plant hormone profiles by targeted mass spectrometry analyses using extraction protocols specifically designed for the analyses of phytohormones (Farrow and Emery, 2012; Urbanová et al., 2013).

NGS and proteome analysis revealed several sugar metabolism and interconversion pathways that are actively ongoing in spur buds, which develop flower meristem, indicating that particular sugar forms might play an important role in flower bud induction and initiation. However, the majority of MS signals obtained from different sugar forms found in metabolic profiles of apple spur buds could not be interpreted because the clear separation of sugar molecules with the same molecular mass using reversed-phase chromatography could not be achieved. In order to enlarge the knowledge about the involvement of simple sugar forms, such as mono-, di-, and trisaccharides in flower induction in apple, sugar analysis in apple buds is also essential. Once performed, it would help to link the activity of genes and corresponding proteins to specific sugar compounds.

The picture of flower bud induction mechanisms in apple is far from complete. In the recent years, the understanding of

plant flower organ formation mechanisms has strongly advanced; however, it is still unclear what the initial trigger for floral meristem formation is and how the fruit may inhibit flower development in the adjacent spur buds. The second question may be answered by determining which metabolites that showed higher abundance in spur buds from ON-trees are originated from the fruit. Once determined, it would narrow down the search of candidate genes, which are affected by the unknown mobile signal. In the apple metabolome, from 1,140 features with robust signals detected by non-targeted metabolomics, only 111 could be annotated with any potential compound from chemical databases, indicating that only 10% of compounds found in apple spur buds could be claimed as "knowns." Besides the 22 compounds presented in the current work, nearly 70 hitherto unknown metabolites were found to be differentially abundant between spur buds from ON- and OFF-trees on a time-series scale.

In summary, the multi-omics approach applied for the identification of flower induction signaling molecules in apple allowed observing complex plant metabolic processes and tracing some pathways from gene to product level. The data suggests that thiamine, chlorogenic acid, and an adenine derivative play a role in metabolic pathways promoting early flower bud development in apple. Tryptophan was found to be more abundant in spur buds collected from high-cropping (ON) trees compared to non-cropping OFF-trees. Cultivar comparison (biennial cultivar 'Fuji' vs. non-biennial cultivar 'Gala') revealed 4.3–5.6-fold higher abundance of chlorogenic acid in 'Gala' spur buds, whereas tryptophan was 10-fold higher abundant in spur buds collected from 'Fuji'. Genes controlling tryptophan biosynthesis were not affected by ON- and OFF-treatments; however, genes regulating tryptophan metabolism to indoleacetate showed significant expression differences between treatments and cultivars. At transcriptomic and proteomic levels, in apple spur buds collected from OFF-trees, metabolic pathways associated with tissue growth and development were detected that potentially result in a promoting effect on early flower bud development. In contrast, in spur buds from ON-trees, the plant hormone signal transduction pathway was enriched, suggesting the involvement of hormonal metabolites in determining the fate of the apple bud meristem.

DATA AVAILABILITY STATEMENT

The proteomic data are available on Proteomics Identifications Database (PRIDE) under the ID PXD021716. The RNAseq data are accessible on ArrayExpress under the ID E-MTAB-9644.

AUTHOR CONTRIBUTIONS

AM and JK carried out the experiment. AM performed the proteomics and metabolomics data analysis with support of SC, IK, JP, JK, and DS and wrote the manuscript with support of JW, HF, and SC. IK and JP conducted MS analysis of proteins and metabolites. SC performed RNA sequencing and RNA-Seq downstream analysis. JW, HF, and MH supervised the project. All authors provided critical feedback and helped to shape the research, analyses, and the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.604810/full#supplementary-material

Supplementary Table 1 | Fuji gene enrichment analysis.

Supplementary Table 2 | Gala gene enrichment analysis.

Supplementary Table 3 | Fuji and Gala protein enrichment analysis.

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Assessing Dynamic Changes of Taste-Related Primary Metabolism During Ripening of Durian Pulp Using Metabolomic and Transcriptomic Analyses

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Durian is an economically important fruit of Southeast Asia. There is, however, a lack of in-depth information on the alteration of its metabolic networks during ripening. Here, we annotated 94 ripening-associated metabolites from the pulp of durian cv. Monthong fruit at unripe and ripe stages, using capillary electrophoresis- and gas chromatography-time-of-flight mass spectrometry, specifically focusing on taste-related metabolites. During ripening, sucrose content increased. Change in raffinose-family oligosaccharides are reported herein for the first time. The malate and succinate contents increased, while those of citrate, an abundant organic acid, were unchanged. Notably, most amino acids increased, including isoleucine, leucine, and valine, whereas aspartate decreased, and glutamate was unchanged. Furthermore, transcriptomic analysis was performed to analyze the dynamic changes in sugar metabolism, glycolysis, TCA cycle, and amino acid pathways to identify key candidate genes. Taken together, our results elucidate the fundamental taste-related metabolism of durian, which can be exploited to develop durian metabolic and genetic markers in the future.

Keywords: ***Durio zibethinus*, metabolome analysis, ripening-associated metabolites, taste precursors, transcriptome analysis**

INTRODUCTION

Durian (*Durio zibethinus* L.) is a highly economically valuable fruit endemic to Southeast Asia, which has recently begun to be distributed globally. Owing to the increasing demand for durian, the price and production of the fruit tend to increase every year. Durian is well known for its outstanding flavor, described as an overpowering sweet taste, with a sweet, fruity odor resulting from high contents of starch, sugar, and saturated fatty acids in the ripe pulp (Charoenkiatkul et al., 2018). More interestingly, ripe durian pulp contains various bioactive compounds, including

carotenoids, flavonoids, and polyphenols, which confer substantial antioxidant properties (Aziz and Jalil, 2019), suggesting that consumption of durian may have potential health benefits. The ripening process contributes to the organoleptic properties of durian pulp.

Fruit ripening is a complex and coordinated developmental process associated with pronounced molecular and biochemical changes. The climacteric fruits, including durians, are usually harvested at the commercial maturity stage. Afterward, the fruits undergo a postharvest ripening process, during which the rates of respiration and ethylene production increase dramatically. During this process, stored carbohydrates are broken down to sugars, and acidity is reduced alongside the increase in taste and aroma volatiles (Gao et al., 2020). Moreover, modifications of amino acids and organic acids are linked with fruit aroma by acting as precursors for the biosynthesis of aroma-forming volatile compounds (Tieman et al., 2006). Taken together, these modifications are the key contributors to fruit flavor.

Over the past decade, the advent of "omics" approaches has significantly contributed to identifying functional metabolites in primary and secondary plant metabolism during fruit development. Metabolomics has been successfully used to identify primary metabolites, including sugars, organic acids, amino acids, and other related compounds, and to provide an understanding of the whole landscape of metabolic alteration during the development and ripening of fruits, such as peach, tomato, strawberry, and grape (Lombardo et al., 2011). Moreover, metabolomics, coupled with transcriptomics, provides information on major metabolic networks and candidate genes controlling the underlying processes; integration of these data with genomics provides new insights into the major metabolic variations and their genetic and biochemical control during development. Such studies have widely been performed on tomato, a popular model fruit, and documented how breeding and genetic selection globally altered tomato fruit metabolite content (Zhu et al., 2018).

Metabolic profiles of ripe durian pulp have been investigated using capillary electrophoresis-time of flight/mass spectrometry (CE-TOF/MS) (Pinsorn et al., 2018). Cultivar-dependent metabolites associated with the sensory traits of durian fruit pulp, such as its odor-related (cysteine and leucine) and ripening-associated (aminocyclopropane carboxylate) metabolites, are of utmost importance. In 2017, the draft genome of *Durian zibethinus* was first published, and genome data integrated with transcriptome data identified methionine-γ-lyase as a key gene involved in controlling sulfur volatile compound production in durian pulp (Teh et al., 2017). This result was consistent with previously identified odor-active compounds in durian pulp, such as methanethiol (Li et al., 2012). Furthermore, volatile-aroma esters, such as ethyl (2S)-2-methylbutanoate and ethyl butanoate, were isolated in the study, but their related pathways have not been studied in durian. The genomic data led to further studies to better understand the durian ripening process. Subsequently, a genome-wide analysis of the Dof (DNA binding with one finger) transcription factor family identified 24 Dofs (*DzDofs*), among which 15 were expressed in the fruit pulp. Functional characterization of *DzDof2.2* suggested that it exerts its effects on fruit ripening by regulating auxin biosynthesis and auxin-ethylene crosstalk (Khaksar et al., 2019). In addition, a member of the auxin response factor (ARF) transcription factor family was identified, showing that DzARF2A mediates durian fruit ripening through transcriptional regulation of ethylene biosynthesis genes (Khaksar and Sirikantaramas, 2020). In this regard, omics analyses can be powerful tools to improve our limited understanding of the mechanisms underlying the durian postharvest ripening process, especially the changes in primary metabolites. In this study, we performed metabolomics, including capillary electrophoresis (CE) and gas chromatography (GC) coupled with time-of-flight mass spectrometry (TOF/MS), and transcriptomics to elucidate the post-harvest ripening-associated metabolic processes of the Monthong cultivar, the most widely cultivated durian in Thailand. Our findings provide comprehensive information on metabolic shifts during these stages. The identification of ripening-associated metabolites, which contribute to its unique flavors, and key candidate genes may be further exploited in durian breeding programs to develop cultivars with altered sensory characteristics or enhanced nutritional value.

MATERIALS AND METHODS

Plant Materials and Sample Preparation

Durian cv. Monthong fruits were harvested with at least three replicates of each stage from an orchard in the Trat province of Thailand in 2016. The three stages of the durian fruit used in this study are as follows: unripe, midripe, and ripe. For the unripe stage, the fruit was harvested at the commercially mature stage of 105 days after anthesis. The fruit at midripe and ripe stages were harvested at the commercially mature stage and kept at room temperature (28 °C) for postharvest ripening until reaching a firmness of 3.4 ± 0.81 N (∼3 days after harvest) and 1.55 ± 0.45 N (∼5 days after harvest), respectively (Khaksar et al., 2019). At each stage, durian pulp was collected from the central seed of each locule, immediately frozen in liquid nitrogen, and ground into powder. The powder was either freeze-dried for CE-TOF/MS, GS-TOF/MS, and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analyses or stored at −80°C for RNA extraction. Another seed from a different locule of the same fruit was used to analyze firmness, using a texture analyzer, to ensure that samples of the same stage from different cultivars were under identical conditions.

Determination of Total Soluble Solids (TSS) and Titratable Acidity

Two sets of a freeze-dried sample (20 mg, at least three replicates) were homogenized in 200 μL of distilled water. The homogenate was centrifuged at 14,000 × *g* at 18 C for 20 min. The supernatant from the first set was used to measure total soluble solids (TSS) using a digital refractometer (Hanna Instruments, Woonsocket, RI, United States). The other set of the supernatants was used to quantify titratable acidity modified from Tan et al. (2019). Briefly, the supernatant was titrated against 0.1 M NaOH mixed

with 2 μL of phenolphthalein until the solution turned pink. Titratable acidity was calculated using 1 mg malic acid/100 mg dried sample, where the milliequivalent factor of malic acid is 0.067. The formula is as follows:

$$\%\text{acid} = \frac{\text{NaOH used } (\mu\text{L}) \times \text{molarity of NaOH used} \times (\text{miliequivalent factor}) \times 100}{\text{Amount of sample used (mg)}}$$

Metabolite Analysis Using CE-TOF/MS

Metabolite extraction was performed according to our previous method (Pinsorn et al., 2018). In brief, the freeze-dried sample (5 mg, five replicates) was mixed with methionine sulfone and camphor 10-sulfonic acid as internal standards (Sigma-Aldrich, St. Louis, MO, United States). CE-TOF/MS analysis was then performed as previously described (Oikawa et al., 2011). Methionine sulfone and camphor 10-sulfonic acid were used for cation and anion analyses, respectively, and their peak areas were used to normalize the peak areas of metabolites, providing the relative intensity of each metabolite. In addition, the CE-TOF/MS data of the ripe-stage durian harvested in 2016 were retrieved from our previous paper (Pinsorn et al., 2018).

Metabolite Analysis Using GC-TOF/MS

Metabolite extraction, derivatization, and chromatography data processing were performed according to the methods described previously (Erban et al., 2020). In brief, the freeze-dried sample (10 mg, three replicates) was mixed with 360 μL methanol-mix that includes $^{13}C_6$-sorbitol as an internal standard, followed by 200 μL chloroform and 400 μL water that are added for phase separation. Solvents were of highest available purity (Merck, Darmstadt, Germany). Samples were vortexed and agitated 15 min at 70 °C after adding the methanol-mix, incubated 5 min at 37 °C after chloroform addition, and thoroughly vortexed after water addition. Phase separation was induced by centrifugation. The upper polar phase (80 μL) was dried. Each sample was chemically derivatized using methoxyamine hydrochloride in pyridine and BSTFA (Macherey-Nagel, Düren, Germany) as previously described (Erban et al., 2020), including *n*-alkanes for retention index calculation. The derivatized samples (1 μL) were analyzed by splitless and 1:30 split-injection modes with a 6890N gas chromatograph (Agilent, Santa Clara, CA, United States) connected to a Pegasus III time-of-flight mass spectrometer (Leco Instruments, St. Joseph, MI, United States). Metabolite annotation was performed by matching mass spectra and retention index information to the Golm Metabolome Database using TagFinder software (Kopka et al., 2005; Luedemann et al., 2008; Erban et al., 2020).

Measurement of Sugar Content Using HPAEC-PAD Analysis

A freeze-dried sample (15 mg, at least three replicates) was extracted in 1 mL of 80% methanol, with 0.3 mg of cellobiose (Wako, Osaka, Japan) as an internal standard for calibration, and incubated at 92 °C for 10 min. The extraction step was repeated twice. The supernatant was transferred to a new tube and evaporated with CentriVap Centrifugal Vacuum Concentrators. The pellet was dissolved in 300 μL of ultrapure (UP) water, 50-fold diluted, and filtered through a 0.22-μm syringe filter for further analysis.

For HPAEC-PAD, Dionex ICS 5000 ion chromatography system (Dionex, Sunnyvale, CA, United States) was used with a Carbo PacTM PA1 high-performance anion-exchange column (4 × 250 mm) (Dionex). The elution buffer system was consisted of 150 mM NaOH (buffer A) and 500 mM CH_3COONa (Kemaus, Cherrybrook, Australia) in 150 mM NaOH (buffer B). The column was equilibrated by buffer A with a flow rate of 1 mL/min at 30 C. The samples were eluted by multistep gradients as follows: constant 100% buffer A at 0–5 min, linear gradient to 5% buffer B at 5–8 min and constant at 5% buffer B to 12 min, linear gradient to 20% buffer B at 12–15 min, linear gradient to 100% buffer B at 15–17 min and hold at 100% buffer B for 2 min, and linear gradient to 100% buffer A at 19–20 min.

Quantification of the targeted sugars, including *myo*-inositol (Phytotechnology Laboratories, Lenexa, KS, United States), galactinol (TCI, Tokyo, Japan), sorbitol (Acros, Antwerp, Belgium), fructose (Sigma), glucose (Univar, Downers Grove, United States), 1-kestose (Wako), maltose (Condalab, Madrid, Spain), raffinose (TCI), and sucrose (Univar), was conducted using mixtures of the reference standards in the ranges of 0.1–2.5 mg/mL.

RNA Extraction and Transcriptome Analysis

Total RNA was extracted (in triplicate) from the samples at unripe and ripe stages, using the PureLink Plant RNA Reagent (Invitrogen Carlsbad, CA, United States) per the manufacturer's instructions. cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, United States) according to the manufacturer's instructions. Next, transcriptome sequencing was performed using the BGISEQ-500 platform (BGI, Shenzhen, China). We used the OmicsBox program (v1.4.1.1) for transcriptome data analysis. Filtering of raw reads was performed, which resulted in paired-end clean reads, and unpaired reads that lost their corresponding sequence partners due to quality control procedures. Then, the *de novo* transcriptome assembly of the cleaned reads was performed using the Trinity (v2.8.5) package of the program with default parameters. The assembled transcripts were identified ORF and annotated with NCBI BLAST against non-redundant protein sequences (Nr v5), InterProScan, Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG). The clean reads were mapped back to the assembled transcripts using the Bowtie2 (v2.3.5.1) package with default parameters, and the expression levels of the transcripts were quantified using the RSEM (v1.3.1) package with default parameters. The read counts were normalized using the reads per kilobase of transcript and per million mapped reads (RPKM) method. Pairwise differential expression analysis was performed using the exact test (FDR < 0.05) of the software package edgeR (v3.28.0) when change in expression was two-fold or higher. The differentially expressed genes (DEGs) were

classified using GO classification and into specific biological pathways using KEGG.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

cDNA was used for gene expression analysis with RT-qPCR; the reaction mix was prepared using the Luna® Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, United States) according to the manufacturer's protocol and the reaction was run on a CFX Real-Time PCR system (Bio-Rad Laboratories, Inc., Hercules, MA, United States). Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), based on the ratio of the expression of candidate genes and elongation factor 1 alpha (*EF1a*), a housekeeping gene (Khaksar and Sirikantaramas, 2020). The primers used for RTqPCR analyses are listed in **Supplementary Table 1**.

Statistical Analysis

The metabolite data were processed by algorithms in MetaboAnalyst 5.0[1] (Pang et al., 2021). Principal component analysis (PCA), which is a dimension-reduction tool, was applied to investigate the relationships between samples. A volcano plot was used to select significant metabolites, which were designated as those with a relative intensity greater than the threshold ($|\log_2(\text{fold change [FC]})| > 1, P < 0.05$) between the two stages. Metabolomics Pathway Analysis (MetPA) was performed to enrich the metabolite set into the relevant pathways. Pathway impact of each metabolic pathway is calculated from the sum of the importance measures of the matched metabolites divided by the su of the importance measures of total metabolites. Pathways that reached cut-off values ($P < 0.05$, pathway impact ≥ 0.1) were considered perturbed (Guo and Tao, 2018). One-way ANOVA followed by Tukey's test in GraphPad Prism version 5 was used to compare the means of each sugar at different ripening stages ($P < 0.05$).

RESULTS AND DISCUSSION

An Overview of Metabolites Involved in Durian Fruit Ripening

Durian pulp undergoes physiological changes during ripening, including slight yellowing of the pulp, during postharvest ripening (**Supplementary Figure 1**). The total soluble solid (TSS) level of durian pulp increased significantly from the unripe stage (**Supplementary Figure 2A**). The accumulation of TSS increases consistently with total sugar content but is negatively related to starch content (Ketsa and Daengkanit, 1998) relative to the sweetness of the ripe pulp. At the same time, the percentage of titratable acidity (%TA) did not change significantly during ripening. However, %TA tended to increase during durian ripening (**Supplementary Figure 2B**). Titratable acidity refers to total acid contents inside the food (Sadler and Murphy, 2010), suggesting that the acidity of durian pulp does not change considerably as it ripens. The changes in the underlying mechanisms during the ripening process contribute to the durian fruit's characteristics. To study the alterations of primary metabolites in these related mechanisms, we performed metabolome analysis in durian cv. Monthong pulp at the unripe and ripe stages. CE-TOF/MS and GC-TOF/MS techniques were used for comprehensive analysis of primary metabolites, which identified 167 and 56 metabolites, respectively (**Supplementary Table 2**). Metabolites were further analyzed using the web-based tool MetaboAnalyst 5.0. PCA plots, showed the total variance of the CE-TOF/MS data was 63% from PC1 and 13.4% from PC2 (**Figure 1A**), while total variance of the GC-TOF/MS data was 60.6% from PC1 and 18% from PC2 (**Figure 1B**). According to these two PCA plots, unripe durian samples were clearly separated from the ripe groups in PC1. As expected, the results showed that ripening greatly influences metabolic changes at the biochemical level and leads to physiological changes, such as the increased flavor and softening of the edible ripe fruit. This phenomenon is likewise observed in other fruit species, such as peach strawberry, tomato, and grape (Carrari and Fernie, 2006; Lombardo et al., 2011), which each constitute different fruit models. To obtain the relevant biological pathways, Metabolomic Pathway Analysis (MetPA) was used. CE-TOF/MS results indicated that of 59 enriched biological pathways identified by KEGG analysis, 29 metabolic pathways were perturbed (**Supplementary Table 3**), including central carbon pathways (glycolysis and TCA cycle) and several amino acid pathways (**Figure 1C**). Meanwhile, GC-TOF/MS results indicated that nine pathways were significantly impacted (**Figure 1D**), including starch and sugar metabolism, as well as some amino acid pathways that were previously found in the CE-TOF/MS analysis (**Supplementary Table 3**). These results demonstrated that primary metabolic pathways were significantly altered during durian ripening, which may involve in flavor precursor biosynthesis.

As observed, there was some overlap in pathways identified by each method. We further investigated metabolites in those pathways. Among these metabolites, 28 were annotated using both techniques (**Supplementary Table 2** and **Supplementary Figure 3**). In total, 195 annotated metabolites were obtained and used for further analysis. Our annotation showed that results from each technique validated those from other techniques because the same metabolites exhibited similar changes in intensity during the ripening period (**Supplementary Table 2**). It is known that both CE-TOF/MS and GC-TOF/MS annotate slightly different kinds of primary metabolites. CE-TOF/MS can separate a wider range of charged metabolites, including amino acids and organic acids, while GC-TOF/MS is more dependent on volatility and can identify uncharged metabolites, such as sugar and sugar derivatives.

Ripening-Associated Metabolites Contributing to Durian Flavor

Out of 195 annotated metabolites, 94 in durian pulp were significantly altered between the unripe and ripe stages (**Figure 2**,

[1] https://www.metaboanalyst.ca/

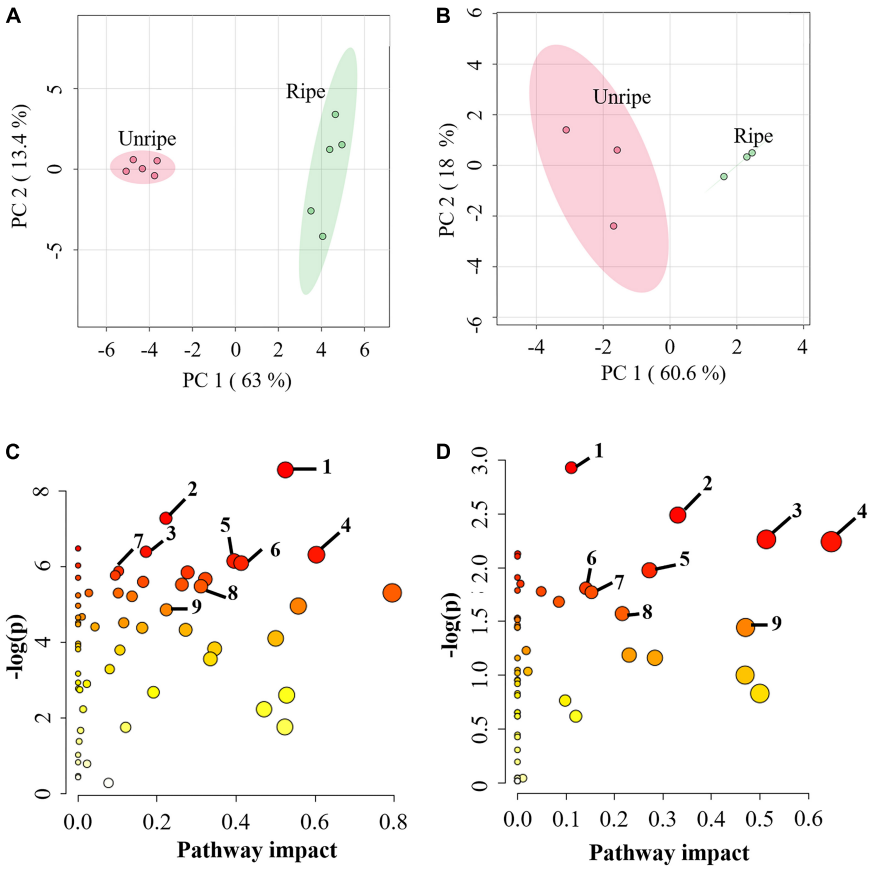

FIGURE 1 | (A) Principal component analysis (PCA) score plot of metabolites from capillary electrophoresis-time of flight/mass spectrometry (CE-TOF/MS) and **(B)** from gas chromatography- time-of-flight mass spectrometry (GC-TOF/MS) in the pulp of durian cv. Monthong at unripe and ripe stages, which are labeled in red and green, respectively. Each dot represents each biological replicate sample, and the circle represents 95% confidence intervals. **(C)** Metabolic pathway analysis (MetPA) of metabolites from CE-TOF/MS and **(D)** from GC-TOF/MS. Matched pathways related to ripening of durian cv. Monthong are displayed as circles. The color and size of the circles represent the *P* value and pathway impact value, respectively. The significantly altered pathways associated with our detected primary metabolites were labeled. Metabolic pathways with pathway impact > 0.1 and $P < 0.05$ were perturbed pathways. The top 7 perturbed pathways of **(C)** are as follows: 1, arginine and proline metabolism; 2, aminoacyl-tRNA biosynthesis; 3, glutathione metabolism; 4, glycine, serine and threonine metabolism; 5, cysteine and methionine metabolism; 6, glyoxylate and dicarboxylate metabolism; 7, vitamin B6 metabolism. The central carbon pathways of **(C)** are: 8, Citrate cycle (TCA cycle); 9, glycolysis. All perturbed pathways of **(D)** are: 1, aminoacyl-tRNA biosynthesis; 2, starch and sucrose metabolism; 3, glycine, serine and threonine metabolism; 4, alanine, aspartate and glutamate metabolism; 5, galactose metabolism; 6, glutathione metabolism; 7, arginine and proline metabolism; 8, tyrosine metabolism; 9, phenylalanine metabolism.

Supplementary Figure 4, and **Supplementary Table 4**). The ripening-associated metabolites were classified into six groups: proteinogenic amino acids, non-proteinogenic amino acids, sugar and sugar derivatives, polyamines, organic acids, and nucleotide and nucleotide derivatives. All others were placed in a miscellaneous group.

Sugar is an important metabolite group responsible for sweetness in fruits. Previously, sucrose, fructose, glucose, and maltose were increased in ripe durian pulp samples (Charoenkiatkul et al., 2018). Using GC-TOF/MS, we confirmed that these four major sugars and a newly annotated sugar, raffinose, were significantly increased during ripening with the $\log_2$ FC ranging between 1.16 and 4.84 (**Figure 2**), while the newly annotated 1-kestose was unchanged. Since sugars were only annotated from GC-TOF/MS in this work, we applied HPAEC-PAD analysis to validate and confirm the result (**Figure 3**). According to the results, the contents of all four major sugars agreed with GC-TOF/MS. However, the content of 1-kestose was significantly increased, while the content of raffinose remained unchanged during the ripening period (**Figure 3**). Disagreements between the two methods could be obtained when the concentrations in the samples were very low, as raffinose and 1-kestose were in this case. When using GC-MS, it is suggested that optimum derivatization and separation are needed before measuring sugars in each individual plant extract (Füzfai et al., 2004). Therefore, in our analysis, quantification by targeted HPAEC-PAD analysis is more reliable than the untargeted analysis, which can lead to the discovery of novel metabolites. Fruits can accumulate different types of sugars, but sucrose is the primary storage sugar in many fruits, such as melon, banana, peach, and strawberry (Yamaki, 2010). Notably, durian pulp contains over 50% sucrose by dry weight (**Figure 3**),

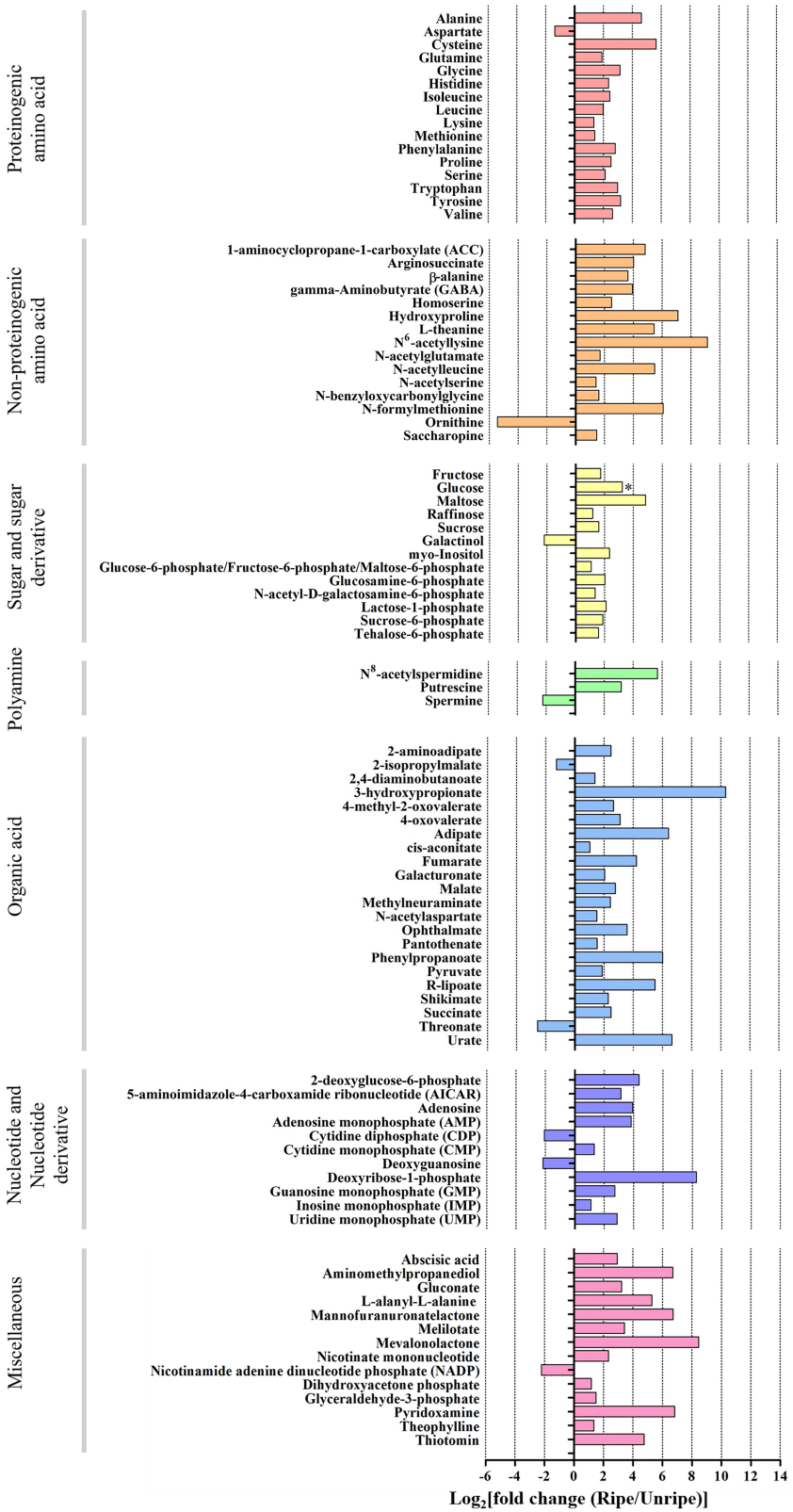

FIGURE 2 | Log_2 of fold change (FC) of metabolite intensity from ripe and unripe stages of durian cv. Monthong. Based on volcano plot ($|\log_2(FC)| > 1$, $P < 0.05$), these metabolites significantly changed during the ripening process. Changes are represented in the bar chart, indicating increased levels (positive values) or decreased levels (negative values) of the metabolite intensity. (* = glucose which is significant under $P = 0.1$).

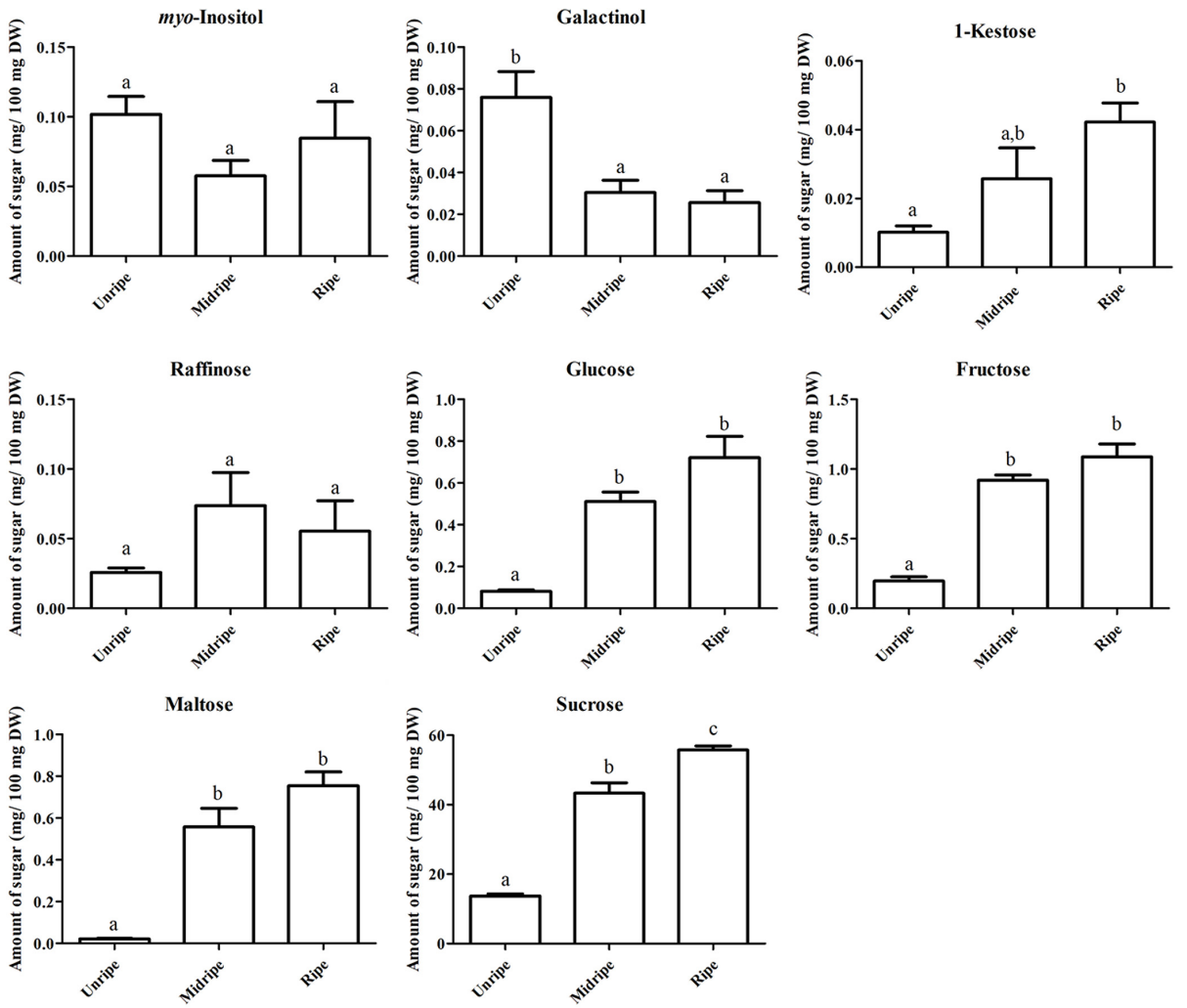

FIGURE 3 | Soluble sugar content based on dry weight, including myo-inositol, galactinol, 1-kestose, raffinose, glucose, fructose, maltose, and sucrose contents (mg/100 mg dry weight [DW]) of the durian pulp at three ripening stages (including unripe, midripe, and ripe). Each bar indicates mean ± standard error (±SEM) values from triplicate experiments and significant difference between stages is denoted by lower case letters.

which makes it the highest sucrose-accumulating fruit among those reported. Since sucrose is the most abundant sugar, this suggests that the major sweetness-providing metabolite in the ripe durian pulp is sucrose.

In addition, we identified two ripening-associated sugar alcohols found at low concentration related to raffinose, galactinol and *myo*-inositol (**Figure 2**). Galactinol content decreased during ripening, which was in agreement with the two analytical methods (**Figure 3**), whereas *myo*-inositol content remained unchanged, as confirmed by HPAEC-PAD. Raffinose and *myo*-inositol are synthesized from galactinol. The decrease in galactinol during the ripening period suggested that it might be stored before ripening then is converted to the downstream sugars later. The accumulation of raffinose-family sugars varies between various fruits (Jovanovic-Malinovska et al., 2014), and their role in fruits has not been studied much to date; however, they may be involved in stress tolerance during ripening. Furthermore, we found an increase in sugar phosphates, including glucosamine-6-phosphate, *N*-acetyl-*D*-galactosamine-6-phosphate, lactose-1-phosphate, sucrose-6-phosphate, and trehalose-6-phosphate in ripe durian pulp with $\log_2$ FC ranging between 1.34 and 2.09 (**Figure 2**). Sugar phosphates are intermediates in different biological pathways. In addition, they also act as signaling molecules. One interesting metabolite is trehalose-6-phosphate, which is an intermediate for trehalose biosynthesis. It is an important signaling molecule for sucrose consumption and a negative feedback molecule for sucrose biosynthesis in plants (Fichtner et al., 2020). Therefore, the increase in trehalose-6-phosphate in durian pulp possibly helps to control the sucrose content and supports trehalose content at the same time.

We detected 22 ripening-related organic acids in the durian pulp. Of these, the levels of 20 organic acids significantly increased during durian ripening, with $\log_2$ FC ranging between

1.38 and 10.33, while those of 2-isopropylmalate and threonate decreased (Log_2 FC = −1.25 and −2.56, respectively) (**Figure 2**). There were five ripening-related organic acids which participated in central carbon pathways, namely *cis*-aconitate, fumarate, malate, pyruvate, and succinate. Malate and succinate were the highest (**Supplementary Table 2**), which was consistent with the results for the ripe durian pulps from other cultivars (Yi et al., 2020). Another abundant organic acid was citrate, and its content was not significantly changed during the ripening period (**Supplementary Table 2**). Therefore, malate and succinate probably contribute to sour taste in the ripe fruit, even if the sweet taste is dominant in the pulp, due to high sugar composition. In addition, we also detected a significant change in 4-methyl-2-oxovalerate and 2-isopropylmalate (**Figure 2**), which are two important intermediates in branched-chain amino acid metabolism that will be elaborated in the section below.

Amino acids serve as important flavor precursors in many fruits, including durian. We detected 31 ripening-associated amino acids, while three amino acids were unchanged: asparagine, glutamate, and threonine. Levels of 15 proteinogenic and 14 non-proteinogenic amino acids increased during durian ripening, with log_2 FC ranging between 1.34–5.65 and between 1.4–9.15, respectively, while only aspartate and ornithine decreased (Log_2 FC = −1.36 and −5.64, respectively) (**Figure 2**). Durian pulp contains several volatile sulfur compounds. The volatiles are associated with amino acids in sulfur-related pathways. Notably, cysteine is the most modulated amino acid in the pathway (**Figure 2** and **Supplementary Table 4**) and is an important precursor for the biosynthesis of methionine, another sulfur-containing amino acid. We found that methionine levels slightly increased in ripening durian pulp (**Figure 2** and **Supplementary Table 4**). Methionine is a central metabolite of the sulfur pathway and ethylene biosynthesis, in which ethylene is an important compound for climacteric ripening. Therefore, this may be the reason for the slight changes in methionine content during ripening. Methionine is also an important precursor for sulfur volatile production, providing strong odor. Previous research on melon demonstrated that exogenous apply of methionine increased the content of sulfur volatiles related to methanethiol (Gonda et al., 2013). Interestingly, methanethiol and other thiol volatiles can be detected at high concentrations in ripe durian pulp using static headspace gas chromatography-olfactometry (SHGC-O) (Li et al., 2012) and headspace solid-phase microextraction GC-MS (Teh et al., 2017). In addition, we also found the accumulation of serine and homoserine (**Figure 2**), which are intermediates in the sulfur pathway, supporting the involvement of the pathway during the ripening process. Therefore, the accumulation of these amino acids may provide key precursors that contribute to the characteristic sulfuryl odor of durian.

In addition to sulfuryl odor, durian pulp also exhibits a fruity aroma during ripening. Branched-chain amino acids, including isoleucine, leucine, and valine, are precursors for volatile alcohols, esters, aldehydes, and lipid-derived compounds (Roze et al., 2010). We found a significant increase in the levels of these three amino acids and the isoleucine-derived keto acid, 4-methyl-2-oxovalerate in ripe durian pulp (**Figure 2**). According to a previous study conducted on strawberry, exogenously applied isoleucine increased the level of ethyl 2-methylbutanoate, a volatile ester (Perez et al., 2002). Furthermore, either exogenously applied isoleucine or 4-methyl-2-oxovalerate elevated the levels of ethyl 2-methylbutanoate, while applying valine increased the ethyl 2-methylpropanoate level (Gonda et al., 2010). These two volatiles, providing fruity odor, were also identified at high concentrations in the durian pulp (Li et al., 2012). Therefore, the accumulation of branched-chain amino acids can be important for the biosynthesis of volatile esters, and this accumulation may affect the expression of durian's fruity aroma during ripening.

For the aromatic amino acid pathway, phenylalanine, tryptophan, and tyrosine are ripening-related amino acids in durian fruit (**Figure 2**). These amino acids are precursors for secondary metabolites such as flavonoids, alkaloids, and indoles. Interestingly, durian pulp contains a high amount of flavonoids at the ripe stage, which positively correlates with the high antioxidant activity of the pulp (Haruenkit et al., 2010). Accumulation of these amino acids may be important for durian pulp to synthesize secondary metabolites during ripening.

Aspartate was the only proteinogenic amino acid whose levels decreased during ripening (**Figure 2**). It can be directly used for the biosynthesis of glutamate and asparagine. Moreover, it is involved in the biosynthesis of lysine, methionine, isoleucine, leucine, and valine, whose contents increased during ripening, and threonine, whose content was unchanged. Therefore, the decrease in aspartate levels during ripening could result from its usage for biosynthesis of other amino acids. Glutamate is a highly abundant amino acid that provides the umami taste in durian pulp (Pinsorn et al., 2018). We found that the glutamate content remained constant during the ripening period (**Supplementary Table 2**). Glutamate can be converted to other metabolites, such as glutamine, arginine, proline, and GABA (Sorrequieta et al., 2010). We observed increased levels of proline and hydroxyproline during ripening. Until now, the roles of these two metabolites in fruits have not been well understood. Previous studies have shown that accumulation of proline is involved in the nitrogen sink mechanism, and it helps to increase the fruit quality traits and yield (Sayed et al., 2014). Regarding the precursors for polyamine biosynthesis, we found that the arginine content was unchanged (**Supplementary Table 2**), while ornithine content decreased during the ripening period (**Figure 2**). Both amino acids can be converted to putrescine, a central metabolite of the polyamine pathway.

For polyamines, we found significant increases in N^8-acetylspermidine and putrescine, while spermidine and spermine were unchanged and decreased, respectively (**Figure 2** and **Supplementary Table 2**). Polyamines are associated with abiotic stress responses in fruits (Chen et al., 2019). Furthermore, previous studies have shown that they are also involved in the modulation of fruit ripening by intricate crosstalk with the ethylene pathway (Tassoni et al., 2006). The role of polyamines in durian pulp still requires further investigation, and their accumulation might be related to the ripening period of durian. Polyamines are also precursors for biosynthesis of GABA, a

health-promoting bioactive compound. Interestingly, GABA was highly enriched in ripe durian pulp (**Supplementary Table 4** and **Figure 2**). Although the role of GABA accumulation in fruit has not been clearly ascertained, previous studies have shown that it is associated with abiotic stress responses in strawberry (Deewatthanawong et al., 2010), tomato (Wu et al., 2020), and apple (Brikis et al., 2018). It has been shown that these fruits accumulate high GABA content under elevated CO_2 and low O_2. Therefore, the role of GABA in durian might also be involved in stress response. Further studies in durian may help to promote its market value by developing appropriate storage conditions for higher GABA content in the future.

The levels of nine nucleotide derivatives increased during ripening, with $\log_2$ FC ranging between 1.32 and 8.28 (**Figure 2**), while those of cytidine diphosphate (CDP) and deoxyguanosine decreased (Log_2 FC = −2.06 and −2.18, respectively). To our knowledge, 3 of 11 nucleotide derivatives are known to be involved in fruit flavor. AMP, IMP, and GMP accumulation is associated with enhancing the umami taste in tomato (Chew et al., 2017). In addition, we also detected 14 additional miscellaneous metabolites with significantly altered levels during durian ripening with $\log_2$ FC ranging between −2.18 and 8.45 (**Figure 2**). Some of these metabolites are food supplements in the durian pulp, such as gluconate and pyridoxamine, the so-called vitamin B group (Pinsorn et al., 2018).

Transcriptome Analysis of Durian cv. Monthong During Ripening

The metabolomic data provided us only the ripening-associated metabolites and the pathways associated with durian flavor. Therefore, to understand the biochemical modification of the pathways at a deeper and more comprehensive level, we integrated transcriptome analysis to study the expression of candidate genes in associated pathways.

Approximately, 10–16 million clean reads from unripe and ripe Monthong were generated. The clean reads were then used to perform *de novo* assembly, which generated 164,618 transcripts with an average length of 1,082.68 bp, and a total of 85,164 coding regions were predicted. Afterward, total reads of each library were mapped to the *de novo* assembled transcriptome (**Supplementary Table 5**). The annotation results of transcripts are shown in **Supplementary Table 6**. The number of the transcripts obtained in this study is similar to the previous report by Teh et al. (2017). The PCA plot shows that the expression of transcripts in the unripe samples were clearly separated from the ripe samples according to PC1 (90.1%) (**Supplementary Figure 5**), suggesting that gene expression changes as the ripening progresses. DEG analysis identified a total of 18,616 DEGs (**Supplementary Table 7**) with 8,145 upregulated genes and 10,471 downregulated genes. Subsequently, we identified the top 10 upregulated genes by FC. Two of those genes were polygalacturonase (PG) and 1-aminocyclopropane-1-carboxylate synthase (ACS) (**Supplementary Table 7**), which are responsible for cell wall degradation and ethylene biosynthesis, respectively. In addition, we identified a key sulfur metabolism gene, methionine gamma-lyase (MGL), which contributes to VSC biosynthesis in sulfur metabolism (Teh et al., 2017). Although MGL did not have a high FC value, it showed a remarkably high RPKM expression value (**Supplementary Table 8**), suggesting its importance during durian ripening. Additionally, a high RPKM value was also found for 1-aminocyclopropane-1-carboxylate oxidase (**Supplementary Table 8**) at the ripe stage. DEGs were then used to perform GO analysis, and most of the GO terms were assigned to biological processes. Within biological processes, the largest proportion was in protein modification by small protein conjugation and ubiquitin-dependent protein catabolic process categories, respectively. Within molecular function, the largest proportion was in the ATPase activity category. Lastly, within the cellular compartment, the largest proportion was in the host cell nucleus category (**Supplementary Figure 6**). To reveal the biological pathways associated with DEG data, DEGs were assigned to KEGG. The results showed that the DEGs participated in 144 biological pathways. The top five pathways are purine metabolism, thiamine metabolism, starch and sucrose metabolism, glycolysis/gluconeogenesis, and pyruvate metabolism (**Supplementary Figure 7**). This result is consistent with the metabolic pathway analysis which found that the change of nucleotide metabolism, sugar metabolism, and central carbon metabolism was highly associated with the durian ripening process.

To validate the DEGs identified from RNA-seq results, we randomly selected 11 key genes (*ALDH* [LOC111284558], *ADC* [LOC111308806], *CM* [LOC111286253], *GABA-TK* [LOC111305652], *GAD* [LOC111281776], *BCAT* [LOC111305652], *MDH* [LOC111293205], *NADP-MEs* [LOC111286064], *OAT* [LOC111278083], *PFK* [LOC111285266], and *TrpAB* [LOC111287933]) from various metabolic pathways possibly related to durian flavor, and their expression levels were analyzed by RT-qPCR using Monthong cDNA at the unripe and ripe stages. According to the RT-qPCR analysis, the expression results of all selected genes agreed with results obtained from RNA-seq analysis showing the reliability of the RNA-seq method for expression quantitation (**Figure 4**).

Identification of Candidate Genes in Metabolic Pathways Contributing to Durian Flavor

Our results demonstrated that sugar and central carbon pathways (glycolysis and TCA cycle) are important pathways during the ripening process (**Supplementary Figure 7**). High sugar accumulation promotes sweet taste in ripe durian pulp. Alternatively, sugars are metabolized into the central carbon pathway, which not only produces cellular energy, but also generates organic acids for fruit acidic taste and precursors for amino acid biosynthesis. In this section, we focused on the modulation of the central carbon pathway and its connection to flavor-related pathways. Therefore, a metabolic network was constructed to elucidate the relationship between changes in metabolite levels and candidate genes (**Figure 5**). Information related to candidate genes, such as locus number and expression level, are provided in **Supplementary Table 8**.

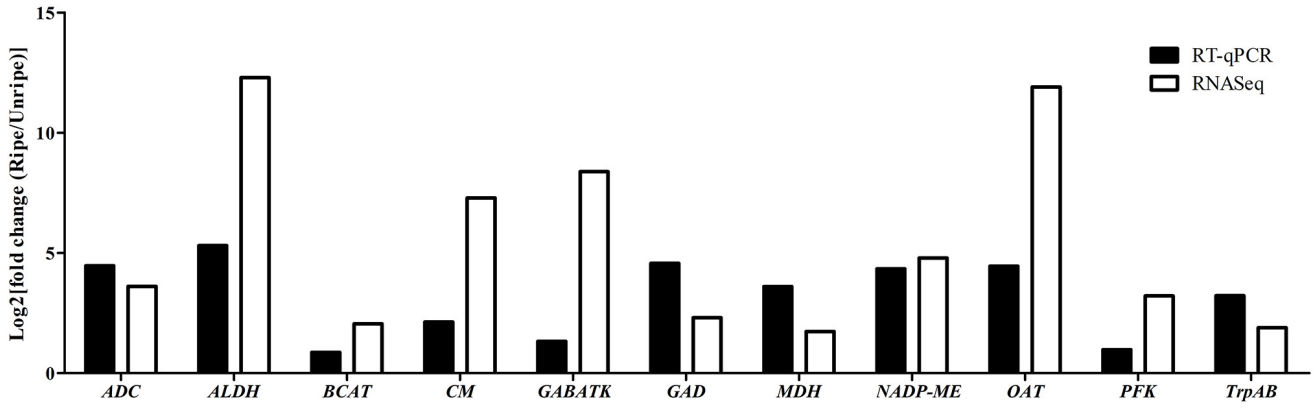

FIGURE 4 | Log_2 fold change in expression values in the ripe pulps relative to unripe pulps, as measured with RNA-seq and RT-qPCR in the 11 selected genes.

Central Carbon Pathway

We identified genes in the glycolytic pathway, including hexokinase (*HK*), phosphofructokinase (*PFK*), and pyruvate kinase (*PK*), which control rate-limiting steps of the glycolytic pathway in fruit (Ball et al., 1991). According to our results, some isoforms, annotated as *HK*, *PFK*, and *PK,* were upregulated with high RPKM values at the ripe stage compared to the downregulated isoforms (**Figure 5** and **Supplementary Table 8**). The expression of these isoforms is consistent with the accumulation of pyruvate, the final product of the pathway (**Figure 5**), suggesting that these candidate genes participate in glycolysis during durian ripening. Afterward, pyruvate is converted to acetyl-CoA and enters the TCA cycle by pyruvate dehydrogenase (*PDH*), whose isoform was annotated as *PDH* acyl-transferring subunit was upregulated in the ripe durian pulp (**Figure 5** and **Supplementary Table 8**). The first step of the TCA cycle is producing citrate, which is controlled by citrate synthase (*CS*). Although *CS* was upregulated, the content of citrate remained constant during ripening (**Figure 5**), suggesting that the rate of citrate biosynthesis is probably equal to citrate consumption in the durian. Furthermore, we also observed the upregulation of other genes in the TCA cycle, including isocitrate dehydrogenase (*IDH*), α-ketoglutarate dehydrogenase (*2-OGDH*), fumarase (*FUM*), and malate dehydrogenase (*MDH*), together with the upregulated metabolites, isocitrate, succinate, fumarate, and malate (**Figure 5**). These results show the activation of the TCA cycle, supporting citrate biosynthesis during ripening. Citrate is used in glutamate metabolism and is bypassed through α-ketoglutarate. We found that the content of α-ketoglutarate was unchanged during the ripening period. Notably, the gene for its biosynthesis, *IDH*, the gene for its degradation, *2-OGDH*, and glutamate dehydrogenase (*GDH*), were upregulated (**Figure 5**). This result suggests that α-ketoglutarate content remains constant because it is converted for the TCA cycle by *2-OGDH* and bypassed to glutamate metabolism by *GDH*. Similar observations were found in other fruits, such as tomato (Yin et al., 2010), showing that the change in citrate is correlated to α-ketoglutarate content during ripening and salt-stress treatment. Although some metabolites in this pathway do not provide the dominant taste found in ripe durian, they serve as important precursors for the biosynthesis of other taste-related compounds, which will be discussed later.

Glutamate and Glutamate-Derived Metabolic Pathways

Glutamate, an amino acid providing umami taste, is linked to the TCA cycle via α-ketoglutarate, which is controlled by *GDH*. We found that the gene was upregulated during the ripening period (**Figure 5** and **Supplementary Table 8**). Additionally, there are three routes for glutamate biosynthesis, including aspartate aminotransferase (*AST*), alanine aminotransferase (*ALT*), and glutamate synthase (*GOGAT*). We found that only *AST* and *GOGAT* were upregulated during the ripening period (**Figure 5**). Notably, the route from *AST* (LOC111284590) contained the highest log_2 FC (4.83) and RPKM expression value compared to the other routes (**Supplementary Table 8**). Therefore, this route is highly activated for glutamate biosynthesis during durian ripening. Glutamate interconverts with aspartate, and this step is controlled by *AST*. We found that the decrease in aspartate content during durian ripening correlated with the upregulation of *AST* (**Figure 5**), suggesting a transfer of the amino group from aspartate to glutamate.

Although the above-mentioned glutamate-related biosynthetic genes were highly upregulated, glutamate content remained unchanged during the ripening period (**Figure 5**), implying an equal rate of biosynthesis and consumption. Interestingly, we found upregulation of several genes related to glutamate conversion. *GS* was correlated with an increase in glutamine content (**Figure 5**). Furthermore, we also found the upregulation of nitrate reductase (*NR*) (**Supplementary Table 8**). It has been reported that GS and NR were upregulated under higher nitrogen supply, resulting in higher accumulation of soluble sugars, free amino acids, and other substances related to fruit quality (Scarpeci et al., 2007; Liao et al., 2019). Therefore, activating the nitrogen metabolism in ripening durian fruits could be important for

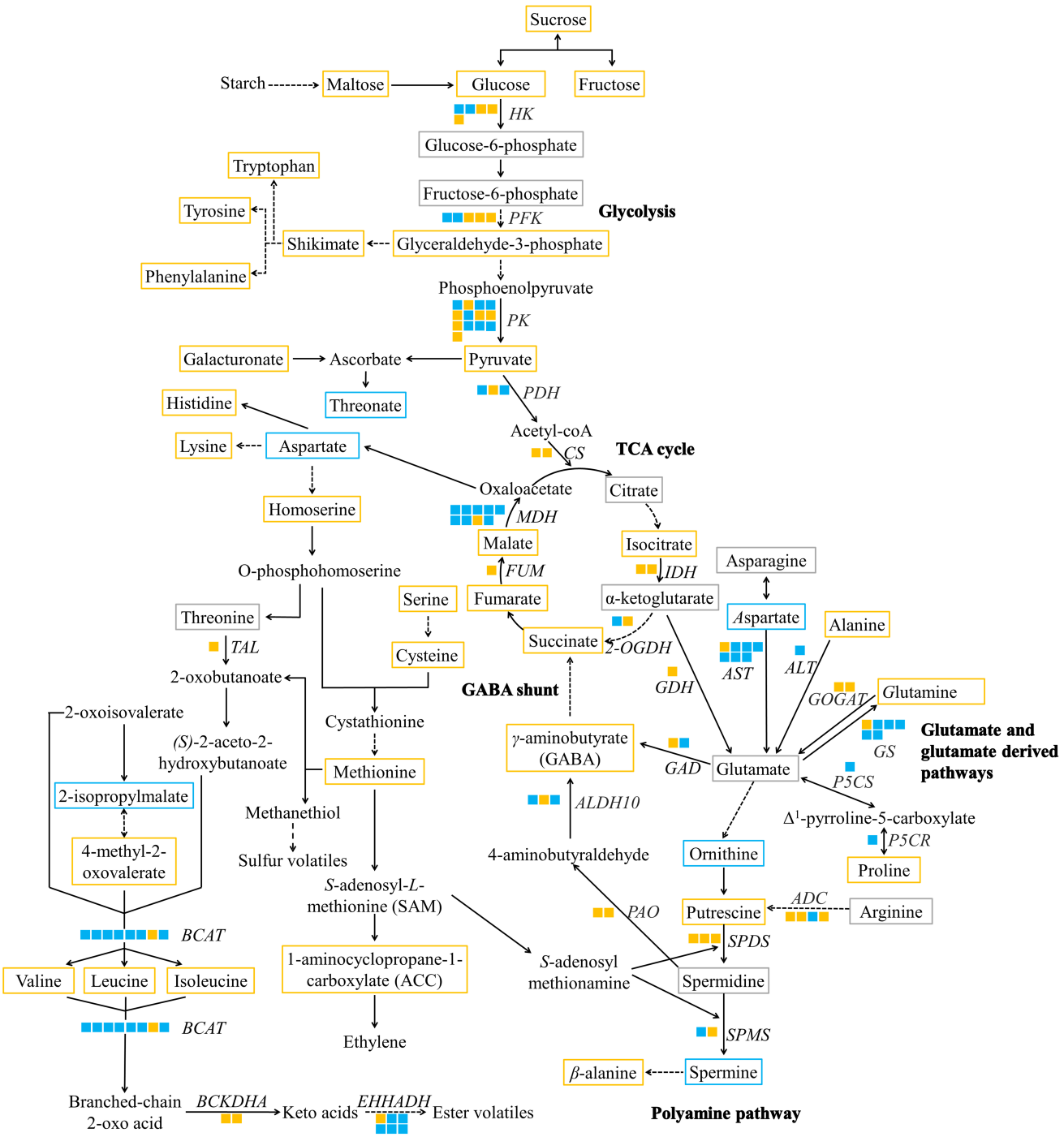

FIGURE 5 | A metabolic map of significantly altered metabolites and DEGs of taste-associated pathways during durian cv. Monthong ripening. Metabolites with significantly increased, significantly decreased, and unchanged contents are outlined in orange, blue, and gray, respectively. Undetectable metabolites are shown without boxes. Isoforms of each putative gene are represented by a solid box, and each isoform is arranged by locus number. Upregulated and downregulated isoforms are colored in orange and blue, respectively. Solid arrows indicate one enzymatic reaction, while dash arrows indicate multiple reactions. *HK,* hexokinase; *PFK*, phosphofructokinase; *PK,* pyruvate kinase*; PDH*, pyruvate dehydrogenase; *CS*, citrate synthase; *IDH*, isocitrate dehydrogenase; *2-OGDH*, α-ketoglutarate dehydrogenase; *FUM*, fumarase; *MDH*, malate dehydrogenase; *AST*, aspartate aminotransferase; *ALT*, alanine aminotransferase; *GDH*, glutamate dehydrogenase; *GOGAT*, glutamate synthase; *GS*, glutamine synthetase; *P5CS,* Δ^1*-pyrroline-5-carboxylate synthetase*; *P5CR*, Δ^1-pyrroline-5-carboxylate reductase; *ADC*, arginine decarboxylase; *SPDS*, spermidine synthase; *SPMS*, spermine synthase; *GAD*, glutamate decarboxylase; *PAO,* polyamine oxidase; *ALDH10*, 4-aminobutanal dehydrogenase; *TAL*, threonine ammonia-lyase; *BCAT, branched-chain amino acid transaminase*; *BCKDHA*, 3-methyl-2-oxobutanoate dehydrogenase; *EHHADH*, long-chain-enoyl-CoA hydratase.

enhancing taste-related characteristics. Glutamate can also be sequentially converted to Δ^1-pyrroline-5-carboxylate and proline by a dual activity enzyme, possessing of Δ^1-pyrroline-5-carboxylate synthetase (*P5CS*) and Δ^1-pyrroline-5-carboxylate reductase (*P5CR*). Only one isoform of the gene was found in our transcriptomic data, and it was downregulated during durian ripening, which was not consistent with the increased proline content (**Figure 5**). A previous report showed that proline content may not be correlated to the expression of *P5CR* in developing tissues of Arabidopsis (Hua et al., 1997). Therefore, this isoform may be the only candidate genes of proline metabolism in the pulp. In addition, we also identified proline dehydrogenase, a gene involved in proline catabolism, was downregulated during durian ripening (**Supplementary Table 8**).

Furthermore, glutamate can be used for the biosynthesis of ornithine and arginine, which are precursors for polyamine biosynthesis. These two amino acids can later be converted to putrescine, a precursor for further polyamine production. We propose that the primary route for polyamine biosynthesis in durian pulp is through arginine route because the key gene arginine decarboxylase (*ADC*) was upregulated with high RPKM value during durian ripening (**Figure 5** and **Supplementary Table 8**), while the expression level of ornithine decarboxylase (*ODC*), controlling the reaction from ornithine to putrescine, was not significantly changed. The arginine route has been found to be a major pathway for polyamine biosynthesis in other fruits such as apple and tomato (Hao et al., 2005). For the polyamine pathway, spermidine synthase (*SPDS*) and spermine synthase (*SPMS*) control steps that convert putrescine to spermidine and spermine, respectively. We found upregulation of *SPDS* and *SPMS* during durian ripening (**Figure 5**), which were not correlated with spermidine and spermine content. Spermidine is either converted to 4-aminobutyraldehyde or is used for spermine biosynthesis. Moreover, spermine can be degraded by β-alanine metabolism. Therefore, these are probably the reasons for the unchanged spermidine content and decreased spermine content during the ripening period.

GABA is a bioactive compound with a biosynthetic pathway that bypasses from α-ketoglutarate. Interestingly, we found a significant increase in GABA levels in the ripe durian pulp (**Figure 2**). There are two main GABA biosynthetic pathways in fruits, which are GABA shunts and alternative pathways from polyamines. For the GABA shunt, we found the upregulation of glutamate decarboxylase (*GAD*) in ripening durian pulp, which is positively correlated with GABA content (**Figure 5**). GABA shunts are the major pathway in many fruits. *GAD* is the key gene that controls the conversion from glutamate to GABA in this pathway. Similar to results from citrus fruit, the activation of GABA shunt is also related to increased transcription of *GDH*, *AST*, and *GS*, which is a major route for citrate catabolism (Katz et al., 2011; Sadka et al., 2019). These findings are consistent with our results in ripening durian. In addition, we found the upregulation of several genes in polyamine-derived-GABA biosynthesis, including polyamine oxidase (*PAO*) and 4-aminobutanal dehydrogenase (*ALDH10*). Therefore, our gene expression results supported the theory that two pathways contribute to GABA production during durian ripening.

Branched-Chain Amino Acid Pathway

An overpowering smell is a well-known characteristic of ripe durian. Besides the sulfuryl aroma, ripe durian also contains a sweet, fruity aroma, and the volatiles providing such aroma are associated with branched-chain amino acid metabolism. Interestingly, we observed high upregulation of threonine ammonia-lyase (*TAL*) (Log_2 FC = 5.52) (**Supplementary Table 8**), which converts threonine to 2-oxobutanoate, an important intermediate for BCAA biosynthesis, during durian ripening (**Figure 5**). Moreover, we also observed high upregulation of genes in the BCAA pathway, such as branched-chain amino acid aminotransferase (*BCAT*) (Log_2 FC = 6.32) (**Supplementary Table 8**), which is a key regulatory gene controlling branched-chain amino acid biosynthesis and degradation (Gonda et al., 2010; Maloney et al., 2010), supporting the activation of this pathway. In addition, branched-chain amino acids are precursors for volatile ester production. Notably, the upregulation of 3-methyl-2-oxobutanoate dehydrogenase (*BCKDHA*) and long-chain-enoyl-CoA hydratase (*EHHADH*), which are key genes in pathways connected to branched-chain amino acid degradation (Kochevenko et al., 2012), was observed during durian ripening (**Figure 5** and **Supplementary Table 8**). Our results support the hypothesis that branched-chain amino acid biosynthesis is upregulated during durian ripening, and that these amino acids are possibly used for volatile ester production, providing the aroma of ripe durian.

In conclusion, our findings provide fundamental knowledge for future durian molecular studies. We expect that these findings will allow comparison of the candidate gene expression and their related metabolites among different durian cultivars, thereby assisting in developing molecular markers for durian breeding that may prove useful the future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: The raw sequences have been deposited into NCBI Sequence Read Archive (SRA) under the project accession number PRJNA683229 and CNGB Sequence Archive (CNSA) of China National GeneBank DataBase (CNGBdb) with project accession number CNP0001432.

AUTHOR CONTRIBUTIONS

SS conceived the original screening and research plan. LS wrote the first draft of the manuscript. AO and RS performed CE-TOF/MS analysis. AE and JK performed GC-TOF/MS analysis. LS and KW performed HPAEC-PAD analysis. LS performed *de novo* transcriptome analysis. LS and PP analyzed the data. LS, GK, PP, AO, AE, JK, RH, KW, MW, TT, KS, and SS revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.687799/full#supplementary-material

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Transcriptomic, Proteomic and Metabolomic Analysis of Flavonoid Biosynthesis During Fruit Maturation in *Rubus chingii Hu*

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Rubus chingii HU, is a medicinal and nutritious fruit, which is very rich in flavonoids. However, the biosynthesis of its flavonoids is poorly understood. This study examined flavonoids and the genes/proteins at four fruit ripening phases using LC-MS/MS and qPCR. Six major kinds of anthocyanins, primarily consisted of flavanol-anthocyanins, which differed in form or concentration from other *Rubus* species. In contrast to other known raspberries species, *R. chingii* had a decline in flavonoids during fruit ripening, which was due to down-regulation of genes and proteins involved in phenylpropanoid and flavonoid biosynthesis. Unexpectedly, anthocyanin also continuously decreased during fruit maturation. This suggests that anthocyanins are not responsible for the fruit's reddish coloration. Flavanol-anthocyanins were derived from the proanthocyanidin pathway, which consumed two flavonoid units both produced through the same upstream pathway. Their presence indicates a reduction in the potential biosynthesis of anthocyanin production. Also, the constantly low expression of RchANS gene resulted in low levels of anthocyanin biosynthesis. The lack of RchF3′5′H gene/protein hindered the production of delphinidin glycosides. Flavonoids primarily comprising of quercetin/kaempferol-glycosides were predominately located at fruit epidermal-hair and placentae. The proportion of receptacle/drupelets changes with the maturity of the fruit and may be related to a decrease in the content of flavonoids per unit mass as the fruit matures. The profile and biosynthesis of *R. chingii* flavonoids are unique to *Rubus*. The unique flavonol pathways of *R. chingii* could be used to broaden the genetic diversity of raspberry cultivars and to improve their fruit quality.

Keywords: flavanol-anthocyanins, gene, enzyme, flavonoid biosynthesis, Chinese raspberry

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INTRODUCTION

Rubus chingii Hu is distributed widely across many Asian countries, such as China, Korea and Japan. It has been documented in ancient Chinese pharmacopeia including "Shen-nong-ben-cao" (Shennong's classic of materia medica) and "Ben-cao-gang-mu" (Compendium of Materia Medica), as well as in Korean pharmacopeia. Its health benefits are believed to include improving renal function (Ding, 2011), protecting hepatocytes (Zhang et al., 2015) and relieving anxiety, pain and inflammation (Sun et al., 2013). Red raspberry (*Rubus idaeus*), black raspberry (*Rubus occidentalis*) and Chinese raspberry (*R. chingii*) all belong to subgenus Idaeobatus. The fruit of *R. chingii* is an aggregate fruit of drupelets (each containing a single seed) around the central receptacle

(Chen et al., 2021). Unlike red or black raspberries, the drupelets of *R. chingii* do not detach from the receptacle at maturity. Typical phases of fruit maturation are "mature green phase (MG)," "green yellow phase (GY)," "orange yellow phase (YO)," and "red phase (RE)." During maturation, MG and GY fruits are both hard but different colors; YO fruits begin to soften and become orange; RE fruits rapidly soften and become red. The unripe fruit is traditionally used in Chinese medicine while the ripe fruit is appreciated by consumers not only for its special flavor but also for its nutritional properties.

Flavonoids occur ubiquitously in dietary and medicinal plants, which mainly consist of anthocyanins, as well as condensed and hydrolyzable tannins. These flavonoids contribute to the taste, flavor, color and pharmaceutical uses such as astringent actions. Prior studies have extensively examined *Rubus* flavonoids. For example, anthocyanin compositions have been identified and quantified in raspberries. Red and black raspberry share the same profile of anthocyanins. Their anthocyanins are predominantly cyanidin glycosides (e.g., glucosides, sophorosides, rutinosides, sambubioside, and glucosyl-rutinosides), but they only contain low to trace levels of pelargonidin glycosides (Mazur et al., 2014; Ludwig et al., 2015; Kula et al., 2016). Flavonols in red and black raspberry, as well as in Chinese raspberry, are mainly kaempferol/quercetin glycosides with the glucosides rutinoside and coumaroylglucoside (Kula et al., 2016; Yu et al., 2019). During the process of fruit ripening, the flavonoids dramatically change in composition and content, which is associated with the transformation of fruit pigmentation and flavor. In recent years, high-throughput sequencing of mRNA has been performed during the fruit ripening process in many *Rubus* species, e.g., red raspberry (*R. idaeus* cv. Nova) (Gutierrez et al., 2017), blackberry (*Rubus* spp. Var. Lochness) (Garcia-Seco et al., 2015) and black raspberry (*Rubus coreanus*) (Hyun et al., 2014; Chen et al., 2018) and (*R. occidentalis*). In red raspberry, an active anthocyanin biosynthesis takes place in the fruits during ripening (Gutierrez et al., 2017). In black raspberry (*R. coreanus*), anthocyanins and flavonols greatly increase during fruit development, while flavanols and proanthocyanidins are only accumulated at the very beginning of fruit set (Chen et al., 2018). The up-regulation of F3′H1, DFR4 and LDOX1 is responsible for the accumulation of cyanidin derivatives during the process (Hyun et al., 2014; Chen et al., 2018). These transcriptomic studies provide important information on genes in flavonoid biosynthesis.

Previous phytochemical studies mainly focused on the immature fruit in *R. chingii* (Guo et al., 2005; Ding, 2011) as only the immature fruit were used in traditional Chinese medicine. However, few studies have been conducted on *R. chingii* flavonoid biosynthesis. The purpose of this study was to investigate major flavonoid components and explore the potential mechanism underlying flavonoid biosynthesis.

MATERIALS AND METHODS

Plant Material

Rubus chingii plants were collected from the wild and grown in a commercial nursery located at LINHAI, Zhejiang, China (**Figure 1A**). The 2 year old plants were grown in 1.5 m rows. The distance between the rows was 2.0 m. Compound fertilizer (N-P_2O_5-K_2O = 15–15–15) was applied to plants. Fruits were handpicked from five to six plants (2 years old) at varying maturation phases, i.e., mature green (MG), green yellow (GY), Yellow orange (YO), and Red (RE) during the growing season (May, 2019). These fruits were put into 50 ml tubes and then immediately frozen in liquid nitrogen. Three biological replicates were designed for all experiments, with each replicate comprised of 100 g of fruit. Fruit weight varied by the maturation phase. To obtain a 100g sample approximately, 12–15 fruits were used for the RE phase, 25 for the YO, 50 for the GY, and 60 fruits for the MG phase.

Anthocyanin, Carotenoid, and Flavonoid Content

Total anthocyanin content was determined via spectrophotometry (Li et al., 2021a). The ground fruit tissue (0.3 g FW) was mixed with 10 mL 1% (*v*/*v*) HCl methanol and incubated for 24 h at room temperature in the dark. After centrifugation, supernatants were measured for absorbance at 530, 620, and 650 nm. Total anthocyanin was estimated as cyanidin-3-glucoside equivalents (mg/g FW).

Total carotenoid was determined via spectrophotometry (Li et al., 2021b). The ground fruit tissue (0.3 g FW) was mixed 10 mL extraction solution (ethanol:acetone = 1:2). The extraction was vortexed and then put in darkness for at least 30 min until the residues became colorless. The absorbance was measured at 440, 645, and 663 nm for carotenoid, and chlorophyll a/b, respectively.

Total flavonoid content was quantified by a colorimetric assay method (Li et al., 2021a). The ground fruit tissue (0.3 g FW) was mixed with 10 mL ethanol, and then centrifuged. Of supernatant, 1 mL was mixed with 2.4 mL ethanol and 0.4 mL $NaNO_2$. After incubation for 6 min, the mixture was added to 0.4 mL 10% $Al(NO_3)_3$ solution. After an additional 6 min, the mixture was added to 4 mL 4% NaOH and brought to volume of 10 mL with 100% ethanol. After 15 min at room temperature the absorbance was determined at 510 nm and measured relative to a blank extraction solvent. A calibration curve was prepared using rutin solution (8–48 μg/mL). Total flavonoid content was estimated as rutin equivalent (mg/g FW).

Fruit Anatomy and Flavonoid *in situ* DPBA Staining

Fruits were immersed and stored in FAA solution (10 formaldehyde/5 glacial acetic acid/35 ethyl alcohol) for 1 month. Radial and Transverse sections were taken and dehydrated in a graded ethanol series (20, 40, 60, 80, 95, 100, and 100% for 30 min per step) followed by paraffin infiltration and embedding using tert-butyl alcohol as an intermediate solvent. Sections of 12–14 μm were obtained using a 0.25-mm steel microtome blade on a rotary microtome and were mounted on glass slides. The mounted sections were deparaffinized and stained with Safranin O and Aniline Blue. Finally, slides were sealed with neutral balsam, observed through a light microscope (Olympus SP 350, Japan) and photographed.

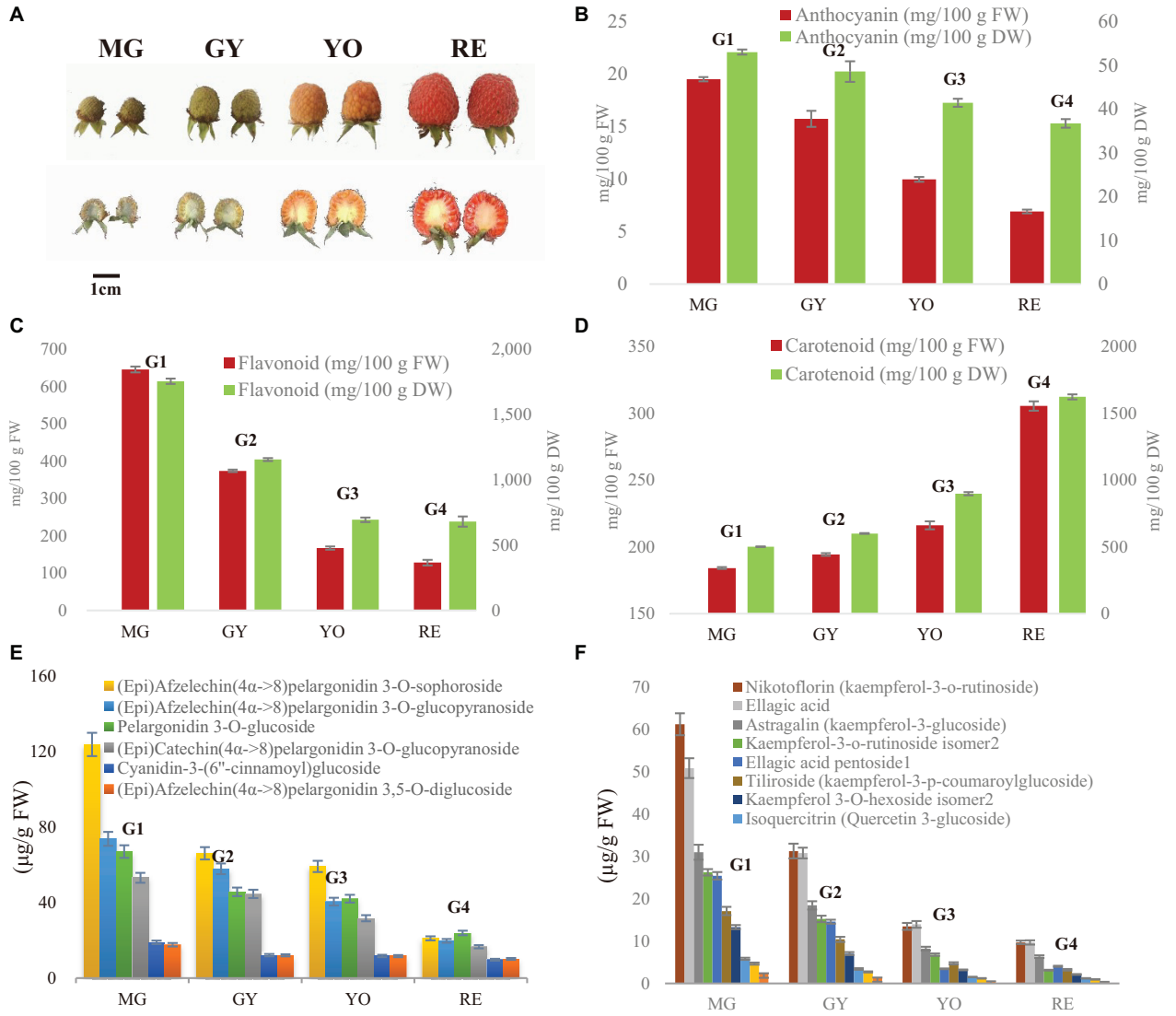

FIGURE 1 | Dynamic change of fruit appearance, anthocyanins and flavonoids during maturation in *R. chingii*. **(A)** Fruit appearance, **(B)** total carotenoid, **(C)** total anthocyanin (expressed as cyanidin 3-glucoside equivalents), **(D)** total flavonoids. The four typic phases of fruit maturation are MG, mature green; GY, green yellow; YO, yellow orange; RE, red. **(E)** Content of flavanol-anthocyanins. **(F)** Content of other flavonoids. Test of between-phases effects: significant ($P < 0.05$); error bar indicates standard deviation (SD); homogeneous subsets in Duncan's Multiple Range test: G1, G2, G3, and G4.

The fresh fruits were separated into several parts and then embedded in medium (SCEM, Section-Lab, Hiroshima, Japan). The surface of tissues was completely covered with adhesive medium, and then immediately frozen at −20°C. The frozen samples embedded in medium were trimmed and then carefully sliced to produce 50–80 μm fresh-frozen sections using a CM1850 Cryostat (Leica microsystems, Wetzlar, Germany) set at −20°C. The sample sections were stained in a freshly prepared aqueous solution of 0.25% (w/v) 2-aminoethyl diphenylborate (DPBA) (Tokyo Chemical industry, Tokyo, Japan) and 0.00375% (v/v) Triton X-100 (Sigma-Aldrich, Shanghai, China) for at least 15 min. A Zeiss LSM880 confocal laser scanning microscope (Carl Zeiss AG, Jena, Germany) excited the sample with 30% maximum laser power at 458 nm. The fluorescence was scanned at 475–504 nm for kaempferol derivates while at 577–619 nm for quercetin derivates (Lewis et al., 2011).

Total RNA Extraction, Library Construction, and Bioinformatic Analysis

Fruit RNAs were extracted by CTAB method (Gambino et al., 2008). Extraction buffer (2% CTAB, 2.5% PVP-40, 2 M NaCl, 100 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0, and 2% of β-mercaptoethanol was added just before use) was prepared at 65°C in a microcentrifuge tube. The fruit tissue powder (150 mg) was added to 900 μL extraction buffer and incubated at 65°C for 10 min. An equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added, vortexed, and then

centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was added to chloroform:isoamyl alcohol and transferred to a new microcentrifuge tube. LiCl (3 M final concentration) was added and incubated in ice for 30 min. RNA was selectively pelleted after centrifugation at 20,000 *g* for 20 min at 4°C. The pellet was resuspended in 500 μL of SSTE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0, 1% SDS, and 1 M NaCl) pre-heated at 65°C. An equal volume of chloroform:isoamyl alcohol was added, mixed and then centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was transfered to a new microcentrifuge tube and the RNA was precipitated with 0.5 volume of cold isopropanol and immediately centrifuged at 20,000 *g* for 10 min at 4°C. The pellet was washed with ethanol (70%), dried and resuspended in DEPC-water. RNA quality was tested with an Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit) (Agilent, Santa Clara, CA, United States) for RNA concentration, and their purity was determined using a NanoDrop™ (Thermo Fisher Scientific, Wilmington, DE, United States).

The libraries construction followed the method described in Li et al. (2013). The mRNAs were isolated from total RNA with oligo(dT) and then fragmented. The first and second strand of cDNA were synthesized, purified and resolved with EB buffer for end repair and adenine (A) addition. After that, the cDNA fragments were connected with adapters and those with suitable size were PCR amplified. Agilent 2100 Bioanaylzer and ABI StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Rockford, IL, United States) were used to quantify and qualify the libraries.

The read data were processed following the procedure (Li et al., 2013). The low-quality reads (>20% of the bases with low quality < 10) and reads with adaptors and unknown bases (N > 5%) were filtered to get clean reads. The clean reads were assembled into unigenes using Trinity, for functional annotation and expression estimation. Data are available via NCBI with accession (PRJNA671545). The relative expression was estimated by Fragments Per Kilobase of transcript per Million mapped reads (FPKM). Based on the relative expression, differential expressed unigenes were defined by threshold (fold Change > 2.00 or <0.5; adjusted P-value < 0.05) and they were subjected to pathway enrichment (FDR < 0.01 are defined as significant enrichment).

Real-Time Quantitative PCR Assay

The expressions of gene involved in flavonoid biosynthesis was determined by qPCR (Li et al., 2015). The reverse transcription reaction was performed with M-MLV (Takara, China), and the reverse-transcribed products were used as the template for qPCR with gene-specific primers. All reactions were assayed in three biological and technical replications, and performed in an ABI PRISM 7900HT (Applied Biosystems, United States) using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, United States). PCR conditions consisted of: pre-denaturation and hot start Taq activation at 95°C for 5 min, then 40 cycles of 95°C for 15 s, and 60°C for 30 s. Actin was used as reference gene. The relative expression was calculated on the basis of $2^{-\Delta\Delta Ct}$.

Protein Extraction, HPLC Fractionation, LC-MS/MS Assay and Bioinformatic Analysis

Fruit proteins were extracted by the method described by Li et al. (2019, 2020). The ground fruit tissue was mixed with four volumes of lysis buffer (8 M urea, 1% Triton-100, 10 mM dithiothreitol, and 1% Protease Inhibitor Cocktail), followed by sonication three times on ice. Debris were removed by centrifugation at 20,000 *g* at 4°C for 10 min. Protein was precipitated with cold 20% trichloroacetic acid (TCA) for 2 h at −20°C and then centrifugated at 12,000 *g* 4°C for 10 min. After the supernatant was discarded, the remaining precipitate was washed three times with cold acetone. The protein was re-dissolved in 8 M urea and the protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific, Wilmington, DE, United States) according to the manufacturer's instructions. The extracted proteins were reduced and alkylated, and then digested by trypsin.

After trypsin digestion, peptide was desalted by Strata X C18 SPE column (Phenomenex, Tianjin, China) and vacuum-dried. Peptide was reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for TMT kit. One unit of TMT reagent was thawed at room temperature and reconstituted in anhydrous acetonitrile (enough for about 100 μg protein). 41 μL of the TMT Label Reagent was added to each 100 μL sample and samples were labeled with different TMT tags. The peptide mixtures were then incubated for 2 h at room temperature, pooled, desalted and dried by vacuum centrifugation.

The labeled peptides were fractionated by HPLC, and the peptides were divided into nine fractions. The peptides were loaded into tandem mass spectrometry (MS/MS), Q Exactive HF-X™ (Thermo Fisher Scientific, Rockford, IL, United States). These processes were performed as described by Li et al. (2019, 2020). The relative expression of protein was estimated by comparing the intensities of the reporter ions. Compared to the expression profile at the MG phase, differential expressed proteins were defined by threshold change (change fold > 1.5 or <0.67 and P < 0.05).

The resulting MS/MS data were processed using the MaxQuant search engine (v.1.5.2.8). Tandem mass spectra were searched against a local database of *R. chingii* transcriptome and concatenated with a reverse decoy database. Trypsin/P was used as a cleavage enzyme allowing up to 2 missing cleavages. The mass tolerance for precursor ions was set as 20 ppm in the first search and 5 ppm in the main search and the mass tolerance for fragment ions was set as 0.02 Da. Carbamidomethyl on Cys was specified as fixed modification and oxidation on Met were specified as variable modifications. FDR was adjusted to <1%. Data are available via ProteomeXchange with identifier (PXD021977).

Analysis of Major Anthocyanins and Flavonoids

Anthocyanins were extracted with 1% (v/v) HCl methanol and concentrated by CentriVap refrigerated Centrifugal

Concentrators at 8°C (Models 73100 Series) (Labconco, Kansas City, MO, United States) and then re-dissolved in 1 mL 1% (v/v) HCl methanol. Flavonoids were extracted with 70% methanol for 2 h at room temperature in the dark, concentrated with refrigerated Centrifugal Concentrators at 8°C (Labconco Models 73100 Series) and then re-dissolved in 1 mL 70% methanol. The extract was passed through a 0.22-μm microporous membrane filter for LC-ESI-MS analysis.

For anthocyanins, the mobile phases were 1% formic acid-water (A) and acetonitrile (B). Gradient conditions were as follows: 0–25 min, 5–35% phase B; 25–37 min; 35–95% phase B. The loading volume was 5 μL, the flow rate was 0.4 mL min^{-1}; the column temperature was 50°C, and the UV detector was set at 530 nm. For flavonoids, the mobile phases were 0.1% formic acid-water (A) and 0.1% formic acid-acetonitrile (B). The linear gradient programs were as follows, 0–5 min, 5–10% phase B; 5–25 min, 10–25% phase B; 25–37 min, 25–95% phase B; Sample injection volume was 5 μL; Column oven temperature was 50°C; flow rate was 0.3 mL min^{-1}; and the UV detector was set at 360 nm. Anthocyanins and flavonoids separated by UPLC were analyzed using a MS AB Triple TOF 5600^{plus} System (AB SCIEX, Framingham, MA, United States) in both negative ion (source voltage at −4.5 kV, and source temperature at 550°C) and positive ion mode (source voltage at +5.5 kV, and source temperature at 600°C). Maximum allowed error was set to ±5 ppm. Declustering potential (DP), 100 V; collision energy (CE), 10 V. For MS/MS acquisition mode, the parameters were almost the same except that the collision energy (CE) was set at 40 ± 20 V, ion release delay (IRD) at 67 and the ion release width (IRW) at 25. The IDA-based auto-MS^2 was performed on the 8 most intense metabolite ions in a cycle of full scan (1 s). The scan range of m/z of precursor ion and product ion were set as 100–2,000 Da and 50–2,000 Da. The exact mass calibration was performed automatically before each analysis employing the Automated Calibration Delivery System.

Statistical Analysis

The averages and standard deviations were calculated in IBM SPSS Statistics 22. The treatments were compared using a two-way analysis of variance, *T*-test and Duncan multiple Test in IBM SPSS Statistics 22. Unless otherwise stated, significant differences were set at the threshold ($p < 0.05$). KEGG enrichment of proteins were determined by a two-tailed Fisher's exact test. The significant threshold was set up (p-value < 0.05) for KEGG enrichment.

RESULTS

Anthocyanins and Flavonoids Composition, Anatomical Structure and Flavonoid Staining

In our previous study on *R. chingii,* anthocyanin unexpectedly decreased as fruit matured, as well as flavonoids, while carotenoids increased (Li et al., 2021a) (**Figures 1B–D**). Anthocyanins were mainly flavanol-anthocyanins consisting of two flavonoid units (Li et al., 2021a) (**Supplementary Figure 1**). Other flavonoids consisted primarily of glycosides of quercetin and kaempferol. These anthocyanins and flavonoids all significantly decreased in content during fruit ripening (**Figures 1E,F**). In this study, anthocyanin and flavonoid showed a similar trend of decreasing during fruit maturation (based on dry weight), while carotenoids increased (**Figures 1B–D**). The water content continuously increased as fruit maturated (**Table 1**). This suggests that decreases of anthocyanin and flavonoid during fruit ripening is probably due to down-regulation of their biosynthesis rather than an increase of water content.

A raspberry fruit is an aggregate fruit composed of drupelets (Li et al., 2021a,b) (**Figure 2**). Each drupelet contains the pericarp and seed. The pericarp is made up of the exocarp, hypodermis, and mesocarp layers; while the seed consists of the episperm, endosperm, and embryo. The exocarp is attached with a layer of epidermal hair and the seed is surrounded by placentae. In fruit cross-sections, DPBA fluorescence showed flavonoid accumulation patterns at various stages of fruit maturation (**Figure 2B**). Flavonol-specific fluorescence was mainly observed in the fruit epidermal hair throughout the entire fruit maturation process, but rarely in fruit flesh including the exocarp, hypodermis and mesocarp (**Figures 2F–M**). As fruit matured, fruit epidermal hair became shorter and thinner. In addition, flavonol-specific fluorescence was seen in the placentae and seed coats of developing seed (**Figures 2C–F**) and the receptacle enlarged, which made up a larger proportion of the fruit than drupelets (including placentae and seed coats). Thus, the proportion of epidermal hairs and placentae (containing most of the flavonols) decrease with the maturity of the fruit, which is probably one of main reasons for a decrease in the content of flavonoids per unit mass.

Profiling of Genes and Proteins Involved in Flavonoid Synthesis

Twelve transcriptomics were developed for MG, GY, YO, and RE fruits (three replicates for each fruit phase). A total of 89,188 unigenes were obtained, and 49,755 (55.79%) and 37,833 (42.42%) were annotated in the non-redundant and KEGG database, respectively. The biggest difference in gene expression

TABLE 1 | Change of water content during fruit ripening.

Phase	*Fresh weight (g) ± *SD*	*Dry weight (g) ± *SD*	*Water content% ± *SD*
MG	$1.06^{a} \pm 0.06$	$0.39^{a} \pm 0.02$	$63.67^{a} \pm 0.27$
GY	$1.39^{b} \pm 0.08$	$0.45^{b} \pm 0.02$	$67.63^{b} \pm 0.35$
YO	$1.91^{c} \pm 0.10$	$0.46^{c} \pm 0.03$	$75.92^{c} \pm 0.25$
RE	$4.78^{d} \pm 0.15$	$0.90^{d} \pm 0.06$	$81.11^{d} \pm 0.54$

MG, mature green; GY, green yellow; YO, yellow orange; RE, red.
DW, dry weight; FW, fresh weight.
**Significant ($P < 0.05$) in test of between-phases effects: Error bar indicates standard deviation (SD); Homogeneous subsets in Duncan's Multiple Range test: a, b, c, and d.*

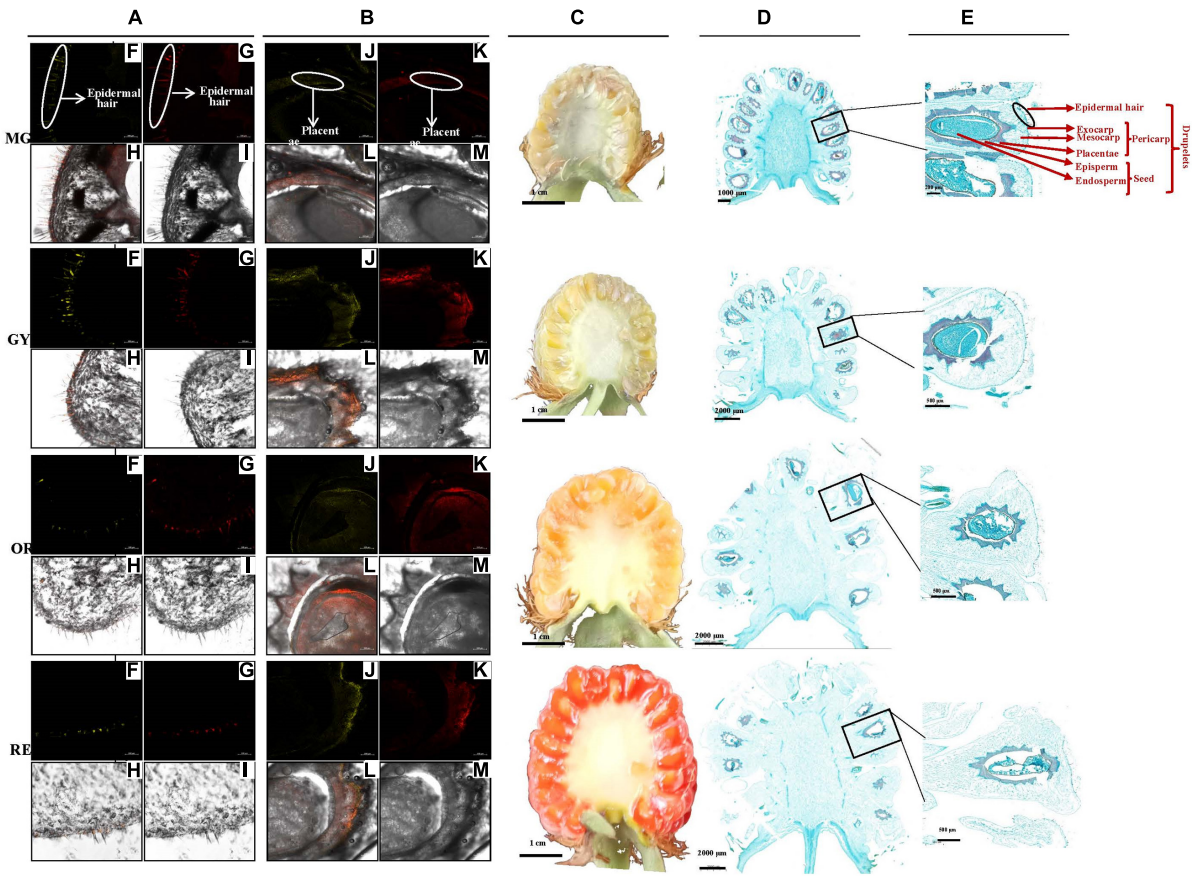

FIGURE 2 | Fruit section and *in situ* flavonoid staining at four maturation phases (MG to GY, YO, and RE) (Li et al., 2021a,b). **(A,B)** Pericarp and seed radial sections by frozen method. **(C)** Fruit radial sections by scalpel and **(D,E)** by paraffin method. Fluorescence was collected at **(F,J)** 475–504 nm for kaempferol and **(G,K)** 577–619 nm for quercetin after fresh-fruit section were stained with diphenylboric acid 2-aminoethylester (DPBA). **(H,L)** Flavonoid localization in inflorescences combing **(F,J)** and **(G,K)**, and **(I,M)** original figure without fluorescence. Flavonoid mainly accumulated in epidermal hair and placentae. The proportion of different tissues gradually changed with the maturity of the fruit.

was between RE/MG (6,502 up-regulated and 5733 down-regulated unigenes) while the smallest difference was between GY/MG (1,965 up-regulated and 1,966 down-regulated unigenes) (**Supplementary Figure 2A**). Accordingly, in twelve proteomes of MG, GY, YO, and RE fruits, 141,036 unique peptides corresponding to 9,478 proteins, and 8,529 quantified proteins were identified. In proteomics, 506 up-regulated and 618 down-regulated proteins were observed between RE/MG while 765 up-regulated and 799 down-regulated proteins were between GY/MG (**Supplementary Figure 2B**). The results suggest that the biggest difference is between RE and MG while the smallest difference is between GY and MG.

Generally, flavonoid products are involved in four pathways, i.e., phenylpropanoid biosynthesis, flavonoid biosynthesis, flavone and flavonol biosynthesis, and anthocyanin biosynthesis. KEGG enrichment was performed to discern the multivariate pattern of up- and down-regulated unigenes/proteins. The unigenes involved in "phenylpropanoid biosynthesis" and "flavonoid biosynthesis" were significantly enriched in GY/MG, YO/MG, and RE/MG and most of them were down-regulated (**Supplementary Figure 3A**). Accordingly, the down-regulated proteins involved in these pathways' biosynthesis were enriched as well (**Supplementary Figure 3B**). However, neither up-regulated or down-regulated unigenes/proteins were enriched in "flavone and flavonol", or "anthocyanin" biosynthesis (**Supplementary Figure 3**). This suggests that "phenylpropanoid biosynthesis" and "flavonoid biosynthesis" are more active in green phases than the other three phases, and responsible for biosynthesis of major flavonoid products during fruit maturation.

Differentially Expressed Genes and Proteins in Flavonoid and Phenylpropanoid Biosynthesis

In phenylpropanoid biosynthesis, two Phe ammonia lyase (PAL) homologs, RchPAL (Unigene4740 and Unigene4485), were phylogenetically grouped together (**Figure 3A**) and significantly down-regulated at the gene/protein level during maturation (**Table 2**). Rch4CL and Rch4CL-like homologs were phylogenetically grouped into two branches, respectively (**Figure 3B**). The 4-coumaroyl-CoA synthase (4CL) homologs were significantly down-regulated at the gene/protein level

during maturation. Rch4CL-like homologs (CL7730.Contig2, CL3087.Contig1, and 8828.Contig1) had low gene expression and their proteins were not detected (**Table 2**). Two cinnamate-4-hydroxylase (C4H) homologs, RchC4H (Unigene9842, and Unigene12468), were phylogenetically grouped into two different branches, i.e., C4H1 and C4H2, respectively (**Figure 3C**), but they had a trend of down-regulation at the gene/protein level during maturation (**Table 2**). Chalcone synthase (CHS) homologs were grouped together (**Figure 3D**) and down-regulated at the gene/protein level during maturation (**Table 2**). These up-stream genes/proteins were down-regulated to decrease conversion from phenylpropanoids to flavonoids (**Figures 4A,B**).

In flavonoid biosynthesis, chalcone isomerase (CHI) homologs were phylogenetically separated into two different branches, i.e., CHI1 and CHI2, respectively (**Figure 3E**), and both of them were down-regulated at the gene/protein level during maturation (**Table 2**). Five bifunctional dihydroflavonol 4-Reductase/flavanone 4-Reductase (DFR) unigenes, belonging to one DFR homolog, were phylogenetically grouped into the DFR branch (**Figure 3F**) and significantly down-regulated during maturation (their proteins were not detected) (**Table 2**). Flavanone-3β-hydroxylase (F3H) and flavonoid-3′-hydroxylase (F3′H) homologs were phylogenetically grouped into the F3H and F3′H branch, respectively, but neither flavonoid-3′,5′-hydroxylase (F3′5′H) unigene nor protein was detected (**Figure 3G**). RchF3H (CL7001.Contig2) was significantly down-regulated at the gene/protein level during maturation, while the RchF3′H (Unigene19522) unigene was maintained at a low level and its protein was not detected (**Table 2**). Flavonol synthase (FLS) homologs were, respectively, separated into FLS and FLS-like groups (**Figure 3H**). The FLS-like group was represented by FLS paralogs without a conserved functional domain (**Figure 3L**). FLS-like homologs were all maintained at low gene expression and none of their proteins were detected. However, RchFLS (Unigene22291) showed a decreasing trend of gene expression but remained expressed at low levels (**Table 2**). These genes/proteins were down-regulated, reducing the biosynthesis of flavone, flavonol, and their derivates (**Figures 4A,B**). Notably, a deficiency of RchF3′5 ′H blocked the conversion from dihydroquercetin to dihydromyricetin, resulting in the absence of leucodelphinidin, myricetin, gallocatechin, and delphinidin glycoside.

In anthocyanin biosynthesis, the leucoanthocyanidin dioxygenase (ANS/LODX) homolog, RchANS/LODX (Unigene7480), was maintained at low gene expression levels and its protein was not detected (**Table 2**). Leucoanthocyanidin reductase (LAR), RchLAR (CL9527.Contig3), and anthocyanidin reductase (ANR), RchANR2 (Unigene7245), were both significantly down-regulated at the gene/protein level during maturation (**Table 2** and **Figures 3I,J**). In contrast, RchANR3 (Unigene17647 and Unigene27757) had very low gene expression and their corresponding proteins were not detected. Three main class of glucosyltransferase were identified, i.e., anthocyanidin 3-*O*-glucosyltransferase (BZ1/UGT78D), anthocyanidin 3-*O*-glucoside 2″-*O*-glucosyltransferase (3GGT/UGT79) and anthocyanidin 5,3-*O* glucosyltransferase (GT1). A phylogenetic tree grouped these glucosyltransferase homologs into three main branches (**Figure 3K**). Two homologs, BZ1/UGT78D-1 and BZ1/UGT78D-2, had different patterns of expression (**Table 2**). RchBZ1/UGT78D-1 (Unigene7678 and Unigene22174) were significantly down-regulated at the gene/protein level as fruit matured, while RchBZ1/UGT78D-2 (Unigene7056) remained constantly expressed at both the gene/protein level and the RchBZ1/UGT78D-2 (CL3164.Contig1) gene/protein was slightly up-regulated. There were three homologs for each Rch3GGT/UGT79 and RchGT1, but they had relatively low expression and did not show a clear trend of change at the gene/protein level. The consistently low level of expression of RchANS suggests a relatively low concentration of anthocyanins. Notably, the genes/proteins of RchLAR and RchANR2 were highly expressed in unripe fruit, resulting in the relative abundance of flavan-3-ols, i.e., (epi) catechin and (epi) afzelechin (**Figures 4A,B**). These flavan-3-ols interacted with each other or with cyanin/pelargonin to generate proanthocyanins or dimeric anthocyanins, respectively.

In conclusion, most of the differently expressed unigenes and proteins in these pathways shared a similar trend of change in expression, which was consistent with the high correlations seen between them (Pearson correlation = 0.956). Additionally, the expression of gene and proteins was validated by qPCR (**Figure 5** and **Supplementary Table 1**). However, the phylogenetically different homologs showed different patterns of gene/protein expression. The results suggest the changes observed in gene expression are consistent with those seen in protein expression, and the homologs are divergent in function and expression.

DISCUSSION

Changes in Flavonoids and Their Localization During Fruit Maturation

Unexpectedly, the total anthocyanins and flavonoids both showed a continuous decrease during the fruit maturation process in *R. chingii*. This pattern was different from any previous report in *Rubus* species including red and black raspberry. In red raspberry, anthocyanin concentration continuously increases throughout fruit ripening, but the flavonoid concentration decreases before veraison stage, and then increases until maturity (Wang et al., 2009). Cyanidin glycosides are the most prominent kind of anthocyanins while quercetin glycosides are constantly present at low concentrations (Stavang et al., 2015). In black raspberry, quercetin glycosides and cyanidin glycosides both increase during ripening, while the content of flavanols and proanthocyanidin dimers decrease (Hyun et al., 2014). Ubiquitously, anthocyanins increase while flavonoids first decrease and then increase during maturation in many berries (Vvedenskaya and Vorsa, 2004; Giribaldi et al., 2007; Song et al., 2015; Li et al., 2019). The increased flavonoids after veraison is mainly due to the substantial increase of anthocyanin concentration (Li et al., 2019). However, the increased anthocyanins were not observed during fruit ripening in *R. chingii*. The continuous down-regulation of anthocyanins was responsible for the continuous decrease of total flavonoids.

TABLE 2 | The temporal change in expression of mRNA unigenes an proteins involved in phenylpropanoid, flavonoid, and anthocyanin biosynthesis during four maturation phases in *Rubus chingii* Hu.

	mRNA unigenes				Proteins			
	MG	GY	YO	RE	MG	GY	YO	RE
	Mean ± *SD*	Mean ± *SD*	Mean ± *SD*	Mean ± *SD*	Mean ± *SD*	Mean ± *SD*	Mean ± SD	Mean ± SD
RchPAL(Unigene4740)	1448 ± 38.35	372.9 ± 21.61*	82.76 ± 2.74*	43.93 ± 1.25*	1.79 ± 0.04	0.84 ± 0.02*	0.64 ± 0.01*	0.52 ± 0.01*
RchPAL(Unigene4485)	261.8 ± 2.12	52.63 ± 6.36*	9.16 ± 1.74*	3.25 ± 0.2*	2.05 ± 0.13	0.54 ± 0.04*	0.56 ± 0.01*	0.56 ± 0.04*
Rch4CL(CL2617.Contig1)	193.5 ± 4.78	49.16 ± 3.54*	31.24 ± 1.51*	30.05 ± 1.34*	1.85 ± 0.04	0.67 ± 0.05*	0.67 ± 0.04*	0.64 ± 0.02*
Rch4CL(CL6300.Contig1)	264 ± 4.91	138.9 ± 2.67*	74.06 ± 1.42*	81.89 ± 1.34*				
Rch4CL(CL6300.Contig2)	235.5 ± 11.2	143.7 ± 10.47*	70.4 ± 2.52*	67.14 ± 3.21*	1.41 ± 0.03	0.96 ± 0.01*	0.77 ± 0.01*	0.82 ± 0.01*
Rch4CL(CL8627.Contig1)	263.4 ± 4.6	143.2 ± 2.01*	20.65 ± 1.35*	1.75 ± 0.43*	1.71 ± 0.04	0.92 ± 0.01*	0.67 ± 0.01*	0.48 ± 0.04*
Rch4CL-like(CL7730.Contig2)	3.65 ± 0.48	1.14 ± 0.24*	0.42 ± 0.12*	0.04 ± 0.04*				
Rch4CL-like(CL3087.Contig1)	1.82 ± 0.08	6.02 ± 0.32*	5.49 ± 0.49*	0.86 ± 0.24*				
Rch4CL-like(8828.Contig1)	3.28 ± 0.19	3.85 ± 0.62	4 ± 0.17	6.62 ± 0.85				
RchC4H(Unigene9842)	692.5 ± 12.81	284.2 ± 10.64*	124.2 ± 3.25*	338.8 ± 6.83*	1.64 ± 0.02	0.83 ± 0.01*	0.71 ± 0.01*	0.67 ± 0.01*
RchC4H(Unigene12468)	274.9 ± 25.84	109.3 ± 6.21*	38 ± 0.76*	7.18 ± 0.53*	1.51 ± 0.01	1.36 ± 0.12*	1.07 ± 0.03*	0.67 ± 0.09*
RchCHS(CL6140.Contig1)	740.4 ± 56.75	43.19 ± 7.71*	21.58 ± 1.69*	13.36 ± 0.73*				
RchCHS(CL6140.Contig2)	234.5 ± 36.02	18.67 ± 7.56*	4.24 ± 0.22*	1.69 ± 1.24*				
RchCHS(CL6140.Contig4)	238.6 ± 26.79	25.21 ± 3.14*	4.08 ± 0.82*	1.61 ± 0.7*				
RchCHS(CL6140.Contig5)	938.4 ± 87.82	68.39 ± 19.58*	21.93 ± 1.95*	12.82 ± 0.19*	2.01 ± 0.06	0.55 ± 0.02*	0.61 ± 0.03*	0.55 ± 0.04*
RchCHI(Unigene14858)	293 ± 3.94	69.41 ± 3.83*	37.66 ± 0.29*	48.17 ± 1.53*	1.82 ± 0.02	0.69 ± 0.02*	0.67 ± 0.01*	0.57 ± 0.01*
RchCHI(Unigene22344)	100.8 ± 1.58	22.08 ± 0.32*	6.96 ± 0.66*	2.18 ± 0.31*	2.66 ± 0.04	0.30 ± 0.01*	0.27 ± 0.03*	0.28 ± 0.00*
RchDFR(Unigene24396)	116.2 ± 6.36	15.79 ± 5.04*	8.31 ± 0.48*	4.45 ± 0.06*				
RchDFR(CL2475.Contig1)	56.67 ± 2.72	9.29 ± 1.79*	4.39 ± 0.7*	1.26 ± 0.03*				
RchDFR(CL2475.Contig4)	41.22 ± 2.74	4.93 ± 0.88*	3.12 ± 0.05*	0.99 ± 0.13*				
RchDFR(CL2475.Contig5)	50.94 ± 0.36	3.44 ± 0.22*	0.59 ± 0.13*	0.36 ± 0.11*				
RchDFR(CL2475.Contig7)	53.15 ± 1.39	2.54 ± 0.18*	0.52 ± 0.08*	0.11 ± 0.05*				
RchF3H(CL7001.Contig2)	367.1 ± 2.13	136 ± 14.02*	48.14 ± 3.59*	9.98 ± 0.74*	1.92 ± 0.05	0.69 ± 0.02	0.62 ± 0.02	0.50 ± 0.03
RchF3′H(Unigene19522)	10.76 ± 0.7	1.67 ± 0.63*	0.15 ± 0*	2.53 ± 0.28*				
RchFLS(Unigene22291)	8.17 ± 1.6	1.98 ± 0.69*	0.57 ± 0.29*	1.07 ± 0.23*				
RchFLS-like(Unigene52413)	2.96 ± 0.16	5.17 ± 0.66	5.44 ± 3.03	3.37 ± 0.18				
RchFLS-like(Unigene52414)	4.99 ± 0.78	5.03 ± 0.17	8.87 ± 2.34	6.96 ± 0.54				
RchFLS-like(CL3345.Contig2)	3.56 ± 0.56	3.95 ± 0.05	5.89 ± 0.35	4.63 ± 0.72				
RchANR2(Unigene7245)	27.39 ± 3.01	6.86 ± 2.01*	3.39 ± 0.05*	2.67 ± 0.65*	1.81 ± 0.08	0.71 ± 0.05*	0.64 ± 0.03*	0.65 ± 0.02*
RchANR3(Unigene17647)	0.1 ± 0.1	1.47 ± 0.06*	1.68 ± 0.02*	0.06 ± 0.06				
RchANR3(Unigene27757)	0 ± 0	0 ± 0	0.43 ± 0.43	0 ± 0				
RchLAR(CL9527.Contig2)	0.36 ± 0.36	1.01 ± 0.58	0.12 ± 0.12	0 ± 0				
RchLAR(CL9527.Contig3)	93.98 ± 10.29	3.06 ± 1.18*	1.53 ± 0.17*	0.41 ± 0.06*	2.28 ± 0.34	0.55 ± 0.12*	0.42 ± 0.15*	0.36 ± 0.13*
RchANS/LODX(Unigene7480)	0.79 ± 0.2	3.41 ± 0.21*	0 ± 0	5.82 ± 0.49*				
RchBZ1/UGT78D-1(Unigene7678)	43.82 ± 4.66	4.17 ± 0.65*	0.12 ± 0.06*	0.03 ± 0.03*	1.87 ± 0.12	0.73 ± 0.07	0.61 ± 0.03*	0.59 ± 0.08*
RchBZ1/UGT78D-1(Unigene22174)	32.5 ± 2.41	4.21 ± 0*	0.84 ± 0.09*	0.11 ± 0*	1.95 ± 0.05	0.79 ± 0.03*	0.62 ± 0.03*	0.46 ± 0.05*
RchBZ1/UGT78D-2(Unigene7056)	2.79 ± 0.01	3.52 ± 0.05	3.9 ± 0.36	0.3 ± 0.03	1.10 ± 0.02	1.14 ± 0.09	1.00 ± 0.03	0.84 ± 0.03
RchBZ1/UGT78D-2(CL3164.Contig1)	8.11 ± 0.48	12.61 ± 0.73	22.92 ± 1.19*	16.59 ± 0.19*	0.81 ± 0.09	1.05 ± 0.05	1.25 ± 0.10*	1.02 ± 0.06
Rch3GGT/UGT79B(CL2207.Contig1)	0.18 ± 0	0.55 ± 0.32	0.09 ± 0.09	0.05 ± 0.05				
Rch3GGT/UGT79B(CL2207.Contig2)	2.92 ± 0.47	4.83 ± 1.47	1.4 ± 0.06	0.01 ± 0.01				
Rch3GGT/UGT79B(CL2207.Contig3)	6.2 ± 0.38	6.46 ± 0.47	5.06 ± 0.13	0.42 ± 0.24	1.09 ± 0.08	1.22 ± 0.04	0.94 ± 0.08	0.81 ± 0.04
Rch3GGT/UGT79B(CL2207.Contig4)	0.72 ± 0.01	1.41 ± 0.04	0.57 ± 0.02	0.13 ± 0.08				
Rch3GGT/UGT79B(CL2207.Contig5)	0.8 ± 0.15	0.35 ± 0.14	0.79 ± 0.14	0.88 ± 0.05				
Rch3GGT/UGT79B(Unigene4033)	0.97 ± 0.21	1.11 ± 0.23	0.25 ± 0.09	0.49 ± 0.16				
Rch3GGT/UGT79B(Unigene4034)	1.68 ± 0.04	2.71 ± 0.59	1.17 ± 0.01	0.23 ± 0.08				
RchGT1(CL10466.Contig1)	7.05 ± 0.11	6.82 ± 0.59	4.53 ± 1.22	0.6 ± 0.45				
RchGT1(CL10466.Contig3)	9.96 ± 0.72	7.4 ± 0.54	6.96 ± 0.42	9.9 ± 1.47				
RchGT1(CL1924.Contig1)	21.29 ± 0.12	68.02 ± 5.49*	86.88 ± 2.18*	53.47 ± 2.1*	0.77 ± 0.03	1.10 ± 0.03	1.13 ± 0.04	1.10 ± 0.01
RchGT1(CL1924.Contig3)	2.08 ± 0.05	1.4 ± 0.23	0.58 ± 0.06	1.59 ± 0.09				
RchGT1(Unigene268)	1.89 ± 0.05	1.07 ± 0.25	1.15 ± 0.25	0.82 ± 0.09				

**Significant difference with fold Change > 2.00 or <0.5 and adjusted P-value < 0.05 for unigenes; with fold > 1.5 or <0.67 and P < 0.05 for proteins. Different homologs were designated with different serial numbers, e.g., two homologs, RcPAL(Unigene4740) and RcPAL(Unigene4485).*

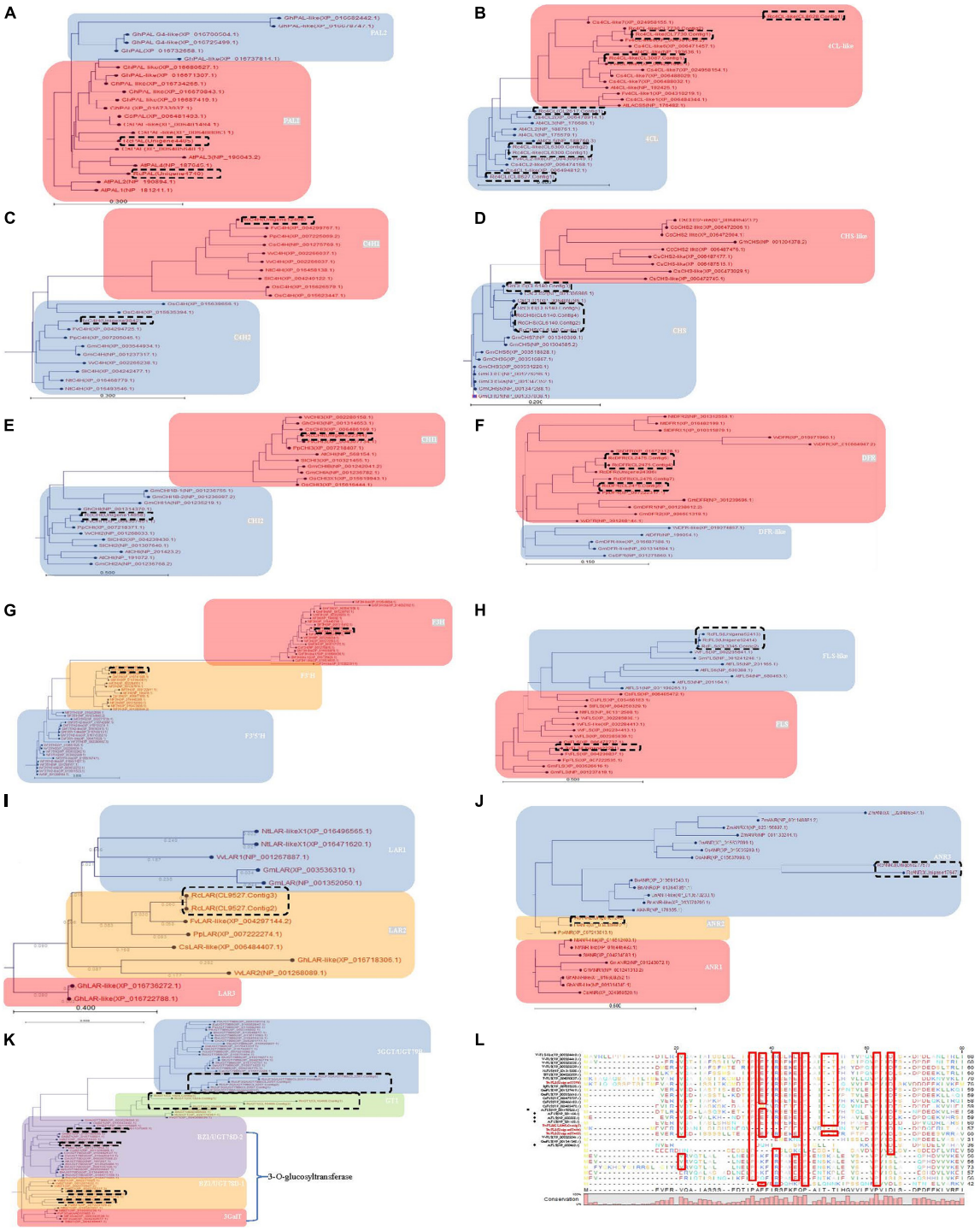

FIGURE 3 | Phylogenetic analysis of the differently expressed genes involved in phenylpropanoid, flavonoid and anthocyanin biosynthesis during four maturation phases in *R. chingii*. Neighbor-Joining with 1,000 bootstrap replicates was used to construct the phylogenetic tree. The distance between deduced proteins was measured with the "Kimura Protein" method. **(A)** Phe ammonia lyase (PAL), **(B)** 4-coumaroyl-CoA synthase (4CL), **(C)** cinnamate-4-hydroxylase (C4H), **(D)** chalcone synthase (CHS), **(E)** chalcone isomerase (CHI), **(F)** bifunctional dihydroflavonol 4-Reductase/flavanone 4-Reductase (DFR), **(G)** flavanone-3β-hydroxylase (F3H), flavonoid-3′-hydroxylase (F3′H), flavonoid-3′,5′-hydroxylase (F3′5′H), **(H)** flavonol synthase (FLS), **(I)** leucoanthocyanidin reductase (LAR) **(J)** anthocyanidin reductase (ANR), **(K)** glucosyltransferase protein. **(L)** alignment of RcFLS in bold with other functionally characterized plant FLS. The residues framed by red boxes are strictly conserved in the various enzyme subclasses. *Arabidopsis* AtFLS (NP_001190266.1) marked by squares having strictly conserved residues while AtFLS (NP_201164.1), AtFLS (NP_680388.1), AtFLS (NP_201165.1), and AtFLS (NP_680463.1) are marked by diamonds and has altered or missing conserved residues. Previous studies indicate that only AtFLS1 (NP_001190266.1) encodes a catalytically competent protein and is the only member of this group that influences flavonoid levels (Owens et al., 2015). Genes in dash-line box were from *R. chingii.* Homologs in gray were down-regulated at the gene/protein level and responsible for the decrease of flavonoids as the fruit matured.

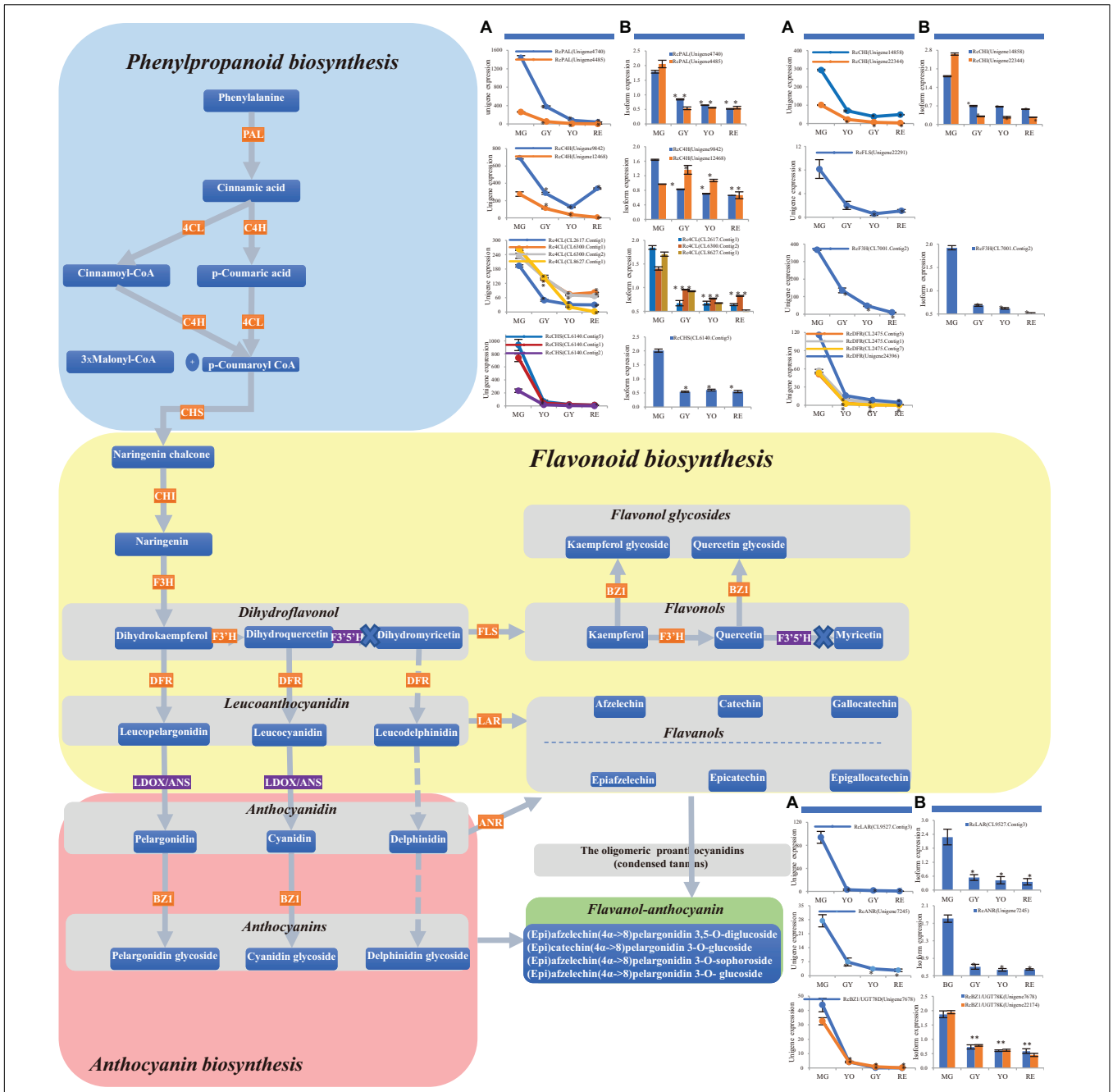

FIGURE 4 | Changes in phenylpropanoid, flavonoid and anthocyanin biosynthesis during four maturation phases in *R. chingii*. **(A)** The change of mRNA unigene expression, **(B)** the change of protein expression. RcANS, shown in the purple rectangle, was expressed at a low gene level while RcF3′5′H was not detected at the gene/protein level. No expression of RcF3′5′H, shown in the purple rectangle, blocked biosynthesis of the dihydromyricetin, leucodelphinidin, (epi) gallocatechin and their corresponding glycosides. Flavanols and anthocyanins were condensed into flavanol-anthocyanins, shown in the green rectangle.

Interestingly, our previous studies showed that the content of total carotenoids increased during fruit ripening (Li et al., 2021a,b). Of them, β-citraurin and its esters, was predominant and quickly accumulated at the late stage of fruit maturation. This indicates that its red coloration is not caused by anthocyanins. β-citraurin is also a color-imparting pigment involved in the reddish color of citrus fruits (Ma et al., 2013). Flavonoid *in situ* staining shows that the flavonoids (i.e., kaempferol and quercetin derivates) predominately accumulate at the same tissues (epidermal hair and placentae) of fruits (Li et al., 2021a). It is highly likely that these flavonoids are synthesized in the cells in which they accumulate. The flavonoids in epidermal hair might function as antioxidants that protect fruit from pests and pathogens, while the ones in seed may function as

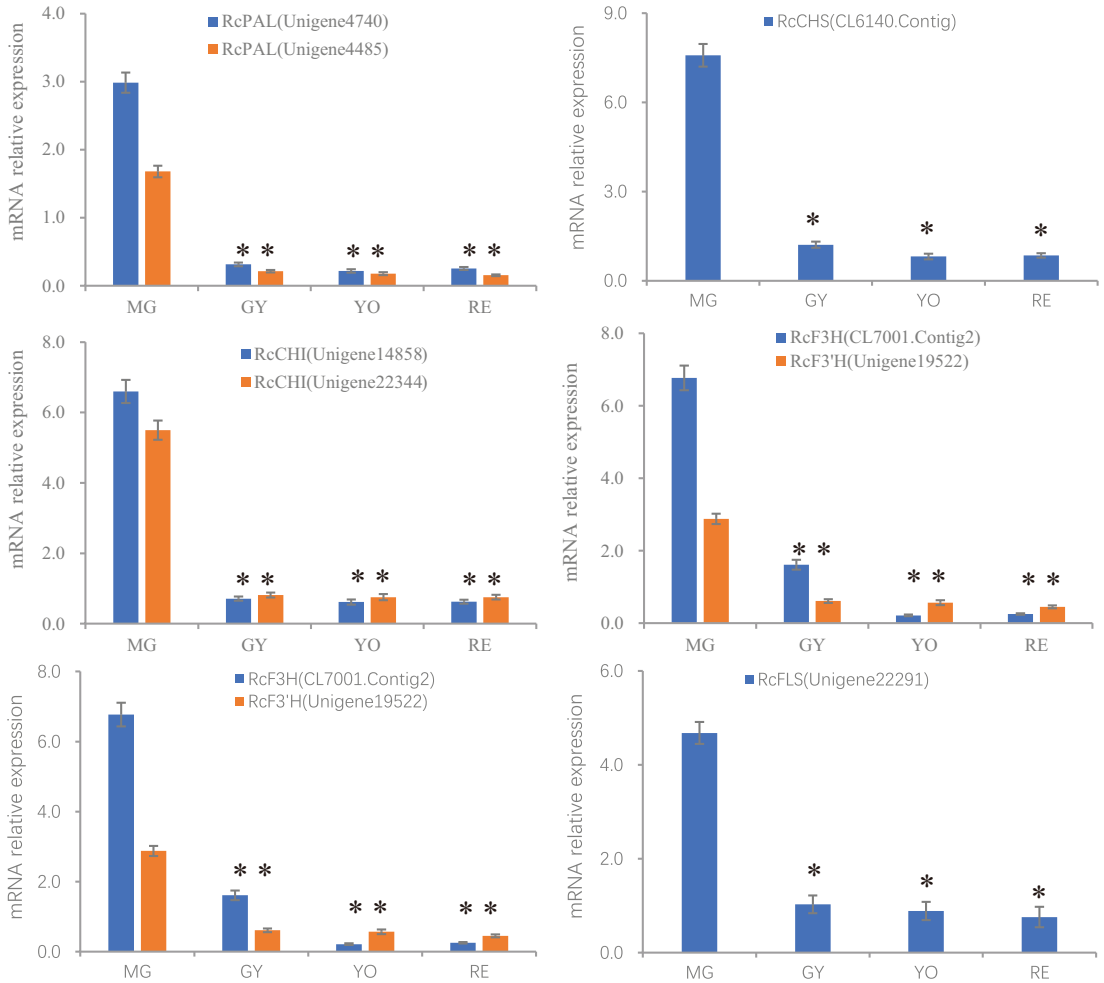

FIGURE 5 | The relative expression of genes (PAL, CHS, CHI, F3H, F3′H, and FLS) involved in flavonoid production during the process of fruit maturation in *R. chingii*. The expression was estimated by real-time PCR. Actin gene was inner control. *indicates comparison between other phases and MG, T-Test, significant ($P < 0.05$).

endogenous regulators of auxin transport that are responsible for seed maturation. As the fruit matured the epidermal hairs became thinner and shorter and many of them fell off (**Figure 3C**) while the receptacle became greatly enlarged makes up a relatively large proportion of the overall fruit (**Figure 3D**). Thus, the proportion of different tissues changes with the maturity of the fruit, which may be one of the main reasons for a decrease in the content of flavonoids per unit mass. Rather than following the regular flavonol pathway in other Rubus (up-regulation during maturation), *R. chingii* utilizes a carotene pathway producing a high level of B-citraurin as its predominant pigment.

Down-Regulated Expression of Genes/Proteins in the Phenylpropanoid Pathway Caused a Decrease in Flux From Phenylpropanoids to Flavonoids

The genes and enzymes involved with phenylpropanoid biosynthesis and the flavonoid biosynthesis have been extensively studied in many plants. Most of these genes are involved in multigene families. Some members are divergent in function and others are redundant or underutilized (Kim et al., 2004). In *Arabidopsis*, two redundant PAL genes (AtPAL1 and AtPAL2) are both expressed in vascular tissues. AtPAL3 is primarily expressed in roots and leaves, albeit at low levels, while AtPAL4 is mainly expressed in developing seed tissue (Raes et al., 2003). These divergent PAL genes respond differentially under various developmental events and environmental stresses (Kumar and Ellis, 2001; Cochrane et al., 2004; Chang et al., 2008). In tomato, only one PAL transcript is induced by pathogen or wounding (Chang et al., 2008). In red raspberry, RiPAL1 is expressed during early fruit ripening, while RiPAL2 is expressed at later stages of flower and fruit development (Kumar and Ellis, 2001). PAL genes also show tissue specific patterns of expression. The expression of RiPAL1 transcripts is much higher than that of RiPAL2 in leaves, shoots, roots, young fruits, and ripe fruits. In blueberry, three PAL genes are up-regulated at the gene/protein level as fruit matures (Li et al., 2019). In this study,

two phylogenetically close RchPALs were both down-regulated at the gene/protein level as fruit matured (**Figures 4A,B**).

4CL isoenzymes exhibit distinct substrate affinities due to their different metabolic functions. In *Arabidopsis*, four 4CL genes are divergent in functions, e.g., At4CL4 exhibits the rare property of activating sinapate and other 4CL substrates (e.g., 4-coumarate, caffeate, and ferulate) (Hamberger and Hahlbrock, 2004). In *Physcomitrella patens*, three 4CLs display the highest catalytic efficiency toward 4-coumarate, which is distinguished from the fourth 4CL (Silber et al., 2008). In blueberry, Vc4CL and Vc4CL-like are both up-regulated as fruit maturates although they are phylogenetically separated (Li et al., 2019). In this study, two phylogenetically distant CL4 homologs (RchCL4 and Rch4CL-like) showed distinct patterns of expression (**Table 2**). 4CLs were significantly down-regulated at the gene/protein level as fruit maturated while 4CL-like genes were expressed at low levels. The result suggests that 4CL rather than 4CL-like functions in down-regulation of the phenylpropanoid pathway in fruit.

C4H belongs to a large group of cytochrome P450 monooxygenases (P450) in plants and exclusively constitute the CYP73 family, a typical group of P450. In citrus, C4H1 and C4H2 are different in both expression patterns and N-termini, suggesting they have specific functions in organelles (Betz et al., 2001). In blueberry, two phylogenetically close C4H homologs (VcC4H2A and VcC4H2B) are dramatically up-regulated from the pink to blue phase (Li et al., 2019). In this study, two phylogenetically related RchC4H homologs (Unigene9842 and Unigene12468) both had down-regulated expression.

Regulation of Genes/Proteins in Flavonoid Biosynthesis Are Responsible for the Diversity in Flavonoid Composition and Concentration

Different CHS genes are associated with the different phenotypes. In genus *Ipomoea*, six CHS genes are regulated by developmental signals. Of these, CHSD and CHSE function in flavonoid biosynthesis, especially CHSD which has dominant effects on floral pigmentation (Clegg et al., 2000). In blueberry, three phylogenetically close CHS genes share a similar pattern of up-regulation (Li et al., 2019). In Korean black raspberry, two CHS genes are both up-regulated during fruit maturation (Hyun et al., 2014). However, in *R. chingii,* the opposite occurs and one of the RchCHS is down-regulated at the gene/protein level during fruit maturation. Chalcone isomerase (CHI) is a rate-determining enzyme in flavonoid biosynthesis. In red-fruited tomato the CHI gene is expressed at low levels and decreases upon ripening while an accompanying accumulation of the CHI substrate, naringenin chalcone, occurs (Bovy et al., 2002). Heterologous expression of petunia CHI gene in tomato results in up to 70-fold increase in flavonols in the fruit peel, and a decrease in naringenin chalcone (Muir et al., 2001). In grape, CHI gene expression gradually decreases with ripening, and later, slightly increases (Wang et al., 2012). In Korean black raspberry, three CHI genes were all up-regulated during fruit maturation (Hyun et al., 2014). In *R. chingii*, two classes of RchCHI (**Figure 3E**) were both down-regulated at the gene/protein level during fruit maturation (**Figure 4**).

F3H, F3′H, F3′5′H and FLS play an important role in the types and quantities of flavonoid biosynthesis, which determines the colors and flavors of fruits (Li et al., 2019). In blueberry, F3H, F3′5′H and FLS are all up-regulated at the gene/protein level during fruit maturation (Li et al., 2019). In Korean black raspberry, two F3H genes are both up-regulated during fruit maturation (Hyun et al., 2014). In contrast, RchF3H was down-regulated at the gene/protein level during fruit maturation. Moreover, the gene expression of RchF3′H, and RchFLS were low and down-regulated during ripening. Interestingly, RchF3′5′H was absent in *R. chingii*. The results suggest that the low expression or down-regulation of these genes/proteins reduces biosynthesis of dihydroflavonol and flavonols, while a deficiency of RchF3′5′H impeded biosynthesis of myricetin and delphinidin glycoside.

DFR is responsible for branch flux from dihydroflavonol into anthocyanin while ANR and LAR converts anthocyanidin leucoanthocyanidins to flavan-3-ols and then to proanthocyanidins (condensed tannins). DFR in Korean black raspberry (Hyun et al., 2014) and blueberry (Li et al., 2019) are both up-regulated at the gene/protein level as the fruit matured. In contrast, two classes of RchDFR genes were both significantly down-regulated during fruit maturation. In blueberry, LAR protein is up-regulated during fruit maturation (Li et al., 2019). The opposite occurred in *R. chingii* with down regulation of RchLAR at the gene/protein level during fruit maturation (**Table 2** and **Figure 4**). The results indicate that the down-regulated expression of RchDFR is responsible for the decrease of leucoanthocyanidins while the down-regulated expression of RchLAR and RchANR is responsible for the decrease of flavanols. However, the flavanols, i.e., (epi) catechin and (epi) afzelechin could be combined with pelargonin for production of dimeric anthocyanins. Notably, the biosynthesis of dimeric anthocyanins requires two flavonoid units, rather than a single flavonoid unit as is needed for monomeric anthocyanins. One unit is produced from anthocyanin biosynthesis while the other is from flavanols biosynthesis, but both share a common upstream pathway. This indicates a reduction in the potential biosynthesis of these dimeric anthocyanins. Also, the constantly low expression of RchANS causes a reduction in overall anthocyanin biosynthesis.

Flavonoid glycosyltransferases have roughly four different functional classes including 3-*O*, 5-*O*, 7-*O* glycosyltransferases and diglycoside/disaccharide chain glycosyltransferases, respectively. 3-*O* glycosyltransferase includes AtUGT78D1 and AtUGT78D2 in *Arabidopsis* (Kim et al., 2012), and CsUGT78A14 and CsUGT78A15 in *Camellia sinensis*, which are responsible for biosynthesis of flavonol 3-*O*-glucosides/galactosides, respectively (Cui et al., 2016). 5-*O* glycosyltransferase includes AtUGT75C1 (anthocyanins 5-*O* glucosyltransferase) in *Arabidopsis* (Gachon et al., 2005), and CsUGT75L12 (flavonoid 5-*O* glycosyltransferases) (Dai et al., 2017). 7-*O* glycosyltransferase includes AtGT-2 (flavonoid

7-*O*-glucosyltransferase) in *Arabidopsis* (Kim et al., 2006), and GmIF7GT (UDP-glucose:isoflavone 7-*O*-glucosyltransferase) (Noguchi et al., 2007). Moreover, there is another class of flavonoid glycosyltransferases, i.e., flavonol 3-*O*-glycoside: 2′′-*O* glucosyltransferase (3GGT/UGT79), an enzyme responsible for the terminal modification of pollen-specific flavonols (Knoch et al., 2018). In blueberry, two 3-*O* glycosyltransferase genes are both up-regulated as fruit matured, while another 5-*O* glycosyltransferase is down-regulated (Li et al., 2019). In Korean black raspberry four 3-*O* glycosyltransferase genes are all up-regulated as fruit matured. In *R. chingii*, two 3-*O* glycosyltransferases (RchBZ1/UGT78D) were all down-regulated at the gene/protein level as the fruit matured, while 5-*O* glycosyltransferase and flavonol 3-*O*-glycoside: 2′′-*O* glucosyltransferase genes were mostly maintained at low expression levels. The results indicate that the down-regulation of 3-*O* glycosyltransferase is responsible for the decreased content of flavonol glycosides (e.g., kaempferol-3-*O*-glucoside and quercetin 3-*O*-glucoside). A diversity in flavonoid glycosyltransferases leads to a variety of flavonoid glycosides, e.g., flavonoid coumaroylglucoside, flavonoid rutinoside, flavonoid sophoroside etc.

CONCLUSION

In *R. chingii*, most flavonoids were located in the fruit epidermal-hair and placentae. In most berries there is an increase in the total flavonoid and anthocyanin concentration near the end of the fruit maturation. However, in *R. chingii* the unripe (mature green) fruit had much higher flavonoid levels, as well as anthocyanin concentrations, than was seen in latter phases of fruit development. The decreases of flavonoid and anthocyanin concentrations in latter phases of *R. chingii* fruit development is due to the down-regulation of phenylpropanoid, and flavonoid biosynthesis. Notably, most of anthocyanins were in flavanol-anthocyanin condensed forms, which is produced from the proanthocyanidin pathway. The mechanisms of flavonoid biosynthesis appear to be unique to *R. chingii,* and have not been reported in other fruit crops. Multiple genes and proteins in these pathways were divergent in function and differently regulated.

DATA AVAILABILITY STATEMENT

The original contributions generated for this study are publicly available. This data can be found here: transcriptomic data are available via NCBI with accession (PRJNA671545). Proteomic data are available via ProteomeXchange with identifier (PXD021977).

AUTHOR CONTRIBUTIONS

XL conceived of the study, performed the experiments, and wrote the manuscript. ZC analyzed mRNA expression. JJ planted *R. chingii* and collected fruit tissues. AJ assisted with writing and revising the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.706667/full#supplementary-material

Supplementary Figure 1 | Composition of anthocyanins and flavonoids in *R. chingii* (Li et al., 2021a). The upper graph(s): electrospray product ion mass spectra; the lower graph(s): fragment ions mass spectra; shown to the right of the graphs: putative molecular structure and cleavage pattern.

Supplementary Figure 2 | Summary of differentially expressed **(A)** genes and **(B)** proteins during four maturation phases in *R. chingii*.

Supplementary Figure 3 | KEGG enrichment of the differentially expressed **(A)** unigenes and **(B)** protein isoforms. Bar size represents the number of genes enriched in a particular pathway. Up-regulated proteins between two phases are marked in red; down-regulated proteins between two phases are marked in blue. The biosynthesis of phenylpropanoid and flavonoid are shown in rectangles.

Supplementary Table 1 | Primers for RT-qPCR analysis.

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The Triple Jags of Dietary Fibers in Cereals: How Biotechnology Is Longing for High Fiber Grains

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Cereals represent an important source of beneficial compounds for human health, such as macro- and micronutrients, vitamins, and bioactive molecules. Generally, the consumption of whole-grain products is associated with significant health benefits, due to the elevated amount of dietary fiber (DF). However, the consumption of whole-grain foods is still modest compared to more refined products. In this sense, it is worth focusing on the increase of DF fractions inside the inner compartment of the seed, the endosperm, which represents the main part of the derived flour. The main components of the grain fiber are arabinoxylan (AX), β-glucan (βG), and resistant starch (RS). These three components are differently distributed in grains, however, all of them are represented in the endosperm. AX and βG, classified as non-starch polysaccharides (NSP), are in cell walls, whereas, RS is in the endosperm, being a starch fraction. As the chemical structure of DFs influences their digestibility, the identification of key actors involved in their metabolism can pave the way to improve their function in human health. Here, we reviewed the main achievements of plant biotechnologies in DFs manipulation in cereals, highlighting new genetic targets to be exploited, and main issues to face to increase the potential of cereals in fighting malnutrition.

Keywords: dietary fibers, cereals, arabinoxylans, β-glucans, resistant starch, crop biotechnologies, health

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HIGHLIGHTS

The application of modern biotechnology to crops is crucial to obtain cereals enriched in dietary fibers, essential bioactive compounds for human health.

INTRODUCTION

Malnutrition is among the main issues in human health and involves three biggest concerns on the population diet: undernutrition (hunger), micronutrient deficiency (hidden hunger), and overnutrition (obesity). In this regard, nutrition research is called to meet the increasing demand for both staple crops and "nutrient-rich foods" (Zhou and Staatz, 2016). In some viewpoints, staple

crops, primarily cereals, are neglected as "nutrient-poor foods" sourcing mostly dietary energy, therefore being responsible for overnutrition issues (Sanchez, 2020). Noteworthy, cereals play a key part in the human diet since they provide more than half of all calories consumed by humans, being a source of macro nutrients, carbohydrates and proteins, and significant amounts of bioactive compounds such as vitamins, minerals, and dietary fibers. The widespread consumption of major cereals makes them ideal to deliver benefits to the most of population due to consolidated diet styles: in this perspective, these crops are called to answer an urgent demand for healthiness by boosting the provision of essential bioactive compounds such as DFs (Poole et al., 2020; **Figure 1**). DFs have received plenty of definitions finely discussed elsewhere (Stephen et al., 2017), the most widely accepted being: "carbohydrate polymers with three or more monomeric units which are neither digested nor absorbed in the small intestine" (Commission Directive 2008/100/EC, 28 October 2018). Major DFs in cereals are βG and arabinoxylans, lignin, fructans, and resistant starch (Shewry et al., 2020).

Health aspects of DF span through many beneficial impacts on colonic function, short-term effects on glycemia, and regulation of blood cholesterol (Anderson et al., 2009) associated with the prevention of hard diseases connected to overnutrition, namely cardiovascular diseases (CVDs), diabetes II and some types of cancer.

These pathologies fall among non-communicable diseases (NCDs) and provoke death globally in 41 million people each year, equivalent to 71% of all deaths (WHO, 2021). DFs are amenable to mitigate major metabolic risk factors like overweight, obesity, hyperglycemia, raised blood pressure by plenty of physiological actions: increasing the viscosity of digesta in the small intestine; promoting prebiotic fermentation in the distal colon; accelerating the intestinal transit; sequestering of carcinogenic cells and cholesterol (Capuano, 2017). Recommendations for DF intake in adults in most countries (Europe and United States, Australia and New Zealand) are far away to be met: the recommended intake is in the order of 30 g/d, while the average intake accounts for 18 g/d in most countries (Stephen et al., 2017).

Cereal food accounts for the largest intake of fiber worldwide, providing from 32 to 48–49% in the United States and Europe respectively (Esteban et al., 2017; Stephen et al., 2017). For instance, the largest proportion of the RS comes from grain products.

That stated, the contribution of each cereal to the total DF diet intake varies greatly depending on several factors: the spread of cereals in the world diet, impact of food processing, distribution of fiber components across the seed layers.

Maize, rice, and wheat account for 89% of total cereal production worldwide, while barley, oat, and rye are categorized as "minor" or "specialty" grains.

In terms of human consumption, rice and wheat are the main crops, while maize production is primarily used in animal feed with only 15% of the grain used for food. Nevertheless, maize is a major staple in such areas as Africa and Latin America with more than 90% used for food (FAO, 2011). Barley is scarcely used for food production (about 6% of the total production), even if, due to its resilience, is still a staple crop in some areas of North Africa and the Near East (Zhou, 2010). Moreover, barley consumption is increasing in developed countries due to the growing awareness of its health properties. Other cereals, like oat and rye, are less important, as their impacts on the global diets are minor. However, rye, the richest in DF among cereals, plays an important role in the diet of several Nordic European countries, associated with relevant health benefits for the population (Jonsson et al., 2018).

Among major cereals, wheat grains contain 12% of DF, followed by maize (7.3%) and brown rice (3.4%); barley (*Hordeum vulgare*) has the highest content in DF (17%) (Prasadi and Joye, 2020). Minor cereals, such as oat and rye, contains 10.5 and up to 20% of total DF. However, DF intake in cereal foods strongly depends on the relationship between their distribution inside kernel layers and seed processing. Cereal grains are composed of several compartments: (i) the external layers (seed coat, pericarp, and aleurone), commonly defined as bran, are the richest in NSP and are removed by milling, whereas are stored in wholegrain products; (ii) the endosperm, mainly composed by starch and proteins, generally contains a lower amount of NSP as well as a trivial amount of resistant starch and represents the most of refined flour; (iii) the embryo, the vital compartment of the seed is removed by milling. If it is true that DFs are much higher in whole grain foods (11–15%), nonetheless consumer preference makes consumption of whole grains inadequate compared to more processed and refined foods. It derives that, beyond promoting education on the benefits of wholegrain foods to consumers, increasing the amount of DF in the endosperm has a great potential to enhance their intake in the diet. Therefore, the distribution of DFs across the seed layers differs widely among different cereals, with some species more amenable to increase the amount of DF in the endosperm.

Dietary fibers fractions majorly represented in the inner compartment of the seed are AXs, βGs, and RS. Arabinoxylans account for 70% of total DF in the wheat endosperm, while βGs are the predominant fiber in barley. In this frame, RS is advantageous for being in the endosperm. It is a starch fraction, whose physiological behavior retraces that of cell wall DFs: it results recalcitrant in amylases hydrolysis thus reducing the level of glucose release in the blood (low glycemic index) and is fermented by the gut microbiota promoting the release of small metabolites, the short chains fatty acids (SCFAs), beneficial for colon health (Regina et al., 2006). The consumption of foods enriched in RS can decrease glycemic and insulin responses and reduce the risk of developing type II diabetes mellitus, obesity, and cardiovascular diseases (Birt et al., 2013; Bird and Regina, 2018; Guo et al., 2020).

Resistant starch content in cereals depends on some major features, among which, the percentage of amylose in the reserve starch. Several studies revealed the existence of a correlation between the amylose content in the kernel and the amount of RS in flours and foods. High-amylose starches are now available in maize, rice, barley, and wheat. A rising number of studies are now evaluating possible beneficial effects on the health of enriched-resistant starch foods (Hogg et al., 2015; Schönhofen et al., 2017; Vetrani et al., 2018; Corrado et al., 2020; Sissons et al., 2020).

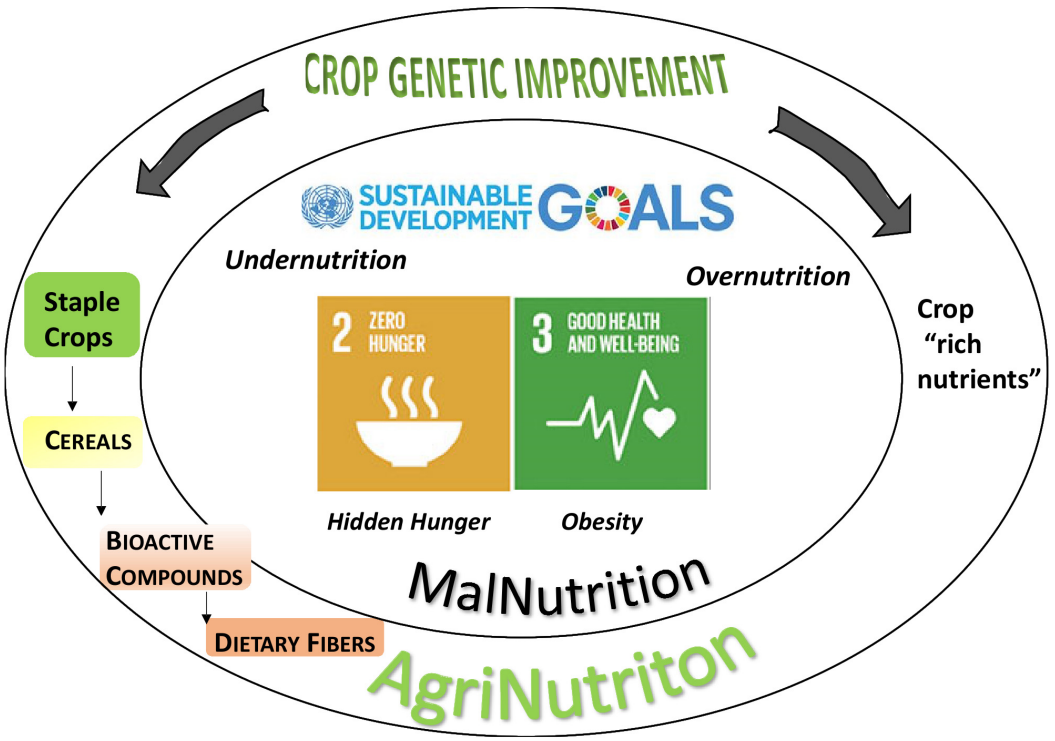

FIGURE 1 | The role of cereals in the fight against malnutrition. The widespread consumption of cereals makes them ideal to deliver health benefits encountering the ask of the Sustainable Development Goals (SDGs) 2 and 3 for Zero Hunger. Dietary fibers are bioactive compounds playing an important role in human health: breeding to increase their content in cereal grains is a promising approach to malnutrition issue; in comparison to "crops rich nutrient," cereals offer a more effective delivery in the diet.

Arabinoxylan, βG, and RS, present at various levels in the endosperm, provide together a good target for crop genetic improvement focused on the enrichment of cereal flours in DF.

Although they share similar physiological behaviors, still, each class is associated with specific functionalities both at health and technological levels: their different chemical nature deeply conditions short-chain fatty acids (SCFAs) production and gut microbiota composition (Tiwari et al., 2019); as well as their differences in solubility profiles greatly affect both food digestibility and its technological properties (Buttriss and Stokes, 2008; Elleuch et al., 2011). Moreover, the amount and percentages of AX, βG, and RS are variable among the different cereals. All considered it seems to us, that to answer to the demand for cereal food enriched in DFs, it is of interest to consider all three groups of these DFs together with the following aims: ideation of strategies able to boost the content of more than one class at a time; finding a good compromise between healthy and technological properties; choosing the best DF target in consideration of the cereal species and the main end-products properties (Stephen et al., 2017).

Dietary fiber enrichment in cereal by genetic approaches relates to the investigation of the metabolic pathways and genetic determinants responsible for their accumulation and structure-functionality relationship in all major species.

An overall analysis of the main actors playing a key role at metabolic and gene levels can help to highlight crucial relationships between the three carbohydrates, useful for appropriate modulation of DF amount in the endosperm.

In this review, we revised studies centered on three main DF classes: βGs, and AXs, and RS. The biosynthetic pathways and key enzymes involved are discussed, highlighting putative main regulators to be considered for future breeding purposes; in addition, studies until so far performed by involving classical and modern crop genetic improvement tools, aiming to influence the amount or modulate the structure of the three DFs, are revised.

Lastly, we highlight novel questions to be addressed and possible targets of interest to be exploited to obtain new cereals biofortified in AXs, βGs, and RS.

DFs IN CEREAL GRAIN

Arabinoxylan, βG, along with cellulose, and the non-carbohydrate component, lignin, are the predominant cell wall polysaccharides in cereals. They occur in different proportions depending on the species and tissue type. Wheat along with maize, is rich in AX, whereas barley and oat contain a modest amount of AX and a high level of βG. AX from wheat and βG from barley and oat are mostly soluble, whereas AX from maize is mainly insoluble (Knudsen, 2014). Solubility impacts their physiological properties with soluble DF (SDF) playing the most of healthy functions (Prasadi and Joye, 2020).

Arabinoxylan and βG are the main components of the cell walls both in the endosperm and aleurone tissue, while in the pericarp there is a large presence of other cell wall constituents.

In barley, βGs are evenly distributed in the sub aleurone layers and endosperm where represent the major endosperm cell wall component (75% of the total cell wall material) (Fincher and Stone, 1986). AXs are the major components of the cell wall in the wheat endosperm reaching up to 67%, whereas the βG content is about 27% (**Supplementary Table 1** and **Supplementary Figure 1**) (Fincher and Stone, 1986). The remaining fraction is mainly constituted by glucomannan and cellulose (Bacic and Stone, 1981). Differently, the third component, the resistant starch, is normally present at low levels in most cereals. Based on the prominence of AXs in wheat endosperm and βG in barley one, these DFs will be mainly discussed in these two cereals. For RS, the discussion will be focused on wheat, rice, maize, and barley, as they were the object of several breeding programs focused on the achievement of genotypes with an elevated amount of amylose and RS.

ARABINOXYLANS, A COMPLEX STRUCTURE

Arabinoxylans consist of a linear backbone of β-1,4-linked D-xylose residues with frequent substitutions with L-arabinose. In the case of mono-substitutions and di-substitutions, L-arabinose is inserted in the O-2 or/and O-3 positions (Courtin and Delcour, 2002). In addition, ferulic acid (FA) or coumaric acid (pCA) can be ester-linked to the O-5 of arabinose (Harholt et al., 2010). FA and pCA are the most abundant phenolic acids in cereals and play a crucial role in the regulation of the properties of the cell wall as cellular interaction and rigidity (Hassan and Burton, 2018). Indeed, the presence of FA esters favors cross-linkages to other FA esters or lignin; these interactions are important for the structure of the cell wall matrix (Grabber et al., 1995; Vogel, 2008). AXs structure shows a high variability inter and intraspecies and among the different seed layers with a great diversity of side chains and composition; although less frequent, single units of α-d-glucuronic acid and 4-*O*-methyl-glucuronic acid can be attached to the backbone at O-2 and O-3 positions, as well as the association of xylose and galactose residues with arabinose, are also found as short sugar units. Notably, AXs of outer layers of the seed, pericarp, and aleurone, where the cell wall material contributes for 40–60% of the dry weight, show a more complex structure with several substituents such as pCA and a higher percentage of FA. Indeed, ester-linked pCA is not detected in pure starchy endosperm tissue dissected from wheat grain (Barron et al., 2007).

Empirically, AXs have been classified in water-extractable (WE-AX) and water-unextractable (WU-AX) fractions. This latter can be extracted using an alkaline solution able to break ester linkages (Courtin and Delcour, 2002; Kiszonas et al., 2013). Differently from WU-AXs, WE-AXs are not retained in the cell walls through covalent and non-covalent interactions with other wall components (proteins, cellulose, and lignin), but they are weakly bound on the cell wall surface. Their water-extractable nature has been not fully elucidated: Courtin and Delcour (2002) hypothesized incomplete cross-linking with other wall components in the kernel. In wheat kernel, WE-AXs constitute about 25–30% of the total AXs (Saulnier et al., 2007). The ratio between arabinose and xylose (A/X) is associated with the polysaccharide solubility; indeed, the presence of arabinose side chains limits the aggregation between the linear xylose chains thus increasing the interaction with the solvent. However, the arabinose is susceptible to esterification by hydroxycinnamic acids, mainly FA, that promotes the crosslinking (di-tri ferulates) between the xylan chains, reducing solvent accessibility. Diferulate cross-linking affects the physio-chemical properties (notably solubility and viscosity) of these polymers (Shewry et al., 2020). The solubility of AXs affects their physiological properties and impacts positively on human health; this correlation is far to be evenly comprised and current knowledge has been recently and widely reviewed in Wang et al. (2020a). Among the three major cereals, wheat is richer in water-soluble AXs (WE-AXs) only found in the cell wall of starchy endosperm.

ARABINOXYLAN BIOSYNTHESIS

The build-up of the AX backbone occurs in the Golgi in a deep dialog of metabolites with the cytosol, mediated by several membrane channels, the (UDP)-sugar transporters. The monomers of the polysaccharide, xylose and arabinose are synthesized both in the cytoplasm and in the Golgi due to the presence of multiple isoforms of the synthetic enzymes. Most of the investigation on AX biosynthesis in cereals focalized on the step of glucan chain construction; nonetheless, the knowledge of the mechanism of xylose and arabinose synthesis can provide new opportunities for the modification of AX fine structure in cereals (**Figure 2A**).

Crosstalk of Nucleoside Diphosphate (NDP) Sugar Precursors Between Cytosol and Golgi

The synthesis of all xylans starts from their precursors, the UDP-sugars, (di)phosphonucleotide-activated sugars, synthesized between the cytosol and Golgi apparatus. The UDP-D-xylose (UDP-Xyl), the monomer of the xylan backbone, is synthesized by the UDP-Xyl synthase (UXS), whose isoforms are localized both in the cytosol and Golgi apparatus, through the decarboxylation of UDP-D-glucuronic acid (UDP- GlcA). Reverse genetic approaches, targeting the genes coding for the UDP-Xyl transporters (UXT), demonstrated that cytosolic UXS isoforms, and not the Golgi-located ones, are essential for the synthesis of xylan chains (Zhao et al., 2018). Arabinose, the major substituent of the xylan chain, is obtained from the conversion of (UDP)-xylose in (UDP)-Arabinopyranose (Ara*p*) by a UDP arabinose epimerase (UXE). There are two and three UXE isoforms in rice and barley respectively (Seifert, 2018). It has been

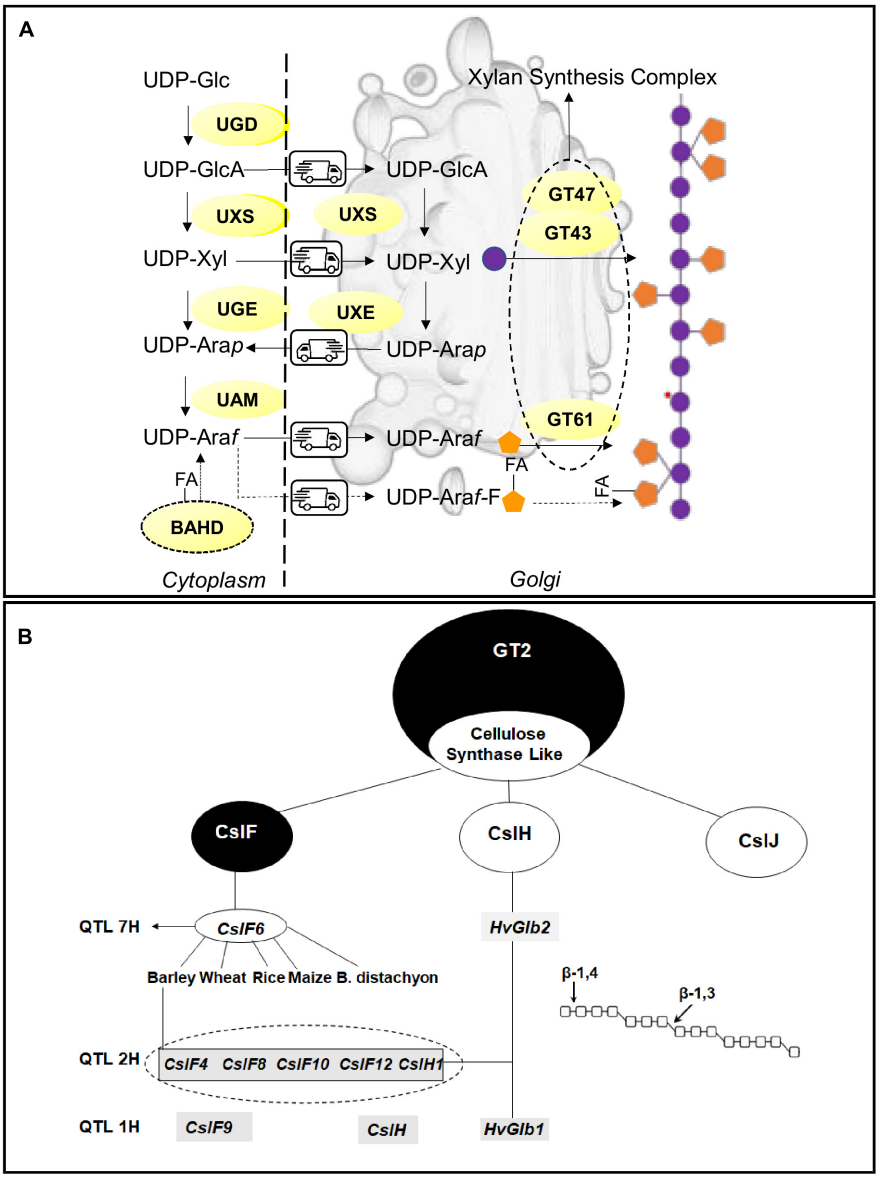

FIGURE 2 | Biosynthetic pathways and key enzymes for **(A)** arabinoxylans and **(B)** β-glucans. **(A)** Schematic representation of arabinoxylan synthesis involving the cytoplasm and Golgi apparatus; nucleotide sugars: Xyl, Xylose; Ara, Arabinose; GlcA, Glucuronic acid; UDP-L-Arap, UDP-L-arabinopyranose; UDP-L-Araf, UDP-L-arabinofuranose. UDP-sugars converting enzymes UAM, UDP-Ara mutase; UGD, UDP-Glc 6-dehydrogenase; UGE, UDP-glucose 4-epimerase; UXE, UDP-Xyl 4-epimerase; UXS, UDP-Xyl synthase; BAHD acyl transferase FA, Ferulic acid. GT47, GT43, GT61 Glycosytransferases. **(B)** Key Enzymes involved in β-glucan synthesis in cereals. The figure shows the main QTLs and associated candidate genes for β-glucan synthesis identified in cereals. The dotted circle underlines the cluster of *CslF* plus *CslH1* genes identified in barley.

reported that the functionality of Golgi-UXS is essential to maintain a normal arabinose content in the cell wall (Saez-Aguayo et al., 2017). Once synthesized in the Golgi, Ara*p* is channeled back to the cytosol where the UAM complex (UDP-arabinopyranose mutase) interconverts UDP-Ara*p* to UDP-Arabinose furanose (Ara*f*); then, Ara*f* is taken back to the Golgi where it will be incorporated in AX structure. The other two suppliers of arabinose in the cells are the fueling caused by the carbohydrate breakdown running in the cytosol and the cytosolic UDP-Xyl conversion operated by the bifunctional enzyme UGE l (UXE/UDP-glucose 4-epimerase) (Kotake et al., 2009).

Feruloylation

Hydroxycinnamic acid derivatives are the AXs' unique feature. Ferulic, dehydrodiferulic, p-coumaric, and sinapic acids esterify the 5-OH of arabinosyl residues. These ferulic acid groups also form covalent bonds to other molecules in the cell wall, such as proteins, lignins, and other glucans (Saulnier et al., 2007). It has previously been suggested that

the dominant characteristic determining AX solubility is the amount of diFA (Saulnier et al., 2007). Ferulic substituents form oxidatively-linked dimers and oligomers with cell wall polymers that result in a covalently linked network within the cell wall. Members of the "Mitchell clade" within the BAHD acyltransferase superfamily are involved in FA and pCA esterification of xylan in monocot cell walls. BAHD acyltransferases presumably transfer FA to an intermediate, such as UDP-Ara*f*, which is then transported into the Golgi and transferred onto the xylan chain by unknown proteins (Bartley et al., 2013).

Synthesis of the Xylan Backbone and Araf Decoration

The building up of the AX structure implicates the cooperation of several enzymes belonging to two major families: the Glycosyltransferases (GTs) and Glycosyl hydrolases (GHs). Several subclasses of GT families exist, and the specificity of the role played by the single protein members is wide far from being elucidated. In addition, it is yet to be established if the biosynthesis of AXs in cereals involves a terminal oligosaccharide at the reducing end which might act as a primer or terminator (York and O'Neill, 2008). GTs transfer the sugar-activated precursors (NDP-sugars) onto a specific acceptor catalyzing the formation of glycosidic bonds. The model proposed for AX synthesis theorizes the existence of a multi-enzyme complex, the xylan synthase complex, composed of at least three members of the two different GT clades. Proteins coded by the three genes *IRX9*, *IRX10* (*GT43*), and *IRX14* (*GT47*) cooperate to the synthesis of the xylan chains in the Golgi apparatus. IRX9 was supposed to play a structural role as its lack of an aminoacidic motif essential for the synthetic activity (Urbanowicz et al., 2014). A hypothesis has been advanced about the involvement of unidentified members of the processive GT2 family in the biosynthesis of (1,4)-β-xylans essential for the processivity of the synthetic process (Bulone et al., 2019).

Enzymes involved in the decoration of the AX backbone with arabinosyl and xylosyl sidechains are members of the GT61 family. Two xylan arabinosyl transferases (XATs) in wheat (TaXAT1, TaXAT2) and rice (OsXAT2, OsXAT3) have been characterized both natively and in heterologous systems (Anders et al., 2012; Zhong et al., 2018). Other GT61 members can transfer xylosyl side chains on the xylan backbone: in rice, xylosyl-arabinosyl substitution by xylan-xylosyltransferase (OsXAX1) mediates the addition of xylose to arabinose units while rice xylan-xylosyltransferase 1 (OsXYXT1) adds xylose sidechains to the xylan backbone (Xyl*p*-1,2-b-Xyl*p*) (Zhong et al., 2018). Xylan-specific arabinosyl-transferase activities of GT61 enzymes in grasses provide new targets for the modification of xylan structure. Members of glycosyl hydrolases have been often associated with AX synthesis; it is postulated that they play a role in the modeling of AX fine structure recalling what happens for starch with debranching enzyme (Fincher, 2009).

β-Glucans: A Simpler Polysaccharide

Within cereals, barley grains are the richest source of βGs with a values range of 2.5–11.5% of its dry weight, while wheat and maize have lower concentrations of the polysaccharide (Izydorczyk and Dexter, 2008; **Supplementary Table 1**). βGs from different sources and tissues present differences in their structure and properties: in barley, βGs from the endosperm have been reported to be 20% soluble in H_2O at 40°C, while in wheat they are completely insoluble.

βGs present a simple primary structure composed of glucose units linked by both β-1,3 and β-1,4 glycosidic bonds. Specifically, 1,4-linked oligosaccharides composed of three (β-cellotriosyl) and four (β-cellotetraosyl-) glucose residues are linked by a single (1,3)-β-linkages. The ratio between (1,4)-β-glucosyl residues and (1,3)-β-glucosyl residues is approximately 3:1 with a wide variation across different species and tissues (Burton and Fincher, 2009). The coexistence of β-1,3 and β-1,4 linkages determines an asymmetry in the conformation of the polysaccharide that prevents the aggregation of the glucan chains increasing the flexibility and the solubility. Hence, the viscosity properties of βGs, associated with their healthy properties, are directly connected with its chemical structure: the ratio between β-cellotriosyl and β-cellotetraosyl (DP3/DP4) residues is a useful predictor of the polysaccharide solubility, where very high or very low ratios indicate low solubility, while ratios around 1.0:1–2.5:1 predict relatively higher solubility (Burton et al., 2010). The manipulation of the distribution of β-1,3 linkages interspaced across β-1,4 linked oligosaccharides represents a main target for the modulations of βGs solubility in cereals. Notably, longer blocks of (1,4)-β-glucosyl residues, up to 12 adjacent, have been found in cereal bran (Burton et al., 2010).

β-Glucan Biosynthesis

In cereals, (1,3,1,4)-βGs are synthesized by enzymes belonging to the Cellulose Synthase Like Superfamily, within the large glycosyltransferase GT2 family (Bulone et al., 2019), specifically belonging to three groups specific for grasses code by the three genes *CslF*, *CslH,* and *CslJ* (Kumar et al., 2016; **Figure 2B**). *CslF6* gene, identified as the key candidate for the synthesis of (1,3;1,4)-βG in the barley endosperm (Burton et al., 2011), has been also studied in wheat, rice, maize, and *B. distachyon* (Nemeth et al., 2010; Coomey et al., 2020). In barley, the comparison of CslF6 with other members of the same clade identified an insertion of 55-amino acid residues essential for the amount and fine structure of the (1,3;1,4)-βG (Schreiber et al., 2014). Dimitroff et al. (2016) were able to identify regions of the enzyme that are important for overall (1,3;1,4)-βG synthesis and for defining the DP3:DP4 ratio of the polysaccharide chain. In detail, the N-terminal region of the CslF6 protein in barley, maize, and sorghum influences total βGs synthesis activity and the C-terminal region appears to influence the ratio of DP3/DP4 linkages.

These findings are of utmost importance for the manipulation and reprogramming of βG structure in cereals. Other enzymes belonging to *Csl* family are supposed to play a major role such as CslF9 (Burton and Fincher, 2009); recently, the targeting by genome-editing of several members of CslF and CslH clades in barley highlighted differences in βGs content only for the CslF6 knockout mutants (Garcia-Gimenez et al., 2020). CslH is

supposed to take part in the remodeling of the polysaccharide, thus contributing to the definition of its fine structure.

Differences among cereal species have been detected concerning the location of βGs synthesis: Golgi-localized synthesis has been suggested for maize, whereas it occurs at the plasma membrane in barley and wheat (Bulone et al., 2019). Here, the synthase enzyme shows an intracellular or intra-organellar active site from which the nascent polysaccharide is extruded to the opposite side of the membrane through a pore formed from six transmembrane α-helices (Bulone et al., 2019).

RESISTANT STARCH IN CEREAL GRAIN

Among the five classes of RSs, RS type 3 is identified as "retrograded amylose." Briefly, starch is overall composed of two distinct populations of α-glucans differing in the degree of polymerization (DP) and level of branching: amylose is a linear chain of α,1-4 linked glucose molecule and rare branches, with DP ranging from 10^2–10^4, while amylopectin is extremely branched due to a high level of α-1,6 side chains (1:12/15 glucose units) and shows a higher degree of polymerization (DP 10^4–10^6) (Zeeman et al., 2010). In cereals, the ratio between the two glucan polymers is mostly around 1:3 (amylose/amylopectin). The modulation of this ratio deeply affects physic-chemical properties of starch, influencing the starch gelatinization, solubility, retrogradation, and end-uses (Blazek and Copeland, 2008). Starch is organized in semi-crystalline structures, called granules, in which amylopectin chains clustered *via* hydrogen bonds forming double-helical structures distributed through layers in a hierarchical order (Buléon et al., 1998; Pérez and Bertoft, 2010). Amylopectin, with its high levels of ramification, is the main responsible for the ordered and crystalline feature of the starch granules. Branching triggers an opened kind-structure with void spaces easily hosting solvent molecules and hydrolytic enzymes: amylopectin is quite soluble in water and easily broken down in glucose units by digestive amylases. Quite the opposite, the linear chains of amylose tightly interact between them, deriving in a refractory arrangement not prone to be solubilized neither to be enzymatically processed: amylose properties reflect in "resistant starch" behavior, so-called since it withstands the enzyme action in the stomach and small intestine promoting lowering of glucose level in the blood after a meal (Sharma et al., 2008; Birt et al., 2013).

STARCH BIOSYNTHESIS

The starch precursor, ADP-glucose, is synthesized mostly in the cytosol by ADP glucose-pyrophosphorylase: glucose-1P reacts with ATP to generate ADP-glucose and PPi; afterward, ADP-glucose is transferred to the plastid by specific transporters.

Starch molecules are the product of a binary mechanism administered by an enzymatic complex including elongating, branching, and debranching enzymes (**Figure 3**). Granule Bound Starch synthase I (GBSSI) catalyzes the α-1,4 linkage between glucose units and is mostly associated with the synthesis of amylose molecules (James et al., 2003; Jeon et al., 2010). To be exact, a few reports have reported the implication of this enzyme also in the production of extra-long amylopectin chains (DP 300–500) (Hanashiro et al., 2008; Crofts et al., 2018). Amylose synthesis is most likely primed by short malto-oligosaccharides (MOSs) (DP 2–7) derived by various sources, processively elongated by GBSSI. Elongated MOSs with DP > 7, constrained into the granule, finally become long amylose chains (Denyer et al., 2001). Among the starch synthases, GBSSI takes on several exceptions: it is deficient in a Carbohydrate-Binding Module (CBM) in its structure; it is internalized within the granule and acts through a processive mode (Hebelstrup et al., 2017; Zhong et al., 2020). Putting together these considerations have helped to theorize that GBSSI acts in the interior of the granule where amylose remains protected by hydrolytic enzymes of the stroma (Seung, 2020); moreover, it may be that, in the absence of the amylopectin-environment, amylose would arrange in an insoluble compound, thus hindering its synthesis. The absence of a CBM module in the GBSSI structure is ascribable to its processive mode of action; a stable interaction between the nascent polymers and the enzyme would not fit with the inter-mobility of the two molecules. Recently, it has been found that the initial localization of GBSSI to the granule is mediated by specific proteins among which, the Protein Targeting to Starch 1 (PTST1) (Seung et al., 2015). In many species the impairing of PTST1 has been associated with low amylose content; in rice, CRISPR/Cas9 generated knocked mutants showing little effects on amylose content in the endosperm (Wang et al., 2020b); differently, starch synthesis was completely abolished in PTST1 knockout barley mutants generated through CRISPR/Cas9 technology (Zhong et al., 2019). Further studies are necessary to better explain the role of these proteins in cereals (Seung, 2020).

Amylopectin synthesis precedes the formation of amylose. It derives from the action of different isoforms of three classes of enzymes: starch synthases (SS), starch branching (SBE), and debranching enzymes (DBE). Four starch synthases (SSI, SSIIa, SSIII, and SSIV) have been characterized and related to the elongation of chains with different DP (Morell et al., 2001); among them, SSIIa elongates medium DP-chains and results essential for starch synthesis. Three isoforms for branching enzymes (SBEI, SBEIIa, SBEIIb) have been extensively characterized: SBEII has two isoforms in grasses with alternative distribution among granule and plastid stroma and results essential for the maintenance of branching pattern in amylopectin (Tetlow and Emes, 2014). SBEI is believed to play a helping role; it has been proposed to be involved in the branching of extra-long chains. Debranching enzymes cleave α-1,6 bonds and result essential for the crystalline structure of the granule: *sugary1* mutants in maize, rice, barley, and wheat, lacking isoamylase (ISA) activity, produce, instead of the starch granules, a water-soluble polyglucan structurally similar to glycogen (Pan and Nelson, 1984; Nakamura et al., 1996; Burton et al., 2002; Sestili et al., 2016). Moreover, branches trimmed by DBE enzymes are a source of malto-oligosaccharides, the substrate for starch synthases, or specific classes of enzymes, such as starch phosphorylase

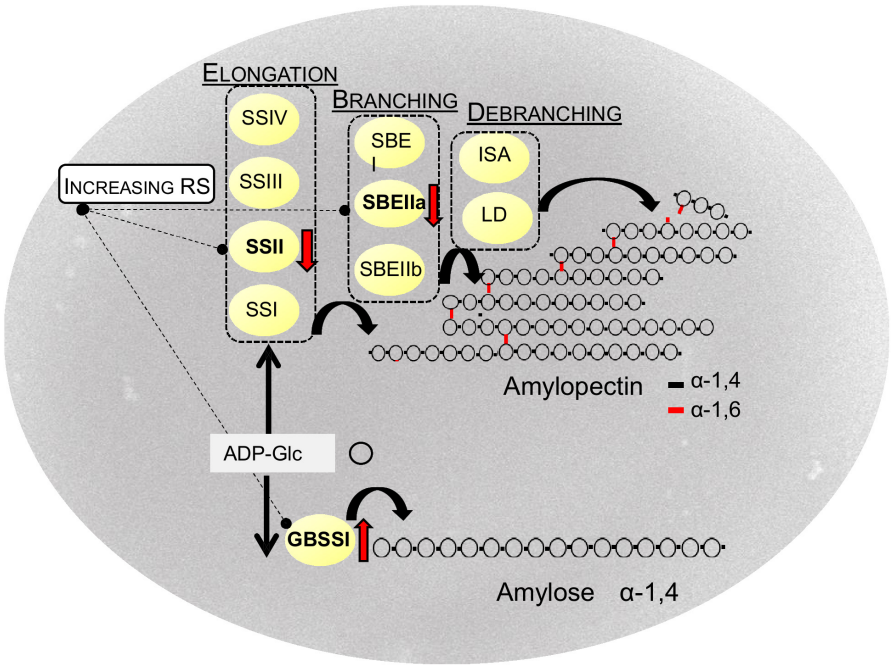

FIGURE 3 | Biosynthesis of (resistant) starch in cereals. SS, starch synthases; SBE, starch branching enzymes; ISA, isoamylases; LD, limit dextrinase. GBSSI Granule Bound Starch Synthase, ADP-Glc: ADP-glucose. Red arrows indicate the main targets to increase RS.

and disproportionating-enzyme involved in granule initiation (Myers et al., 2000).

Transcriptional Regulation of Starch Biosynthesis

Several transcription factors (TFs) regulating starch synthesis have been reported in cereals. Most of them have been identified by co-expression analysis of starch synthetic genes that have largely increased thanks to NGS-based gene expression studies. Detailed analysis of the mode of action for each of these regulators has been reported: overall, it appears a coordinated mechanism in which each TF regulates the expression of many related starch genes. In barley *HvSUSIBA2*, involved in sucrose-mediated control of starch synthesis, directly interacts with *ISA* and *AGPase* genes (Sun et al., 2003). TFs of the bZIP family such as MYB, NAC (for NAM, ATAF and CUC) or AP2/EREBP families have been associated to the regulation of starch related genes (López-González et al., 2019).

OsbZIP58, a basic leucine zipper transcription factor, regulates positively the expression of *OsAGPL3*, *OsWx*, *OsSSIIa*, *OsSBE1*, *OsBEIIb,* and *OsISA*2 in rice (Wang et al., 2013); OsbZIP58 null mutants highlighted an abnormal seed morphology with reduced contents of total starch and amylose. Differently, OsRSR1, an APETALA2/ethylene-responsive element-binding protein family TF, acts as a negative regulator of starch gene expressions in rice seeds (Fu and Xue, 2010): it's silencing increased amylose content, seed size, and yield; on the opposite, its overexpression repressed the expression of starch synthetic genes. In maize, many TFs have been associated with the regulation of key genes involved in starch synthesis (ZmABI4, ZmbZIP91r ZmMYB14 ZmDof3 ZmNAC128, ZmNAC36, and ZmNAC130) (López-González et al., 2019). ZmMYB14 also regulated the expression of the *Brittle 1* (*BT1*) gene, which encodes an ADP-glucose-transporter crucial for starch synthesis. Ethylene signaling has recently been linked in the transcriptional control of starch synthesis in rice, involving the ethylene receptor ETR and the AP2/EREBP family transcription factor (López-González et al., 2019). In wheat and rice endosperms the transcription factor TaNAC019-A1 negatively regulates starch synthesis. Its overexpression reduced significantly starch content, kernel weight, and kernel width (Liu et al., 2020). Recently, Song et al. (2020) identified a transcriptional activator of starch synthesis (TabZIP28) in wheat.

BIOTECHNOLOGICAL RESOURCES FOR DIETARY FIBER IN CEREALS

In the era of genome editing, biotechnology promises an ultra-fine modulation of the genomes without introduction of foreign DNA. The inside potential of this technology is limitless, and much progress has been gained a few times. Single gene targeting has quickly been overcome by multiple loci manipulation through polycistronic designs, thus encountering the need to piece together multiple traits at one time (Camerlengo et al., 2020). Nowadays, we can edit genes precisely "correcting" even one single amino acid or the whole protein sequence (Wada et al., 2020). If this is true, that CRISPR-Cas can afford the challenge to precisely plan gene editing, at now, it is overall meant for reverse genetics purposes aiming at generating "loss of function"

mutations in the genes of interest. The Non-Homologous End Joining mechanism, the most efficient in plants, repairs specific cuts run by Cas enzyme, generating small *in-del* in the sequence that prevents the gene functionality (Schmidt et al., 2019). Notably, in rice a collection of loss of function mutants spanning the genome has been generated, converting CRISPR-Cas into a high-throughput tool for mutagenesis (Lu et al., 2017). This approach is of interest to generate further genetic variability essential in plant genetic improvement (Bigini et al., 2021); in the coming years, the advantages of a CRISPR-Cas induced mutagenesis would be investigated in comparison with other well-established resources such as Targeting Induced Local Lesions IN Genomes (TILLING) (McCallum et al., 2000). Although CRISPR-Cas has revolutionary perspectives, it cannot be done without more classical resources that, over the decades, have allowed to exploit and tag the genome of the organisms associating DNA features to the phenotype: molecular markers have evolved incessantly assuming many forms that, the in last decade, have enormously taken advantage by high-throughput sequencing technologies (**Figure 4**). Considered that in plants, the most are complex quantitative traits relying on the cooperation of multiple loci in the genome, the use of molecular markers is essential to unravel the genetic base of phenotypic variability. The combination of marker technology and NGS allowed the detection of a huge number of DNA markers within a short time frame. The discovery of thousands of genetic markers across the whole genomes resulted largely advantageous either for the study of the genetic variability in wild populations, landraces, and cultivar collections or for the rapid genotyping of hundreds of individuals in a mapping cross focused on the identification of quantitative trait loci (QTLs). Largescale SNP datasets from biparental and multi-parental crossing populations (Borrill et al., 2018), and association panels are now free to access web resources available at CerealsDB (Wilkinson et al., 2016) and Ensembl Plants (Bolser et al., 2016). Genome-Wide Association Study has evolved promptly in the exploitation of natural variability in plant collections picking into the genome the polymorphic sequences responsible for variation in the trait of interest. Other sources of genetic variability, such as induced mutagenesis and TILLING, have also been benefited by NGS: exome capture has been used to sequence the genomes of thousands of individuals of mutagenized plant collections providing enormous advantages to the research of the genotypes of interest (Krasileva et al., 2017; Mo et al., 2018). Similarly, a multitude of RNA-Seq datasets has being generated and made available on the web platform expVIP (Ramírez-González et al., 2018; [1]) supporting the elucidation of key regulatory pathways and genes involved in different traits of interest (**Figure 4**).

Once identified genetic determinants, a main objective of functional genomics is the functional annotation. In this context, reverse genetic tools such as TILLING and Eco-TILLING remain efficient and competitive strategies to validate gene function and generate new genetic variability (McCallum et al., 2000; Comai et al., 2004). In Eco-TILLING, the coexistence of multiple polymorphisms across the plant genomes can allow the identification of plant haplotypes carrying meant polymorphisms at any locus involved in quantitative traits. Expression data are also useful to narrow down candidate genes identified through conventional QTL mapping or functional genomic approaches using a natural and induced variation. Classical transgenic approaches such as gene silencing and RNA-interference, have played a key role in functional genomics overall meant for polyploid organisms, such as wheat, where more than one gene copy should be switched off for perceptible phenotypes (Kumar et al., 2020).

SEARCHING FOR HIGH AX and WE-AX CONTENT QTLs, META-QTLs, AND MOLECULAR MARKERS

Among cereals, AX has been mainly investigated in wheat. The wide network of metabolic pathways involved in AX modeling makes it difficult to target key enzymes to tune their structure and increase their amount. At now, the main work has been done in the exploitation of natural variability in AX content and the identification of related QTLs. Biparental population and large wheat collections have been phenotyped: extensive screening of the extract viscosity, mainly associated with WE-AX content, along with the A/X ratio, the amount of WE-AX and total AX, has been associated with a multitude of genetic loci spread throughout the wheat genome (Marcotuli et al., 2020). Combining the data of extract viscosity from five different wheat populations, Quraishi et al. (2009) optimized the work of different groups through the theorization of a "meta-QTL" including three loci on the chromosomes 1BL, 3D, and 6B. However, several reports analyzing large collections of wheat (inbred, diversity collections) identified many QTLs that mostly account for relatively little variation in AX content (lower than 15.2%). Most of these studies identified a QTL on chromosome 1BL as the most highly associated with the WE-AX content or WE-AX-related properties. At now, the coincidence of various QTLs on this chromosome has not yet been established: Yang et al. (2016) showed that the 1BL QTL, identified in a collection of 240 inbred lines, derived from the 1BL/1RS translocation from rye, known to be absent in modern wheats. More recently, an effective KASP marker for the 1BL QTL has been validated by Lovegrove et al. (2020). Here, two high AX content cultivars Yumai 34 (with high value in WE-AX; Tremmel-Bede et al., 2017) and Valoris, have been intercrossed and crossed with the other two cultivars. The four crosses revealed two major QTLs located on chromosome 1BL from Yumai-34 and chromosome 6B from Valoris. Similarly, Ibba et al. (2021) developed and validated four KASP markers for the 1BL QTL identified through a GWAS study across a collection of 175 wheat lines. Inquiring of the genomic regions surrounded by the associated markers identified a candidate gene involved in AX synthesis. Notably, while most of the research has been conducted on wholegrain material, these last two works focused on white flour.

The discovery of key genes for the manipulation of AX in cereals is challenging also due to the presence of multiple isoforms of the biosynthetic enzymes. In the studies of the gene

[1] www.wheat-expression.com/

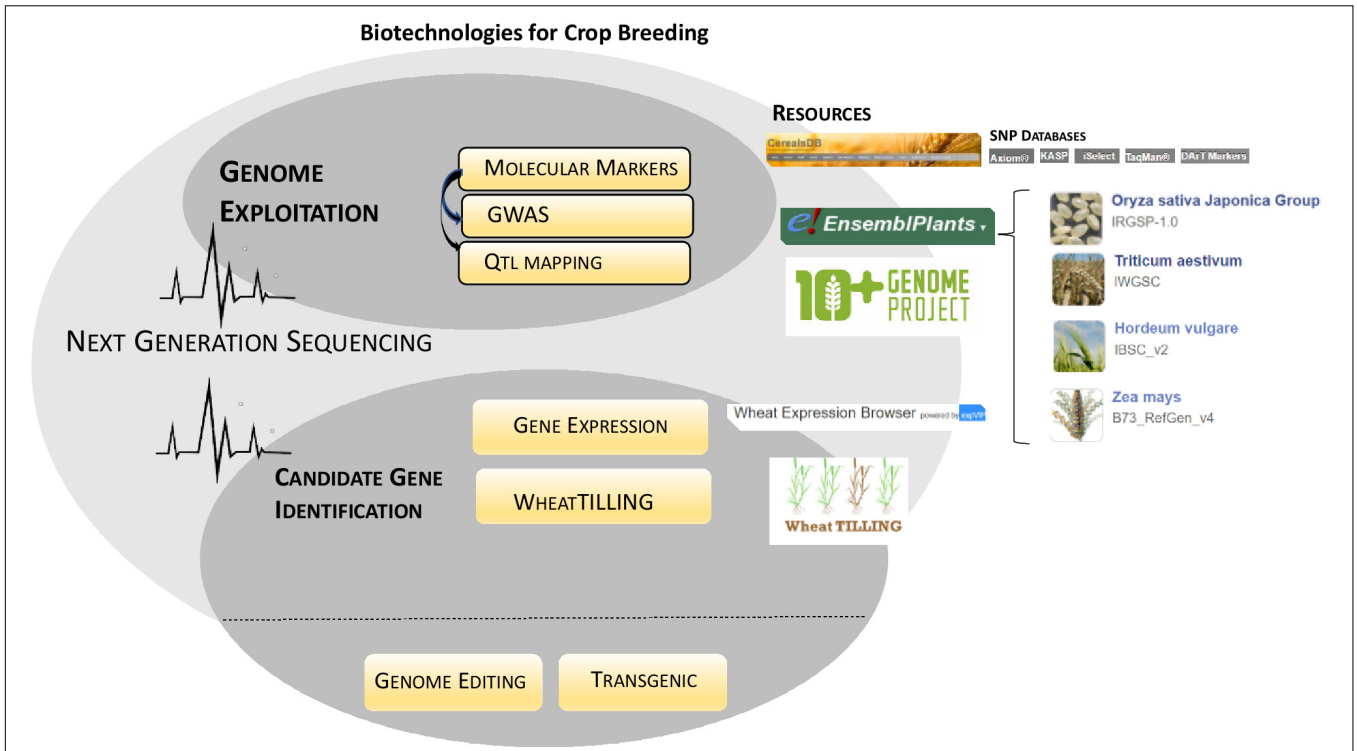

FIGURE 4 | Biotechnologies for crop breeding. The figure summarizes the role played by advanced biotechnologies in crop breeding indicating novel resources now available (Resources). NGS has accelerated both genome exploitation and candidate gene identification by boosting molecular markers research (GWAS/QTL mapping) and transcriptomic/genetic variability studies, respectively.

function, pleiotropic effects of the not-targeted isoforms need to be considered (Bulone et al., 2019). In this regard, omics studies run both on the genomic and transcriptomic scale can help to clarify the role played by different isoenzymes.

β-GLUCAN GENETIC VARIABILITY: A SOURCE TO BOOST β-GLUCAN CONTENT AND VISCOSITY AND TO FINE-TUNE ITS STRUCTURE

The concentrations of βG and DP3:DP4 ratios are significantly influenced by the genotype (Cory et al., 2017). In this regard, βG content and structure are complex traits controlled by a multitude of loci positioned on different chromosomes of the genomes. Major QTLs located on the chromosomes 1H, 2H, 5H and minor loci localized on 3H, 4H, and 6H have been identified (Molina-Cano et al., 2007; Szűcs et al., 2009; Pauli et al., 2014; Shu and Rasmussen, 2014). The QTL on chromosome 7H, localized in the centromeric region, is widely reported and accounts for up to 39% of variation βG content (Li et al., 2008a); an extra QTL located on the distal part of the same chromosome explained a 12.5% of variation; 1H and 5H impacted for the 7–15%. By the synteny with the QTLs identified in barley, key genes involved in βG synthesis were isolated in the rice genome, the first to be annotated among cereals. Many genes belonging to the two major families involved in βG synthesis, *CslF,* and *CslH,* have been isolated in genomic regions concerned by βG associated QTLs. A QTL on chromosome 2H in barley was associated with a cluster of five *Csl*F and one *CslH* gene: *CslF3*, *CslF4*, *CslF8*, *CslF10, CslF12,* and *CslH1*, while in rice a similar cluster of six *OsCslF* genes and two truncated *OsCslF* pseudogenes was detected on rice chromosome 7*; CslF6* was also mapped in the region of the major QTL on chromosome 7H; *CslF9* and a *CslH* isoform were associated to a QTL located on the 1H chromosome (Houston et al., 2014). Single-marker analyses suggested that the genetic control of β-glucan amount and DP3:DP4 ratio was linked to distinct chromosomal regions in the barley genome (chromosome 7H and 1H) (Cory et al., 2017).

In tetraploid wheat, QTLs have been identified on chromosomes 1A, 2A, 2B, 5B, and 7A (Marcotuli et al., 2016) which are associated with genes involved in starch, AX, and fructose metabolism, suggesting a significant role of carbon partitioning in the control of βG content, such as: a starch synthase SSIIa and an isoamylase essential in the elongation and modeling of amylopectin chains; a (1,4)-β-xylan endohydrolase involved in the definition of AX fine structure; a fructan 1-exohydrolase (1-FEH), which is involved in the hydrolysis of fructans have been located in the identified QTLs. Carbon partitioning is known to affect βG content in cereals. Diverse reports in barley, bread, and durum wheat have discussed pleiotropic effects on βG and AX content in starch mutants (Morell et al., 2003; Botticella et al., 2016, 2018).

A GWAS study, performed in barley, reported a common QTL regulating amylose, amylopectin, and βG concentration identified in an operon-like structure on chromosome 7H (Shu and Rasmussen, 2014).

The investigation of the main regulators of the relationships between the metabolisms of the three DFs should be considered in future cereal research as an optimized approach to boost DFs content.

Candidate Genes in β-Glucan Manipulation

The key role of some *CslF* genes has been validated in barley as well as in wheat and rice, by classical gene function studies: a mutation on the *HvCslF6* gene generated plants with a complete lack of βGs in the grain (Tonooka et al., 2009; Taketa et al., 2012); whereas the overexpression of *HvCslF6,* under the control of an endosperm-specific promoter, nearly doubled the content of βGs in the grain (Burton et al., 2011). In wheat transgenic plants with suppressed *TaCslF6*, βG content was decreased by 30–52% (Nemeth et al., 2010). In barley, the overexpression of *CslF4* increased grain βG content by 50% (Burton et al., 2011); differently, the transgenic expression of *CslF9* resulted ineffectively.

As regards the β-glucan structure, *CslF4* overexpression was associated with an increase in DP3:DP4 ratio (from 2.8:1 to 3.1:1), while the downregulation of the *CslF6* gene in the same genetic background decreased the DP3:DP4 ratio to 2.1:1.15. Silencing of endogenous *CslF6* in bread wheat did not affect the DP3:DP4 ratio but reduced the molecular weight of (1,3;1,4)-β-glucan (Nemeth et al., 2010). Additional candidate genes influencing the synthesis of βGs are *HvGlb1* and *HvGlb2* encoding (1,3;1,4)-β-d-glucan endohydrolase isoenzyme EI and EII and co-localized with significant QTLs at the pericentromeric region of 1HL (Han et al., 1995) and the distal end of 7HL, respectively (Li et al., 2008a; Houston et al., 2014). Although the essential role of *CslF6* in βG synthesis has been ascertained, the attempts to associate variation in βG content to polymorphisms in the gene sequence have been unsuccessful (Wong et al., 2015; Garcia-Gimenez et al., 2019). In a panel of 1,336 barley accessions showing a wide variation in βG content, the high conservation in the gene sequence was ascribed to the essential role played by CslF6 in grain vitality; further, the few detected polymorphisms did not explain any variation in βG content. Crossing between parents with extreme values in βG content did not detect stable molecular markers for *CslF6* across different environments and different cultivars (Cory et al., 2012, 2017; Wong et al., 2015). The comparison of gene expression of *CslF6* and other key genes involved in βG synthesis among different cultivars highlighted, in such case, different levels between high and low βG content barley genotypes; however, the mechanism and the genomic regions accounting for these differences are still under investigations (Wong et al., 2015). Different studies suggested a key role for the *CslH* gene family coding for (1,3;1,4)-β-endoglucanases, two isoenzymes co-localized with major QTLs at the pericentromeric region of 1HL (Han et al., 1995) and the distal end of 7HL (Li et al., 2008a; Houston et al., 2014). These genes were found differentially expressed between high and low βG content cultivars; β-endoglucanases could play a role in βG remodeling and be essential to provide a source of glucose from βG degradation in germinating seeds. It is generally assumed that other unknown proteins or regulatory factors are likely to be involved in the determination of βG content in seeds.

To enhance βG content in wheat, different groups tried to introgress key genes from barley through the creation of sets of interspecific addition/substitution lines carrying the chromosome with major QTLs (Danilova et al., 2019; Colasuonno et al., 2020). This approach was unable to balance barley βG content in wheat, supporting the hypothesis that multiple QTLs cooperate in the control of βG content, thus multiple key sequences need to be identified and joined to define a complete haplotype.

MAKING CEREAL STARCH "RESISTANT"

High amylose genotypes have been produced in all the major cereals (**Supplementary Table 2**; Vineyard et al., 1958; Yano et al., 1985; Fujita et al., 1999; Regina et al., 2006; Botticella et al., 2011, 2018; Sestili et al., 2015). Strategies to manipulate the amylose/amylopectin ratio have pointed to target key enzymes involved in starch biosynthesis; two approaches have mainly been exploited: the overexpression of amylose synthesizing enzyme, GBSSI, and the silencing of enzymes involved in elongation and branching of amylopectin chains, namely SS and SBE. The first strategy increased the amount of GBSSI protein but did not increase the amylose content in durum wheat grain (Sestili et al., 2012). In contrast, Itoh et al. (2003) introduced a *GBSSI* transgene into a waxy rice mutant, obtaining transgenic lines with varying levels of amylose content up to 45%. In rice, amylose content ranges from 0 to ∼30% depending on the presence of different *Waxy* alleles, with Wx^a (high AC—more than 20%) and Wx^b (intermediate AC- 14 to ∼18%) being the major alleles found in the indica and japonica varieties, respectively (Teng et al., 2012). Wx^b harbors a mutation at the first nucleotide of intron 1 leading to a low expression of the gene; Crofts et al. (2018) reported that the lower amount of amylose in the genotypes harboring $\underline{Wx^b}$ allele is due to the lower percentage of extra-long amylopectin chains synthesized by the less active GBSSI enzyme.

Although each enzyme showed to affect starch structures, the isoforms able to majorly advantage amylose content were identified in SS class II isoform a (SSIIa) and SBE class II (Wang et al., 2017). Elimination of SSIIa in cereals promotes amylose content from low to a medium level according to different species; moderate increase in amylose was found for maize *sugary2* (*su2*) and wheat *SSIIa* mutant (40–50%) (Yamamori et al., 2000; Zhang et al., 2004; Sestili et al., 2010a; Botticella et al., 2016). In barley *sex6* mutants, amylose increased up to 50% (Morell et al., 2003; Sparla et al., 2014). All these genotypes possess natural or induced mutations identified in wild/landraces collections and TILLING populations (Sestili et al., 2014): the selection of null SSIIa genotypes has been advantaged by the feasibility to visualize starch synthase by simple electrophoresis assays. Recently, the

combination of SNPs, responsible for changes in key amino acids, in the three genes *GBSSI*, *SSIIa,* and *SSIIIa* was associated with a RS content of 8% (26% in amylose) in rice (Gurunathan et al., 2019). This finding highlights the potential of missense mutations to fine-tune traits of interest.

Superior outcomes arise from the suppression of SBEII activity. SBEII defective genotypes have been derived essentially by the screening of mutant collections or by transgenic silencing technology. Among cereals, SBEIIb isoform is the major in maize and rice, while in wheat and barley the most abundant is the "a" isoform (SBEIIa) (Regina et al., 2005). This difference well correlates with the diverse results in the modification of starch composition consequent to the suppression of the "a" or "b" isoforms among the different species. Inactivation of SBEIIb, which is 50 times more abundant than "a" in maize, promotes amylose content from 25–30 to 61–67% in a*mylose-extender* (*ae*) mutants (Li et al., 2008b). In rice, SBEIIb/SBEIIa ratio is much lower (5:1) thus reducing the extent of the rise in amylose to 15% in the *ae* mutant (Nishi et al., 2001). Another *ae*-like mutant *Goami2*, from a *japonica* rice variety, showed about a twofold increase in amylose (Kang et al., 2003) but it has been imputed to the incidence of a further mutation not yet discovered.

On the other hand, SBEIIa is the most significant isoform in wheat and barley (Botticella et al., 2012): removal of its activity by gene silencing raised amylose content to 75% in transgenic bread and durum wheat (25–30% in the control) (Regina et al., 2006; Sestili et al., 2010b); the outcome was different in barley, where a substantial increase in amylose (65%) was obtained only in lines where the expression of both SBEII isoforms resulted decreased by 80% (Regina et al., 2010). The two isoenzymes in barley share the branching activity with distinctive patterns of chain length being transferred by each other in the amylopectin backbone. High amylose wheats have also successfully been derived by the use of TILLING platforms both in bread and durum wheat (Botticella et al., 2011, 2018; Hazard et al., 2012; Slade et al., 2012; Sestili et al., 2015): amylose content increased from 26–33% of wild type to 55–70% in the two *SBEIIa* mutants derived from bread wheat cultivars Express and Cadenza, respectively; similarly, the silencing of *SBEIIa* genes in durum wheat cultivars, Kronos and Svevo, raised the amount of amylose from 24–30 to 47–52%, respectively. In all these lines the increase in amylose was associated with a strong increase in the RS fraction: up to 11–12% and 7% in bread and durum wheat mutants, respectively, compared to less than 1% detectable in wild type sib lines.

A further improvement was achieved by targeting more SBE isoforms in the same plant. In barley, an "amylose only" genotype was produced through the simultaneous suppression of both SBEII and SBEI isoforms through RNA interference (Carciofi et al., 2012).

Crossing between mutant lines derived from two mutagenized wheat populations, one obtained by treatment with physical agents in the cv Chara and the other by treatment with chemical agents in the cv Sunstate, confirmed a major impact for SBEIIa enzyme but also that, double mutation of the two isoforms, SBEIIa and SBEIIb, in wheat is cooperative for the increase in amylose and RS. RS raised to 16.6% of whole flour in the line with 84% in amylose content (Regina et al., 2015). Similarly, Li et al. (2019) produced bread wheat with 93.3% in amylose content (36.7% in the sib line), by combining null mutations in *SBEIIa* and *SBEIIb* genes derived by chemical mutagenesis.

Combining predicted defective allelic variants of SBEIIa and SBEIIb isoforms did not highly enhance the amylose in durum wheat cultivar Kronos (Hazard et al., 2014). The reasons for this discrepancy still need to be investigated. Recently, CRISPR-Cas9 technology was used to produce high amylose rice, raising RS up to 9% in SBEIIb defective mutant lines (Sun et al., 2017). The same approach has been successfully adopted by Li et al. (2021) to target *SBEIIa* genes in both winter and spring wheat varieties, generating transgene-free wheats with high-amylose (up to 69.7% of total starch) and high RS (up to 15%).

FUTURE PERSPECTIVES

Considering the notions here reviewed, much work could be done to produce high fiber cereals by adopting biotechnological tools. Generally, the digestibility of polysaccharides depends on the structure, molecular weight, the nature of glycosidic bonds, and the grade of crosslinking among glucans. The presence of side chains and the coexistence of more types of linkages among monomers increase the solubility of polymers that results in higher accessibility to the solvent and hydrolytic enzymes produced by microflora. For starch, where glucose moieties are linked by α-type bonds, the linearity and molecular weight positively correlate with a decrease in the susceptibility to the human's amylases thus increasing the fraction (RS) available for fermentation by microbiota.

One challenge to be accomplished remains the identification of the main genetic determinants of DFs' fine structure.

As regards AX, the manipulation of arabinose content can positively affect the solubility of the polymer; for this purpose, targeting enzymes involved in both synthesis and transport of Golgi-derived UDP-sugar precursors can help to increase arabinose side chains in the AX structure. However, some arabinose residues may represent substrates for esterification by ferulic acid that reduces its solubility. Consequently, it would be of interest to distinguish isoenzymes responsible for the incorporation of arabinose residues at different positions in the polysaccharide chain. To this aim, primarily reverse genetics tools (i.e., mutagenesis, genome editing, transgenesis) will be essential to identify the key isoenzymes. Knowledge of gene sequences and the elucidation of the pangenome of cereal species (Della Coletta et al., 2021) coupled with functional investigations will facilitate the selection of the best candidate genes to be genetically manipulated. Therefore, further advances can derive by QTLs identification accelerated by NGS-based molecular markers. Similarly, βGs solubility is mainly ascribed to DP3/DP4 ratio; here the elucidation of the role played by specific members of CslF and CslH along with other unknown regulators can be pursued by functional omics studies based on the comparison of high and low content genotypes; moreover, the investigation of the catalytic properties of single isoenzymes by *in vitro* assays could give an important contribution. Also, regards RS, many opportunities can arise by a better understanding of

mechanisms regulating starch fine structure; indeed, molecular weight and branching patterns are finely regulated by a complex of enzymes where the exact role played by each member in the diverse cereals has not yet completely pointed out. In this sense, the targeting of specific enzymes coupled with a detailed investigation of the resultant changes in the fine structure will provide novel opportunities for starch applications in the food industry.

A further issue to face regards the mitigation of side effects associated with high DF content genotypes. Indeed, it has been ascertained that the manipulation of their biosynthetic pathways can affect the metabolic networks causing carbon re-allocation among the compounds in the seed (Morell et al., 2003; Botticella et al., 2018). Occasionally, high amylose starch genotypes display a decreased total starch content with a yield penalty. For this purpose, one strategy can be to introgress yield-associated alleles in such genotypes as well as crossing them with high yield cultivars. Furtherly, for polyploids species such as wheat, it is of interest to evaluate the phenotypes of partial mutants, having mutations in some homoeologous, to find a good balance between improved quality and acceptable yield. The other two traits to be studied in high DF genotypes are the resistance to biotic stress and seed germination rate, since changes in the cell wall structure, as well as the reduction in starch digestibility, can impact both physiological processes; for instance, changes in starch and βGs, essential nutriments for seed development, can impact on seed germination rate.

A further side effect in starch mutants is the change in hardness: in wheat, hardness is an important trait for the technological and qualitative properties of flour. The increase in hardness of high amylose genotypes can have negative impacts in milling with a reduced flour yield and a high percentage of damaged starch (Botticella et al., 2018).

To address this issue, the introgression of the high amylose character in soft cultivars could be ideal for the creation of high RS lines.

Several investigations have found an association between the three DFs here discussed: AXs and b-glucan were increased in high and low amylose lines in several species (Morell et al., 2003; Botticella et al., 2018) but still few studies have investigated this relationship at the metabolic level and the key regulators are still unknown. For this purpose, new knowledge will permit the development of new crop improvement programs aimed to increase the content of more than one type of DFs in the same plant.

Considering the urgency to fight malnutrition through a global approach, a further objective is to combine multiple healthy traits in the same genotype; increasing the content of essential micronutrients such as microelements or vitamin precursors in high fiber genotypes will produce super crops aimed at satisfying multiple nutrition needs at the same time. At this purpose, genome editing technologies, able to target multiple genes simultaneously, may greatly accelerate breeding programs; these new approaches accomplished to the advent of "speed breeding" (Watson et al., 2018) will permit to respond rapidly and efficiently to the new challenges, such as the climate changes, the necessity to increase crop yield and to realize new plants more nutritious and healthy for humans.

CONCLUSION

NGS and the novel biotechnological tools have enormously accelerated the elucidation of nutrient metabolism and the identification of key loci responsible for their accumulation in the plant, representing one of the best approaches to gain biofortification of staple crops. Genotyping of hundreds of individuals has become feasible in a short time frame, allowing the identification of multiple loci determining complex traits and, overall, increasing the possibility of genetic variability exploitation in complex crops such as cereals. Among DFs, βGs and AXs are cell wall polysaccharides more concentrated in the outer layers of the seed, while RS is essentially associated with high amylose starches only found in the endosperm. If candidate genes for RS content have been identified and successfully targeted to increase its content, both for AXs and βGs this is far longer to be achieved also due to the high number of members belonging to the large family of the biosynthetic enzymes. Nonetheless, the high potential of modern omics and biotechnological tools is accelerating the identifications of major QTLs and key biosynthetic enzymes thus paving the way for the development of novel high fiber genotypes in major cereals.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.745579/full#supplementary-material

Supplementary Figure 1 | Dietary fibers across seed layers in major cereals. The figure reports the major dietary fibers and their localization in the different kernel layers. AX, arabinoxylans; βG, βglucans; RS, resistant starch.

Supplementary Table 1 | Amount of DFs in different seed tissues of major cereals.

Supplementary Table 2 | High Resistant starch genotypes in major cereals.

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Modifications of Grapevine Berry Composition Induced by Main Viral and Fungal Pathogens in a Climate Change Scenario

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The grapevine is subject to high number of fungal and viral diseases, which are responsible for important economic losses in the global wine sector every year. These pathogens deteriorate grapevine berry quality either directly *via* the modulation of fruit metabolic pathways and the production of endogenous compounds associated with bad taste and/or flavor, or indirectly *via* their impact on vine physiology. The most common and devastating fungal diseases in viticulture are gray mold, downy mildew (DM), and powdery mildew (PM), caused, respectively by *Botrytis cinerea*, *Plasmopara viticola*, and *Erysiphe necator*. Whereas *B. cinerea* mainly infects and deteriorates the ripening fruit directly, deteriorations by DM and PM are mostly indirect *via* a reduction of photosynthetic leaf area. Nevertheless, mildews can also infect berries at certain developmental stages and directly alter fruit quality *via* the biosynthesis of unpleasant flavor compounds that impair ultimate wine quality. The grapevine is furthermore host of a wide range of viruses that reduce vine longevity, productivity and berry quality in different ways. The most widespread virus-related diseases, that are known nowadays, are Grapevine Leafroll Disease (GLRD), Grapevine Fanleaf Disease (GFLD), and the more recently characterized grapevine red blotch disease (GRBD). Future climatic conditions are creating a more favorable environment for the proliferation of most virus-insect vectors, so the spread of virus-related diseases is expected to increase in most wine-growing regions. However, the impact of climate change on the evolution of fungal disease pressure will be variable and depending on region and pathogen, with mildews remaining certainly the major phytosanitary threat in

most regions because their development rate is to a large extent temperature-driven. This paper aims to provide a review of published literature on most important grapevine fungal and viral pathogens and their impact on grape berry physiology and quality. Our overview of the published literature highlights gaps in our understanding of plant-pathogen interactions, which are valuable for conceiving future research programs dealing with the different pathogens and their impacts on grapevine berry quality and metabolism.

Keywords: grapevine, biotic stress, *Plasmopara viticola*, leafroll virus, fanleaf virus, *Erysiphe necator*, *Botrytis cinerea*

INTRODUCTION

The European grapevine *Vitis vinifera* L., by far the main *Vitis* species used for wine production in the world, is host of a multitude of biotic adveristies from insects and fungi to viruses and bacteria. Downy and powdery mildew (DM and PM) are the major fungal pathogens in most wine-growing regions worldwide. Because these two pathogens were accidentally imported into Europe from North America rather recently, at the end of the 19th century, their host, the European grapevine, did not co-evolve with them and consequently does not possess natural resistances against them. Thus, in order to guarantee sustainable yield and fruit quality, viticulture depends on relatively high amounts and frequency of pesticide application, compared to other agricultural crops. Because the grapevine has been propagated vegetatively for thousands of years, it is host of a very high number (currently >80) of graft- and vector-transmitted viral diseases that can cause important economic losses in all wine-growing regions worldwide, with the most important ones being grapevine fanleaf disease (GFLD), grapevine leafroll disease (GLRD), and the recently characterized grapevine red blotch disease (GRBD; Maliogka et al., 2015; Bragard et al., 2019; Fuchs, 2020).

The effects of global climate change on disease pressure are not unambiguous. Some models predict a decreasing fungal disease pressure and, consequently, as predicted by Zito et al. (2018) for PM and DM in the region of Burgundy, mainly due to lower precipitations during the growing season. Other modelling approaches show that the increase in temperature advances the outbreak time of diseases, such as DM, leading to more severe infections and more infection cycles, due to the polycyclic nature of the pathogen (Francesca et al., 2006; Bove et al., 2020). The higher temperature during the months of May and June create also a more favorable environment for mildew development, counterbalancing the effects of precipitation reductions, which alone would have diminished the disease pressure (Salinari et al., 2007). In any case, at the global level, mildews will very likely remain the major phytosanitary threat under future climatic conditions (Bois et al., 2017). In this context, the increasingly widespread use of single-site fungicides to control DM accelerated the development of *P. viticola* strain with resistance to most of the fungicide classes (Massi et al., 2021). The predicted increase in temperature caused by global warming is already leading to advances of the development of the grape berry moth whose larvae feed on ripening grape berries, thereby providing "entry-gates" for *B. cinerea* infection (Reineke and Thiéry, 2016; Santos et al., 2020). This could consequently result in increasing berry mold infections if pest management strategies are not adapted. As for DM, rainfall and relative humidity are key factors for the onset of gray mold (Molitor et al., 2016). The increasing risk of mold infection could thus be counterbalanced by decreasing precipitations in some regions. Higher temperatures will also favor the development of insect vectors of bacterial and viral diseases and thus augments the spread of viral diseases in most growing regions (Bois et al., 2017). A better understanding of pathogen-host interactions is of upmost importance for elaborating efficient disease-management strategies in order to guarantee high quality and sustainable wine production in an evolving environment.

The lifecycle of the most common fungal diseases is well-characterized, as is their negative impact on grapevine physiology; yet, for most diseases, the molecular mechanisms that underpin the deterioration of berry quality remain to be elucidated. Recent advances in metabolomics led to the discovery of new odorous and non-odorous compounds produced either directly by pathogens or released by leaves or berries following infection (Darriet et al., 2002; Ky et al., 2012; Pons et al., 2018; de Ferron et al., 2020). These compounds can be linked to deleterious effects on wine quality. However, molecular data regarding the interactions between pathogens and the berry are scarce. Nevertheless, the rapid development and improvement of omic tools continuously increase our insights into plant–berry pathogen interactions (Blanco-Ulate et al., 2015, 2017; Rienth et al., 2019b; Ghaffari et al., 2020; Toffolatti et al., 2020; Pimentel et al., 2021). The first part of this review aims to summarize the most important and recent studies dealing with the effects of gray mold, PM, and DM on berry quality and metabolism. Subsequently, we review the literature describing the effects on berry physiology of the most important virus-related diseases, such as grapevine fanleaf, grapevine leafroll, and red blotch disease (a general overview of modulated metabolites is provided in **Figure 1** and a more detailed summary of modulated compounds and transcripts in **Supplementary Table 1**). Although a major attention was paid on the effects on primary and secondary metabolism, we did not review the general biosynthetic pathways because this would have gone beyond the scope of the paper. For more detailed description of biosynthetic pathways in the grape berry we invite the reader to consult recent reviews on berry development and physiology (Conde et al., 2007;

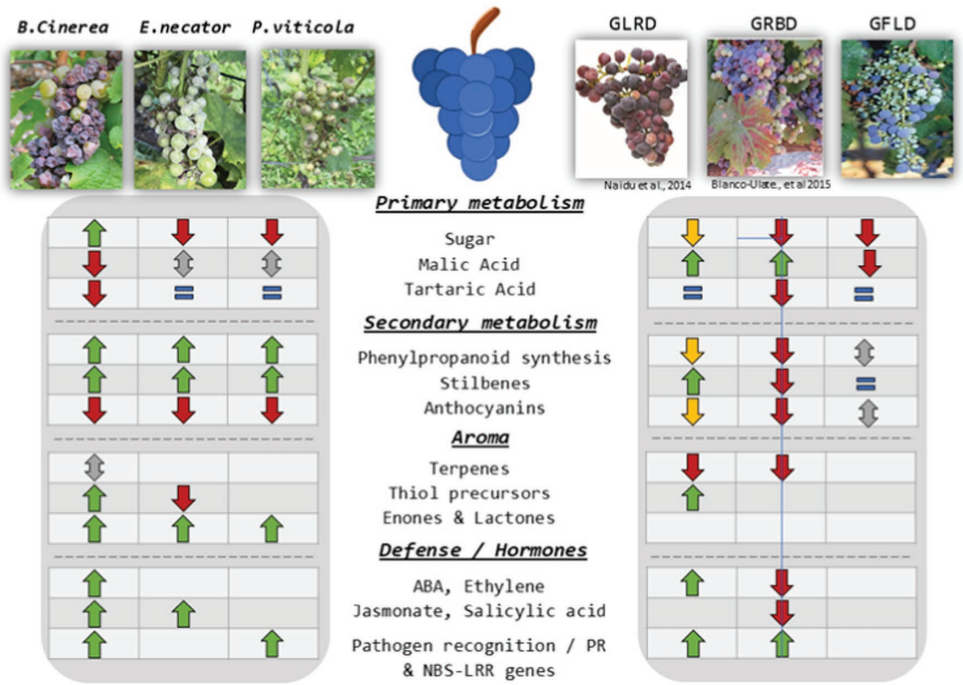

FIGURE 1 | General overview of most important metabolites and mechanisms modulated by the reviewed pathogens: ↓: decrease; ↑: increase; ↕: opposed results reported; ↓: decrease only in some studies likely due to sampling strategy, thus decrease putatively observed due to a delay in phenology; =: no differences.

Kuhn et al., 2013) as well as primary (Burbidge et al., 2021; Walker et al., 2021) and secondary metabolism (Azuma, 2018; Lin et al., 2019; Rienth et al., 2021).

BOTRYTIS CINEREA

Botrytis cinerea is among the most relevant berry-infecting fungal pathogens and is responsible for important reductions in yield and quality of grapes worldwide. *Botrytis cinerea* is responsible for both the so-called prejudicial gray mold and noble rot that will both be discussed in subsequent sections. Whether *B. cinerea* infection cause noble rot or gray mold is principally dependent on weather post-infection. Wet or humid conditions lead berries infected with *B. cinerea* to develop gray mold, whereas the development of noble rot requires moist nights and foggy mornings with dry and warm days.

Noble Rot

Noble rot is used to produce highly valued sweet dessert wines, such as Sauternes, Tokaji Aszu, and Amarone. The positive effects of noble rot are not only due to the concentration of sugar and aroma components through *B. cinerea*-induced water loss of the berry, but also from an enhanced synthesis of aromatic metabolites and their precursors, such as odorous lactones (γ and δ lactones) and their precursors (Miklósy and Kerényi, 2004; Tosi et al., 2012; Stamatopoulos et al., 2014; Lopez Pinar et al., 2017a,b; Stamatopoulos et al., 2018). In particular, this has been shown for S-conjugates of glutathione and cysteine, the precursors of varietal thiols reminiscent of citrus, grapefruit/passionfruit, or boxwood aroma. Their concentration can increase more than 100-fold upon *B. cinerea* infection (Sarrazin et al., 2007; Bailly et al., 2009; Thibon et al., 2009, 2011). The concomitant presence of S-conjugates of glutathione and cysteine in musts from infected berries as well as in botrytized berries highlights an activation of the glutathione biosynthetic pathways, a known strategy of plants as a general response to biotic (pathogenic attack) or abiotic stresses (injury, oxidative stress etc.; Hasanuzzaman et al., 2017). This has also been shown for grapevine leaves and berries exposed to cold and heat shock as well as UV irradiation (Kobayashi et al., 2011). Biosynthesis of S-conjugated amino acids and subsequent metabolites results from an increased release of reactive and toxic aldehydes, such as trans-2-hexenal reacting with glutathione to form glutathione S-conjugates, such as S-3-(hexan-1-ol)-glutathione, which in turn will be metabolized into S-conjugate cysteine and ultimately cleaved into sulfanylalcohols during fermentation by ß-lyase activity of *Saccharomyces cerevisiae* (Tominaga et al., 1998).

Other odorous volatile compounds, such as furanone, methional, and phenylacetaldehyde, have been found in botrytized berries (Kikuchi et al., 1983; Miklósy and Kerényi, 2004; Tosi et al., 2012; Stamatopoulos et al., 2014; Lopez Pinar et al., 2017a,b; Stamatopoulos et al., 2018). These compounds, with γ and δ lactones and varietal thiols, can contribute in a desirable way to the flavor of sweet wines produced from noble rot-infected grapes (Schreier et al., 1976; Sarrazin et al., 2007; Tosi et al., 2012). Organic acids, such as tartaric and malic, are metabolized by the fungi and wines from botrytized berries have generally lower total acidity (Shimazu et al., 1984).

Gray Mold

Gray mold can be very detrimental to fruit and wine quality due to the degradation of a number of grape berry components (Pallotta et al., 1998). In particular, phenolic compounds (anthocyanins, hydroxycinnamic acids, and flavanols) are oxidized by the polyphenol oxidase (laccase) activity of *B. cinerea* (Dubernet et al., 1977), which leads to quinones, that are highly reactive with glutathione and volatile odorous thiols. Ky et al. (2012) showed that all phenolic compounds (anthocyanins and proanthocyanidin monomers, dimers and trimers) decreased drastically in gray mold-infected grape skins as well as the mean degree of polymerization of the proanthocyanidin polymeric fraction.

Some aromatic components, like monoterpenes that play a major role in the aromas of Muscat grape cultivars and wines, are transformed into less odorous compounds (Boidron, 1978; Bock et al., 1986). Ethyl esters of fatty acids, which contribute to the fermentation aromas of wine, are hydrolyzed by the esterase activity of *B. cinerea* (Dubourdieu et al., 1983). Moreover, the development of gray mold, associated with abundant sporulation, as well as the presence of spores in the must, leads to the production of mushroom and earthy off-odors in the resulting wines (La Guerche et al., 2006; Lopez Pinar et al., 2017a,b). Compounds responsible for such off-odors have been identified as 1-octen-3-one, 1-octen-3-ol, 2-heptanol, and 2-octen-1-ol with mushroom notes, or 2-methylisoborneol with strong earthy notes. Sometimes, a certain proportion of botrytized grapes (< 1–5%) are co-contaminated with other saprophytic fungi belonging to the genus *Penicillium* spp., particularly *P. expansum*, as well as *Mucor* spp., *Trichotecium* spp., *Cladosporium* spp., *Aureobasidium* spp., *Alternaria* spp., etc., which may develop inside the grape clusters, resulting in secondary rot with yellow, green, or pink shades. Their presence is generally favored by their ability to grow at lower temperatures than *B. cinerea* (10–15°C). In this context, other off-odors may be detected in wines, associated with the formation of potent, malodorous compounds, particularly caused by the presence of *Penicillium* spp., in particular *Penicillium expansum*, and its production of (−)-geosmin (Darriet et al., 2000; La Guerche et al., 2006, 2007; Steel et al., 2013; Behr et al., 2014).

Berry Transcriptomic Modulations by *B. cinerea*

The four most recent whole-genome transcriptomic studies on *B. cinerea*-infected berries used either Microarrays (Agudelo-Romero et al., 2015; Kelloniemi et al., 2015) or RNA-seq (Blanco-Ulate et al., 2015; Lovato et al., 2019a,b) to detect differentially expressed genes upon *B. infection*. Varieties, sampling- and infection protocols vary in all studies, making it difficult to draw general conclusions. Agudelo-Romero et al. (2015) and Blanco-Ulate et al. (2015) investigate gray mold infection on Semillon berries sampled at maturity (23.9°Brix; Blanco-Ulate et al., 2015) and Trincadeira at two different stages of berry development (green berries: EL 33 and at véraison: EL 35; Agudelo-Romero et al., 2015). Both studies draw samples directly *in situ*. Kelloniemi et al. (2015) and Lovato et al. (2019b) studied noble rot on post-harvest-infected berries from Marselan berries cut at version and maturity (Kelloniemi et al., 2015) and on Muller-Thurgau and Garganega (Lovato et al., 2019b). Furthermore, the latter authors carried out a meta-analysis of transcriptomic data from all aforementioned studies on *B. cinerea*-infected grapes. They could identify only 17 commonly upregulated transcripts in all varieties and stages that were specific to *B. cinera* infection. When looking only at either gray mold or noble rot and only after véraison, commonly upregulated genes were found to be higher: 129 and 173, which seems still relatively low in comparison with other biotic (Rienth et al., 2019a) and abiotic stress (Rienth et al., 2014b; Lecourieux et al., 2017) transcriptomic studies. Obviously, this relatively low number of commonly regulated genes can, to some degree, be explained by the fact that gray mold and noble rot infections were compared from different varieties. However, it is very likely that the different, not very precisely defined and characterized berry sampling stages prevent the detection of more commonly genes modulated specifically by *B. cinerea*. It has been shown that a unprecise definition of berry sampling stages can biases biotic and abiotic stress to a high extent, mainly when studies include the stage of véraison (which is considered when 50% of berries change color), where a large transcriptomic reprogramming occurs (Terrier et al., 2005; Rienth et al., 2014a) within 24 h from green to soft (and colored) berries. Thus mixing green and colored berries from a cluster at véraison can introduce unquantifiable biases that could cover important transcriptomic regulations (Carbonell-Bejerano et al., 2016; Shahood et al., 2020).

Nevertheless when looking at different metabolic pathways one can summarize that in all studies, *B. cinerea* caused an induction of secondary metabolism. Notably, the phenylpropanoid pathway was generally highly activated, as indicated by the upregulation of *R2R3-MYB*, *VviMYB5a/b* transcription factors (TFs), known as key regulators of phenylpropanoids, and the downstream key genes involved in the production of flavonoids, such as chalcones, flavonols, and anthocyanins, procyanidins, lignin, and lignans (Blanco-Ulate et al., 2015; Lovato et al., 2019a,b).

In particular, the biosynthesis of stilbenes, major defense compounds (Gindro et al., 2012a), is highly activated upon *B. cinerea* infection, as highlighted by the induction of *VvMYB14*, *VvSTSs* and *PAL* expressions in samples of Lovato et al. (2019a,b) which was similar to that of berries sampled at the véraison stage by Kelloniemi et al. (2015). Interestingly, in the white cultivar Semillon, noble rot caused by *B. cinerea* induced the expression of transcriptional regulators normally expressed in the skins of red cultivars, including five *R2R3-MYBs* that regulate stilbene (*VvMYB14,VvMYB15*), proanthocyanidin (*VvMYBPA1*), and phenylpropanoid (*VvMYBC2-L1, VvMYB4a*) metabolism, as well as other potential regulators of ripening, such as *VvNAC33, VviNAC60*, a zinc-finger transcription factor, a MYB transcription factor, and an *AP2/ERF* transcription factor (Blanco-Ulate et al., 2015).

Transcriptomic data regarding volatile compounds biosynthesis highlight a cultivar-dependent response. For

example, Blanco-Ulate et al. (2015) observed a general upregulation of several terpene synthases, *VvTPS*, in Sémillon botrytized berries, which was, however, not confirmed in Trincadeira (Agudelo-Romero et al., 2015) or Muller-Thurgau and Garganega (Lovato et al., 2019b), where terpene biosynthetic genes were generally downregulated by the infection. A further, more general physiological response of berry metabolism upon *B. cinerea* infection is the upregulation of oxidative stress-related transcripts, abscisic acid, ethylene, jasmonate, and salicylate pathways, and genes encoding resistance (PR-genes; Agudelo-Romero et al., 2015; Coelho et al., 2019; Lovato et al., 2019b). Coelho et al. (2019) compared the hormonal response in *Botrytis*-infected berries of susceptible (Trincadeira) and resistant (Syrah) varieties and showed that high basal levels of salicylic acid (SA) and indoleacetic acid (IAA) at an early stage of ripening, together with activated SA and IAA metabolism and signaling seem to be important in providing a fast defense response leading to grape tolerance against *B. cinerea*.

Interestingly, a group of sugar transporter (*SWEETs*) seems to play an important role during *B. cinerea* infections as shown for the first time by Chong et al. (2014), who demonstrated an involvement of the grapevine *SWEET transporter 4* (*VvSWEET4*). Moreover, they further showed that *VvSWEET4* is a glucose transporter localized in the plasma membrane, which is upregulated by inducers of reactive oxygen species and virulence factors from necrotizing pathogens. This led authors to the hypothesis that stimulation of expression of a developmentally regulated glucose uniporter by reactive oxygen species production and extensive cell death after necrotrophic fungal infection could facilitate sugar acquisition from plant cells by the pathogen. Later, Breia et al. (2020b) highlighted the role of several other *VvSWEETs* in grapevine berries upon pathogen infections. Notably the mono- and disaccharide transporter *VvSWEET7* was strongly upregulated during *B. cinerea* infection of grape berries. This induction may be caused by the pathogen itself to promote leakage of sugars into the apoplastic space for nutrition, or, as a defense-related process to improve sugar remobilization which can trigger signaling cascades that activate plant defense mechanisms. The same authors showed as well that grapevine's sucrose transporter *Early-Response to Dehydration six-like 13* (*VvERD6l13*) was strongly upregulated in response to *B. cinerea* but as well by *E. necator* infection (Breia et al., 2020a).

As mentioned above, several studies report increased thiols, which correlate with the detoxification pathway and increased lactone content in wines and musts produced from *B. cinerea*-infected grapes. Together with the upregulation of the phenylpropanoid, anthocyanin, stilbene, and terpene pathways, and with the induction of defense phytohormones pathway, this highlights a deep metabolic reprogramming as a plant defense mechanism against *B. cinerea* infection. However, transcriptomic studies are unambiguous regarding volatile synthesis. More studies including more varieties and precise definitions of berry sampling and infection protocols are required in the future to better characterize stage and variety dependent responses to *B. cinerea* which could also give more insight on genotypic plasticity of tolerance to infection of some varieties.

DOWNY MILDEW – *PLASMOPARA VITICOLA*

Downy mildew infections on leaves can cause important losses of leaf surface, and thus negatively affect carbon status at the vine level. This leads to ripening delay, slacking sugar accumulation, and malic acid respiration in berries. Therefore, grapes from vines with high infections of *P. viticola* have in general lower total soluble solids, less juice color, and higher acidity compared to fruits from healthy grapevines. DM can also infect berries at early stages, as well as the pre-bloom flowers, pedicel, and rachis (Kennelly et al., 2005). Mature berry infection is strongly restricted by ontogenic resistance due to the progressive modifications of stomata into lenticel at véraison (Gindro et al., 2012b). After véraison, sporulation can still be seen on pedicel and rachis with little to no consequence for berry quality. Probably, because of this almost-absence of DM infection after fruit set, few studies were conducted to understand the effect of *P viticola* on berries. To understand the resistance mechanism of different cultivars, Gindro et al. (2012a) analyzed stilbene content in grape clusters of susceptible cultivars, such as Chasselas and Merlot, and resistant ones, such as Solaris and the hybrid 2091, after *P. viticola* infection. At the inflorescence stage (BBCH 53), in non inoculated conditions, susceptible cultivars presented a high basal content of piceid compared to the resistant ones. A shift in stilbene content occurred after inoculation for all cultivars. Upon infection susceptible cultivars showed a high accumulation of piceid while other stilbenes remained at lower concentrations. For Solaris, a higher accumulation of piceid, resveratrol, δ- and ε-viniferins, and pterostilbene compared to the control is observed. The 2091 hybrid showed a high accumulation of piceid and resveratrol and δ-viniferin and a lower accumulation of ε-viniferin and pterostilbene. At later stage (end of flowering and berry pea-sized stages), a gradual diminution of stilbene content was observed, which was longer in susceptible cultivars, especially for Chasselas, with a high accumulation of resveratrol at the end of flowering that would later be metabolized at the berry pea-sized stage. Beside the stilbene-related plant defense response, DM berry infections modify the grape aroma profile. These modifications are associated with increased concentrations of lactones in wines, such as γ-octalactone, γ-nonalactone, and γ-decalactone, as well as a significant proportion of volatile compounds issued from unsaturated fatty acid degradation, such as (*Z*)-1,5-heptadien-3-one and (*Z*)-1,5-octadien-3-one (geranium leaves descriptors; Pons et al., 2018). Furthermore, 3-methyl-2,4-nonanedione (MND), a powerful β-diketone identified in prematurely aged red wines marked with an intense prune flavor, is more abundant in wines produced from grapes that include diseased berries. Finally, Merlot wines produced with increased levels of *P. viticola*-infected berries presented a higher intensity of the "cooked fruit" character and sometimes

green nuances (Pons et al., 2018). Recently, Poitou et al. (2021) evidenced the impact of Methyl salicylate (MeSA), which induces plant defense resistance and is an odorous volatile compound presenting green nuances in wine. The latter authors could show that *P. viticola*-infected grapes had higher concentration of MeSA than healthy ones and showed that this aroma compound contributes in the expression of fresh green aromatic nuances in red wines, e.g., "pharmaceutical," "camphor," or "menthol" aromas. With the exception of stilbene-related defense mechanisms and lactone biosynthesis, the impact of *P. viticola* on berries remains elusive and requires deeper investigation, in particular from a molecular point of view.

POWDERY MILDEW – *ERYSIPHE NECATOR*

Together with DM, PM is the most important fungal disease infecting mainly grapevine leaves. Berry infections can occur during the green growth phase and are particularly detrimental to quality when they occur around fruit set. Berry infections during ripening are reported to occur until soluble solids levels reach 8°Brix (Gadoury et al., 2003). As for DM, foliar infections can cause important losses of photosynthetic leaf area, which leads mainly to a delay in ripening, thus lower sugar concentration and higher acidity. When grape berries are directly infected with PM, they mostly show similar soluble solids content as healthy grapes but with significantly lower yields (Pool et al., 1984; Gadoury et al., 2001; Stummer et al., 2003, 2005; Calonnec et al., 2004; Pimentel et al., 2021). Contradictory results are reported concerning titratable acidity of infected berries, with some authors reporting an increase (Calonnec et al., 2004) and others a decrease (Lopez Pinar et al., 2016). Anthocyanin concentration was lower in Cabernet Sauvignon (Calonnec et al., 2004) and Sangiovese (Piermattei et al., 1999) in wines produced with PM-contaminated berries. Total phenolics, hydroxycinnamates, and flavonoids were higher in wines from infected grapes from the white cultivar Chardonnay (Stummer et al., 2003, 2005).

Studies about effects on aroma compounds are scarce. Calonnec et al. (2004) report a decrease of the thiol 3-sulfanylhexan-1-ol (3SH) in Sauvignon blanc wines and high concentrations of lactones (γ-butyrolactone, γ-dodecalactone, and γ-decalactone) similarly to what also observed in Riesling when berries were affected with PM (Lopez Pinar et al., 2017a,b).

Another important aspect of PM development on grapes concerns the presence of 1-octen-3-one (mushroom-like notes), (*Z*)-1,5-octadien-3-one (geranium leaf-like notes), phenylacetic acid (honey notes), and (R)-carvone (spearmint notes). The enone aroma-related compounds mentioned above generally diminish and sometimes disappear during winemaking, a phenomena related to the enzymatic reduction of main off-odors [1-octen-3-one, (*Z*)-1,5-octadien-3-one] to less odorant compounds [3-octanone, (*Z*)-5-octen-3-one] by enone reductase of *Saccharomyces cerevisiae* (Wanner and Tressl, 1998; Darriet et al., 2002).

Transcriptomic studies on PM infection of grapevine berries are scarce. Up to our knowledge, the only comprehensive metabolomic and whole-genome transcriptomic study on PM-infected berries was conducted on Grenache berries on developmental stages EL32 (green berry at bunch closer) and EL35 (véraison) by Pimentel et al. (2021). They report a strong indication of defensive mechanisms upon PM infection indicated by higher levels of jasmonates and salicylic acid together with the secretion of effectors related to effector-triggered susceptibility, such as PR1 genes and Enhanced disease susceptibility 1 (EDS 1). PM infection lead as well to an upregulation of carbohydrate-active enzymes, fatty acid and nitrogen uptake and the increase of metabolites, such as gallic, eicosanoic and docosanoic acids, and resveratrol, which could serve as potential metabolic biomarkers, that could be used to monitor the early stages of the infection (Pimentel et al., 2021). Furthermore, PM infection induced an activation of key phenylpropanoid pathway genes (*PAL, C4H, 4CL, CHS, F3H*) and accumulation of catechins, resveratrol and an overexpression of the respective transcripts, such as *LAR/ANS* and *STS*s. Anthocyanins transcripts (*F3'5'H, F3'H,UFGT*) were also found to be upregulated in early stages of berry development, which goes along with previous studies on Chardonnay (Stummer et al., 2003, 2005). These transcripts were particularly upregulated in the green berry (EL32) and correlated with higher anthocyanin content. This was however not significant anymore at EL35. Since anthocyanin accumulation starts at later stages after véraison a clear conclusion on the impact on anthocyanins at maturity cannot be drawn on the basis of these results.

Infection of berries with secondary fungi can be responsible not only for off flavors in grape and wine but also for high concentration of phytotoxin, such as ochratoxin A (OTA) or fumonisin B2 (FB2). Indeed, in an interesting study, it was seen that Negroamaro berries, infected with PM were significantly more susceptible to both *Aspergillus niger* and *Aspergillus carbonarius* colonization which produce FB2 and OTA (Cozzi et al., 2013).

VIRUS INFECTIONS

The present section will summarize the impact on berry composition of the most important viral diseases, GLRD, GFLD, as well as the recently discovered and described Grapevine red blotch and Pinot Gris virus (Sudarshana et al., 2015; Blanco-Ulate et al., 2017; Adiputra et al., 2018; Reynard et al., 2018; Cieniewicz et al., 2020).

In general, symptoms of virus infections in plants strongly vary according to genotype and, up to a great extent, to pedoclimatic conditions (Cretazzo et al., 2010). The symptoms differ as well between plant tissues, sometimes triggering completely opposite metabolic responses in berries (Vega et al., 2011) or leaves as was shown for GLRD (Perrone et al., 2017).

Grapevine Leafroll Disease

Grapevine leafroll disease is considered the most widespread and devastating virus-associated disease. So far, nine serologically distinct virus types from the *Closteroviridae* family were associated with GLRD, named grapevine leafroll associated virus (GLRaV) types 1–9, with the most widespread ones being GLRaV-1 and GLRaV-3 (Maree et al., 2013; Velasco et al., 2014). GLRD causes important reductions in yield, vigor, and longevity of vines. It also delays fruit ripening, reduces sugar accumulation, and impairs fruit pigmentation (Guidoni et al., 1997; Maree et al., 2013; Naidu et al., 2014, 2015; Alabi et al., 2016). Decreases in anthocyanin and total flavonoid concentrations due to GLRD infections were related to the downregulation of anthocyanin-related transcripts, such as *VvUFGT*, *VvMYABA1,* and other phenylpropanoid genes, such as *VvCHS*, *VvFLS1,* and *VvMYAPA1* (Vega et al., 2011; Vondras et al., 2021). Vega et al. (2011) also observed a viral repression of sugar transporters, which translated to lower sugar concentration in grapes. Interestingly, a study using a different berry sampling approach, which accounts for potential phenological shifts and intracluster berry heterogeneity, has demonstrated that the downregulation of genes belonging to primary and secondary metabolite pathways was mainly due to the berry phenological delay induced by GLRD infection and not to a direct effect of the viral infection (Rienth et al., 2019b; Ghaffari et al., 2020). In a very comprehensive study on Carbernet Franc over 2 years on different rootstocks, Vondras et al. (2021) observed a rootstock specific transcriptomic response of berries from GLRaV-infected vines. Latter authors observed the modulation of genes related to pathogen detection, for example NBS-LRR genes that confer resistance to powdery and downy mildew (DM) in grapevine (Riaz et al., 2011; Zini et al., 2019), as well as abscisic acid (ABA) signaling, phenylpropanoid biosynthesis, and cytoskeleton remodeling similar to previous studies of Vega et al. (2011) and Ghaffari et al. (2020). Interestingly the increase of ABA abundance in GLRD-infected berries (Vondras et al., 2021) was different than that observed for red blotch virus-infected berries, in which ABA abundance and *NCED* expression decrease in infected berries after véraison (Blanco-Ulate et al., 2017).

In leaves, some studies report a similar upregulation of defense-related genes and a concomitant accumulation of phenylpropanoids, such as resveratrol, which led to an enhanced resistance to downy mildew (Repetto et al., 2012). This highly interesting observation was somehow confirmed in a very elegant experiment, where authors transmitted GFLaV and grapevine rupestris stem pitting-associated virus (GRSPaV), by *in vitro*-grafting, to Nebbiolo and Chardonnay. Upon subsequent downy and powdery mildew infection, GFLV-infected plants showed a reduction in severity of the diseases caused by powdery and downy mildews in comparison to virus-free plants, which highlights a potential upregulation of plant innate immunity by GLRD infection (Gilardi et al., 2020).

Grapevine Red Blotch Disease

Grapevine red blotch disease is caused by the Grapevine red blotch-associated virus (GRBaV) and was discovered in 2008 in California. GRBD has recently become a major economic problem for the wine industry in many growing regions (Sudarshana et al., 2015; Adiputra et al., 2018; Reynard et al., 2018). GRBaV infections result in the appearance of red patches on the leaf blades, veins, and petioles in red grape varieties, whereas in white grape varieties, they cause irregular chlorotic areas on the leaf blades. Detrimental effects of red blotch disease are similar to GLRD. GRBaV affects berry physiology, causing uneven ripening, higher titratable acidity, and lower anthocyanin and sugar content (Girardello et al., 2019, 2020; Rumbaugh et al., 2021) putatively due to an impairment of carbon import into the berry (Martínez-Lüscher et al., 2019). Rumbaugh et al. (2021) observed a general decrease of fruity aroma compounds in Cabernet Sauvignon, mostly linked to a reduction of monoterpenes, such as limonene, β-myrcene, α-terpinene, geranial, and p-cymene. Higher titratable acidity has been attributed to lower acid respiration as shown by Pereira et al. (2021), who found 56% higher malic acid and lower tartaric acid and phenolic compounds, such as the flavan-3-ols, catechin, epicatechin and the flavonol quercetin-glucoside, in Cabernet Sauvignon infected with GRBaV.

Using RNA-seq for differential gene expression analysis, Blanco-Ulate et al. (2017) associated the GRBD-induced deterioration of grape quality with a downregulation of key genes of the phenylpropanoid pathway, which confirms aforementioned phenotypic observation. Furthermore, Blanco-Ulate et al. (2017) showed that GRBD disturbs berry development and induces stress responses by altering transcription factors (e.g., *VviNACs*, *VviMYBs*, and *VviAP2-ERFs*) and phytohormone networks causing an inhibition of the ripening process thus reducing color, flavor, and aroma compounds in berries.

Grapevine Fanleaf Disease

Grapevine fanleaf disease is one of the oldest known viral diseases of grapevines and has been found in all wine-growing regions around the world (Raski et al., 1983; Silva et al., 2017). GFLD has been reported to cause significant economic losses by reducing grape yield due to reduction of both cluster weight and berry weight, shortening the longevity of vines, and affecting fruit quality by decreasing the sugar content and titratable acidity (Raski et al., 1983). More detailed studies focusing on berry physiology and quality are not very numerous and often ambiguous. It has been shown that GFLD can also affect the anthocyanin levels in a cultivar-dependent manner. In the variety Manto Negro, GFLD reduced the anthocyanin level (Cretazzo et al., 2010), while in Schioppettino, an increase in anthocyanin content is observed and the relative proportions between di- and tri-hydroxylated or -methylated derivatives of anthocyanins seem to be affected *via* the upregulation of *VviF3H1* and *VviF3'H5* and downregulation of *VviF3H* (Rupnik-Cigoj et al., 2018). Further studies on berries from GFLD-infected vines, including several developmental stages and cultivars, are utterly needed to better understand the effect on fruit quality of this widespread pathogen.

New Emerging Virus-Associated Diseases

Recently, several emerging viruses have been described (Cieniewicz et al., 2020), with the Pinot Gris Virus

(Giampetruzzi et al., 2012) being the most threatening one. It is present in most wine-growing regions from North (Al Rwahnih et al., 2016) and South America (Debat et al., 2020) to almost all the European countries, such as Italy (Gentili et al., 2017), Germany (Messmer et al., 2021), Spain (Ruiz-García and Olmos, 2017), and France (Renault-Spilmont et al., 2018). Although leaf symptoms are well-characterized, its impact on berry physiology has not yet been characterized in detail. The most important detrimental impact of Pinot Gris virus in viticulture is certainly the great reduction in yield, which can range between 66 and 85%, as shown for Glera and Pinot Noir; however, it causes no significant alteration of fruit quality (Bertazzon et al., 2017).

CONCLUSIONS

The consequences of the most important fungal and viral diseases on grapevine performance, longevity, yield, and berry and wine quality are mostly well known. However, fundamental molecular studies, aiming to characterize the underpinning mechanisms involved in berry–pathogen interactions are scarce.

The most studied fungal pathogen, due to its multiplicity of host plants and fruits and its partly beneficial effects in the production of noble rot wines, is *B. cinerea*. For powdery and downy mildew and their rather indirect impact on berry development and quality, published studies focus almost entirely on the leaves' transcriptome and metabolome without considering berry development and metabolism. More molecular studies are therefore needed to gain more insight into the berry–pathogen interactions and to better characterize the negative effect of fungal pathogens on leaves as well as on berries. Furthermore, it would also be useful that future studies on berry–pathogen interactions focus more on a precise definition of the berry developmental stage and account for post-véraison berry heterogeneity (Carbonell-Bejerano et al., 2016; Rienth et al., 2021). This would improve comparability and thus yield better insights in molecular berry–pathogen interactions.

It would also be interesting from a growers' and scientific point of view to conduct more studies investigating the combined effects of fungal pathogens and their corresponding conventional or organic fungicides on berry composition and metabolism, as investigated by Rantsiou et al. (2020).

A growing concern in worldwide viticulture is grapevine trunk diseases, due to a lack of studies on them. Common consequences on berry quality seem to be lower sugars and phenolic compounds, such as catechin, epicatechin, and anthocyanins (Lorrain et al., 2012; Fontaine et al., 2016). However, detailed physiological and omic studies are missing thus far. Viral diseases cause a global perturbation of the plant physiology with strong effects on primary and secondary metabolism that leads to interference with the ripening of berries. Characterization of the broad effect of viral disease is still in its infancy, but an increasing number of studies is being published using state-of-the-art molecular tools to provide valuable knowledge on plant/fruit-pathogen interactions. However, more coordinated efforts at genomic, transcriptomic, and metabolomic levels, in particular including epigenetics, should be deployed to understand and better characterize the differences among *V. vinifera* cultivars and their different responses to diseases. Furthermore, integrative studies comprising multiple simultaneous infections with different viral and fungal pathogens (Gilardi et al., 2020), even with abiotic stresses and multiple genotypes, should be considered in the future to better anticipate disease impact in a climate change scenario. A deeper understanding of defense response mechanisms in various *V. vinifera* as well as other *Vitis* spp. could help identify new resistance traits essential for improving breeding programs as well as for the development of biopesticides and biostimulants.

AUTHOR CONTRIBUTIONS

MR, SC, and PD devised the main body and structure and content of the manuscript. CS, CBu, CBo, NV, RW, and FF provided valuable ideas and corrections and triggered fruitful discussions *via* helpful comments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.717223/full#supplementary-material

Supplementary Table 1 | Selection of important metabolites, genes, and transcription factors that have been shown to be modified by different pathogens.

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