FRUIT RIPENING: FROM PRESENT KNOWLEDGE TO FUTURE DEVELOPMENT

EDITED BY: José M. Palma, Francisco J. Corpas, Luciano Freschi and Victoriano Valpuesta

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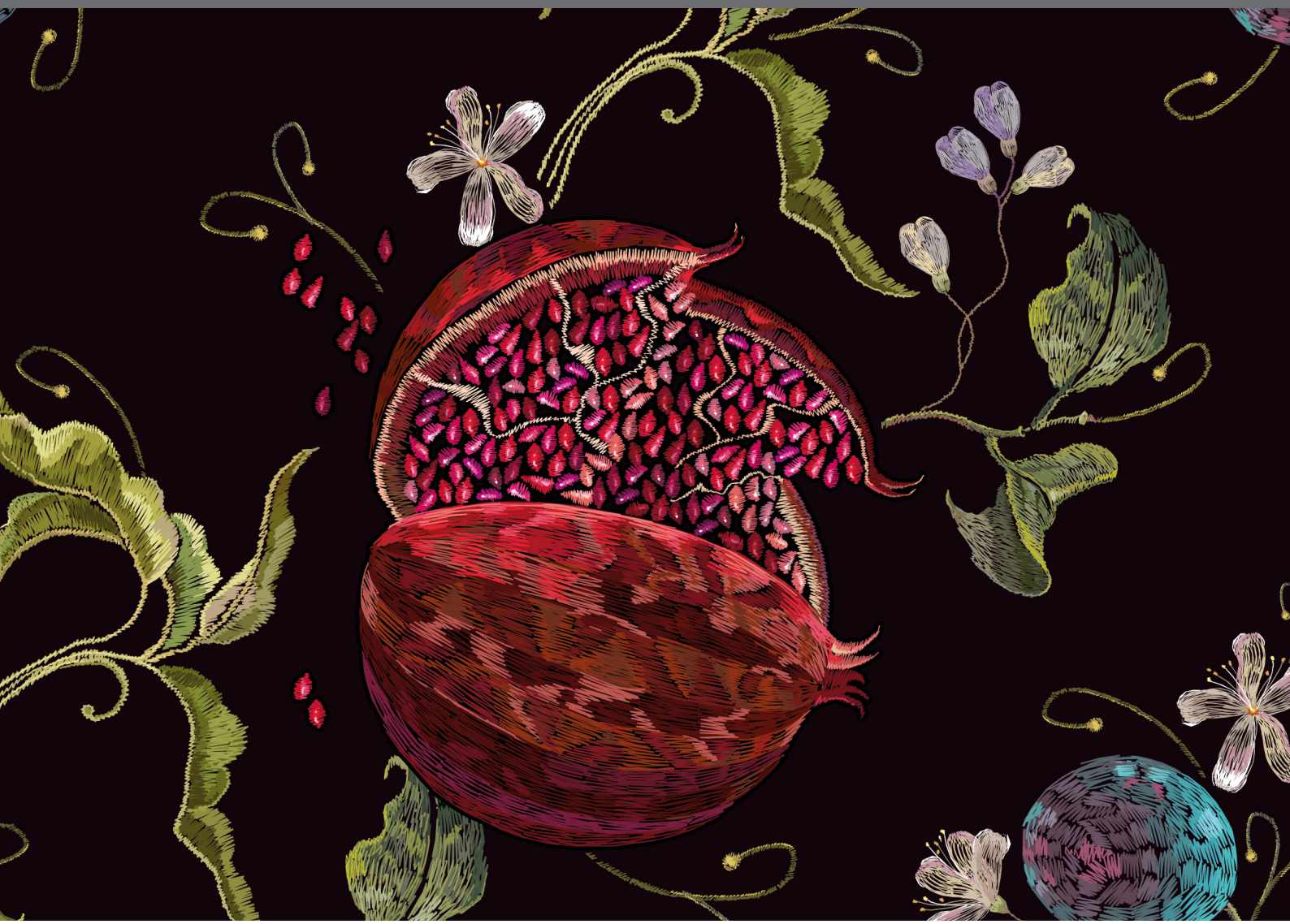

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FRUIT RIPENING: FROM PRESENT KNOWLEDGE TO FUTURE DEVELOPMENT

Topic Editors:
José M. Palma, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Spain
Francisco J. Corpas, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Spain
Luciano Freschi, University of São Paulo, Brazil
Victoriano Valpuesta, University of Málaga, Spain

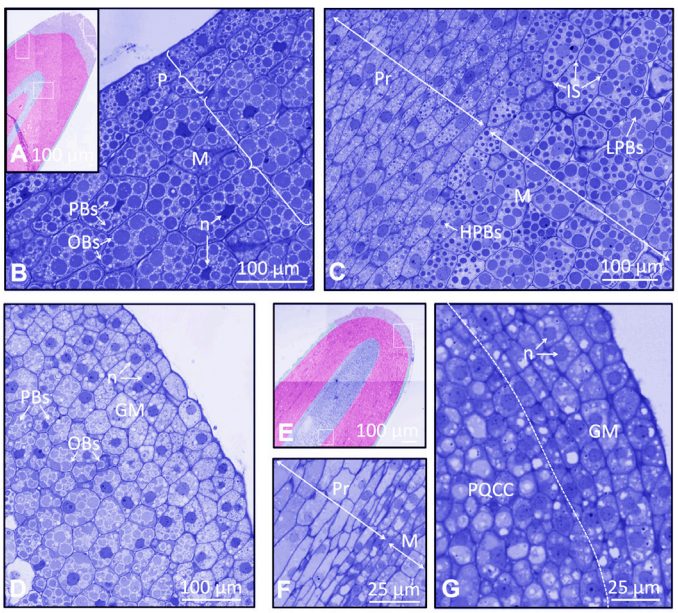

FIGURE 8

Figure 8 from Zafra et al. (2018).

Zafra A, M'rani-Alaoui M, Lima E, Jimenez-Lopez JC and Alché JdD (2018) Histological Features of the Olive Seed and Presence of 7S-Type Seed Storage Proteins as Hallmarks of the Olive Fruit Development. Front. Plant Sci. 9:1481. doi: 10.3389/fpls.2018.01481

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This Research Topic compiles the most recent advances made in cutting-edge research on fruit ripening events, including crop species such as fig, watermelon, tomato, peach, berries, olive, etc. From the regulation of metabolic pathways of physiological relevance for fruits to genetic and molecular approaches, this piece

of work covers current bio-technology cues like CRISPR/Cas9, metagenomics, metabolomics, transcriptomics, microRNA, and others oriented towards future improvement of fruit nutritional value. The editors hope the readers enjoy this work and acknowledge the authors' great contributions to this Research Topic.

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EDITORIAL
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Editorial: Fruit Ripening: From Present Knowledge to Future Development

José M. Palma[1], Francisco J. Corpas[1], Luciano Freschi[2] and Victoriano Valpuesta[3]*

[1] Group of Antioxidants, Free Radicals and Nitric Oxide in Biotechnology, Food and Agriculture, Estación Experimental del Zaidín, CSIC, Granada, Spain, [2] Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil, [3] Departamento de Biología Molecular y Bioquímica, Instituto de Hortofruticultura Subtropical y Mediterránea (IHSM), Consejo Superior de Investigaciones Científicas- Universidad de Málaga, Málaga, Spain

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Editorial on the Research Topic

Fruit Ripening: From Present Knowledge to Future Development

Fruit ripening is a very well-orchestrated physiological process of Angiosperm species which is under developmental, hormonal and epigenetic regulation and is finely tuned by environmental stimuli (**Figure 1**; Palma et al., 2011; Bianchetti et al., 2018; Corpas et al., 2018). Over the years, a number of signaling molecules (e.g., phytohormones) have been implicated in controlling fruit ripening, some of them playing very well-recognized roles like ethylene, abscisic acid (ABA) and reactive oxygen species (ROS), and others emerging only more recently as driving forces of this physiological process, such as nitric oxide (NO) and NO-derived molecules (reactive nitrogen species, RNS), hydrogen sulfide (H_2S) and melatonin (Corpas and Palma, 2018; Corpas et al., 2018, 2019; Mukherjee, 2019). Among these newcomers, NO has received comparatively more attention, and it has been found that during fruit ripening this species promotes post-translational modifications (PTMs) through protein nitration and protein *S*-nitrosation of proteins (Corpas and Palma, 2018). Globally, fruit ripening has been a main focus of the plant research community, motivated not only by its biological and evolutionary significance in seed development and dispersal, but also by its implications on determining the quality and nutritional value of some of the most worldwide consumed foods (Agius et al., 2005; Palma et al., 2015, 2018; Karasawa and Mohan, 2018).

From this overall view, many efforts have been dedicated by plant biologists to understanding fruit ripening, not only as a physiological phenomenon but also as a target to promote human health. This Research Topic is a faithful reflection of how plant research has been increasingly devoted to deciphering fruit ripening biology. Contributions made by 88 scientists from 11 countries belonging to 4 continents for this special issue have accumulated together more than 23,900 views until mid-March 2019. Using the most recent cutting-edge approaches, the authors have focused their work on major ripening-related topics, embracing distinct disciplines such as chemistry, physiology, genetics, biochemistry and molecular biology. Genome editing, metagenomics, metabolomic and transcriptomic profiles, transcriptional regulation, gene expression, histology, signaling processes, and the antioxidant metabolism have been some of the tools used to build up this compendium about fruit biology.

In line with the interest of expanding the investigation of the role exerted by hormones on fruit ripening, several papers included in this Research Topic have dealt with the ripening-associated signaling network. For example, the proteomic quantification of the main proteins involved in the ethylene signal transduction pathway has been achieved by Mata et al., revealing that transcriptomic and proteomic patterns of some, but not all, these proteins positively correlated during tomato fruit

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****Correspondence:***
José M. Palma
josemanuel.palma@eez.csic.es

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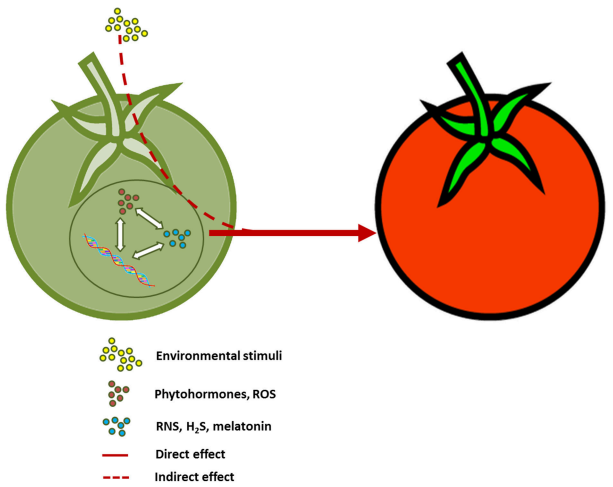

FIGURE 1 | Integrated model for fruit ripening. A close interaction occurring in immature fruits among phytohormones, reactive oxygen and nitrogen species (ROS and RNS, respectively), hydrogen sulfide (H_2S), melatonin and genes leads to a controlled physiological process (ripening) which can be also affected by the interaction with environmental stimuli.

ripening. Moreover, by the exogenous addition of ABA to the non-climacteric bilberry (*Vaccinium myrtillus*) fruits, Karppinen et al. demonstrated that this phytohormone plays a significant role in regulating processes linked to ripening such as anthocyanin biosynthesis and cell wall modification. It was also proved that sugars, either sucrose or glucose, have minor regulatory functions in the ripening of bilberry fruits (Karppinen et al.), in contrast with other non-climacteric fruits such as strawberry and grape, where both these sugars promote fruit ripening in a hormone-like signaling manner in coordination with ABA (Jia et al., 2013, 2017). Plant hormones, particularly ethylene and auxin, and light signaling have been proposed to establish a dynamic crosstalk, being identified as essential regulators of carotenoid biosynthesis during tomato fruit ripening. In this Research Topic, the potential involvement of ethylene and auxins in light-mediated regulation of tomato fruit carotenogenesis was investigated by comparing the impacts of light treatments and the light-hyperresponsive *high pigment-2* (*hp2*) mutation on both carotenoid synthesis and hormonal signaling (Cruz et al.).

The quality as an index of the fruit physiological condition has also been covered by this Research Topic. The biosynthesis and regulation of the vitamin C content during fruit ripening has been reviewed, with the perspective of the critical role exerted by ascorbate in the activation of epigenetic mechanisms controlling cell differentiation and dysregulation events, which eventually might lead to the development of diverse types of cancer. Thus, the different strategies to boost the ascorbate contents in crops, with special emphasis on fruits, have being reviewed by Fenech et al. The profiles of distinct organic acids during fruit development and ripening are also analyzed as qualitative and quantitative traits of crop fruits. In this context, it appears that citrate and malate play major roles on those physiological processes, as they usually are accumulated in numerous climacteric and non-climacteric fruits (Batista-Silva et al.). The relationship between peach (*Prunus persica* L.) fruit quality and β-galactosidases (BGALs), which are cell wall hydrolases critically important for fruit softening, has also been analyzed. The down-regulation of two *BGAL* genes in peach via virus-induced gene silencing (VIGS) delayed fruit softening by reducing the activity of polygalacturonases and pectinmethylesterases, which are known to promote cell wall degradation (Liu et al.). The regulation of anthocyanin production-related gene expression was also addressed in this special issue. Transcriptomic analysis revealed a significant down-regulation of genes encoding phenylpropanoid and flavonoid biosynthetic enzymes in young fig (*Ficus carica*) fruits of the color mutant "Purple peel" compared to "Green Peel," whereas a simultaneous up-regulation in almost all of the anthocyanin and flavonoid pathway-related genes took place in the mutant at the mature stage. Metabolomic data also showed that anthocyanins, particularly cyanidins, are the major responsible for the distinctive purple phenotype of the mutation (Wang et al.).

Another interesting perspective included in this special volume concerns the plant microbiome, which is a research theme that has been increasingly gaining attention in recent years. The plant microbiome is considered a key determinant for plant health and productivity, thus having profound impacts on fruit quality. The link between the host and the fruit-associated microbiome was investigated in watermelon (*Citrullus lanatus*) using the carbohydrate metabolism as an index of the beneficial consequences of such interaction. The use of the holobiont concept to incorporate the associated microbiomes to the breeding programs is proposed (Saminathan et al.). Finally, product quality was also the driving force behind the study on storage proteins in olive (*Olea europaea*). The accumulation of seed storage proteins of the 7S-type (β-conglutin), analyzed by biochemical and immunocytochemical methods, suggests that these molecular markers could be used to facilitate assessing the appropriate ripening stage of olives for commercial and industrial purposes (Zafra et al.).

Molecular aspects of fruit ripening regulation and technological advances to improve ripening-associated traits are also among the topics included in the present issue. For example, the regulation of gene expression and transcript abundance via non-coding small (microRNAs among them) and long chain RNA (lncRNA) was reviewed in the context of ovule, seed and fruit development and ripening (de Oliveira-Correa et al.). The regulation of a conserved kinase complex was demonstrated to participate in the control of the ripening process in tomato fruits. In tomato, the sucrose non-fermenting-1-related protein kinase 1 (SnRK1) was shown to interact with several transcription factors and this event regulated the expression of downstream ripening-related genes, thereby promoting fruit ripening (Yu et al.). Finally, in the context of applying genome editing tools to improve commercially relevant crop species, Martín-Pizarro and Posé discuss the use of CRISPR/Cas9 technology not only for functional research but also to generate plants with improved fruit quality traits (Martín-Pizarro and Posé).

All these subjects addressed in this Research Topic provide new and complementary information to the model depicted

in **Figure 1**, but they also open up new windows for future investigation on fruit ripening, highlighting some of the most promising areas for both basic and applied research. Thus, the potential interactions among NO, H_2S, H_2O_2 and melatonin depicts a scenario where many intermediate players can be envisaged including small regulatory molecules but also antioxidant and stress-related enzymes (Muñoz-Vargas et al., 2018; Corpas et al., 2019; Mukherjee, 2019). The precise modulation through these novel molecules in coordination with other endogenous signaling compounds (i.e., phytohormones) in the different ripening-associated events (e.g., change in firmness, degradation of chlorophyll, synthesis of new pigments and flavonoids, etc.), and how it is governed in either climacteric and non-climacteric fruits remain to be totally understood and some critical avenues for upcoming research on the regulation of fruit metabolism and development are presented in this issue. In the last years, the regulation of the ascorbate metabolism by NO, and H_2S has initiated new ways to establish direct connection among these signaling molecules and the antioxidant biochemistry in plants (Rodríguez-Ruiz et al., 2017; Shan et al., 2018). Therefore, fitting molecules like ascorbate and other players implicated in redox homeostasis and signaling, especially H_2O_2, NO, and H_2S, within this regulatory framework may further clarify the mechanisms behind fruit ripening control in both climacteric and non-climacteric species.

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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ORIGINAL RESEARCH
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Ethylene Receptors, CTRs and EIN2 Target Protein Identification and Quantification Through Parallel Reaction Monitoring During Tomato Fruit Ripening

*Clara I. Mata[1], Bertrand Fabre[2†], Harriet T. Parsons[2], Maarten L. A. T. M. Hertog[1], Geert Van Raemdonck[3], Geert Baggerman[3,4], Bram Van de Poel[5], Kathryn S. Lilley[2] and Bart M. Nicolaï[1]**

[1] Postharvest Group, Division of Mechatronics, Biostatistics and Sensors, Department of Biosystems, KU Leuven, Leuven, Belgium, [2] Cambridge Centre for Proteomics, Cambridge Systems Biology Centre, University of Cambridge, Cambridge, United Kingdom, [3] Centre for Proteomics and Mass Spectrometry, University of Antwerp, Antwerp, Belgium, [4] Flemish Institute for Technological Research (VITO), Mol, Belgium, [5] Molecular Plant Hormone Physiology, Division of Crop Biotechnics, Department of Biosystems, KU Leuven, Leuven, Belgium

Ethylene, the plant ripening hormone of climacteric fruit, is perceived by ethylene receptors which is the first step in the complex ethylene signal transduction pathway. Much progress has been made in elucidating the mechanism of this pathway, but there is still a lot to be done in the proteomic quantification of the main proteins involved, particularly during fruit ripening. This work focuses on the mass spectrometry based identification and quantification of the ethylene receptors (ETRs) and the downstream components of the pathway, CTR-like proteins (CTRs) and ETHYLENE INSENSITIVE 2 (EIN2). We used tomato as a model fruit to study changes in protein abundance involved in the ethylene signal transduction during fruit ripening. In order to detect and quantify these low abundant proteins located in the membrane of the endoplasmic reticulum, we developed a workflow comprising sample fractionation and MS analysis using parallel reaction monitoring. This work shows the feasibility of the identification and absolute quantification of all seven ethylene receptors, three out of four CTRs and EIN2 in four ripening stages of tomato. In parallel, gene expression was analyzed through real-time qPCR. Correlation between transcriptomic and proteomic profiles during ripening was only observed for three of the studied proteins, suggesting that the other signaling proteins are likely post-transcriptionally regulated. Based on our quantification results we were able to show that the protein levels of SlETR3 and SlETR4 increased during ripening, probably to control ethylene sensitivity. The other receptors and CTRs showed either stable levels that could sustain, or decreasing levels that could promote fruit ripening.

Keywords: ethylene signal transduction, ethylene receptors, targeted proteomics, parallel reaction monitoring, ripening, tomato

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Francisco J. Corpas, Consejo Superior de Investigaciones Científicas (CSIC), Spain

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Georg Groth, Heinrich Heine Universität Düsseldorf, Germany
Francisco Javier Romera, Universidad de Córdoba, Spain

****Correspondence:***
Bart M. Nicolaï
bart.nicolai@kuleuven.be

†Present address:
Bertrand Fabre, Technion Integrated Cancer Center, Rappaport Faculty of Medicine and Research Institute, Haifa, Israel

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INTRODUCTION

Worldwide, tomato is the second most important vegetable crop in terms of production (Food and Agriculture Organization of United Nations, 2016). It is widely used as a model organism to study fleshy fruit development and climacteric fruit ripening (Giovannoni, 2004; Osorio et al., 2011). The ripening of tomato, and of climacteric fruit in general, is regulated by the plant hormone ethylene, which also regulates numerous aspects of plant growth and development including responses to biotic and abiotic stress (Van de Poel et al., 2015; Wen, 2015). Climacteric fruit is characterized by a burst in respiration which coincides with a burst in ethylene production at the onset of ripening, decreasing both afterward when the fruit becomes ripe (Lelievre et al., 1997).

Post-harvest control of ethylene is of great importance to assure proper storage conditions and to control fruit quality. Thus, a good understanding of ethylene perception by the fruit is essential to eventually improve post-harvest practices. The ethylene signal transduction pathway starts with the perception of ethylene by a family of receptors spanning the membrane of the endoplasmic reticulum (Chen et al., 2002; Zhong et al., 2008). In tomato there are seven ethylene receptors (ETRs), with the seventh only recently been validated by phylogenetic analysis (Wilkinson et al., 1995; Lashbrook et al., 1998; Tieman and Klee, 1999; Klee and Tieman, 2002; Liu et al., 2015). The receptors are homologous to bacterial two-component histidine kinases, formed of a sensory histidine kinase and a response regulator domain (Chang et al., 1993). Depending on their histidine kinase activity, the receptors have been classified into two subfamilies. Three ethylene receptors (SlETR1-SlETR3) are classified into subfamily I containing a well-conserved histidine kinase domain, and four receptors (SlETR4-SlETR7) into subfamily II, missing some of the residues to act as histidine kinases (Klee, 2002; Liu et al., 2015). Mutant analyses have shown that the receptors are negative regulators of the ethylene response, meaning that in the presence of ethylene the receptors are inactivated, which leads to the induction of ethylene signaling (Hua and Meyerowitz, 1998; Tieman et al., 2000). The ethylene receptors interact with the downstream CTR-like protein kinases (Zhong et al., 2008). Four of these tomato CTR-like proteins are homologous to the Raf-like kinase CONSTITUTIVE TRIPLE RESPONSE1 of *Arabidopsis*, which is also a negative regulator of the ethylene response (Kieber et al., 1993; Adams-Phillips et al., 2004; Zhong et al., 2008). ETRs maintain the conformation of CTR1, which in this state is able to phosphorylate and inhibit ETHYLENE INSENSITIVE 2 (EIN2), another endoplasmic reticulum spanning protein (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). The generally accepted model is that ethylene binding to the receptors reduces their phosphorylation levels, which results in receptor degradation through the proteasome (Chen et al., 2007; Kevany et al., 2007; Kamiyoshihara et al., 2012). As a consequence, CTR1 is inactivated and EIN2 ceases to be phosphorylated, which results in the cleavage and translocation of the EIN2 C-terminal part to the nucleus (Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012). In the nucleus the C-terminal part of EIN2 stabilizes EIN3 and EIN3-like proteins (EILs), preventing them from proteasomal degradation mediated by the F-box proteins ETHYLENE BINDING FACTOR 1 (EBF) and EBF2 (Guo and Ecker, 2003; An et al., 2010). Alternatively, the EIN2-C terminal end can also control ethylene sensitivity via a non-nuclear mechanism, through the translational repression of EBF1 and EBF2 synthesis (Li et al., 2015; Merchante et al., 2015). The nuclear transcription factors EIN3 and EILs promote the expression of ethylene response factor (ERF) family genes which are downstream regulators of the ethylene responses (Fujimoto et al., 2000; Tieman et al., 2001; Tournier et al., 2003; Liu et al., 2016).

Several studies have analyzed gene expression of the ethylene receptors during tomato fruit ripening, showing, in general, an increase in expression at the onset of ripening for *SlETR3*, *SlETR4,* and *SlETR6* (Kevany et al., 2007; Rugkong et al., 2011; Osorio et al., 2012; Liu et al., 2015). Recently, Mata et al. (2018) showed peaks in expression at the onset of ripening for the receptors *SlETR2-SlETR6* and *SlCTR1* and *SlCTR2*. Previous transcriptional analysis of *CTRs* in tomato revealed that only *SlCTR1* was ethylene induced during ripening, while the *SlEIN2* expression levels, which are not so well-documented, did not change during ripening (Zegzouti et al., 1999; Leclercq et al., 2002; Adams-Phillips et al., 2004; Lin et al., 2008; Liu et al., 2015). Recent studies have shown that the transcribed mRNA and translated protein are not necessarily directly correlated, as changes in gene expression are frequently not reflected at the protein level (Ghazalpour et al., 2011; Vogel and Marcotte, 2012; Peng et al., 2015). This might be due to factors such as different half-lives, post-transcriptional modifications or protein degradation, amongst others. Therefore, transcript analyses need to be supplemented by protein quantification to fully understand the underlying regulation. To date, only three ethylene receptor proteins have been quantified in tomato pericarp through western blot analyses. Two studies showed high protein levels for SlETR3 (also called Never Ripe), SlETR4 and SlETR6 in immature fruit, which significantly decreased during the onset of ripening (Kevany et al., 2007, 2008), while a third study showed increasing protein levels for SlETR3 and SlETR4 during ripening (Kamiyoshihara et al., 2012).

Western blotting, a semi-quantitative technique, is a common method to quantify proteins through the binding of specific antibodies (Towbin et al., 1979). However, the assay relies on the specificity of the antibodies which can be limited by cross-reactivity and unspecific binding to other proteins, leading to the production of an imprecise identification and quantification (Mann, 2008; Liebler and Zimmerman, 2013). Furthermore, the quality of the antibodies cannot always be easily verified. Nowadays, liquid chromatography mass spectrometry (LC–MS) provides an improved alternative to western blotting in terms of protein identification and quantification as it measures multiple signals (multiple peptides per protein, multiple fragment ions per peptide, and multiple measurements of each signal) as opposed to the intensity of a single band. Moreover, mass spectrometry has the power of multiplexing, that is, to

simultaneously measure multiple proteins in a single run at high-throughput.

A few LC–MS discovery studies in *Arabidopsis* have found, among the total proteins identified, some AtETRs, AtCTRs, and AtEIN2 (Maor et al., 2007; Baerenfaller et al., 2008, 2011; Marondedze et al., 2014). Chen et al. (2011) and Qiao et al. (2012) used mass spectrometry to specifically study the cleavage site of AtEIN2 and its phosphorylation status. Recently, two studies have identified some of the ethylene signaling elements in tomato through mass spectrometry (Mata et al., 2017; Szymanski et al., 2017). Both studies used an untargeted data dependent acquisition (DDA) approach. Shotgun proteomics attempts to identify and quantify as many proteins as possible, but is inherently biased toward the most abundant peptides (Gilmore and Washburn, 2010). To focus on the identification and quantification of low abundant proteins, targeted proteomics techniques such as selected reaction monitoring (SRM) and parallel reaction monitoring (PRM) have been developed (Lange et al., 2008; Peterson et al., 2012). These techniques have become the gold standard in targeted proteomics (Gillet et al., 2012; Aebersold and Picotti, 2013). Unlike in shotgun proteomics, in SRM and PRM acquisition modes, peptides of interest must be defined in advance. The first mass analyzer selects a narrow mass window around the m/z of the ions of interest, thereby discarding other ions and thus increasing the signal to noise ratio (Liebler and Zimmerman, 2013). In PRM mode all transitions are acquired and measured in the second mass analyzer, while in SRM mode an extra selection of the transitions to be measured in the MS2 is applied (Gallien et al., 2012; Peterson et al., 2012). Moreover, synthetic peptides with an amino acid sequence identical to the targeted peptides are used for a first identification screening, while spiking of the samples with a known concentration of isotopically labeled peptides can deliver absolute peptide quantification (Kirkpatrick et al., 2005).

The objective of the present work was to develop a targeted LC–MS based method to identify and quantify ethylene receptors, CTRs and EIN2 proteins of the ethylene signal transduction pathway in tomato pericarp, to study their dynamics during fruit ripening and eventually their regulation at the gene expression level. Up to date, this work has not been done due to the difficulty of the identification of such very low abundant proteins (Mata et al., 2017). Our previous results from an extensive LC–MS shotgun approach (Mata et al., 2017) were taken as a starting point. In this targeted assay, a specific microsomal membrane protein extraction followed by fractionation of the protein samples through SDS-PAGE was used to reduce the complexity of the tomato pericarp samples. After protein digestion, the peptides were analyzed on the LC–MS in PRM mode to be able to counteract the low abundance problem. Subsequently, the proteins were absolutely quantified in tomato fruit of four different ripening stages using heavy labeled peptides. To complement the proteomics data, gene expression of the targeted proteins was investigated using real-time qPCR. This enabled a comparison of protein abundance and gene expression levels for the targeted proteins of the ethylene signal transduction pathway during tomato fruit ripening.

MATERIALS AND METHODS

Plant Material

Tomato plants (*Solanum lycopersicum* L. cv. Bonaparte) were grown in a greenhouse at the Research Station for Vegetable Production of Sint-Katelijne-Waver (Belgium). Plants were hydroponically cultivated on rockwool under natural light. Twelve biological replicates from each maturity stage (mature green, breaker, orange, and red) were harvested (April 2016) after visual inspection. Mature green corresponded to fully developed tomatoes that had not started the ripening process yet; breaker, to tomatoes in which ripening was initiated and the first degreening was visible; orange, to the ones in which no green color was visible anymore and red tomatoes, to the ones which matched the final red-ripe stage. Pericarp tissue of 24 tomatoes (six biological replicates for each ripening stage) were directly homogenized and processed for protein extraction. The pericarp tissues of the other 24 samples (six biological replicates for each ripening stage) were flash frozen in liquid nitrogen, crushed with a grindomixer (Retsch, Haan, Germany) and stored at −80°C for gene expression analysis.

Protein Extraction

The protein extraction method was adapted from Kamiyoshihara et al. (2012). The pericarp tissue of each sample was homogenized at 4°C using a high speed disperser (IKA Labortechnik, Staufen, Germany) in 2 volumes of homogenization buffer (100 mM Tris-HCl [pH 8.2], 300 mM NaCl, 20 mM EDTA, 20% [v/v] glycerol, 5 mM dithiothreitol [DTT] with complete EDTA-free protease inhibitor cocktail [Roche, Basel, Switzerland]), and centrifuged at 5,000 *g* for 15 min at 4°C. The supernatants were filtered over Miracloth (Merc Millipore, Darmstadt, Germany), and centrifuged at 100,000 *g* for 1 h at 4°C. The pellets were re-suspended in homogenization buffer containing 10% SDS, 10 mM Tris pH 7.5, and the samples were boiled at 95°C for 5 min. Protein concentrations of solubilized pellets were determined with a DC protein assay kit (Bio-Rad, Hercules, CA, United States) using bovine serum albumin as standard.

Reduction Alkylation, SDS-PAGE Fractionation and In-Gel Digestion

Hundred μg of protein per sample were denaturated and reduced by addition of Laemmli buffer for 5 min at 95°C and then alkylated by addition of 60 mM iodoactetamide for 30 min at RT in the dark. The samples were loaded on an SDS-PAGE gel (4% stacking and 12% resolving) and were migrated until the smallest protein band of the pre-stained protein standard (New England BioLab, Ipswich, MA, United States) reached the end of the gel. Proteins were stained overnight with colloidal blue Coomassie staining. For each gel lane, one band fraction containing the proteins ranging from 163 to 52 kDa was excised from the gel and cut into small pieces. Gel pieces were de-stained in 25 mM ammonium bicarbonate/50% acetonitrile (ACN) at 37°C, then incubated in ACN for 15 min. Gel pieces were dried in a speed-vac until the ACN was completely evaporated. Gel pieces were incubated overnight in 500 ng of trypsin in 50 mM

ammonium bicarbonate at 37°C. Next, 200 μL 10% formic acid (FA) and 200 μL 100% ACN were added to the gel pieces and incubated during 15 min at 37°C. The supernatant was retained, and gel pieces were re-incubated with 200 μL 100% ACN and 200 μL 10% FA. Supernatants were pooled and dried in a speed-vac. Finally, the pellets were re-suspended in 2% ACN and 0.1% FA and the peptide concentration determined with a Pierce Quantitative Colorimetric Peptide Assay (Thermo Scientific, Waltham, MA, United States).

Design of the Targeted Proteomics Experiment

Parallel reaction monitoring assays were developed using Skyline version 4.1 (University of Washington, United States, MacLean et al., 2010). *In silico* tryptic digestions of protein sequences obtained from UniProt (Bateman et al., 2015) were performed. Target peptides were selected using the following criteria: peptide mass between 7 and 25 amino acids, no missed cleavages, absence of methionines, cysteines, and histidines and RP KP (prolines after the arginines and lysines). Modifications were set to carbamidomethylation of cysteines, oxidation of methionines and N-terminal acetylation, tolerating three possible modifications per peptide and one neutral loss. Uniqueness of the targets was verified using the tomato proteome (downloaded from UniProt on December 2015, 40,069 sequences, Bateman et al., 2015). The following settings were used to select the transitions: precursor charges 2 and 3, ion charges 1 and 2, ion types y, b, and p (precursor), 3 product ions from m/z to precursor, ion match tolerance 0.5 Da, pick 10 product ions, isotope peaks included COUNT, precursor mass analyzer Orbitrap, peaks 3, resolving power 60,000 at m/z 400, acquisition method targeted, product mass analyzer Orbitrap, use only scans within 5 min of MS–MS IDs.

Non-labeled and Labeled Synthetic Peptides

Unlabeled synthetic peptides (SpiketidesTM) for assay development were purchased from JPT Innovative Peptide Solutions (Berlin, Germany, Schnatbaum et al., 2011). A list with all the unlabeled peptides tested can be found in **Supplementary Table 1**. The labeled peptides (SpikeTides_TQL) for the combined identification and quantification, purchased from the same company, were heavy-isotope labeled on the C-terminal lysine or arginine and absolutely quantified using a proprietary Quanti-Tag. **Table 1** presents the list of peptides monitored for the quantification and their corresponding labeled peptides. The proteotypic labeled peptides were pooled and digested with trypsin to be released from the tag.

LC–MS and Parallel Reaction Monitoring (PRM) Acquisition

Samples (1 μg) were analyzed in PRM acquisition mode on a Q Exactive Plus mass-spectrometer (Thermo Scientific, Waltham, MA, United States), using a 75 μm × 2 cm, C18, 3 μm, 100 Å trapping column (Acclaim PepMap, Thermo Scientific) and an Easy nLC 1000 system (Thermo Scientific). Peptides were separated with a 50 μm × 15 cm, nanoViper, C18, 2 μm, 100 Å column (Acclaim PepMap) retrofitted to a NanoSpray Flex source with a flow rate of 300 nL/min (buffer A: HPLC grade H_2O, 0.1% FA, buffer B: 100% ACN, 0.1% FA). Samples were run using a 60 min gradient from 5% up to 35% solvent B. Analytes were transferred to the gaseous phase with positive ion electrospray ionization at 2.0 kV. Precursors were targeted with a 2 m/z selection window around the m/z of interest. Precursors were fragmented in high-energy collisional dissociation mode with normalized collision energy of 28. A single MS1 scan was performed at a mass resolution of 17,500, an automatic gain control (AGC) target of 10^6 ions and a maximum C-trap fill time of 200 ms. Subsequently, 10 PRM scans were performed at a resolution of 70,000, an AGC target of 10^5 ions and a maximum injection time of 200 ms. Initial screening for targets transitions was unscheduled but retention-time scheduling of PRM (sPRM) was adopted for peptide quantification, allowing analysis of 42 peptides in a single acquisition.

Provisional Peptide Identification

For the first screening and provisional identification of the endogenous peptides in the samples, a PRM analysis of a pooled sample of the unlabeled peptides was performed, followed by PRM analyses of endogenous peptides from tomato samples. The individual raw-files were imported into Skyline, and precursor and product ion chromatograms were extracted. MS–MS spectra were analyzed in Skyline with manual validation comparing the extracted ion chromatogram (XIC) of the unlabeled peptides and the endogenous peptides of the tomato sample. Peptide identification was based on retention time, the presence of the main transition ions and a low mass error (less than 5 ppm). Labeled synthetic versions were ordered for candidate peptides with the most consistently detectable transitions.

Preparation of the Labeled Synthetic Peptides Mix

The labeled synthetic peptides were spiked into endogenous peptides digests (six aliquots of 1 μg) from tomato samples at the following concentrations: 0, 1, 5, 10, 100, and 200 fmol. The aliquots were measured by LC–MS in PRM mode using retention time scheduling. Based on a comparison of the XIC of the labeled and endogenous peptides, final concentrations of labeled peptides were chosen for absolute quantitation experiments such that signal intensity was similar to that of endogenous peptides.

This experiment was also used to evaluate the linearity of the dilution curves for the individual peptides. The ratio of sum of the area-under-the-curve (AUC) of the transitions (**Table 1**) of the heavy labeled peptide to the sum of the AUC of the transitions of the endogenous peptide contained in the tomato peptide pool was calculated to correct for run to run variation of the different LC–MS analysis of the spiking concentrations. The dilution curves are provided as (**Supplementary Figure 1**).

Peptide Identification and Quantification

After spiking the samples with labeled peptides, two sets of precursor ions were detected upon PRM analysis: heavy-isotope

labeled (mass difference + 8 if containing a lysine or + 10 if containing an arginine) and non-labeled (from digested endogenous protein). The XIC from each individual peptide was manually checked in Skyline to ensure the correct identification of the peptide across biological replicates. Furthermore, the mProph algorithm was used to calculate the FDR (q-value) of the targeted peptide identifications trained with the second best peak option. The information extracted from this analysis is provided in **Supplementary Table 2**. It was found that 83.7% of the transitions groups were identified with q-values < 0.01 (FDR of 1%). About 13.9% had a q-value between 0.01 and 0.05, some of which were eliminated from the analysis and 2.4% displayed q-values higher than 0.05, which were directly removed. For the quantification, the ratio of sum of the AUC of the transitions

TABLE 1 | List of the proteins identified and quantified, their peptides and their corresponding labeled peptides monitored in PRM analysis, the precursor's m/z and charge, the product ions used for the quantification, the average retention time (RT) of their extracted ion peaks and the amount of labeled peptide in fmol used to spike into the samples for the quantification of the endogenous peptide.

Protein	Peptide sequence	Precursor m/z (charge state)	Product ions for PRM	RT	Amount of labeled peptide used for quantification (fmol)
ETR1	ISPNSPVAR ISPNSPVA[Heavy R]	470.7642 (+ 2) 475.7683 (+ 2)	$y7^{+}$, $y6^{+}$, $y5^{+}$, $y3^{+}$, $y7^{++}$,	13.25	10
	EGNVSISAFVAK EGNVSISAFVA [Heavy K]	611.3273 (+ 2) 615.3344 (+ 2)	$Y8^{+}$, $y7^{+}$, $y6^{+}$	23.30	50
ETR2	YIPGEVVAVR YIPGEVVAV[Heavy R]	551.8164 (+ 2) 556.8205 (+ 2)	$y8^{+}$, $y7^{+}$, $y6^{+}$, $y5^{+}$, $y4^{+}$, $y8^{++}$	20.47	5
ETR3	YIPPEVVAVR YIPPEVVAV[Heavy R]	571.8320 (+ 2) 576.8362 (+ 2)	$y8^{+}$, $y7^{+}$, $y6^{+}$, $y5^{+}$, $y4^{+}$, $y8^{++}$, $y7^{++}$, $b2^{+}$	21.69	10
	VPLLHLSNFTNDWAELSTR VPLLHLSNFTNDWAELST[Heavy R]	738.3832 (+ 3) 741.7193 (+ 3)	$y8^{+}$, $y7^{+}$, $y6^{+}$, $y5^{+}$, $y4^{+}$, $y3^{+}$, $b12^{++}$	34.25	100
	LIQTLLNVAGNAVK LIQTLLNVAGNAV[Heavy K]	727.4405 (+ 2) 731.4476 (+ 2)	$y12^{+}$, $y10^{+}$, $y9^{+}$, $y8^{+}$, $y7^{+}$, y4, $y3^{+}$, $b4^{+}$, $b5^{+}$	30.31	400
ETR4	DSSFNSAYNLPIPR DSSFNSAYNLPIP[Heavy R]	790.8888 (+ 2) 795.8929 (+ 2)	$y9^{+}$, $y8^{+}$, $y7^{+}$, $y4^{+}$	29.25	15
	SDPDVIQVK SDPDVIQV[Heavy K]	500.7691 (+ 2) 504.7762 (+ 2)	$y7^{+}$, $y6^{+}$, $y5^{+}$, $y7^{++}$	16.15	15
	VLPESVSR VLPESVS[Heavy R]	443.7533 (+ 2) 448.7574 (+ 2)	$y6^{+}$, $y5^{+}$, $y4^{+}$, $y6^{++}$	14.61	10
ETR5	SLSINDPDVLEITK SLSINDPDVLEIT[Heavy K]	772.4143 (+ 2) 776.4214 (+ 2)	$y9^{+}$, $y8^{+}$, $y7^{+}$	28.93	50
ETR6	FWLNQEVEIVR FWLNQEVEIV[Heavy R]	716.8828 (+ 2) 721.8869 (+ 2)	$y8^{+}$, $y7^{+}$	31	25
	GVEVLLADYDDSNR GVEVLLADYDDSN[Heavy R]	783.3757 (+ 2) 788.3799 (+ 2)	$y9^{+}$, $y8^{+}$, $y7^{+}$	27.9	100
ETR7	SLPIDDPDVLEITK SLPIDDPDVLEIT[Heavy K]	777.9167 (+ 2) 781.9238 (+ 2)	$y9^{+}$, $y8^{+}$, $y12^{++}$	30.51	15
	GLQVLLADDDDVNR GLQVLLADDDDVN[Heavy R]	771.8916 (+ 2) 776.8957 (+ 2)	$y9^{+}$, $y8^{+}$, $y7^{+}$, $b8^{+}$	25.98	100
CTR1	IPSIESLR IPSIESL[Heavy R]	457.7689 (+ 2) 462.7731 (+ 2)	$y7^{+}$, $y6^{+}$, $y5^{+}$, $y4^{+}$, $y7^{++}$	21.5	15
	LNPPQVIAAVGFNR LNPPQVIAAVGFN[Heavy R]	748.4226 (+ 2) 753.4268 (+ 2)	$y10^{+}$, $y9^{+}$, $y8^{+}$, $y12^{++}$, $y11^{++}$	29.76	15
CTR2	YAPNEVPR YAPNEVP[Heavy R]	473.2431 (+ 2) 478.2472 (+ 2)	$y6^{+}$, $y5^{+}$, $y4^{+}$, $y6^{++}$	14.35	10
	LVIPAYVDQLNSR LVIPAYVDQLNS[Heavy R]	744.4145 (+ 2) 749.4186 (+ 2)	$y10^{+}$, $y9^{+}$, $y8^{+}$, $y7^{+}$, $y10^{++}$, $b3^{+}$	28.38	10
CTR3	ASASAASAETLSHR ASASAASAETLSH[Heavy R]	679.8366 (+ 2) 684.8407 (+ 2)	$y8^{+}$, $y7^{+}$, $y6^{+}$	12.78	5
EIN2	GVSENAQSFISDGPGSYK GVSENAQSFISDGPGSY[Heavy K]	921.9289 (+ 2) 925.9360 (+ 2)	$y11^{+}$, $y10^{+}$, $y9^{+}$, $y5^{+}$	23.66	50
	VESSAYIPSGSAR VESSAYIPSGSA[Heavy R]	662.3306 (+ 2) 667.3347 (+ 2)	$y9^{+}$, $y8^{+}$, $y7^{+}$, $y6^{+}$	16.31	5

of the endogenous peptide to the sum of the AUC of the transitions of the heavy labeled peptide was used to calculate the absolute concentration of the peptide in the sample, also known as single point calibration quantification (Gallien et al., 2013). **Supplementary Figure 2** displays the absolute quantification of the individual peptides of the target proteins.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from tomato fruit pericarp samples. Ground tissue samples (500 mg) were homogenized in 800 μL of extraction buffer containing cetyltrimethylammonium bromide, as described previously (Gasic et al., 2004). The mixture was incubated vigorously shaking at 65°C for 10 min. Chloroform (800 μL) was added and mixed by inversion, and the mixture was centrifuged at 21,000 *g* for 10 min at room temperature. The supernatant was transferred to a gDNA eliminator spin column (Plant RNeasy Extraction Kit, Qiagen, Hilden, Germany) and centrifuged at 8,000 *g* for 2 min at room temperature. Half a volume of ethanol was added to the effluent, then the mixture was loaded and washed through the RNeasy mini column (Plant RNeasy Extraction Kit) and finally the RNA was eluted with RNAse free water. The amount of total RNA extracted was measured by spectrophotometry using the NanoDrop 2000 (Thermo Scientific, Waltham, MA, United States) and its purity determined by the 260/280 or 260/230 nm ratio. RNA integrity was checked on an ethidium bromide stained 1% agarose gel. One microgram of purified RNA was reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen) in a total volume of 20 μL following the manufacturer's protocol.

Gene Expression Analysis by Reverse Transcription-qPCR

Gene expression studies were performed following Minimum Information for publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). Real-time qPCR was carried out with SYBR® Green PCR Master Mix (Thermo Scientific, Waltham, MA, United States) on a Rotor Gene Q (Qiagen GmbH, Hilden, Germany). The selected primers, designed with the Primer3 web tool[1], are listed in **Supplementary Table 3**. All RT-qPCR reactions contained 1 μL of cDNA template (50 mg/L), 7.5 μL of Absolute QRT-PCR SYBR Green Mix (Thermo Scientific), and 1 μL of 0.375 μM primer pairs, in a final volume of 15 μL. The cycling conditions were as follows: denaturation step at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 63°C for 20 s, and extension at 72°C for 20 s. Primer pair specificity was performed for every run using a melting curve analysis ranging from 55 to 95°C, with temperature increasing in steps of 0.5°C/s. Furthermore, a standard dilution curve, based on cDNA pooled from all samples, was included in every run to calculate the efficiency of the amplification. The relative quantification of expression levels was performed using a modified Ct method as previously described (Mellidou et al., 2012). All RT-qPCR expression data were normalized against the average expression of three reference genes: Actin, Elongation factor1, and Glyceraldehyde-3-phosphate dehydrogenase. Results presented are the mean ± standard error (SE) of six independent biological replicates.

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

Statistical Analyses

Given an individual protein was represented by up to three different peptides, protein data were analyzed using the mixed models procedure. In this approach 'ripening stage' was considered a fixed categorical factor while 'peptide' was treated as a categorical random factor introducing a repeated structure 'sample' to account for the fact that the various peptides were covariates measured on the same fruit samples. In the case of a single peptide per protein the classical one-way ANOVA was applied. In both cases, Tukey's honestly significant difference (HSD) test ($p < 0.05$) was used to compare between ripening stages. Statistical differences in gene expression between ripening stages were analyzed with the one-way ANOVA procedure and Tukey's HSD test ($p < 0.05$). All analyses were performed using JMP 12 statistical software (SAS Institute, Cary, NC, United States).

Correlation between protein and gene expression levels was calculated and can be visualized in **Supplementary Figure 3**. Given protein and gene expression levels were measured on different biological replicates their structural correlation is not known. To approximate this relationship, 1500 random data sets were generated with the same distribution properties (average and standard error of the mean) based on which the correlation coefficients were calculated. Using a Fisher transformation, the 95% confidence interval was calculated and from that, the significance of the correlation coefficient was determined. The protein, gene expression data and their standard errors were normalized for visualization.

RESULTS

Identification of the Proteins Through PRM

In Mata et al. (2017) we provided the identification of 8588 tomato pericarp proteins, including four ethylene receptors (SlETR1, SlETR3, SlETR4, and ETR7), three CTRs (SlCTR1-SlCTR3) and SlEIN2. The approach taken, shown schematically in **Figure 1A**, consisted of the extraction of the pericarp proteins from a red tomato through a microsomal membrane isolation protocol, followed by in-gel digestion and fractionation of the subsequent peptides through off-line high pH reverse phase. The 60 sub-fractions obtained through the fractionation were analyzed on a Q Exactive and a Triple-TOF 6600 mass spectrometers in shotgun mode. This was the starting point of our current research as: (i) it allowed us to prove the identification of some of our proteins of interest through LC–MS and (ii) it helped to prioritize the peptides to follow in targeted mode.

The approach taken in the current work is shown schematically in **Figure 1B**. After *in silico* digestion of the

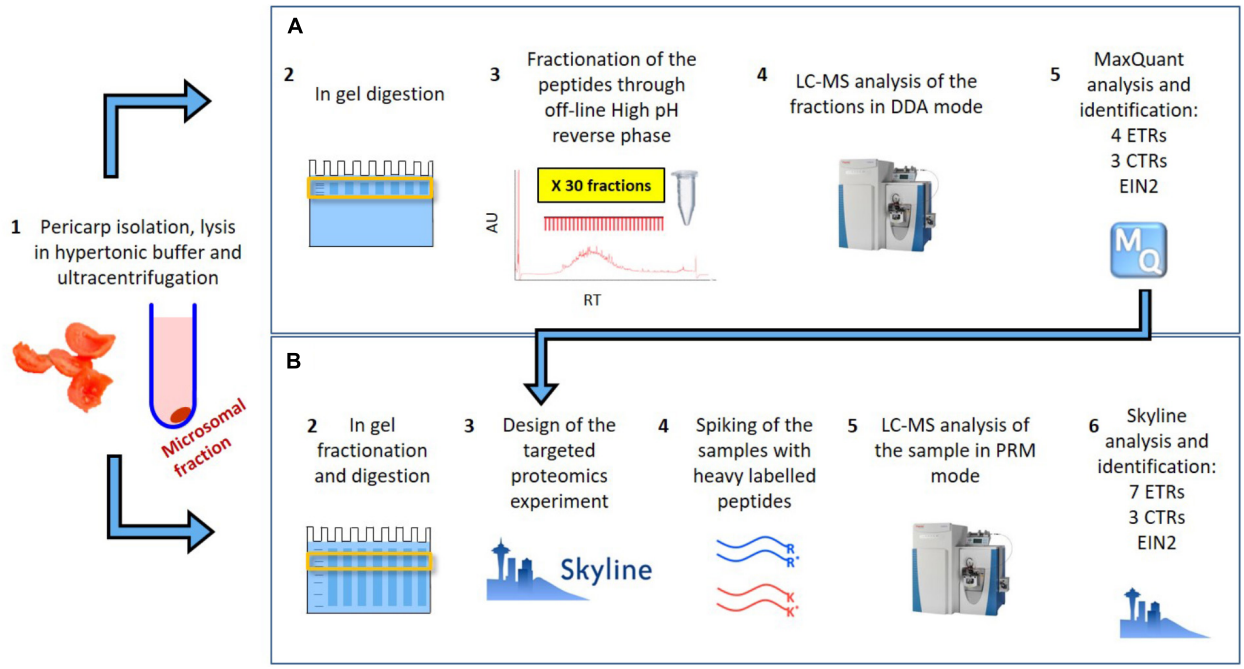

FIGURE 1 | Schematic representation of **(A)** the extensive fractionation analyzed though shotgun approach which allowed to identify 8588 proteins and between them 4 ETR, 3 CTR and EIN2 in tomato pericarp (Mata et al., 2017) and **(B)** the targeted proteomics approach followed in this work which allowed the identification and quantification of the 7 ETR, 3 CTRs and EIN2.

target proteins (7 SlETRs, 4 SlCTRs and SlEIN2) a list of unique peptides was established. Those unique peptides that also followed the criteria for being identifiable in MS1, were combined with a selection of unique peptides identified during the previous shotgun approach (Mata et al., 2017), resulting in a list of 88 unique peptides for the 12 proteins targeted (**Supplementary Table 1**). An unscheduled PRM analysis was conducted on unlabeled, synthetic versions of these 88 peptides. By comparing retention times, fragment ions, and mass errors of their MS2 spectra with those of native peptides derived from different ripening stages of tomato, we identified promising candidate peptides for all seven ethylene receptors, three CTRs (1–3) and EIN2 (**Supplementary Table 1**). This approach is exemplified in **Figures 2A,B** where similarities in transitions and retention times, with low mass errors, were observed between a synthetic and endogenous SlETR4 peptide.

To confirm the identification and to be able to quantify the endogenous peptides, heavy labeled C-terminal lysine or arginine peptides of 21 of the peptides candidates were ordered afterward and were spiked in tomato samples from four different ripening stages, from mature green to red. The PRM analysis of these samples proved the legitimate identification of all the 21 endogenous peptides and, therefore, of the 11 ethylene signaling proteins. The PRM.raw data and Skyline results files are available via ProteomeXchange in PeptideAtlas/PASSEL repository (PASS01249) and the output of the mProphet analysis can be found in **Supplementary Table 2**. An example of the XIC of the fragment ions of one of the identified peptides of the protein SlETR4 and its corresponding labeled peptide is shown in **Figures 2C–E**. This figure shows that retention time, fragment ions and the intensity order of the fragment ions are the same for endogenous and labeled peptide confirming its identification.

The location of the 21 peptides, used for the quantification, in the specific protein sequences can be checked in **Supplementary Table 4**. As it can be appreciated, the quantified peptides came from different protein domains, as in the case of SlETR1 in which one of the peptides derived from the predicted GAF domain and the other from the kinase domain. Qiao et al. (2012) revealed for *Arabidopsis* the amino acid residue where the proteolytic cleavage of the C-terminal domain of EIN2 is produced after ethylene binds to the receptor-CTR complex. We performed a Clustal alignment with UniProt between the EIN2 protein of *Arabidopsis* and tomato and both proteins only have 48% sequence similarity (**Supplementary Table 5**). There is no information about the proteolytic residue of the SlEIN2, but based on the alignment, the first tomato peptide identified in this study may contain the proteolysis residue. The second tomato peptide identified likely belongs to the C-terminal end of SlEIN2.

Absolute Quantification of the Protein Levels

The representation of the absolute quantification of the individual peptides of the target proteins, in fmol of target protein/μg of total membrane proteins, is shown in **Supplementary Figure 2**. Most proteins were identified with two peptides (SlETR1, SlETR6, SlETR7, SlCTR1, SlCTR2, and

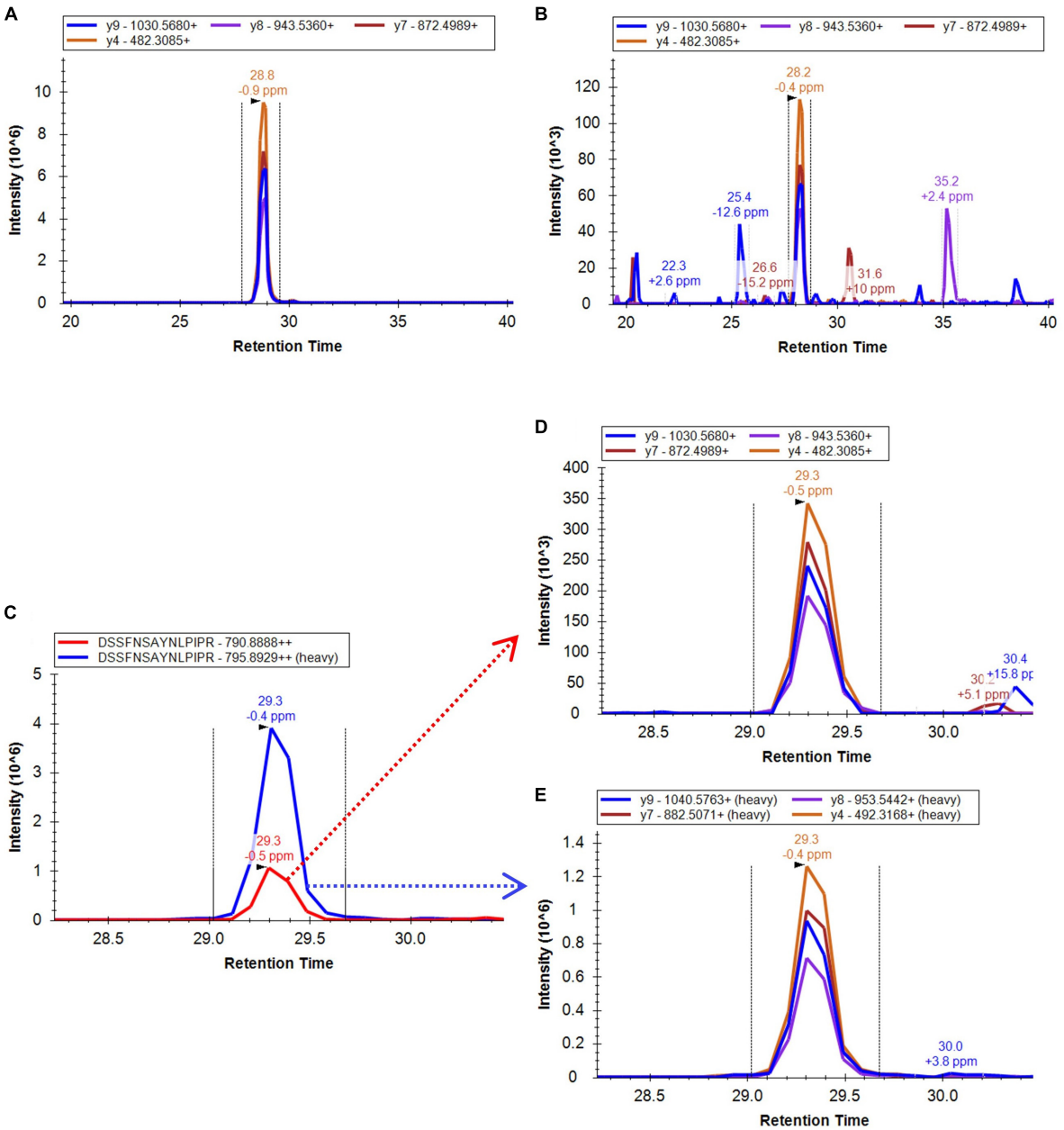

FIGURE 2 | Extracted ion chromatogram of the PRM four most intense fragment ions identified from **(A)** the synthetic non-labeled peptide DSSFNSAYNLPIPR, **(B)** an endogenous peptide sample derived from a mature green tomato. **(C)** XIC of the combined fragment ions of the endogenous peptide (red peak) DSSFNSAYNLPIPR of the protein SlETR4 in a mature green tomato peptide sample and the combined fragment ions of its heavy labeled peptide (blue peak) spiked in the sample. **(D)** XIC of the four most intense fragment ions used for quantification of the endogenous peptide and **(E)** the equivalent fragments for the heavy labeled peptide. All data were analyzed by the Skyline software.

SlEIN2), while some proteins with one (SlETR2, SlETR5, and SlCTR3) or three (SlETR3 and SlETR4) peptides. It can be observed that for some of the proteins identified with more than one peptide, the absolute concentration levels of their peptides are variable, highlighting the limit of absolute quantification using spiked peptides. For these proteins identified with multiple peptides, the absolute peptide quantifications were combined in a final protein quantification representation through the use of mixed models. **Figures 3**, **4** shows the graphical representation of the absolute protein quantification of the 11 proteins identified, for the four ripening stages of tomato, in combination with their gene expression levels measured in the same ripening stages. SlCTR4 protein levels could not be quantified, probably because of the low abundancy of this protein, so only its gene expression levels are shown (**Figure 4**).

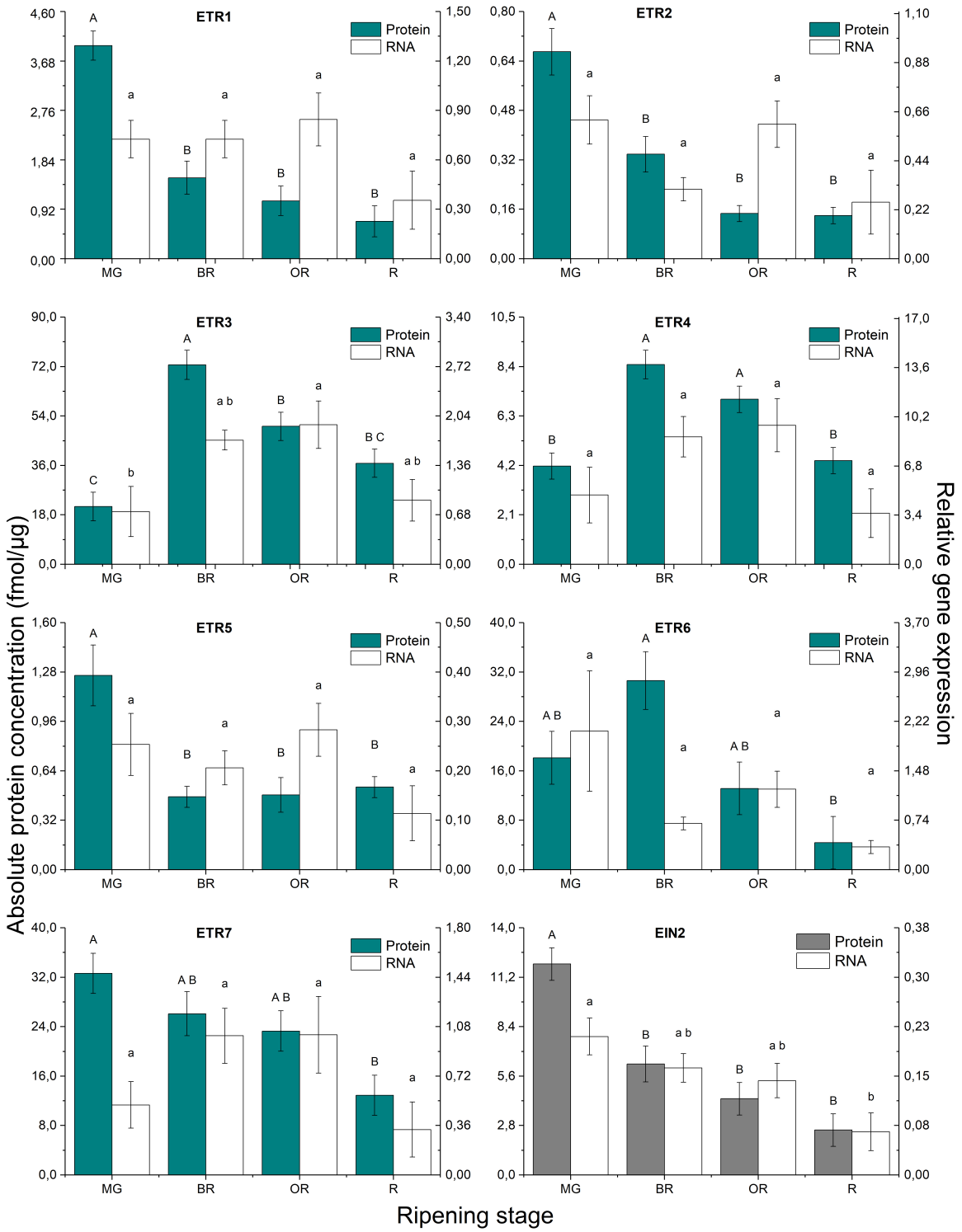

FIGURE 3 | Absolute protein quantification (fmol of target protein/µg of total membrane proteins) and relative gene expression of ETR1-ETR7, and EIN2 during tomato fruit ripening. MG, mature green; BR, breaker; OR, orange; R, red tomatoes. Error bars represent the standard error of the mean based on six biological replicates. Difference uppercase letters indicate significant differences between the absolute protein concentration levels of the four tomato ripening stages determined by Tukey's HSD test ($p < 0.05$). Different lowercase letters indicate significant differences between the relative gene expression levels of the four tomato ripening stages determined by Tukey's HSD test ($p < 0.05$).

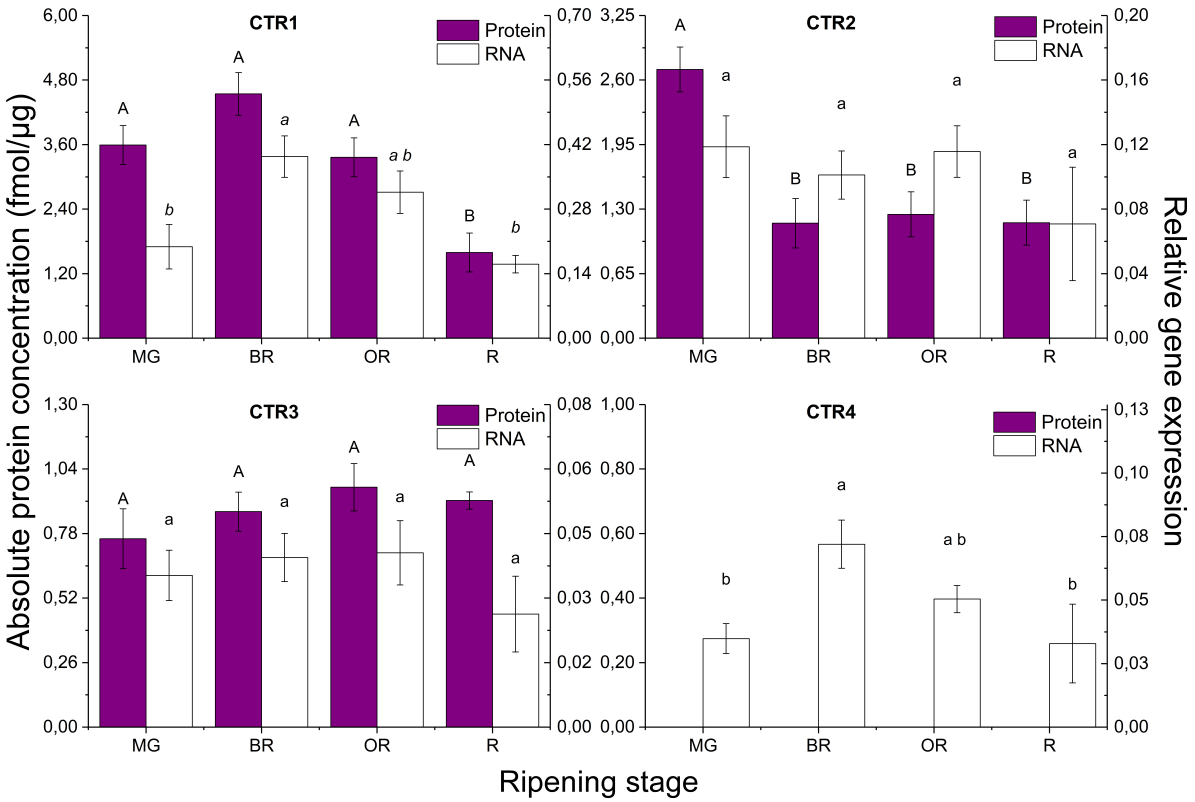

FIGURE 4 | Absolute protein quantification (fmol of target protein/µg of total membrane proteins) and relative gene expression of CTR1–CTR4 during tomato fruit ripening. MG, mature green; BR, breaker; OR, orange; R, red tomatoes. Error bars represent the standard error of the mean based on six biological replicates. Difference uppercase letters indicate significant differences between the absolute protein concentration levels of the four tomato ripening stages determined by Tukey's HSD test ($p < 0.05$). Different lowercase letters indicate significant differences between the relative gene expression levels of the four tomato ripening stages determined by Tukey's HSD test ($p < 0.05$).

Figure 3 demonstrates that the most abundant ethylene receptor proteins are SlETR3, SlETR6 and SlETR7, followed by SlETR4, SlETR1, SlETR5 and finally SlETR2. SlCTR1 is the most abundant SlCTR protein, followed by SlCTR2 and SlCTR3 (**Figure 4**). SlETR3 and SlETR4 are the only proteins whose abundance profiles seemed to follow a climacteric protein pattern, both increasing significantly at the onset of ripening followed by a subsequent decrease toward the red ripening stage. The receptors SlETR1, SlETR2 and SlETR5, SlCTR2 and SlEIN2 proteins are most abundant during the mature green stage, decreasing significantly at the start of ripening and maintaining low levels during the breaker, orange and red stages. On the other hand, the protein abundance of SlETR6, SlETR7, and SlCTR1 only decrease during the red ripening stage, so at the onset of ripening no significant changes are observed. SlCTR3 abundance is maintained constant throughout fruit ripening.

Analysis of the Transcripts Levels

Figure 3 demonstrates that *SlETR4* shows the highest expression of all the *SlETRs*, followed by *SlETR3* and *SlETR6,* and then *SlETR7*, *SlETR1,* and *SlETR2*. The expression level of *SlETR5* is the lowest. Within the *SlCTRs*, *SlCTR1* and *SlCTR2* are more expressed compared to *SlCTR3* and *SlCTR4* (**Figure 4**). None of the *SlETRs* show significant changes in gene expression between different ripening stages, except for *SlETR3* of which transcript levels are higher in the orange ripening stage compared to the mature green fruit. Both *SlCTR1* and *SlCTR4* expression levels show a climacteric expression pattern, while *SlCTR2* and *SlCTR3* do not significantly change during the four ripening stages. The mRNA levels of *SlEIN2* are significantly higher in mature green fruit as compared to red fruit.

When comparing the correlation between gene expression and protein levels (**Supplementary Figure 3**) a significant correlation is found only for SlETR3, SlCTR1, and SlEIN2.

DISCUSSION

Benefits and Limitations of the Ethylene Signaling Protein Quantification

Szymanski et al. (2017) performed a proteomics discovery experiment similar to the one of Mata et al. (2017) as outlined in **Figure 1A**, identifying SlETR3, SlETR4, SlCTR2 and SlEIN2, and showed that SlETR3 has a climacteric profile during ripening. However, such methods are not ideal for the quantification of low abundant proteins in a large number of samples, because

the production and MS analysis becomes very costly due to the fractionation required. Furthermore, some of the peptides used for the identification of the proteins appeared in more than one sub-fraction which might generate quantification and reproducibility issues. However, such preliminary discovery work provided a solid starting point on which the current targeted proteomics workflow was based (**Figure 1B**). The current workflow provides a simplified protein fractionation step through SDS-PAGE, without the need of producing extra sub-fractions, and provides a targeted search of the proteins on the LC–MS which, thanks to the increased sensitivity and signal to noise ratio, allows the identification and quantification of low abundant proteins of interest (Gallien et al., 2012). Furthermore, it is a relatively easy and reproducible technique.

The introduction of isotopically labeled peptides provided a strong identity confidence and allowed an absolute quantification of the endogenous peptides in the sample. However, spiking of the samples can only be done just before the LC–MS analysis, and is therefore not accounting for any technical variance nor protein losses during earlier steps. As a result, the estimated absolute protein levels can still be prone to errors. For some proteins, the endogenous peptides resulted in considerably different concentrations (**Supplementary Figure 2**). We hypothesize that this could be due to (i) different trypsin digestion efficiency in different parts of the protein, (ii) incomplete re-solubilization of the labeled peptides during their initial preparation, and/or (iii) partial adsorption of the labeled peptides onto vials. The tryptic digestion efficiency problem would produce an underestimation of some of the endogenous peptides due to their incomplete digestion, while the incomplete re-solubilization or adsorption of the labeled peptides would cause an overestimation, as the calculated spiking concentrations would be smaller in reality. It would, therefore, be interesting to also test QCAT proteins, which is a concatenation of standard tryptic peptides encoded by an artificial gene, and PSAQ which are isotope-labeled full length proteins with the same amino acid composition as the endogenous proteins (Beynon et al., 2005; Brun et al., 2007). These proteins can be incorporated earlier during sample processing and should display biochemical properties more similar to the endogenous proteins (Brun et al., 2007).

Ethylene Receptor Abundance Is Linked to Fruit Ripening of Tomato

Our quantitative analyses demonstrated that SlETR3, SlETR4, SlETR6, and SlETR7 were the most abundant receptors during tomato fruit ripening. Our gene expression results also showed that these receptors were the most expressed. These results are in accordance with the high expression levels for *SlETR3* and *SlETR4* observed in other studies (Kevany et al., 2007; Yan et al., 2013; Liu et al., 2015; Mata et al., 2018). It is thus plausible that these receptors are the most important and thus play an important role in regulating ethylene sensitivity during climacteric fruit ripening of tomato. Both protein abundance and gene expression data showed that SlCTR1 was the most abundant member of the SlCTR family during fruit ripening. Our gene expression data for S1CTR1 are similar to data from Adams-Phillips et al. (2004) and Liu et al. (2015). The high expression and protein abundance data for SlCTR1 might indicate that SlCTR1 is the main fruit ripening specific SlCTR member. Previous work demonstrated that transgenic antisense tomato lines with a reduced expression of *SlETR3*, *SlETR4,* and *SlETR6* showed an increased ethylene sensitivity and an accelerated ripening phenotype (Tieman et al., 2000; Kevany et al., 2007). Fu et al. (2005) also demonstrated that silencing *SlCTR1* expression using virus-induced gene silencing, promoted fruit ripening in green tomatoes. Because the receptors and SlCTRs act as negative regulators of ethylene signaling (Kieber et al., 1993; Hua and Meyerowitz, 1998; Tieman et al., 2000; Lin et al., 2008), a higher abundance of these proteins would lead to a reduced ethylene sensitivity.

Receptor phosphorylation has been also linked to ethylene sensitivity, as Kamiyoshihara et al. (2012) showed that both SlETR3 and SlETR4 are differentially phosphorylated during fruit ripening and by an ethylene, 1-MCP or 2,5-norbornadiene treatment, likely influencing receptor stability or activity. So, it seems that ethylene receptor turnover, but also receptor activity, is most likely regulated by specific post-translational modifications and by the hormone itself.

Climacteric Protein Levels of SlETR3 and SlETR4 Control Fruit Ripening

Kevany et al. (2007) showed that an ethylene treatment of tomato resulted in a rapid decline in receptor protein abundance of SlETR3, SlETR4 and SlETR6, likely caused by protein degradation through the proteasome-dependent pathway. They also quantified receptor abundance during ripening, using western blot, and hypothesized that the decreasing protein levels during ripening were caused by receptor degradation (Kevany et al., 2007). Our mass spectrometry quantification analysis reported results more similar to the ones of Kamiyoshihara et al. (2012), which showed by western blot that SlETR3 and SlETR4 receptor abundance increased during tomato fruit ripening. Specifically, in our results SlETR3 and SlETR4 showed a peak in the protein levels, suggesting that the concentration of these proteins follows the climacteric ethylene production levels observed during ripening. This bring us to the hypothesis that receptor degradation of SlETR3 and SlETR4 after ethylene binding, cannot counteract the high synthesis rate of new receptors during the onset of ripening. Therefore, as the receptors are negative regulators of the ethylene response, both the climacteric increase in the protein levels of SlETR3 and SlETR4 and their high abundance suggest that these receptors might control and reduce ethylene sensitivity at the onset of fruit ripening and as a consequence, control the timing and rate of fruit ripening. The increase in receptor abundance during ripening may allow the fruit to bind more ethylene which is autocatalytically produced and so control ethylene sensitivity and its downstream responses. On the other hand, the drop of SlETR3 and SlETR4 receptor abundance at the end of ripening, when tomatoes have turned red, might be related to the decline in ethylene production levels after the climacteric peak. When

less free ethylene is produced, fewer receptors are necessary to control ethylene sensitivity and control ripening. During this post-climacteric ripening stage, it is possible that receptor degradation is higher than *de novo* synthesis.

The positive feedback that ethylene exerts on receptor abundance during ripening is likely caused by an increase in receptor gene expression. Our results showed that the mRNA levels of *SlETR3* increased during ripening, while the mRNA levels of *SlETR4* followed a climacteric trend but did not show significant differences during ripening. However, it seems odd to find an increase in the SlETR4 protein levels during the onset of ripening without any increase in the mRNA levels (**Figure 3**). When studying the correlation between gene expression and protein abundance levels during ripening, only SlETR3 was significantly correlated (**Supplementary Figure 3**). The expression of both *SlETR3* and *SlETR4* have been studied the most during tomato fruit ripening, confirming an increase in expression during fruit ripening for both genes (Kevany et al., 2007; Osorio et al., 2012; Yan et al., 2013; Liu et al., 2015; Mata et al., 2018). Assuming a short change in gene expression can induce a longer lasting response at the protein level, our interpretation is that the current four ripening stages were too coarse to identify such short lasting significant changes at the transcript levels for SlETR4. Adding intermediate ripening stages would have helped to provide a more accurate picture of this regulation, like in the case of Mata et al. (2018).

Steady State Protein Levels Sustain Fruit Ripening

Protein levels of the receptors SlETR6 and SlETR7 and SlCTR1 and SlCTR3 stayed constant during ripening, only showing a perceivable decrease when the fruit reached its red ripe stage, except for SlCTR3. SlETR6 protein abundance seemed to increase in breaker fruit compared to mature green, but this change was not significant. Furthermore, the gene expression levels of both receptors (SlETR6 and SlETR7) and *SlCTR3* displayed no significant changes during ripening. The correlation between gene expression and protein abundance levels was not significant either, indicating that the protein turnover is possibly driven by post-translational modifications including protein degradation, instead of by gene expression directly. A possible explanation for the trend observed for these receptors and SlCTR3 could be that constant protein levels were sustained as a mechanism to control ethylene sensitivity in a more gentle way than through receptors 3 and 4, thus they would sustain the ripening process. The final low protein levels in the red stage would again be the consequence of the end of ripening, where no extra action would be needed to control the process.

In the case of *SlCTR1* the increase in the expression levels is not reflected at the protein level. However, a significant correlation between both kind of data was found during ripening, indicating that the protein abundance was directly controlled by gene expression. We hypothesize that in this specific case, the high transcription was counteracted by a fast rate of protein degradation of the newly formed protein after the binding of ethylene to the receptor-CTR complex. This could be the reason why no peak in protein levels was observed. Given SlCTR1 is the most abundant CTR and because of its specific behavior, it might be the strongest regulator of the tomato CTRs. Likewise the transcript levels of *SlCTR4* behaved, but its low abundancy did not allow its identification in spite of using the highly sensitivity targeted acquisition proteomics method PRM.

Decreasing Protein Levels Enable the Onset of Fruit Ripening

It is remarkable that SlETR1, SlETR2 and SlETR5 and SlCTR2 protein levels rapidly declined as soon as ripening started in the breaker stage. However, no comparative decline of their transcript levels could be observed during ripening, neither correlation between protein and mRNA. This suggests that protein abundance of these signaling components is likely controlled by post-translational modifications, like degradation, and not by a transcriptional regulation. Although SlETR1, SlETR2, and SlETR5 are the three least abundant ethylene receptors, it is possible that their higher protein levels in the mature green stage influence ethylene sensitivity by restraining ethylene signaling in this maturity stage due to their negative action. Their subsequent decrease in abundance during ripening could release this inhibitory action of ethylene sensitivity and perhaps eventually trigger fruit ripening. In this scenario, these receptors together with SlCTR2, could influence the initiation of tomato fruit ripening.

EIN2 Levels Might Control Ethylene Sensitivity During Ripening

EIN2, on the other hand, is a positive regulator of ethylene signaling and is believed to play a central role in transmitting the ethylene signal from the ER to the nucleus (Alonso et al., 1999; Zheng and Zhu, 2016). Transgenic tomato plants in which *SlEIN2* expression is silenced, show a delayed fruit ripening phenotype, confirming that SlEIN2 is a positive regulator of ethylene signaling in tomato (Fu et al., 2005; Hu et al., 2010; Wang et al., 2016). We show now that SlEIN2 protein levels decreased directly in the breaker stage suggesting that ethylene sensitivity is gradually lost during fruit ripening. SlEIN2 protein abundance is directly correlated to *SlEIN2* expression, which also declines, but the drop became only significant in the red stage. Contrarily, Liu et al. (2015) reported, based on publicly available gene expression data, that *SlEIN2* expression did not change much during ripening, which does not match our findings using qPCR.

SlEIN2 is the largest protein analyzed in this work and in theory, based on the alignment with AtEIN2 (**Supplementary Table 5**), the C-terminal end of SlEIN2 could, given its size, also be present in the fractionated gel part. However, due to the microsomal membrane protein extraction used in this study, it is unlikely that the C-terminal cytosolic soluble portion was co-extracted with the membrane fraction, unless it had a strong membrane association. Therefore, what we can assure is the quantification of the complete protein SlEIN2, but not of its C-terminal portion, which anyway would be present in a lower

percentage than the intact SlEIN2 protein. The fact that the quantification was mainly of the intact protein would mean that SlEIN2 levels are declining during ripening, possible through the ETP-mediated degradation (Qiao et al., 2009). This would explain why the decrease in the protein levels could already be seen in the breaker stage, while for the gene expression, levels became only significant at the red stage. Hence, the apparent discrepancy between the more constant transcription levels and the falling protein levels of SlEIN2. The discovery of the exact cleavage site of SlEIN2 in tomato, as well as the retirement of additional peptides that are exclusively located in the N-terminal part, would allow us to distinguish the abundance of both the N- and C-terminal part of EIN2, and give more insight in the regulatory dynamics of this enigmatic protein.

CONCLUSION

This work describes a feasible and reproducible technique to identify and quantify the low abundant ethylene signaling proteins ethylene receptors (ETRs), CTRs and EIN2 in tomato pericarp. The strategy is composed of (i) microsomal membrane extraction, (ii) fractionation of the protein sample through 1-D gel, (iii) in-gel tryptic digestion and (iv) identification and absolute quantification through the monitoring of several unique peptides of the target proteins by PRM. The combined quantification of protein and mRNA levels of the ethylene signaling components during ripening has revealed different patterns between gene expression and protein abundance which might collectively modulate and control ethylene sensitivity and thus the timing and rate of fruit ripening. Our hypothesis is that some receptors would largely control the ethylene sensitivity and, therefore, the ripening process, like SlETR3 and SlETR4 with the help of SlCTR1, some of the most abundant proteins, and possibly SlCTR4. Other signaling components such as SlETR6, SlETR7, and SlCTR3 show an unaltered protein abundance during the onset of ripening and might therefore be important to sustain the ripening process. Finally, proteins such as SlETR1, SlETR2, SlETR5, and SlCTR2 show a rapid decline in protein abundance, which might suggest that they could control the initiation of ripening. SlEIN2, being a positive regulator of ethylene signaling, also show a declining abundance profile, and could therefore also control ethylene sensitivity during climacteric fruit ripening of tomato. In conclusion, it seems that ethylene sensitivity is differently controlled by a balanced turnover of the different components of the ethylene signaling pathway, combining positive and negative feedback regulations.

Future mass spectrometry analyses are needed to reveal the specific proteolytic cleavage site of SlEIN2 and to study the phosphorylation dynamics of both the receptors and SlEIN2 during ripening. Finally, a broad quantitative proteomics study including additional downstream signaling transcription factors such as the EILs and ERFs could help us to better understand ethylene sensitivity and signaling during climacteric fruit ripening of tomato.

AUTHOR CONTRIBUTIONS

CM, BF, HP, MH, and KL designed the experiments. CM carried out the experiments, data analysis, prepared the figures, and wrote the manuscript. CM, BF, HP, MH, GVR, GB, BVdP, KL, and BN helped to improve the manuscript and participated in discussions. All authors provided feedback on the manuscript and gave their final approval for submission.

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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.2018.01626/full#supplementary-material

FIGURE S1 | Dilution curves for the PRM analysis of 0–200 fmol/μL of the selected heavy labeled peptides for the proteins SlETR1-SlETR7, SlCTR1-SlCTR3 and SlEIN2 and linearity expressed by coefficient of determination (R2).

FIGURE S2 | Absolute quantification (fmol of target protein/μg of total membrane proteins) of the peptides of SlETR1-SlETR7, SlCTR1-SlCTR3 and SlEIN2 during tomato fruit ripening. MG, mature green; BR, breaker; OR, orange; R, red tomatoes. Error bars represent the standard error of the mean based on six biological replicates. Different uppercase letters indicate significant differences between the absolute protein concentration levels of the four tomato ripening stages determined by Tukey's Honestly Significant Difference (HSD) test ($P < 0.05$).

FIGURE S3 | Correlation between protein and gene expression levels of the SlETR1-SlETR7, SlCTR1-SlCTR3 and SlEIN2. Significant correlations are represented with an asterisk in the chart title and non-significant correlation with the letters N.S. The protein, gene expression data and their standard errors were normalized for visualization.

TABLE S1 | List of unlabeled peptides tested for the assay development. The ones marked in yellow were the identified and quantified peptides (labeled peptides were order afterwards for these ones). The ones in green were promisingly identified but labeled peptide for them were not obtained.

TABLE S2 | Selected results output of the use of the mProphet algorithm of the targeted peptide identifications trained with the second best peak option.

TABLE S3 | RT-qPCR primers for the 12 ethylene signaling and 3 reference genes used in this study. Primers were designed with the Primer3 web tool (http://bioinfo.ut.ee/primer3/). Primer specificity was checked by BLAST-ing against all tomato EST's and known cDNA sequences.

TABLE S4 | Amino acid sequences of the proteins SlETR1-SlETR7, SlCTR1-SlCTR3 and SlEIN2 obtained from Uniprot (Bateman et al., 2015). Their Uniprotannotated transmembrane domains are underlined, their possible phosphorylation sites are highlighted in green and their GAF domain, kinase domain and response regulatory domains are represented in green, blue and orange fonts, respectively. The peptides used for the quantifications of the proteins in the current study are highlighted in yellow.

TABLE S5 | Cluster alignment of Q9S814 (AtEIN2) and Q6Q2C1 (SlEIN2) with the alignment tool of Uniprot. The C-terminal part of AtEIN2 is highlighted in green based on the results of Qiao et al. (2012). The two peptides of SlEIN2 identified and quantified in this work are highlighted in yellow.

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Abscisic Acid Regulates Anthocyanin Biosynthesis and Gene Expression Associated With Cell Wall Modification in Ripening Bilberry (*Vaccinium myrtillus* L.) Fruits

*Katja Karppinen[1,2], Pinja Tegelberg[1], Hely Häggman[1] and Laura Jaakola[2,3]**

[1] Department of Ecology and Genetics, University of Oulu, Oulu, Finland, [2] Climate laboratory Holt, Department of Arctic and Marine Biology, UiT The Arctic University of Norway, Tromsø, Norway, [3] Norwegian Institute of Bioeconomy Research (NIBIO), Ås, Norway

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***Correspondence:**
Laura Jaakola
laura.jaakola@uit.no

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Ripening of non-climacteric bilberry (*Vaccinium myrtillus* L.) fruit is characterized by a high accumulation of health-beneficial anthocyanins. Plant hormone abscisic acid (ABA) and sucrose have been shown to be among the central signaling molecules coordinating non-climacteric fruit ripening and anthocyanin accumulation in some fruits such as strawberry. Our earlier studies have demonstrated an elevation in endogenous ABA level in bilberry fruit at the onset of ripening indicating a role for ABA in the regulation of bilberry fruit ripening. In the present study, we show that the treatment of unripe green bilberry fruits with exogenous ABA significantly promotes anthocyanin biosynthesis and accumulation both in fruits attached and detached to the plant. In addition, ABA biosynthesis inhibitor, fluridone, delayed anthocyanin accumulation in bilberries. Exogenous ABA also induced the expression of several genes involved in cell wall modification in ripening bilberry fruits. Furthermore, silencing of *VmNCED1*, the key gene in ABA biosynthesis, was accompanied by the down-regulation in the expression of key anthocyanin biosynthetic genes. In contrast, the treatment of unripe green bilberry fruits with exogenous sucrose or glucose did not lead to an enhancement in the anthocyanin accumulation neither in fruits attached to plant nor in post-harvest fruits. Moreover, sugars failed to induce the expression of genes associated in anthocyanin biosynthesis or ABA biosynthesis while could elevate expression of some genes associated with cell wall modification in post-harvest bilberry fruits. Our results demonstrate that ABA plays a major role in the regulation of ripening-related processes such as anthocyanin biosynthesis and cell wall modification in bilberry fruit, whereas sugars seem to have minor regulatory roles in the processes. The results indicate that the regulation of bilberry fruit ripening differs from strawberry that is currently considered as a model of non-climacteric fruit ripening. In this study, we also identified transcription factors, which expression was enhanced by ABA, as potential regulators of ABA-mediated bilberry fruit ripening processes.

Keywords: *Vaccinium*, non-climacteric fruit, berry ripening, hormonal regulation, signaling molecules, anthocyanins, abscisic acid, sucrose

INTRODUCTION

Fleshy fruits and berries have important roles in human health and nutrition, and therefore their ripening regulation have been intensively studied. Development and subsequent ripening of fleshy fruits are complex processes including major metabolic and structural changes, such as accumulation of pigments, flavor and aroma compounds as well as changes in fruit texture. These processes are controlled by a series of signaling events regulated by plant hormones. Fleshy fruits are physiologically defined as either climacteric or non-climacteric according to the differences in respiration rate and production of plant hormone ethylene during ripening (Osorio et al., 2013). A burst of ethylene accompanied by an increase in the respiration rate at the onset of ripening has long been known to be a critical signal controlling ripening of climacteric fruit, such as tomato, mango, melon, apple, and peach. In contrast, ripening mechanisms of non-climacteric fruits, lacking the burst of respiration and ethylene production, have remained less understood (Cherian et al., 2014).

Studies have shown that plant hormone abscisic acid (ABA), in addition to its central role in plant growth and development and in the adaptation to stress conditions (Vishwakarma et al., 2017), is a major regulator of non-climacteric fruit ripening (Cherian et al., 2014; Leng et al., 2014). ABA has been indicated as a ripening promoter in many non-climacteric fruits, such as strawberry (Jia et al., 2011; Li et al., 2011; Kadomura-Ishikawa et al., 2015), grape (Koyama et al., 2010; Jia et al., 2017), sweet cherry (Luo et al., 2014; Shen et al., 2014), cucumber (Wang et al., 2013), citrus (Zhang et al., 2014), pear (Dai et al., 2014), and litchi (Singh et al., 2014) but also for climacteric fruits, such as tomato, peach, melon, and mango (Zhang et al., 2009; Soto et al., 2013; Sun et al., 2013; Zaharah et al., 2013; Mou et al., 2015). The direct molecular level evidence for the role of ABA in fruit ripening was shown in strawberry by suppressing the expression of the key ABA biosynthetic gene, *FaNCED1*, blocking ABA biosynthesis and leading to partly uncolored strawberry fruits that could be rescued by exogenous ABA (Jia et al., 2011).

Progress has been made during recent years in the understanding of the molecular mechanisms underlying ABA perception and signal transduction during non-climacteric fruit ripening (Li et al., 2011; Cherian et al., 2014; Leng et al., 2014). The ABA receptors were identified by down-regulating the expression of receptors *FaPYR1* and *FaCHLH/ABAR* that led to delay in strawberry fruit ripening and fruit coloring that could not be rescued by exogenous ABA (Chai et al., 2011; Jia et al., 2011). Studies have also identified some ABA-regulated fruit ripening-related transcription factors (TFs) belonging to different gene families, such as MADS, MYB, and bZIP (Daminato et al., 2013; Nicolas et al., 2014; Medina-Puche et al., 2016). However, the ABA-mediated regulatory network promoting non-climacteric fruit ripening still remains poorly understood.

Sugars have traditionally been considered as a carbon and energy source for plants, and in fruits sugars have been thought merely to affect fruit quality. A growing number of studies have indicated that sugars, such as glucose and sucrose, can act as signaling molecules and possess hormone-like signaling functions in plant development and stress responses (Van den Ende and El-Esawe, 2014; Huang et al., 2016). Especially sucrose-specific signaling pathway has been proposed in the regulation of anthocyanin biosynthesis, and in *Arabidopsis* anthocyanin biosynthesis was shown to be up-regulated by sucrose (Solfanelli et al., 2006; Loreti et al., 2008). Fruit ripening signals have been studied extensively in strawberry, which is currently considered as a model of non-climacteric fruit ripening (Li et al., 2011; Cherian et al., 2014). In strawberry, studies have demonstrated that especially sucrose but also glucose promotes fruit ripening (Jia et al., 2011, 2013a,b). Currently, sucrose in co-operation with ABA are indicated as the core signaling molecules regulating strawberry fruit ripening (Jia et al., 2011, 2013b). The coordinated regulation of fruit ripening by ABA and sucrose has recently been suggested also for grapes (Jia et al., 2017).

Bilberry (*Vaccinium myrtillus* L.) is one of the most abundant wild berries in the Northern Europe and it is valued for its nutraceutical and health-beneficial properties (Kolehmainen et al., 2012; Jimenez-Garcia et al., 2013; Törrönen et al., 2013). Ripening of non-climacteric bilberry fruit is characterized by a high accumulation of health-beneficial anthocyanins both in peel and flesh providing deep blue color to the ripe fruits. Anthocyanins are biosynthesized via the well-known phenylpropanoid/flavonoid pathway consisting of a number of enzymatic steps that catalyze a sequential reaction leading to the production of different anthocyanin classes (**Supplementary Figure S1**). Our earlier studies have identified altogether 33 different anthocyanins in bilberry fruits belonging to delphinidin, cyanidin, petunidin, peonidin, and malvidin classes (Zoratti et al., 2014).

So far, there are no studies concerning the role of ABA or sugars on the regulation of fruit ripening and anthocyanin biosynthesis in bilberry fruit. Furthermore, earlier studies have given contradictory results on the role of ABA in the anthocyanin accumulation in other *Vaccinium* species (Percival and MacKenzie, 2007; Forney et al., 2009; Buran et al., 2012; Oh et al., 2018). In our previous study, an increase in endogenous ABA level accompanied by an increase in the expression of *VmNCED1*, the key gene in ABA biosynthesis, was demonstrated at the onset of bilberry fruit ripening suggesting a role for ABA in bilberry ripening regulation (Karppinen et al., 2013).

The aim of the current study was to examine the role of ABA and various sugars on bilberry fruit ripening and ripening-related processes. For this purpose, the effect of exogenous ABA and sugars on bilberry fruit ripening and anthocyanin accumulation was examined in both pre- and post-harvest experiments. The effects of the post-harvest treatments on the expression of the key genes in anthocyanin, ABA and sucrose biosynthesis as well as the expression of the genes encoding major cell wall modifying enzymes was studied. The role of ABA in anthocyanin biosynthesis was further examined by silencing *VmNCED1* in ripening bilberry fruits. Finally, we also identified potential TFs in ABA-regulated bilberry fruit ripening processes.

MATERIALS AND METHODS

Plant Material

Bilberry (*V. myrtillus* L.) plant material used for the experiments was originated from the natural forest stands in Oulu (65°01′ N, 25°28′ E) and Tromsø (69°42′ N, 18°51′ E). For the virus-induced gene silencing (VIGS) experiments, bilberry plants with their root system were harvested at the stage when fruits were unripe and green. The plants were placed in boxes (50 cm × 70 cm) with forest peat soil and watered well. The five developmental stages of bilberry fruit were collected as described earlier (Karppinen et al., 2013).

Pre-harvest Treatments With ABA and Sugars

In order to study the effect of exogenous ABA on bilberry fruits still attached to plants, an experiment was conducted on field conditions with bilberries growing on the natural forest stand in Oulu, Finland July 2014. For the treatments, ABA [(±)-abscisic acid; Sigma, St. Louis, MO, United States] at concentrations of 0.5 and 2 mM with 0.5% (v/v) Tween 20 were utilized. A solution containing water with 0.5% (v/v) Tween 20 was used as a control treatment. The solutions were applied individually on unripe green bilberry fruits by spraying until run-off with a hand-held sprayer on alternate days for 6 days (three times) in the late afternoon to minimize ABA photo-degradation. Approximately 50 berries were utilized per treatment with four replicates by employing around 15 m^2 areas adjacent to each other. Berries were evaluated for their color 7 days from the beginning of the first treatment. Berry samples were collected after 0, 24, 48, 96, and 168 h (7 days) from the first treatment, immediately frozen in liquid nitrogen and stored at −80°C until used for RNA extraction and determination of anthocyanin content.

The effect of glucose and sucrose on bilberry fruits still attached to plants was studied on field conditions with bilberries growing on the natural forest stand in Tromsø, Norway August 2015. Sucrose and glucose at concentration of 200 mM with 0.5% (v/v) Tween 20 were used. A solution containing water with 0.5% (v/v) Tween 20 was used as a control treatment. The solutions were applied individually on unripe green bilberry fruits similarly as described above by spraying until run-off with a hand-held sprayer on alternate days for 6 days (three times). Approximately 50 berries per treatment were utilized with four replicate areas as described above. When obvious induction in berry ripening was not detected after 7 days from the first treatment, the treatments were repeated (three times on alternate days). Berries were evaluated for their color and collected after 0, 7, and 19 days from the beginning of the first treatment, immediately frozen in liquid nitrogen and stored at −80°C until used for the determination of anthocyanin content.

Post-harvest Treatments With ABA and Sugars

For studying the effect of ABA and sugars on detached bilberry fruits, fruits at unripe green stage were harvested from natural forest stand in Oulu, Finland July 2017. Fruits of similar size and color with absence of physical injuries or insect infections were selected for the experiment. The experiment was set-up aseptically under a laminar flow. After rinsing the fruits three times with sterile distilled water, the fruits were randomly divided and immersed with their pedicels (Roubelakis-Angelakis and Kliewer, 1986; Jia et al., 2013a) into the following filter sterilized solutions in sterile Petri plates: 0.5 and 2 mM ABA [(±)-abscisic acid; Sigma], 50 and 200 mM sucrose (VWR International, Lutterworth, United Kingdom), 50 and 200 mM glucose (Sigma), 50 and 200 mM fructose (Merck, United States), 200 μM fluridone (Sigma), 0.5 mM ABA + 200 mM sucrose, and water (control). All solutions contained 0.5% (v/v) Tween 20. Three replicate Petri plates with approximately 50 berries per plate were employed. The plates were placed at 18°C under 30 μmol m^{-2} s^{-1} light. The berries were evaluated for their color on the 4th and 6th day from the beginning of the experiment. Berry samples were collected after 0, 24, 48, 96, and 144 h (6 days) from the beginning of the experiment, immediately frozen in liquid nitrogen and stored at −80°C until used for RNA extraction and determination of anthocyanin content.

Construction of *VmNCED1* VIGS Vector and *Agrobacterium*-Mediated Infiltration

A 165 bp cDNA fragment of *VmNCED1* (GenBank accession no. JX982599) was PCR-amplified using forward primer 5′-GGATCCCGATCAGCAAGTGGTGTTTA-3′ (*Bam*H1 site is underlined) and reverse primer 5′-TGGAAGCTTAATGTATCCGGACACTCG-3′ (*Hind*III site is underlined) under standard PCR conditions. The PCR product was gel-purified, digested with *Bam*HI and *Hind*III and ligated into pTV00 vector. The resulting pTV00-*VmNCED1* vector was confirmed by sequencing and transformed into *Agrobacterium tumefaciens* strain GV3101 by the freeze-thaw method.

Agrobacterium-mediated infiltration by syringe injection with a needle into bilberry fruits was performed as described earlier by Jaakola et al. (2010). Briefly, a 5 ml cultures of *Agrobacterium* strain GV3101 containing pTV00-*VmNCED1* and strain C58c1 containing pBINTRA6 were grown overnight at 28°C in liquid Luria-Bertani (LB) medium (pH 5.6) with appropriate antibiotics. The overnight cultures were inoculated into 50 ml of LB medium containing 10 mM MES, 20 μM acetosyringone and appropriate antibiotics and grown at 28°C until the OD_{600} of the cultures reached 1.0–1.3. The cells were collected by centrifugation (3500 rpm, 5 min, 20°C), resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, 200 μM acetosyringone) to a OD_{600} of approximately 1.5 and incubated at room temperature at least for 2 h. *Agrobacterium* mixture containing pTV00-*VmNCED1* and pBINTRA6 (1:1 ratio) was injected into unripe green bilberry fruits at two spots on the same side of the berry by a 1-ml syringe with a needle. As a control, only *Agrobacterium* with pBINTRA6 was injected into the fruits. The bilberry plants were placed at 18°C with 60% humidity and 125 μmol m^{-2} s^{-1} light intensity. Fruits were evaluated 4 weeks after injection, then

frozen in liquid nitrogen and stored at −80°C until used for RNA extraction.

Isolation of RNA and cDNA Preparation

Total RNA was isolated from berries according to the method described earlier for bilberry (Jaakola et al., 2001). The cDNA was synthesized from the total RNA by using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. The cDNA was purified from the contaminating genomic DNA by using the method described by Jaakola et al. (2004).

Relative Quantification of Gene Expression

Real-time quantitative reverse transcription PCR (qRT-PCR) analyses were performed with a LightCycler 480 instrument and software (Roche Applied Sciences, Indianapolis, IN, United States). The transcript abundance of the genes was detected using a LightCycler® SYBR Green I Master qPCR Kit (Roche). The qRT-PCR conditions were an initial incubation at 95°C for 10 min followed by 40 cycles at 95°C for 10 s, 60°C for 20 s, and 72°C for 10 s. The studied genes we identified from the publicly available *Vaccinium* transcriptome databases. The gene-specific primer sequences used for the qRT-PCR analyses are listed in **Supplementary Table S1**. Glyceraldehyde-3-phosphate dehydrogenase (*VmGAPDH*; GenBank accession no. AY123769) was employed as a reference gene for the relative quantification of the PCR products. The results were calculated with LightCycler® 480 software (Roche), using the calibrator-normalized PCR efficiency-corrected method (Technical note No. LC 13/2001, Roche). The amplification of only one product in qRT-PCR was confirmed by a melting curve analysis.

Determination of Total Anthocyanins

Frozen berries were ground to fine powder with a mortar and pestle in the presence of liquid nitrogen. Berry powder of 0.1 g was extracted with methanol acidified with 0.1% HCl (v/v) by sonication in the dark for 10 min followed by shaking at room temperature in the dark for 1 h. After centrifugation, the supernatant was collected and the total anthocyanin content was determined according to the pH differential method (Lee et al., 2005, 2008) that has been tested for bilberry material (Dandena et al., 2012). Analyses were performed with three to four biological replicates. The results were expressed as mg (cyanidin-3-glucoside equivalent) g^{-1} fresh weight.

Statistical Analysis

The quantitative results of gene expression and measurements of anthocyanins in bilberry fruits were analysed either with Student's *t*-Test or one-way analysis of variance (ANOVA) followed by Tukey's HSD test by using SPSS Statistics program, version 25 (IBM, New York, NY, United States).

RESULTS

Effect of Pre-harvest Treatments With ABA and Sugars on Bilberry Fruit Ripening and Anthocyanin Accumulation

To investigate the role of ABA on bilberry fruit ripening and anthocyanin accumulation, exogenous ABA was sprayed three times on alternate days on unripe green bilberry fruits still attached to plants. Seven days after the first treatment with 0.5 mM ABA, and especially with 2 mM ABA, most of the fruits had turned red/blue indicating fruit ripening and anthocyanin accumulation while most of the control fruits treated with water were still green (**Figure 1A**). The anthocyanin content was significantly higher at day seven in ABA treated fruits compared to control fruits sprayed with water (**Figure 1B**). Also, both the ABA treatments up-regulated the expression of the anthocyanin biosynthetic genes *VmCHS*, *VmANS*, and *VmUFGT* during the 7 days experiment (**Figure 1C**).

Glucose and sucrose were similarly applied by spraying on attached unripe green bilberry fruits to investigate their effect on bilberry fruit ripening and anthocyanin accumulation. Seven days after the beginning of the experiment, the fruits were still green in color and neither 200 mM glucose nor 200 mM sucrose had induced significant changes in the fruit anthocyanin content compared to control fruits treated with water (**Figure 2A**). Therefore, the treatments were repeated second time by again spraying berries with sugars three times on alternate days. After 19 days of the first treatment, there were only slightly more red/blue berries in the sugar treatments compared to control berries treated with water (**Figure 2B**). There were no significant differences between the treatments in the anthocyanin content of the berries at day 19, however, there was a high variation between individual berries in the response to sucrose (**Figure 2A**).

Effect of Post-harvest Treatments With ABA and Sugars on Bilberry Fruit Ripening and Anthocyanin Accumulation

To examine in more detail the role of ABA and sugars on bilberry fruit ripening and anthocyanin accumulation and verify the results attained with attached fruits, unripe green bilberry fruits were harvested and submerged into various post-harvest treatments in Petri plates. The treatments were: ABA (0.5 and 2 mM), glucose (50 and 200 mM), fructose (50 and 200 mM), sucrose (50 and 200 mM), 0.5 mM ABA + 200 mM sucrose, 200 μM fluridone (ABA biosynthesis inhibitor) or water (control). An increase in red coloration in bilberry fruits treated with 2 mM ABA was obvious already after 1 day from the beginning of the treatment. After 4 days, all the fruits in 2 mM ABA treatment and most of the fruits in 0.5 mM ABA and 0.5 mM ABA + 200 mM sucrose treatments had obtained red coloration indicating anthocyanin accumulation (**Figures 3A,B**). The fruits treated with water (control), fluridone or different types and concentrations of sugars were still mostly unripe and green with only few individual berries obtained some red coloration

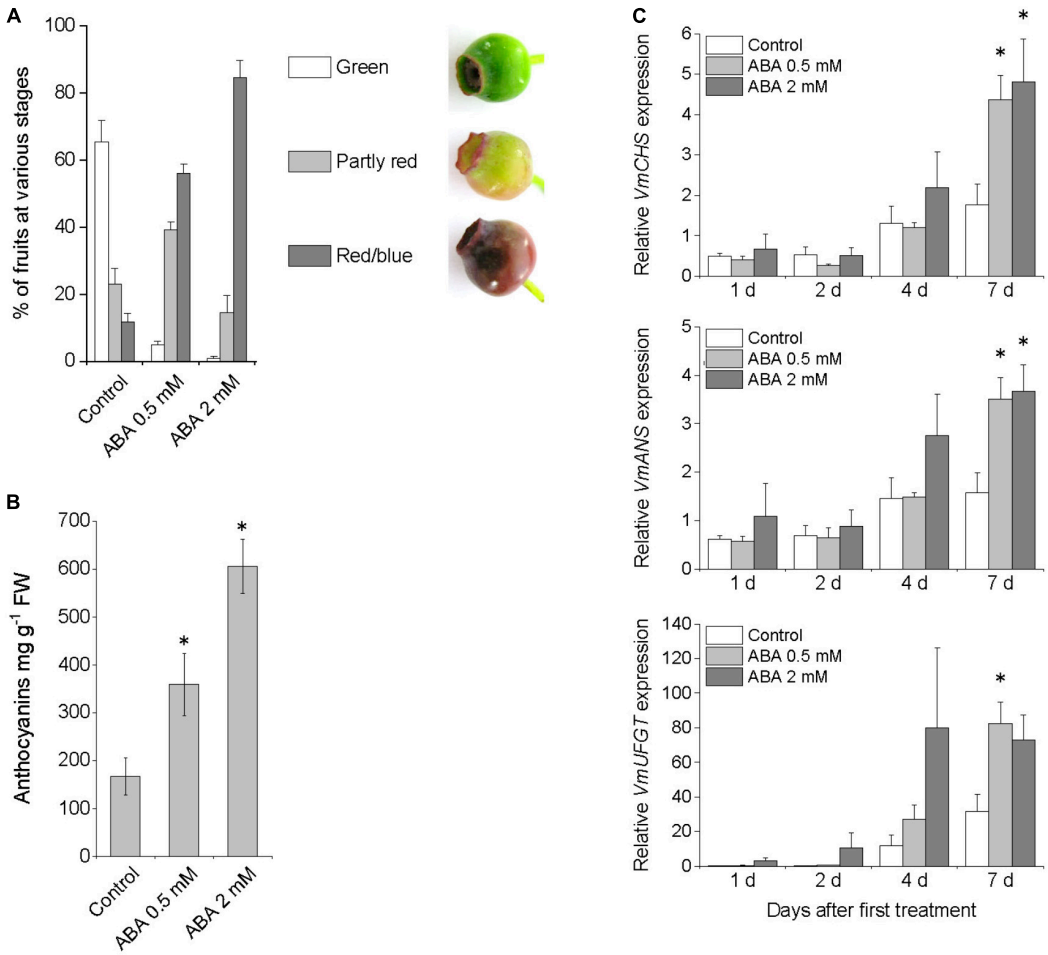

FIGURE 1 | Effect of pre-harvest treatment with ABA on bilberry fruit color **(A)**, anthocyanin content **(B)**, and expression of anthocyanin biosynthetic genes **(C)**. Unripe green berries attached to plants were sprayed with 0.5 mM ABA, 2 mM ABA or water (control). Fruit color and anthocyanin content was evaluated after 7 days from the beginning of the experiment. Total anthocyanin content is expressed as milligrams of cyanidin-3-glucoside equivalents g^{-1} FW. Relative expression of the genes was quantified by qRT-PCR and normalized to *VmGAPDH*. Values represent means ± SEs of four replicates. Asterisks indicate significant differences from control in Student's *t*-Test ($P \leq 0.05$).

(**Figures 3A,B**). The berries treated with either 0.5 mM or 2 mM ABA had significantly higher levels of anthocyanins compared to control berries in water after 4 days from the beginning of the experiment (**Figure 3C**). The treatment either with glucose, fructose or sucrose, except 50 mM glucose, did not significantly increase the anthocyanin content in bilberry fruits (**Figure 3C**). The increase with 50 mM glucose was due to increase in anthocyanin accumulation in few individual berries and the increase was not seen with 200 mM glucose. Berries treated with fluridone had significantly lower level of anthocyanins compared to control fruits in water (**Figure 3C**). Furthermore, the fruits treated with 0.5 mM ABA + 200 mM sucrose did not have significantly higher anthocyanin level compared to the berries treated only with 0.5 mM ABA, suggesting that sucrose does not significantly enhance the effect of ABA in bilberry anthocyanin accumulation (**Figure 3C**).

After 6 days from the beginning of the experiment, almost all the ABA treated berries had turned fully red/blue while most of the berries in the treatment with water (control), fluridone or sugars were still unripe and green (**Supplementary Figure S2**). None of the berries in fluridone treatment had reached the fully red/blue coloration. The anthocyanin content in berries treated with 0.5 mM ABA had highly increased from day 4. Instead, the anthocyanin accumulation in berries in 2 mM ABA treatment had slowed down from day 4 and the anthocyanin content was significantly lower in berries in 2 mM ABA compared to berries in 0.5 mM ABA treatment (**Supplementary Figure S2**) indicating over-ripening of the berries in 2 mM ABA treatment and cease in anthocyanin biosynthesis.

Expression of Anthocyanin Biosynthetic Genes in Response to Post-harvest Treatments With ABA and Sugars

The transcript levels of anthocyanin biosynthetic genes (**Supplementary Figure S1**) were examined by qRT-PCR in the

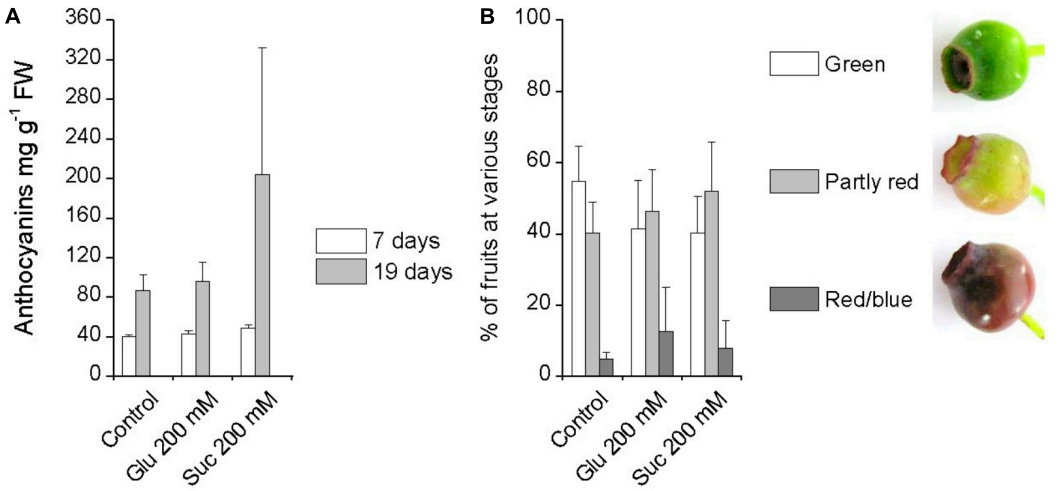

FIGURE 2 | Effect of pre-harvest treatments with glucose and sucrose on bilberry fruit anthocyanin content **(A)** and fruit color **(B)**. Unripe green berries attached to plants were sprayed with 200 mM glucose, 200 mM sucrose or water (control). Total anthocyanin content in fruits was measured after 7 and 19 days from the beginning of the experiment and is expressed as milligrams of cyanidin-3-glucoside equivalents g^{-1} FW. Fruit color was evaluated after 19 days from the beginning of the experiment. Values represent means ± SEs of four replicates.

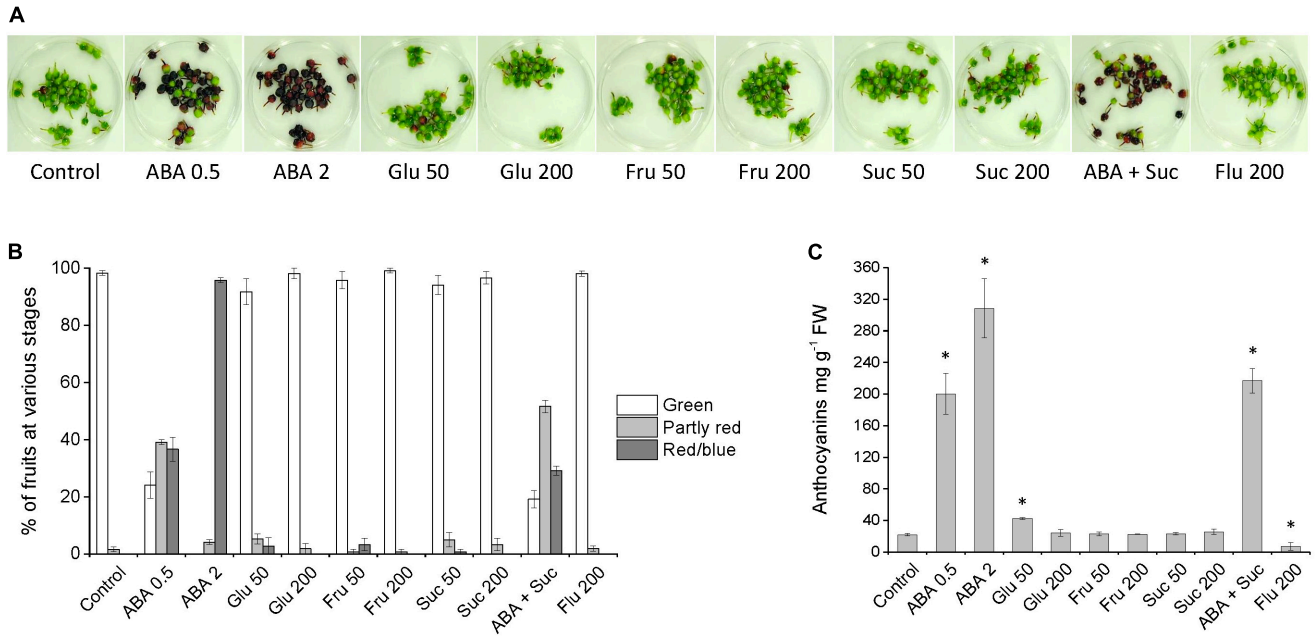

FIGURE 3 | Effect of post-harvest ABA and sugar treatments on bilberry fruit color and anthocyanin accumulation. Detached unripe green berries were immersed into solutions containing ABA (0.5 and 2 mM), glucose (50 and 200 mM), fructose (50 and 200 mM), sucrose (50 and 200 mM), 0.5 mM ABA + 200 mM sucrose, 200 μM fluridone or water (control). After 4 days from the beginning of the experiment, fruits in Petri plates **(A)** were evaluated for their color **(B)** and measured for their anthocyanin content **(C)**. Total anthocyanin content is expressed as milligrams of cyanidin-3-glucoside equivalents g^{-1} FW. Values represent means ± SEs of three replicates. Asterisks indicate significant differences from control in Student's *t*-Test ($P \leq 0.05$).

detached berries during 4 days in different treatments in Petri plates. Significant induction in the expression of all anthocyanin biosynthetic genes was detected in berries in ABA treatments indicating a major role for ABA as a positive regulator of bilberry anthocyanin biosynthesis (**Figure 4**). Especially the transcripts levels of *VmCHS*, *VmF3H*, *VmF3′H*, *VmF3′5′H*, *VmANS*, and *VmUFGT* were significantly elevated ($P \leq 0.001$) by both ABA treatments at day 4 and 2 mM ABA treatment already at day 2. For example, after 4 days in 2 mM ABA treatment the up-regulation of *VmCHS*, *VmF3′5′H*, and *VmUFGT* were 180-, 460-, and 850-fold, respectively, compared to water control. Instead, although slight elevation in gene expression

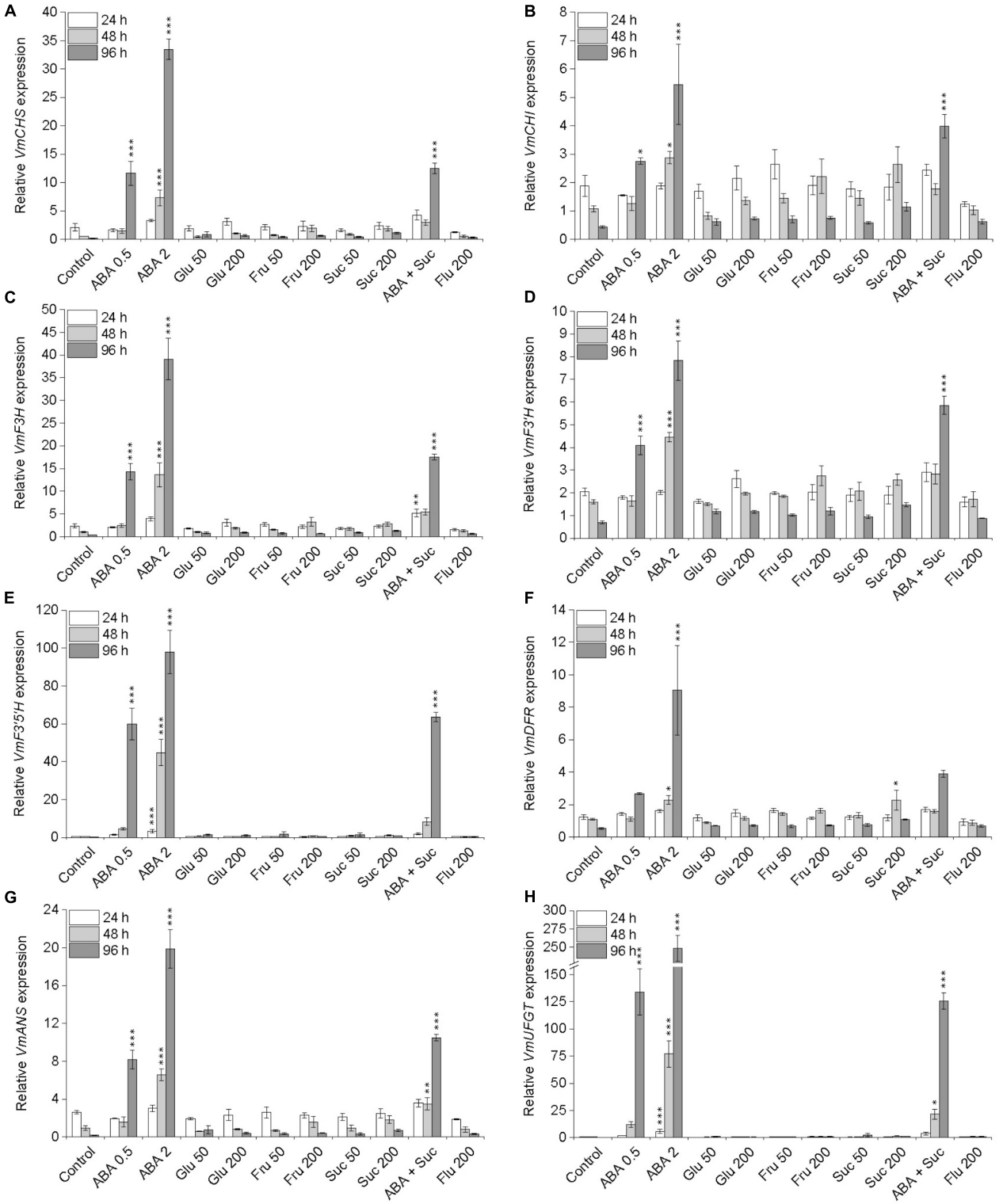

FIGURE 4 | Effect of post-harvest ABA and sugar treatments on the expression of anthocyanin biosynthetic genes *VmCHS* **(A)**, *VmCHI* **(B)**, *VmF3H* **(C)**, *VmF3′H* **(D)**, *VmF3′5′H* **(E)**, *VmDFR* **(F)**, *VmANS* **(G)**, and *VmUFGT* **(H)** in bilberry fruit. The treatments were: ABA (0.5 and 2 mM), glucose (50 and 200 mM), fructose (50 and 200 mM), sucrose (50 and 200 mM), 0.5 mM ABA + 200 mM sucrose, 200 μM fluridone or water (control). Relative expression of the genes was quantified by qRT-PCR and normalized to *VmGAPDH*. Values represent means ± SEs of three replicates. Asterisks indicate significant differences from respective control (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, one-way ANOVA with Tukey's HSD test).

was detected in sugar treatments at day 2 and 4 compared to control fruits, no obvious induction in the expression of the anthocyanin biosynthetic genes in fruits was detected (**Figure 4**). In fact, the expression of the anthocyanin biosynthetic genes was more or less decreased during the 4 days in sugar treatments indicating that sugars have less obvious positive signaling role to induce bilberry anthocyanin biosynthesis compared to ABA (**Figure 4**).

Expression of ABA and Sucrose Biosynthetic Genes in Response to Post-harvest Treatments With ABA and Sugars

In order to examine the interaction between ABA and sucrose, the transcript levels of ABA and sucrose biosynthetic genes was analyzed in post-harvest bilberry fruits in the different treatments. Bilberry fruits in 0.5 and 2 mM ABA treatments had significantly elevated transcript levels of *VmNCED1*, the key gene in the ABA biosynthetic pathway, compared to control fruits in water (**Figure 5A**) indicating autocatalytic biosynthesis of ABA. In the berries in treatments with different sugars the expression of *VmNCED1* was not induced but decreased during the 4 days experiment. The expression of the two key genes in the sucrose metabolism, *VmSS* and *VmSPS* was also studied, especially in response to ABA treatments. The expression of *VmSS* was significantly elevated in berries in 2 mM ABA treatment compared to control berries after 4 days indicating sucrose degradation while no significant increase in the expression of the gene was observed in other treatments (**Figure 5B**). The expression profiles of the three identified bilberry *SPS* genes slightly differentiated from each other. The expression of *VmSPS1* and *VmSPS2* showed initially up-regulation in berries in ABA treatments and then down-regulation after 96 h compared to control berries, while the *VmSPS3* expression was significantly down-regulated by ABA (**Figures 5C–E**) indicating that ABA does not advance sucrose formation in bilberry fruits.

Silencing of *VmNCED1* in Bilberry Fruit by Virus-Induced Gene Silencing (VIGS)

The effect of ABA on bilberry fruit ripening and anthocyanin biosynthesis was further studied by silencing *VmNCED1*, the key gene in ABA biosynthetic pathway. *VmNCED1*-VIGS vector was injected into unripe green bilberry fruits attached to bilberry plants. After 4 weeks of injection, chimeric fruits with green sectors at the site of injection were found (**Figure 6A**). The transcript levels of the *VmNCED1* were confirmed to be suppressed in these fruits compared to control fruits as well as in green sectors of the chimeric fruits compared to red sectors (**Figure 6B**). The silencing of *VmNCED1* was accompanied by the down-regulation in the expression of anthocyanin biosynthetic genes *VmCHS*, *VmF3H*, *VmF3′5′H*, *VmANS*, and *VmUFGT* in intact bilberry fruits injected with *VmNCED1*-VIGS vector as well as in green sectors of the fruits compared to red sectors (**Figure 6B**).

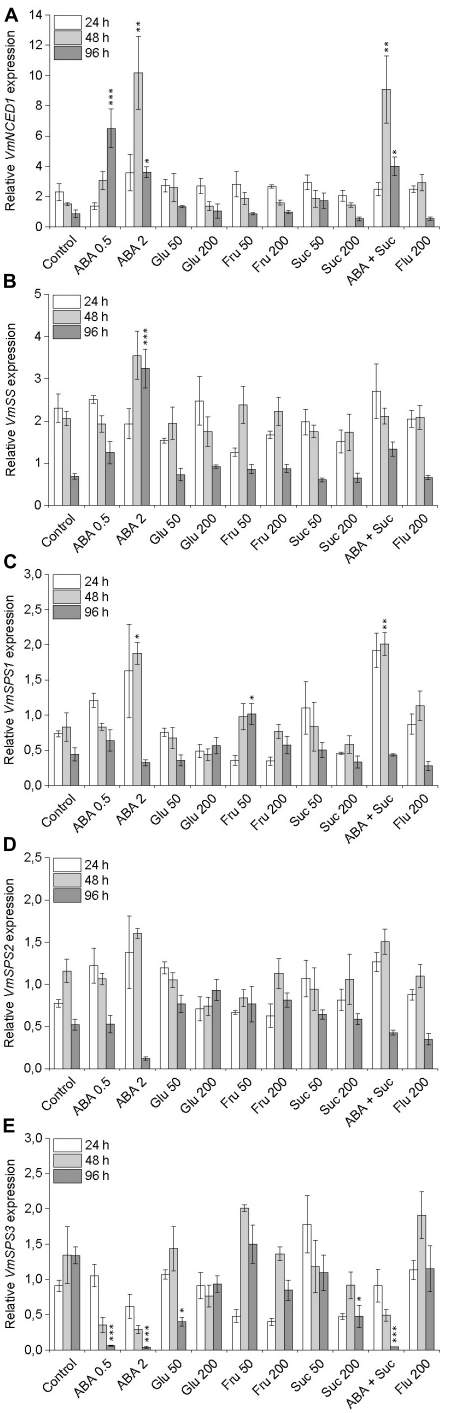

FIGURE 5 | Effect of post-harvest ABA and sugar treatments on the expression of key ABA and sucrose biosynthetic genes *VmNCED1* **(A)**, *VmSS* **(B)**, *VmSPS1* **(C)**, *VmSPS2* **(D)**, and *VmSPS3* **(E)** in bilberry fruit. The treatments were: ABA (0.5 and 2 mM), glucose (50 and 200 mM), fructose (50 and 200 mM), sucrose (50 and 200 mM), 0.5 mM ABA + 200 mM sucrose, 200 μM fluridone or water (control). Relative expression of the genes was quantified by qRT-PCR and normalized to *VmGAPDH*. Values represent means ± SEs of three replicates. Asterisks indicate significant differences from respective control (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, one-way ANOVA with Tukey's HSD test).

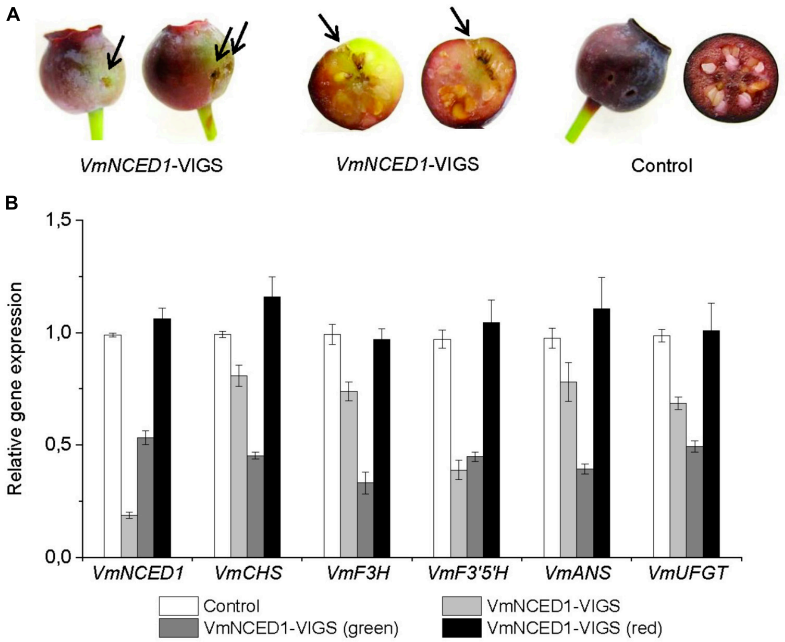

FIGURE 6 | Effect of *VmNCED1* silencing on anthocyanin biosynthesis in ripening bilberry fruit. Green unripe fruits still attached to the bilberry plants were injected with *VmNCED1*-VIGS vector or pBINTRA6 vector only (control). Arrows indicate injection sites. Fruits were evaluated 4 weeks after injection for color **(A)**, and the expression of *VmNCED1* and the key anthocyanin biosynthetic genes in intact fruits as well as in green and red sectors of chimeric fruits **(B)**. Relative expression of the genes was quantified by qRT-PCR and normalized to *VmGAPDH*. Values represent means ± SDs of three replicates.

Expression of Genes Associated With Cell Wall Modification in Response to Post-harvest Treatments With ABA and Sugars

In order study the effect of ABA and sugars on other ripening-related processes, we analyzed the expression of several genes encoding cell wall modifying enzymes in bilberry fruit in response to the post-harvest treatments. As shown in **Figure 7**, ABA significantly increased the expression of some of the genes associated with cell wall modification, including genes indicated in pectin modification *VmPL*, *VmRGLyase*, *VmβGAL1*, and *VmβGAL2* as well as genes involved in the depolymerization of hemicellulose *VmXTH*, *VmCEL*, and three expansins (*VmEXP1*, *VmEXP2*, and *VmEXP3*). The tested isoforms of *PE*, *PG,* or *XYL* responded to ABA treatment by down-regulating their expression. Instead, the sugar treatments could elevate expression of some genes associated with cell wall modification and the treatment with glucose and fructose significantly elevated the expression of *VmPE1* and *VmPG1* (**Figure 7**).

Effect of Post-harvest Treatments With ABA and Sugars on the Expression of Potential Bilberry Fruit Ripening Regulators

In order to get an insight into the ABA signaling transduction in bilberry fruit ripening-related processes, we identified from the publicly available *Vaccinium* transcriptome databases the closest homologs for the genes encoding TFs that have earlier been demonstrated to have a role in ripening regulation or anthocyanin biosynthesis in other fruits. From the tested TFs, expression of *VmSCL8*, *VmMADS18*, *VmMADS9*, *VmSHP,* and *VmBL* were significantly up-regulated by ABA indicating their potential involvement in ABA-regulated fruit ripening processes in bilberry (**Figure 8A**). Also *VmTDR4*, that has earlier been shown to be involved in anthocyanin accumulation in ripening bilberry fruit (Jaakola et al., 2010), was significantly up-regulated by ABA treatments. Expression of *VmMADS18* and *VmBL* were also significantly up-regulated by fructose and sucrose, respectively (**Figure 8A**).

The expression of these TFs was further analyzed during bilberry fruit development and ripening. The expression of *VmSCL8* and *VmMADS18* was significantly elevated in ripe fruit (*VmMADS18* also at stage 2) indicating a role in ripening processes at late stage of bilberry fruit ripening (**Figure 8B**). The expression of the genes *VmMADS9, VmSHP,* and *VmBL* was significantly up-regulated ($P \leq 0.001$) earlier during the fruit development and at the onset of fruit ripening together with *VmTDR4* (**Figure 8B**).

DISCUSSION

ABA Is a Positive Regulator of Bilberry Fruit Ripening Processes

A central role for the plant hormone ABA in promoting fruit ripening has been demonstrated during recent years. Exogenous

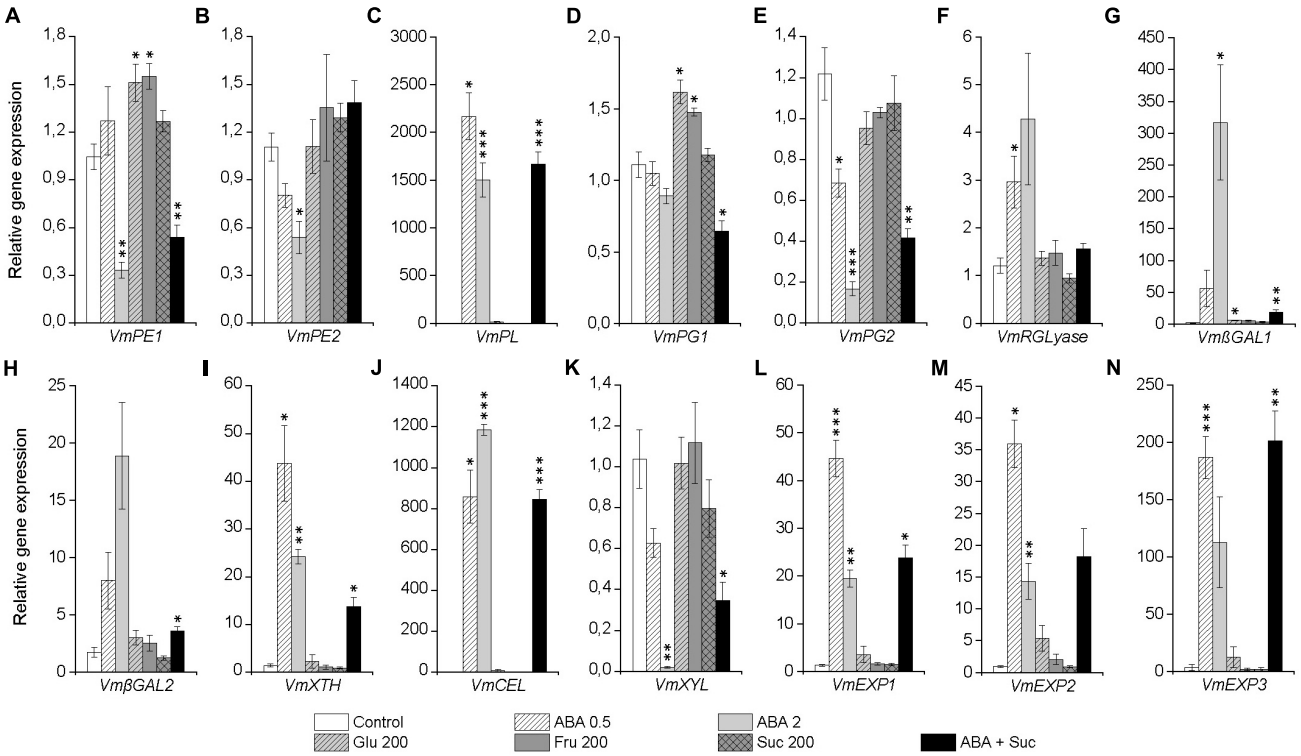

FIGURE 7 | Effect of post-harvest ABA and sugar treatments on the expression cell wall modifying genes *VmPE1* **(A)**, *VmPE2* **(B)**, *VmPL* **(C)**, *VmPG1* **(D)**, *VmPG2* **(E)**, *VmRGLyase* **(F)**, *VmβGAL1* **(G)**, *VmβGAL2* **(H)**, *VmXTH* **(I)**, *VmCEL* **(J)**, *VmXYL* **(K)**, *VmEXP1* **(L)**, *VmEXP2* **(M)**, and *VmEXP3* **(N)** in bilberry fruit. The treatments were: ABA (0.5 and 2 mM), glucose (200), fructose (200 mM), sucrose (200 mM), 0.5 mM ABA + 200 mM sucrose, or water (control). Relative expression of the genes was quantified by qRT-PCR after 4 days of the beginning of the experiment and normalized to *VmGAPDH*. Values represent means ± SEs of three replicates. *PE*, pectin esterase; *PL*, pectate lyase; *PG*, polygalacturonase; *RGLyase*, rhamnogalacturonate lyase; *βGAL*, *β*-galactosidase; *XTH*, xyloglucan endotransglycosylase/hydrolase; *CEL*, endo-*β*-1,4 glucanase: *XYL*, *β*-xylosidase; *EXP*, expansin. Asterisks indicate significant differences from control in Student's *t*-Test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

application of ABA has been shown in many studies to advance especially non-climacteric fruit ripening and the associated anthocyanin accumulation in grape berries (Wheeler et al., 2009; Koyama et al., 2010; Villalobos-González et al., 2016), strawberries (Chai et al., 2011; Jia et al., 2011; Kadomura-Ishikawa et al., 2015; Chen et al., 2016), sweet cherries (Luo et al., 2014; Shen et al., 2014), and litchi fruit (Wei et al., 2011; Singh et al., 2014). Moreover, treatments with inhibitors of ABA biosynthesis, such as fluridone and nordihydroguaiaretic acid (NDGA), delay fruit ripening and decrease anthocyanin accumulation (Jia et al., 2011; Shen et al., 2014; Kadomura-Ishikawa et al., 2015).

In bilberry, the accumulation of anthocyanin pigments is an important indicator of fruit ripening. Our previous studies have demonstrated an increase in ABA content and ABA biosynthesis at the onset of bilberry fruit ripening preceding anthocyanin accumulation (Karppinen et al., 2013, 2016). Similarly, Zifkin et al. (2012) demonstrated a substantial increase in ABA level in highbush blueberries (*V. corymbosum*) at the initiation of fruit ripening suggesting a role for ABA in fruit ripening regulation. However, contradictory results on the effect of ABA in anthocyanin accumulation have been reported when fruits of genus *Vaccinium* have been treated with ABA. Oh et al. (2018) demonstrated that exogenous application of ABA increased northern highbush blueberry (*V. corymbosum*) fruit coloration and accumulation of anthocyanins, especially malvidin, delphinidin, and petunidin glycosides. Instead, exogenous ABA application delayed ripening of southern highbush blueberries (*V. darrowii*; Buran et al., 2012) while ABA had no effect on the anthocyanin accumulation in lowbush blueberry (*V. angustifolium*; Percival and MacKenzie, 2007) or in white cranberries (*V. macrocarpon*; Forney et al., 2009).

The data from the current study clearly demonstrates that ABA induces bilberry fruit anthocyanin biosynthesis. Exogenous ABA applied to unripe bilberry fruits either as pre- or post-harvest treatment promoted berry coloration and anthocyanin accumulation. In addition, the post-harvest treatment with ABA biosynthesis inhibitor, fluridone, delayed bilberry fruit coloration and reduced fruit anthocyanin content. The anthocyanin accumulation in bilberry fruit was supported by the gene expression data demonstrating that all the genes related to anthocyanin biosynthesis were significantly induced by ABA. Especially the expression of *VmUFGT* and *VmF3′5′H* were highly induced in bilberry fruit by post-harvest ABA treatment. At the branch point in flavonoid biosynthetic route, F3′H leads

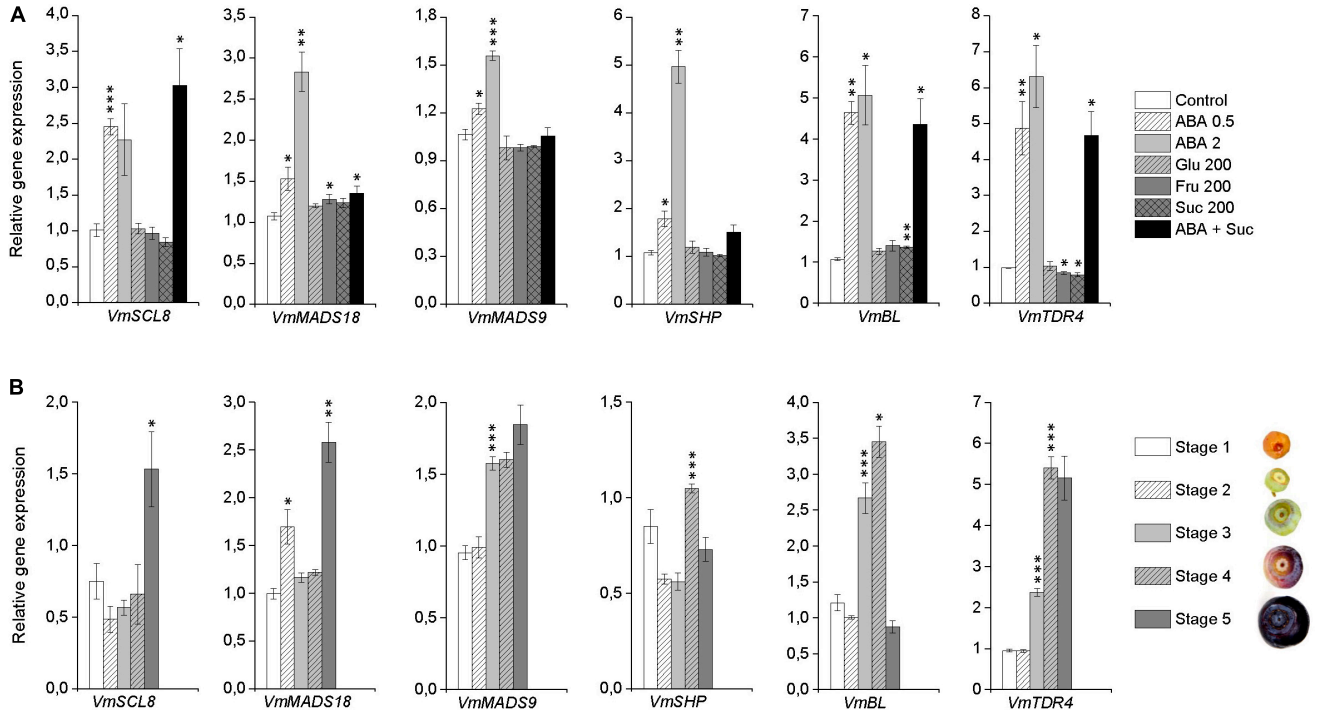

FIGURE 8 | Expression of potential ripening-related transcription factors (TFs) in response to post-harvest ABA and sugar treatments **(A)** and during bilberry fruit development **(B)**. The gene expression was analyzed 4 days after the beginning of the treatments. The treatments were: ABA (0.5 and 2 mM), glucose (200 mM), fructose (200 mM), sucrose (200 mM), 0.5 mM ABA + 200 mM sucrose, or water (control). Relative expression of the genes was quantified by qRT-PCR and normalized to *VmGAPDH*. Values in **(A)** represent means ± SEs of three replicates and asterisks significant differences from control in Student's *t*-Test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). Values in **(B)** represent means ± SEs of four replicates and asterisks significant increase from previous developmental stage in Student's *t*-Test (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). Stages 1–5 indicate the bilberry fruit developmental stages from flower to ripe berry.

to cyanidin-derived anthocyanins while F3′5′H activity leads to delphinidin-derived compounds (**Supplementary Figure S1**). Earlier, exogenous ABA treatment has been shown to modify fruit anthocyanin profile (Koyama et al., 2010; Singh et al., 2014; Ju et al., 2016). Our results may imply that in bilberry ABA promotes especially biosynthesis of delphinidin-derived compounds similarly as reported in highbush blueberries by Oh et al. (2018). Also in grape berries, the treatment with high ABA concentration increased the ratio of delphinidin-derived anthocyanins to cyanidin-derived anthocyanins (Ju et al., 2016). The earlier reported contradictory results concerning the role of ABA as a regulator of anthocyanin accumulation among different *Vaccinium* fruits may reflect the differences among the species or a dose of ABA. Also, timing of ABA application has been reported to be critical in ripening promotion among other fruits (Soto et al., 2013; Wang et al., 2013; Luo et al., 2014).

Our results of silencing of *VmNCED1* gene also evidenced the role of ABA in bilberry anthocyanin biosynthesis. 9-*cis*-epoxycarotenoid dioxygenase (NCED), catalyzing the oxidative cleavage of 9-*cis*-isomers of violaxanthin and neoxanthin to xanthoxin, is considered as the key enzyme responsible for ABA biosynthesis (Leng et al., 2014), also in fruits of genus *Vaccinium* (Zifkin et al., 2012; Karppinen et al., 2013). In the present study, silencing of *VmNCED1* in bilberry fruits by virus-induced gene silencing (VIGS) resulted in chimeric fruits with green sectors at the site of infection. The silencing of *VmNCED1* was accompanied by the down-regulation in the expression of the key anthocyanin biosynthesis genes. Our results are similar to previously reported. Previously, Shen et al. (2014) showed that silencing of *PacNCED1* led to the decrease in anthocyanin biosynthesis and resulted in partly colorless sweet cherries. In strawberries, the down-regulation of *FaNCED1* by VIGS was demonstrated to reduce ABA accumulation, delay fruit ripening and anthocyanin biosynthesis, and to lead partly uncolored fruits (Jia et al., 2011; Medina-Puche et al., 2014; Kadomura-Ishikawa et al., 2015). Furthermore, the suppression of *SlNCED1* expression in tomato has been shown to slow down ripening, elongate fruit shelf life and enhance fruit firmness (Sun et al., 2012; Ji et al., 2014).

In the current study, post-harvest ABA treatment was also found to increase expression of *VmNCED1* indicating that ABA regulates its own biosynthesis in bilberry fruit. Earlier, similar results have been reported and externally applied ABA has been found to elevate *NCED* gene expression and ABA synthesis in grapes (Wheeler et al., 2009), cucumber (Wang et al., 2013), and sweet cherry (Luo et al., 2014). Up-regulation of *FaNCED1* expression by ABA has also been demonstrated in strawberry fruits (Chen et al., 2016; Medina-Puche et al., 2016) and it was suggested that the autocatalytic biosynthesis of ABA may be

necessary for the induction of high increase in ABA production at fruit ripening (Medina-Puche et al., 2016).

Both transcriptomic and proteomic level studies have revealed that ABA regulates many aspects of non-climacteric fruit ripening (Giribaldi et al., 2010; Li et al., 2015; Medina-Puche et al., 2016; Rattanakon et al., 2016). In addition to pigment formation, fruit ripening is associated with other ripening-related processes, including fruit softening. Structural changes in cell wall polysaccharides pectin, hemicellulose and cellulose due to the action of hydrolytic enzymes and expansins lead to a loss of firmness of fruit pulp at late stage of fruit ripening (Goulao and Oliveira, 2008; Payasi et al., 2009). Studies in non-climacteric grapes (Koyama et al., 2010), strawberry (Li et al., 2014), and cherries (Luo et al., 2014) have implicated that the fruit ripening-related softening is regulated by ABA, and in northern highbush blueberry exogenous ABA was shown to decrease fruit firmness (Oh et al., 2018). In climacteric tomato, involvement of ABA in cell wall degradation was proven by silencing of *SlNCED1* leading to higher pectin content, enhancement in fruit firmness and down-regulation in expression of genes encoding cell wall degrading enzymes (Sun et al., 2012).

Bilberry fruit has a short post-harvest shelf life due to relatively rapid softening. However, gene expression associated with ripening-related fruit softening has not been studied previously in bilberry or other *Vaccinium* species. In the present study, post-harvest ABA treatment during bilberry fruit ripening led to the induction in the expression of genes associated with cell wall modifications. Among these, ABA induced genes encoding pectin-modifying enzymes *VmPL*, *VmRGLyase*, *VmβGAL1*, and *VmβGAL2* as well as genes involved in depolymerization of hemicellulose, including *VmXTH*, *VmCEL*, and three expansins (*VmEXP1*, *VmEXP2*, and *VmEXP3*). Earlier, fruit softening related gene expression has been studied extensively in strawberry having also a short shelf life. Indications that ABA activates expression of *FaPL*, *FaCEL*, *FaRGlyase, FcXTH1, FaβGal4, FaXYL1* and expansins have been reported (Bustamante et al., 2009; Molina-Hidalgo et al., 2013; Opazo et al., 2013; Chen et al., 2016; Medina-Puche et al., 2016; Paniagua et al., 2016). However, earlier studies have shown that the textural changes occurring during fruit ripening are characteristic to particular fruit species, and are due to differences in type and extent of cell wall modifications and the expression of the modifying enzymes during ripening (Goulao and Oliveira, 2008; Payasi et al., 2009). Overall, our results indicate that the expression of many genes involved in cell wall disassembly is enhanced during bilberry fruit ripening by ABA while the expression of some of the genes/gene isoforms is not induced by ABA.

Sugars Do Not Induce Bilberry Fruit Ripening Processes

Soluble sugars have been indicated during recent years as fruit ripening regulators in some non-climacteric fruits acting as signaling molecules rather than a carbon source. The role of sugars in fruit ripening has been studied in strawberry and grapes where especially sucrose has been shown to accelerate fruit ripening and anthocyanin biosynthesis. In strawberry, the signaling function of sucrose in fruit ripening was proposed when exogenous sucrose injected at 50 mM dramatically accelerated the fruit ripening while glucose had smaller but also obvious role in ripening and anthocyanin accumulation (Jia et al., 2011, 2013b). Exogenous sucrose at 100 mM was shown to accelerate strawberry fruit ripening in both pre- and post-harvest experiments with injected and immersed berries, respectively (Jia et al., 2013a). Similar results have been reported for some grape varieties (Lecourieux et al., 2014). Recently, Jia et al. (2017) showed that treatments with sugars, especially sucrose, induced anthocyanin biosynthesis and fruit softening in detached Fujiminori grapes. Also, Zheng et al. (2009) demonstrated elevated anthocyanin biosynthesis and accumulation in Cabernet Sauvignon grape berry disks after immersion in solutions containing 50–200 mM glucose, sucrose, or fructose. Spraying with sucrose either alone or in combination with ABA has been shown to increase anthocyanin biosynthesis and accumulation in Crimson Seedless grape berries (Ferrara et al., 2015; Olivares et al., 2017).

The role of sugars in bilberry fruit ripening and anthocyanin biosynthesis has not been studied earlier. Based on the results of the present study, immersion of bilberry fruits into 50 mM or 200 mM glucose, fructose, or sucrose solution does not induce anthocyanin biosynthesis and accumulation but slightly elevates the expression of some genes associated with cell wall modification. Furthermore, sucrose in the ABA + Suc treatment did not enhance the ripening response induced by ABA alone. These results indicate that the role of sugars in the regulation of bilberry fruit ripening differentiates from that reported to strawberry and grapes, and sugars seem to have less important regulatory role in bilberry fruit ripening. However, intensity of the response may also vary between different studies based on the application method of the sugar solutions.

A cross-signaling between ABA and sucrose in anthocyanin biosynthesis has been suggested (Loreti et al., 2008). In strawberry fruit, exogenous sucrose has been shown to stimulate ABA accumulation by promoting dramatically the expression of *FaNCED* (Jia et al., 2011, 2013a,b). Also, silencing of sucrose transporter *FaSUT1* in strawberry led to a decrease in both sucrose and ABA content indicating connection between the two signaling routes (Jia et al., 2013b). In fact, a model for interaction of these two signaling molecules as the core mechanism in regulation of strawberry fruit ripening was recently suggested (Cherian et al., 2014; Jia et al., 2016). Jia et al. (2013b) proposed that sucrose functions as a signal upstream of ABA and induces strawberry fruit ripening both through ABA-dependent and ABA-independent pathways. The effect of ABA on sugar metabolism has also been shown in grape fruits (Çakir et al., 2003; Pan et al., 2005).

However, in the current study, sucrose or other sugar treatments had no inducing effect on *VmNCED1* expression suggesting that sugars do not induce ABA biosynthesis in bilberry during fruit ripening. We also studied the effect of ABA on sucrose biosynthetic gene expression. These results do not support the assumption that ABA might induce sucrose biosynthesis in bilberry fruit. Sucrose phosphate synthase (SPS), catalyzing sucrose synthesis, and sucrose synthase (SS),

catalyzing reversible conversion of sucrose to monosaccharides, are indicated as the key enzymes affecting sucrose accumulation in different fruits (Choudhury et al., 2009; Dai et al., 2011; Feng et al., 2012; Li et al., 2012; Tian et al., 2012; Jia et al., 2016). High rate of sucrose accumulation during strawberry ripening was demonstrated to be accompanied by the high expression level of *FaSPS* genes and low expression of *FaSS* gene (Jia et al., 2016). Furthermore, *FaSS1* was suggested as important regulator of strawberry fruit ripening which expression was significantly inhibited by ABA and sucrose treatments (Zhao et al., 2017). Based on our studies, ABA does not induce expression of the *VmSPS* genes in bilberry fruit but increases expression of *VmSS* that is contrast to the results reported in strawberry (Zhao et al., 2017).

Overall, our results indicate that the role of sugars in bilberry fruit differs from strawberry, the current model of non-climacteric fruit ripening. In bilberry fruit, glucose, fructose, or sucrose seem not to act as major signaling molecules to clearly regulate and induce anthocyanin biosynthesis. Despite that strawberry is not an ovary-derived fruit and, thus, considered as a false fruit deviating from bilberry fruit, the regulatory mechanisms of fruit development and ripening has been considered to be conserved among angiosperms (Daminato et al., 2013; Karlova et al., 2014). In order to further clarify the fruit ripening regulation and signaling in bilberry, the signaling route of ABA-mediated bilberry fruit ripening needs to be studied in more detail in the future.

ABA Up-Regulates Expression of Potential Bilberry Fruit Ripening Regulators

Several types of TFs belonging to different families have earlier been identified as regulators of fruit ripening and anthocyanin biosynthesis, and some of them are regulated by ABA. Fruit ripening regulation has been studied extensively in tomato and in fruits of Rosaceae family. In the current study, we aimed to identify potential TFs of ABA-regulated bilberry fruit ripening processes by searching the closest homologs of functionally characterized TFs of fruit ripening/anthocyanin biosynthesis from publicly available *Vaccinium* transcriptome libraries. Some of the TFs showed highly increased expression in bilberry fruits after ABA treatment. Furthermore, we found elevated TF transcript levels in ripening or ripe fruits indicating a potential role in bilberry fruit ripening-related processes.

One of them was *VmSCL8*, the closest bilberry homolog for *FaSCL8* that is similar to *AtSCL8* in *Arabidopsis*, a member of SCARECROW-LIKE gene family, which members are known to have general roles in plant development. *FaSCL8* expression has been shown to be induced in strawberry receptacle at fruit ripening increasing further in ripe red fruit indicating a role as a regulator of fruit ripening (Pillet et al., 2015). Furthermore, silencing *FaSCL8* in strawberry resulted in lower transcript accumulation of *PAL*, *CHS*, *CHI*, *F3H*, *UFGT,* and *MYB10* but increased *F3′H* and *ANR* transcripts suggesting a role as a general modulator of flavonoid pathway possibly affecting cyanidin-pelargonidin balance by enhancing expression of flavonoid regulating MYB TFs (Pillet et al., 2015). Recently, Medina-Puche et al. (2016) showed that the *FaSCL8* expression in strawberry is elevated by ABA. In the current study, *VmSCL8* also showed elevated expression after ABA treatment. The expression of *VmSCL8* was highest in ripe bilberry fruit similarly to *FaSCL8* indicating a possible role in the ABA-regulated bilberry fruit ripening at the late stages of ripening.

Similarly, elevated expression after ABA treatments in bilberry fruits was observed for three MADS-box genes, *VmMADS18, VmMADS9,* and *VmSHP*, the bilberry homologs for *PyMADS18*, *FaMADS9,* and *FaSHP*, respectively. MADS-box genes represent highly conserved TF family in plants and have been shown to play important roles in floral and fruit development. While the expression of *VmMADS18* was high in this study both in small green and ripe bilberry fruit, the expression of *VmMADS9* increased in large green stage and *VmSHP* in ripening fruit, indicating their differential roles in bilberry fruit. The *PyMADS18* has been suggested to be involved in the regulation of anthocyanin biosynthesis in pear (Wu et al., 2013). During the fruit ripening, expression of *PyMADS18* was shown to increase at early stages of development, after that decreasing until it was up-regulated again at the end of fruit maturation period (Wu et al., 2013) resembling the expression pattern obtained in this study for *VmMADS18*.

SEPALLATA (*SEP*)-like MADS-box TFs have been indicated to play central roles in ripening of both climacteric and non-climacteric fruits, best known example being *LeMADS-RIN* (Seymour et al., 2011). *FaMADS9*, a fruit-related *SEP1/2-like* gene was indicated as a positive regulator of both development and ripening of strawberry fruit with its expression up-regulated at white stage of strawberry fruit development (Seymour et al., 2011). Silencing of the gene led to inhibition of normal development of strawberry fruit (Seymour et al., 2011). The gene was shown to be ABA-inducible later by Daminato et al. (2013). Also *FaSHP,* a C-type MADS-box gene belonging to a SHATTERPROOF group was indicated as a positive regulator of strawberry fruit ripening with its expression induced by ABA (Daminato et al., 2013; Medina-Puche et al., 2016). The expression of the *FaSHP* increases in strawberry fruit after large green stage due to ABA control being highest at pink stage (Daminato et al., 2013) similarly to *VmSHP* shown in our study. Overall, our results indicate that *VmMADS18, VmMADS9,* and *VmSHP* have potential roles in the ABA-regulated bilberry fruit development and ripening.

The fourth studied MADS-box gene in the present study was *VmTDR4* belonging to a SQUAMOSA group. *VmTDR4* was earlier demonstrated to have a role in the anthocyanin accumulation during bilberry fruit ripening with its expression especially associated in flesh of ripening fruit (Jaakola et al., 2010). Silencing of *VmTDR4* in bilberry fruit resulted in chimeric berries with decreased expression of *CHS*, *DFR,* and *ANS* but elevated expression of *ANR* indicating modulation of flavonoid pathway through flavonoid regulating MYB TFs (Jaakola et al., 2010). Also tomato homolog for *TDR4* induced anthocyanin biosynthesis when expressed in *Arabidopsis* siliques (Jaakola et al., 2010). The response of *VmTDR4* to ABA has not been studied earlier but

the present study demonstrates that *VmTDR4* is highly induced by ABA indicating its role in the ABA-regulated fruit ripening associated anthocyanin accumulation.

Also NAC family TFs have been proposed as activators of fruit ripening with some of their expression shown to be increased by ABA (Zhu et al., 2014; Jiang et al., 2017; Moyano et al., 2018). Recently, a *PpBL* gene (BLOOD) was indicated as a key regulator of anthocyanin biosynthesis in maturing blood-fleshed peach fruit (Zhou et al., 2015). It was shown to act as a heterodimer with another NAC family member, *PpNAC1* (Zhou et al., 2015). In our study, the closest bilberry homolog for the peach *BL* gene, *VmBL*, showed a significant induction in its expression by ABA treatments as well as increased expression at the onset of bilberry fruit ripening slightly before *VmTDR4*. This suggests that *VmBL* could have a role in the regulation of bilberry fruit ripening and/or anthocyanin biosynthesis through ABA-mediated signaling.

CONCLUSION

This is the first report regarding the role of ABA and sugars on the regulation of bilberry fruit ripening. By using both pre- and post-harvest experiments and a molecular approach, we showed that ABA is an important positive regulator of bilberry fruit ripening processes, inducing anthocyanin biosynthesis and fruit softening. However, based on our results, sugars (glucose, fructose, and sucrose) have minor roles in the regulation of bilberry fruit ripening as sugars failed to induce anthocyanin or ABA biosynthesis in bilberry fruit but could elevate expression of some genes associated with cell wall modification. Moreover, sucrose did not enhance the effect of ABA in ripening responses. Our results suggest that the ripening regulation may be different in bilberry fruit compared to the current model of non-climacteric fruit ripening, strawberry, in which the coordinated regulation by the two signaling molecules, ABA and sucrose, have been proposed to have a key role in fruit ripening.

AUTHOR CONTRIBUTIONS

KK and LJ designed the experiments. KK was responsible for conducting the experiments and analyses. PT and KK conducted pre-harvest ABA treatments and subsequent gene expression analyses. KK was responsible for the writing of the manuscript with contribution of LJ and HH. 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.2018.01259/full#supplementary-material

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Light, Ethylene and Auxin Signaling Interaction Regulates Carotenoid Biosynthesis During Tomato Fruit Ripening

*Aline Bertinatto Cruz[1], Ricardo Ernesto Bianchetti[1], Frederico Rocha Rodrigues Alves[1], Eduardo Purgatto[2], Lazaro Eustaquio Pereira Peres[3], Magdalena Rossi[1] and Luciano Freschi[1]**

[1] Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil, [2] Departamento de Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, Brazil, [3] Departamento de Ciências Biológicas, Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Piracicaba, Brazil

Light signaling and plant hormones, particularly ethylene and auxins, have been identified as important regulators of carotenoid biosynthesis during tomato fruit ripening. However, whether and how the light and hormonal signaling cascades crosstalk to control this metabolic route remain poorly elucidated. Here, the potential involvement of ethylene and auxins in the light-mediated regulation of tomato fruit carotenogenesis was investigated by comparing the impacts of light treatments and the light-hyperresponsive *high pigment-2* (*hp2*) mutation on both carotenoid synthesis and hormonal signaling. Under either light or dark conditions, the overaccumulation of carotenoids in *hp2* ripening fruits was associated with disturbed ethylene production, increased expression of genes encoding master regulators of ripening and higher ethylene sensitivity and signaling output. The increased ethylene sensitivity observed in *hp2* fruits was associated with the differential expression of genes encoding ethylene receptors and downstream signaling transduction elements, including the downregulation of the transcription factor *ETHYLENE RESPONSE FACTOR.E4*, a repressor of carotenoid synthesis. Accordingly, treatments with exogenous ethylene promoted carotenoid biosynthetic genes more intensively in *hp2* than in wild-type fruits. Moreover, the loss of *HP2* function drastically altered auxin signaling in tomato fruits, resulting in higher activation of the auxin-responsive promoter *DR5*, severe down-regulation of *AUXIN/INDOLE-3-ACETIC ACID* (*Aux/IAA*) genes and altered accumulation of *AUXIN RESPONSE FACTOR* (*ARF*) transcripts. Both tomato *ARF2* paralogues (*Sl-ARF2a* and *SlARF2b*) were up-regulated in *hp2* fruits, which agrees with the promotive roles played by these ARFs in tomato fruit ripening and carotenoid biosynthesis. Among the genes differentially expressed in *hp2* fruits, the additive effect of light treatment and loss of *HP2* function was particularly evident for those encoding carotenoid biosynthetic enzymes, ethylene-related transcription factors, Aux/IAAs and ARFs. Altogether, the data uncover the involvement of ethylene and auxin as part of the light signaling cascades controlling tomato fruit metabolism and provide a new link between light signaling, plant hormone sensitivity and carotenoid metabolism in ripening fruits.

Keywords: fruit ripening, auxin, ethylene, photomorhogenesis, tomato, climacteric, high pigment mutant, light–dark

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****Correspondence:***
Luciano Freschi
freschi@usp.br

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INTRODUCTION

Light plays a dual role during plant development, providing energy for photosynthesis and information for adjusting plant growth, development and reproduction. Processes as diverse as seed germination, seedling de-etiolation, phototropism, flowering, fruit pigmentation and entrainment of circadian rhythms are intrinsically regulated by light stimuli (Azari et al., 2010a; Llorente et al., 2016a). In tomato, a model crop for fleshy fruits, multiple photomorphogenic mutants have been identified over the years, greatly facilitating the deciphering of the relevance of light signaling in fruit biology and quality traits (Levin et al., 2006; Azari et al., 2010b). Among these genotypes, the tomato *high pigment* (*hp*) mutants *hp1* and *hp2* have been instrumental in illustrating the positive role of light signaling in fruit metabolism and nutritional composition. These mutants are characterized by their exaggerated light responsiveness, over-accumulation of chlorophyll and chloroplasts in leaves and immature fruits as well as intense red fruit pigmentation (Mustilli et al., 1999; Levin et al., 2003, 2006). Compared to their WT counterparts, *hp* ripe fruits display higher levels of health-promoting substances, including carotenoids, flavonoids, tocopherol (vitamin E) and ascorbic acid (vitamin C) (Yen et al., 1997; Liu et al., 2004; Kolotilin et al., 2007). Fruit carotenogenesis is particularly up-regulated in *hp* mutants, which agrees with the positive influence of light on isoprenoid metabolism in both fruit and vegetative tissues (Piringer and Heinze, 1954; Alba et al., 2000; Schofield and Paliyath, 2005).

Genetic analysis of *hp1* and *hp2* alleles revealed mutations in tomato homologs of the nuclear proteins UV-DAMAGED DNA BINDING PROTEIN1 (DDB1) and DEETIOLATED1 (DET1), respectively, two negative regulators of light signal transduction (Mustilli et al., 1999; Schroeder et al., 2002; Levin et al., 2003; Lieberman et al., 2004; Liu et al., 2004). Confirming these findings, silencing of *Sl-DDB1/HP1* or *Sl-DET1/HP2* greatly promotes plastid biogenesis and carotenoid accumulation in fruit tissues (Davuluri et al., 2004, 2005; Wang et al., 2008). Besides *Sl-DDB1/HP1* and *Sl-DET1/HP2,* other components of the light signaling cascade have also been implicated in controlling tomato fruit metabolism, including the E3 ubiquitin-ligases CULLIN4 (CUL4) and CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1), as well as the transcription factors ELONGATED HYPOCOTYL 5 (HY5) and PHYTOCHROME-INTERACTING FACTORs (PIFs) (Liu et al., 2004; Davuluri et al., 2005; Wang et al., 2008; Llorente et al., 2016b). Constitutive silencing of tomato *CUL4*, *COP1* or *PIF1a* generates fruits with increased carotenoid levels (Liu et al., 2004; Wang et al., 2008; Llorente et al., 2016b), whereas the opposite phenotype is caused by the suppression of the light-signaling effector *HY5* (Liu et al., 2014). Significant alterations in carotenoid biosynthesis have also been observed in ripening fruits of transgenic plants with fruit-specific silencing of phytochrome (PHY)-encoding genes (Bianchetti et al., 2018), as well as in cryptochrome1a (CRY1a)-deficient mutants and *CRY1a*-overexpressing lines (Liu et al., 2018).

Virtually all fruit metabolic processes influenced by light are also strictly controlled by an integrated, multi-hormonal signaling network (Giovannoni, 2004; Karlova et al., 2014; Liu M. et al., 2015). Compelling data implicate ethylene as a primary regulator of multiple ripening-related physiological, biochemical, and molecular processes (Barry and Giovannoni, 2007; Pech et al., 2012). Accordingly, disturbed ethylene biosynthesis, perception or signal transduction directly impact fruit ripening initiation and progression (Liu M. et al., 2015). Without undermining the role of ethylene, auxins have also been shown to interfere with fruit ripening and carotenoid accumulation, as revealed by the delayed ripening phenotype and the down-regulation in carotenoid biosynthesis observed in IAA-treated tomato fruits (Su et al., 2015).

Although light signaling and plant hormones, such as ethylene and auxins, are essential regulators of tomato fruit ripening and carotenogenesis, whether and how the light and hormonal signaling cascades crosstalk to control these metabolic processes remains poorly elucidated. Here, the potential involvement of ethylene and auxins in the light-mediated regulation of tomato fruit ripening and carotenogenesis was investigated by comparing the impact of light and dark treatments, isolated or combined with the loss of *Sl-DET1/HP2* function, on both carotenoid synthesis and hormonal signaling.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Wild-type (WT) *Solanum lycopersicum* L. (cv. Micro-Tom), a near-isogenic line (NIL) harboring the mutation *high pigment-2* (*hp2*), and transgenic plants carrying the synthetic auxin-responsive (*DR5*) or ethylene-responsive (*EBS*) promoters fused to the reporter gene *uid* (encoding a β-glucuronidase, GUS) were obtained from the tomato mutant collection maintained at ESALQ, University of São Paulo (USP), Brazil (Carvalho et al., 2011). Crosses and successive screening were performed to generate the double mutants *hp2-DR5::GUS* and *hp2-EBS::GUS*. Plants were grown in 6-L rectangular pots containing a 1:1 mixture of commercial substrate (Plantmax HT, Eucatex, São Paulo, Brazil) and expanded vermiculite, supplemented with 1 g L^{-1} of NPK 10:10:10, 4 g L^{-1} of dolomite limestone ($MgCO_3$ + $CaCO_3$) and 2 g L^{-1} thermophosphate (Yoorin Master®, Yoorin Fertilizantes, Brazil) in greenhouse under automatic irrigation at an average mean temperature of 25°C, 11.5 h/13 h (winter/summer) photoperiod and approximately 250–350 μmol m^{-2} s^{-1} PAR irradiance.

Light Treatments

Fruits at mature green (MG) stage were harvested about 30 days after anthesis (dpa) and transferred to continuous white light or maintained under absolute darkness (D) until reaching distinct ripening stages. White light was delivered at around 50 μmol m^{-2} s^{-1} and supplied by an array of SMD5050 Samsung LEDs mounted in a temperature-controlled growth chamber maintained at 25 ± 1°C and air relative humidity at 80 ± 5%. Top and bottom illumination were applied to homogenize the light environment surrounding the fruits. Fruits were placed into a 0.5-L sealed transparent vessel and continuously flushed with

ethylene-free, humidified air (1 L min^{-1}) to avoid accumulation of ET inside the containers. Samples from light- or dark-incubated fruits were harvested under white light or dim green light, respectively. Harvesting was performed at the same daytime to avoid possible fluctuations in the parameters due to circadian rhythm. Pericarp samples (without seeds, columella, placental tissues and locule walls) were harvested as soon as the fruits had reached the following ripening stages: MG (displaying jelly placental tissues, 2 days after harvesting), Bk (breaker, showing the first external yellow color signals) and Bk1, Bk3, Bk6, and Bk12, corresponding 1, 3, 6, and 12 days after Bk, respectively. Fruits at distinct treatments achieved each ripening stage at a different number of days of treatment. Four biological samples composed of at least five fruits each were harvested at each sampling time. Ethylene emission analysis and quantitative *in vitro* GUS activity assays were performed immediately after harvesting. For all other analyses, samples were frozen in liquid N_2, powdered and stored at −80°C until use.

Hormonal Treatments

Fruits harvested at the MG stage were submitted to ethylene or auxin treatment at 25°C in the presence of white light (50 μmol m^{-2} s^{-1}). For the ethylene treatment, fruits were kept inside transparent sealed tubes in the presence of 50 ppm of ethylene, whereas control fruits were maintained in ethylene-free air. For the auxin treatment, fruits were injected with a buffer solution containing 10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) pH 5.6, 3% sorbitol (w/v) and 100 μM of indole-3-acetic acid (IAA) whereas control fruits were treated with buffer without IAA (Su et al., 2015). After 6 h treatment, fruit pericarp samples were collected before snap freezing in nitrogen.

Chlorophyll Quantification and Carotenoid Profile

Chlorophyll extraction and quantification were carried out as described in Bianchetti et al. (2018). Carotenoids (namely lycopene, β-carotene and lutein) were extracted and analyzed by high-pressure liquid chromatography (HPLC) with photodiode array detector (PDA). Carotenoid extraction was performed precisely as described by Bianchetti et al. (2018). Chromatography was carried out on an Agilent Technologies series 1100 HPLC system on a normal-phase column Phenomenex (Luna C18; 250 × 4.6 mm; 5 μm particle diameter) with a flow rate of 1 mL min^{-1} at 25°C. The mobile phase was a gradient of ethyl acetate (A) and acetonitrile:water 9:1 (v/v) (B): 0–4 min: 20% A; 4–30 min: 20–65% A; 30–35 min: 65% A; 35–40 min: 65–20% A. Eluted compounds were detected between 340 and 700 nm and quantified at 450 nm. The endogenous metabolite concentration was obtained by comparing the peak areas of the chromatograms with commercial standards.

Fruit Surface Color Measurement

Fruit surface color was assessed with a using Konica Minolta CR-400 colorimeter, using the D65 illuminant and the L*, a*, b* space, and the data were processed to obtain hue and chroma values. Three measures were taken at the equator of each fruit and average values were calculated. The hue angle (in degrees) was calculated according to the following equations: hue = tan−1 (b*/a*) if a > 0 and 180 + tan−1 (b*/a*) if a < 0 (Ecarnot et al., 2013).

Antioxidant Activity

Antioxidant activity was measured using the method of Trolox equivalent antioxidant capacity (TEAC). Frozen pericarp samples (approximately 200 mg FW) ground in liquid nitrogen were homogenized with 1 mL of 100 mM sodium acetate buffer (pH 5) and shaken for 30 min at 4°C. After centrifugation (4°C, 5000 *g*, 10 min), the supernatant was discarded, 0.5 mL of hexane was added to the pellet, and the suspension was kept shaking for 30 min at 4°C. After centrifugation (4°C, 5000 *g*, 10 min), the supernatant was collected, and the same process was repeated twice. The lipophilic antioxidant extract was concentrated and suspended in 150 μL of hexane. Absorbance was read at 734 nm after 2 h of incubation under darkness. The activity of the extract was determined by the deactivation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^+$) compared to a standard curve of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox).

Auxin Measurements

Endogenous levels of indole acetic acid (IAA) were determined by gas chromatography-tandem mass spectrometry-selecting ion monitoring (GC-MS-SIM) as described by Santana-Vieira et al. (2016). Frozen pericarp samples (approximately 100 mg FW) were ground in liquid nitrogen and homogenized with 1 mL of isopropanol:acetic acid (95:5, v/v). Precisely 0.5 μg [$^{13}C_6$]-IAA (Cambridge Isotopes, Inc.) was added to each sample as internal standards. Samples were incubated at 4°C for approximately 2 h. After centrifugation (4°C, 16.000 *g*, 20 min), the supernatant was collected, and 100 μL of ultrapure water and 500 μl of ethyl acetate were added. After centrifugation (4°C, 16.000 *g*, 5 min) the supernatant was collected, and this step was repeated. The extract was completely vacuum dried and suspended in 50 μL methanol followed by a 30-min derivatization step at room temperature using 40 μL (trimethylsilyl)diazomethane.

The analysis was performed with a gas chromatograph coupled to a mass spectrometer (model GCMS-QP2010 SE, Shimadzu) in selective ion monitoring mode. One microliter of each sample was automatically injected (model AOC-20i, Shimadzu) on splitless mode, using helium as the carrier gas at a flow rate of 4.5 mL min^{-1} through a fused-silica capillary column (30 m, 0.25 mm ID, 0.50-μm-thick internal film) DB-5 MS stationary phase in the following program: 2 min at 100°C, followed by gradients of 10°C min^{-1} to 140°C, 25°C min^{-1} to 160°C, 35°C min^{-1} to 250°C, 20°C min^{-1} to 270°C and 30°C min^{-1} to 300°C. The injector temperature was 250°C, and the following MS operating parameters were used: ionization voltage, 70 eV (electron impact ionization); ion source temperature, 230°C; and interface temperature, 260°C. Ions with a mass ratio/charge (m/z) of 130 and 189 (corresponding to endogenous IAA) and 136 and 195 (corresponding to [$_{13}C^6$]-IAA) were

monitored. Endogenous concentrations were calculated based on extracted chromatograms at m/z 130 and 136.

Ethylene Emission

Ethylene emission was analyzed by gas chromatography with a flame-ionization detector (GC-FID) as described in Melo et al. (2016). Intact tomato fruits (typically 4 individuals) were enclosed in a sealed transparent tube for 1 h under specific experimental conditions. After incubation, 9-mL gas samples were collected from tubes and injected into a glass vial headspace previously flushed with ethylene-free air (1 L min^{-1}) for 1 min. At least three 1-mL aliquots of each sample were injected in a headspace coupled to a Trace GC Ultra gas chromatography (Thermo Electron) fitted with a flame ionization detector (GC-FID) using an RT-alumina Plot column (Restek Corporation). Nitrogen was used as the carrier gas at a flow rate of 3 mL min^{-1}, and commercial standard mixtures of ethylene were used for the calibration curves. Column, injector and detector temperatures were 34, 250, and 250°C, respectively.

1-Aminocyclopropane-1-Carboxylic Acid (ACC) Measurement

ACC was extracted and subsequently quantified as described by Bulens et al. (2011). Frozen pericarp samples (approximately 1 g FW) were ground in liquid nitrogen and homogenized with 4 mL of a 5% (w/v) sulfosalicylic acid aqueous solution. Extracts were shaken for 30 min at 4°C at 180 rpm in the dark. The supernatant was collected after centrifugation at 4°C, 5000 *g*, for 10 min. The reactions were performed by adding 1.4 mL of extract to a reaction mixture composed of 0.4 mL of 10 mM $HgCl_2$ and 0.2 mL of a 2:1 (v/v) solution of NaOCl 5%:NaOH 6 M. The final product of this reaction, ethylene, was measured by GC-FID as described above.

ACC Oxidase (ACO) Activity

ACO extraction and activity assay were performed according to Bulens et al. (2011). Frozen pericarp samples (approximately 100 mg FW) were ground in liquid nitrogen and homogenized with extraction buffer composed of 300 mM Tris-HCl (pH 8.0), 15 mg mL^{-1} insoluble polyvinylpolypyrrolidone (PVPP), 10% (v/v) glycerol and 30 mM ascorbic acid. After centrifugation (4°C, 20000 *g*, 20 min), 200 μL of the supernatant was added to 1.8 mL of reaction medium composed of 100 mM Tris-HCl (pH 8.0), 10% (v/v) glycerol, 30 mM ascorbic acid, 100 μM $FeSO_4$, 50 mM $NaHCO_3$, 5 mM DTT and 2 mM ACC. ACO activity was estimated by measuring the ability of the extract to convert exogenous ACC to ethylene after incubation at 30°C for 60 min. The ethylene formed during the reactions was measured by GC-FID as described above.

Quantitative GUS Activity Assay

GUS activity was assayed according to Melo et al. (2016). Frozen pericarp samples (approximately 500 mg FW) were ground in liquid nitrogen and homogenized in 1 mL extraction buffer composed of 50 mM Hepes-KOH (pH 7.0), 5 mM DTT and 0.5% (w/v) PVP. After centrifugation (4°C, 20.000 *g*, 20 min), 200 μL aliquots of the supernatant were mixed with 200 μL of an assay buffer composed of 50 mM HEPES-KOH (pH 7.0), 5 mM DTT, 10 mM EDTA and 2 mM 4-methylumbelliferyl-β-D-glucuronide (MUG) and incubated at 37°C for 30 min. Subsequently, aliquots of 100 μL were taken from each tube and the reactions were stopped with 2.9 mL of 0.2 M Na_2CO_3 (pH 9.5). Fluorescence was measured using 365 nm excitation and 460 nm emission wavelength (5 nm bandwidth) by using a spectrofluorometer (LS55, Perkin Elmer). The same instrument settings were maintained throughout the experiments.

Gene Promoter Analyses

Promoter sequences were retrieved from Sol Genomics Network[1] and analyzed using PlantPAN 2.0 platform[2] (Chow et al., 2016) to identify the regulatory motifs. Fragments of 3 kb upstream from the initial codon ATG were analyzed for the presence of PBE-box (CACATG), G-box (CACGTG), CA-hybrid (GACGTA) and CG-hybrid (GACGTG) motifs, which are recognized by HY5 and/or PIFs (Martínez-García et al., 2000; Song et al., 2008).

RNA Isolation and Quantitative RT-PCR Analyses

Total RNA extraction was performed using ReliaPrep™ RNA Tissue Miniprep System (Promega) according to manufacturer's instructions for fibrous tissues. Total RNA and integrity of samples were determined using spectrophotometer and 1% (w/v) agarose gel. Only RNA samples with 260/280 and 260/230 ratio values within 1.8–2.2 were used for the subsequent steps. Approximately 1 μg of total RNA was treated with DNase (DNase I Amplification Grade, Thermo Fisher Scientific) for 30 min at room temperature and complementary DNA (cDNA) was synthesized using SuperScript® IV Reverse Transcriptase kit (Thermo Fisher Scientific) according to manufacturer's instructions. Only cDNA samples free of DNA contamination were used in the subsequent steps.

Quantitative reverse-transcriptase PCR (qPCR) reactions were performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems) using 10 μl mix reaction composed of 5 μL Power SYBR green 2X (Thermo Fisher Scientific), 2 μL cDNA sample and 200 nM of forward and 200 nM of reverse primer. The amplification program consisted of 10 min initial step at 95°C, followed by 40 cycles with 15 s 95°C, 30 s 55/60°C and 30 s 72°C. Melting curve was analyzed to detect unspecific amplifications and primer dimerization. The primer sequences used in this study are listed in **Supplementary Table 1**. Fluorescence data were analyzed using LingReg PCR software, and expression values were normalized against mean values of two references genes: *Sl-EXPRESSED* and *Sl-CAC*, which have been already successfully used to normalize data from fruit development and ripening experiments (Expósito-Rodríguez et al., 2008; Bianchetti et al., 2018).

[1] https://solgenomics.net/

[2] http://plantpan2.itps.ncku.edu.tw/

Statistical Analysis

Two-way analysis of variance (ANOVA) was performed to determine effects of genotype, light treatment and their interactions, using JMP statistical software package (14th edition)[3]. One-way ANOVA with Tukey's test or Student's *t*-test was used to discriminate means of samples within and between genotypes, respectively. Comparisons with $P < 0.05$ were considered statistically significant. Carotenoid-related data were also compared via principal component analysis (PCA) using JMP statistical software package.

RESULTS

Light Treatment and Loss of *Sl-DET1/HP2* Function Promote Fruit Carotenoid Biosynthesis

The impacts of *Sl-DET1/HP2* knockout or knockdown on tomato fruit carotenogenesis have been exclusively investigated in fruits ripening on-the-vine under greenhouse conditions (Davuluri et al., 2004; Kolotilin et al., 2007; Azari et al., 2010a; Enfissi et al., 2010; Sestari et al., 2014). However, after reaching the MG stage, tomato fruits are also able to ripen off-the-vine (i.e., isolated from the plant), a frequent commercial practice in harvesting tomato fruit for human consumption (Sorrequieta et al., 2013).

Here, we demonstrated that the loss of *Sl-DET1/HP2* function promotes carotenogenesis even when tomato ripening occurs separated from the plant under either light or absolute dark conditions (**Figure 1** and **Supplementary Figure 1**). Two-way ANOVA showed that both the *hp2* mutation and the light treatment had a significant ($P < 0.05$) effect on carotenoid biosynthesis and accumulation (**Supplementary Table 2**). In both light- and dark-treated fruits, lutein and β-carotene levels were significantly higher in *hp2* than in the WT at virtually all sampling stages (**Figure 1B**). Moreover, lycopene levels of dark-treated *hp2* fruits were higher than the WT at the final stages of ripening (i.e., Bk6 and Bk12). In agreement, the genes encoding key carotenoid biosynthesis-related enzymes such as geranylgeranyl diphosphate synthase (GGPS), phytoene synthase 1 (PSY1) and phytoene desaturase (PDS) were strongly up-regulated during the climacteric phase (i.e., Bk to Bk6) in both light- and dark-treated *hp2* fruits compared with WT counterparts (**Figure 1C** and **Supplementary Figure 1**). Overall, *Sl-GGPS*, *Sl-PSY1* and *Sl-PDS* transcripts were less abundant in fruits maintained under dark than under light conditions, and this dark-induced reduction in mRNA levels was less marked in the *hp2* mutant compared to the WT (**Figure 1C**). Genes encoding the chloroplast- and chromoplast-specific β-lycopene cyclases (LYCβ and CYCβ, respectively) were also up-regulated in *hp2* fruits compared to the WT, particularly when ripening occurred under light conditions. Among the carotenoid biosynthesis-related genes differently expressed in *hp2* fruits, the additive effect of light treatment and loss of *Sl-DET1/HP2* function was particularly observed at the Bk, Bk1 and Bk12 stages (**Supplementary Figure 1**). Interestingly, lycopene levels were slightly higher in *hp2* fruits ripened in the dark than in light-treated ones (**Figure 1B**), which is very likely due to the accumulation of this carotenoid because the opposite pattern was observed for the transcript levels of *Sl-PSY1* and *Sl-PDS*, *i.e.*, higher mRNA levels in the light than in the dark conditions (**Figure 1C**).

In line with the increment in carotenoid content observed in *hp2* fruits, lipophilic extracts obtained from either dark- or light-incubated fruits of the mutant exhibited higher values of antioxidant capacity than the WT counterparts, a response intensified under light conditions (**Figure 1D**). The influence of the *hp2* mutation on lycopene, β-carotene and antioxidant capacity was moderated by the light treatment, as indicated by a significant genotype x light treatment interaction ($P < 0.0001$, **Supplementary Table 2**). Moreover, when PCA was performed with carotenoid data, the model explained 62.2% of the data variance for these conditions, displaying *hp2* samples separated from WT independently of the developmental stage or light condition, and a strong positive correlation between the changes in mRNA levels of genes encoding carotenoid biosynthetic genes with the fruit carotenoid composition and antioxidant capacity was confirmed (**Supplementary Figure 2**).

At MG, *hp2* fruits displayed a distinctive dark-green coloration, increased chlorophyll levels and higher color saturation (chroma, which is indicative of color intensity) compared to the WT (**Supplementary Figure 3**). In line with the higher content of pigments in *hp2* than in WT fruits, an overall trend of higher values of fruit color intensity was observed in the mutant fruits during ripening (Bk to Bk12) regardless of the light conditions (**Supplementary Figure 3**).

As dark-incubated *hp2* fruits showed carotenoid levels and lipophilic antioxidant capacity higher than dark- or even light-treated WT fruits, this mutation seems to represent a valid strategy to promote fruit nutritional quality even when the light stimulus is not present during fruit ripening.

Light-Hypersensitivity Influences Tomato Fruit Ripening

To investigate whether the loss of *Sl-DET1/HP2* function impacts tomato fruit ripening initiation and progression, we first monitored the ripening-associated changes in fruit color in both the *hp2* and WT genotypes (**Figure 2A**). Hue angle values revealed that light-incubated fruits acquired the distinctive red coloration faster and more intensively than those kept under complete darkness. Moreover, the ripening-associated fruit color transition occurred slightly faster in *hp2* than in WT fruits, particularly under dark conditions (**Figure 2A**).

In line with these results, mRNA levels of genes encoding the master regulators of ripening RIPENING INHIBITOR (RIN), NON-RIPENING (NOR), FRUITFULL1 (FUL1), APETALA2a (AP2a) and TOMATO AGAMOUS-LIKE1 (TAGL1) were significantly higher in *hp2* than in WT fruits ripening either under light or dark conditions (**Figure 2B**). Overall, the impact of the loss of *Sl-DET1/HP2* function on the transcript abundance of these ripening-associated genes was influenced by the light treatment, as indicated by a significant genotype x

[3]http://jmp.com

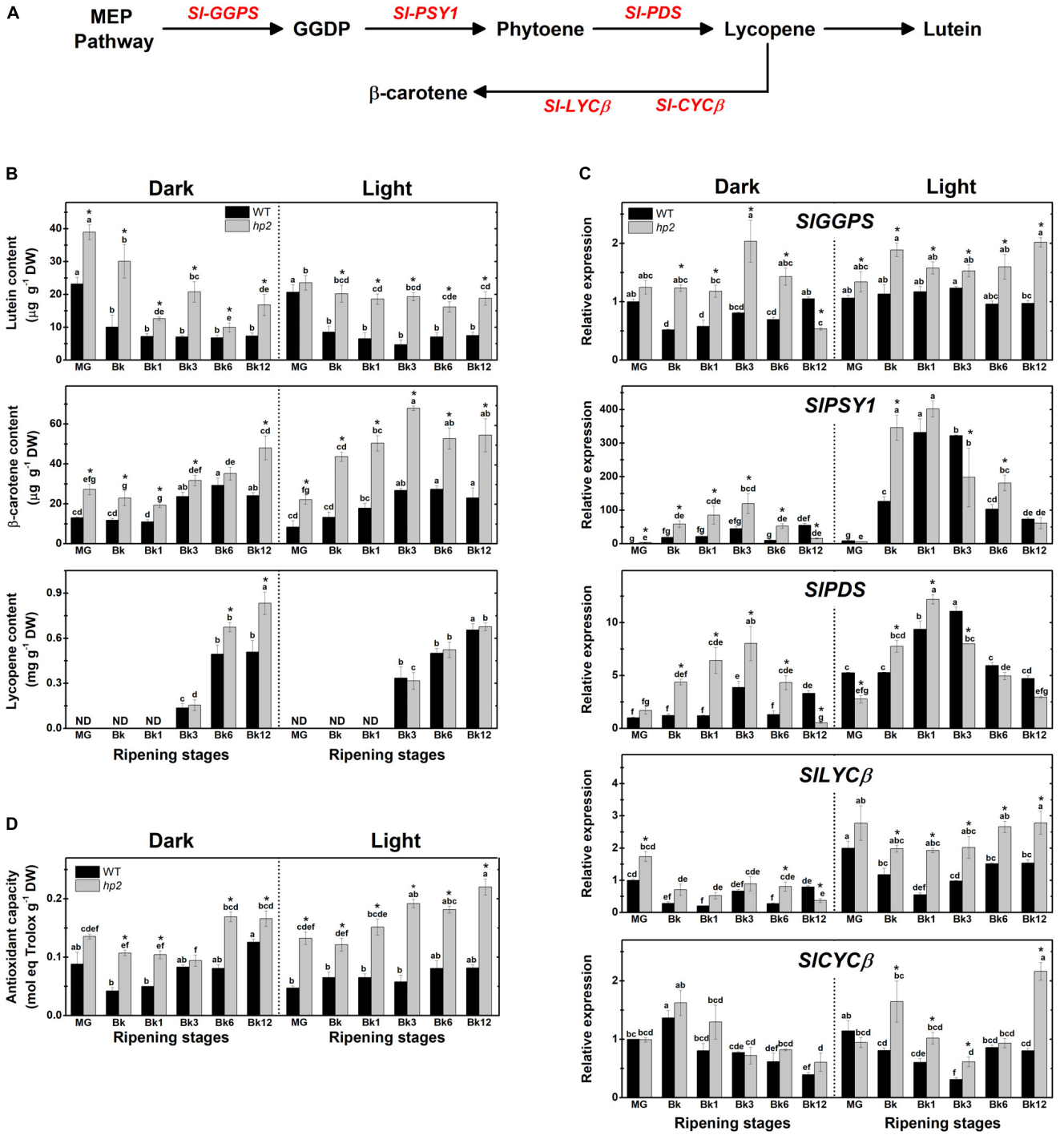

FIGURE 1 | Loss of *Sl-DET1/HP2* function promotes tomato fruit carotenoid biosynthesis and antioxidant capacity in both dark- and light-ripened fruits. Wild-type (WT) and *high pigment-2* (*hp2*) fruits harvested at mature green (MG) stage were left to ripen under constant light (L) or dark (D) conditions. Pericarp samples were harvested at MG (2 days after the beginning of treatment), breaker (Bk), Bk1 (1 day after Bk), Bk3, Bk6, and Bk12 stages. **(A)** Schematic representation of carotenoid biosynthetic pathway in tomato. Intermediate reactions are omitted. **(B)** Lutein, β-carotene and lycopene content in pericarp tissues. **(C)** Relative mRNA levels of carotenoid biosynthesis genes. Mean relative expression was normalized against wild-type (WT) samples at mature green (MG) stage under dark conditions. **(D)** Trolox equivalent antioxidant capacity (TEAC) content in lipophilic extracts. Data are means (±SE) of at least three biological replicates. Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$) within each genotype. Asterisks indicate statistically significant differences (Student's *t*-test, $p < 0.05$) between genotypes. MEP, Methylerythritol 4-phosphate; GGDP, Geranylgeranyl diphosphate; GGPS, GGDP synthase; PSY, Phytoene synthase; PDS, Phytoene desaturase; LCYβ, Chloroplast-specific β-lycopene cyclase; CYCβ, Chromoplast-specific β-lycopene cyclase.

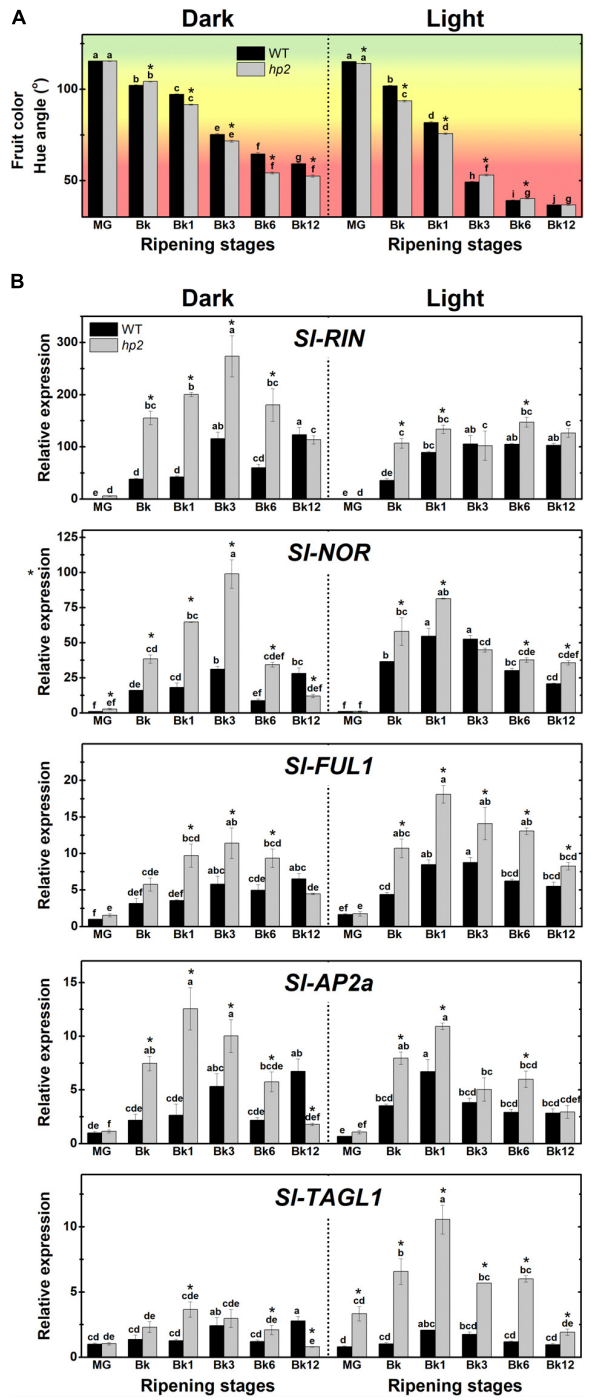

FIGURE 2 | Light signaling influences tomato fruit ripening. Treatment details as described in **Figure 1**. **(A)** Ripening-related changes in fruit color (Hue angle). **(B)** Transcript abundance of ripening regulator genes in dark- and light ripened fruits. Mean relative expression was normalized against wild-type (WT) samples at mature green (MG) stage under dark conditions. Data are means (±SE) of at least three biological replicates. Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$) within each genotype. Asterisks indicate statistically significant differences (Student's *t*-test, $p < 0.05$) between genotypes. *hp2*, *high pigment-2*; Bk, Breaker; RIN, ripening inhibitor; NOR, non-ripening; FUL1, fruitfull1; AP2a, apetala2a; TAGL1, tomato agamous-like1.

light treatment interaction ($P < 0.05$, **Supplementary Table 2**). Therefore, a positive correlation was observed between the up-regulation of the master regulators of ripening and the carotenoid overaccumulation observed in *hp2* ripening fruits. The promotive impact of the loss of *Sl-DET1/HP2* function on the expression of master regulators of ripening may also be linked to the slightly faster fruit color transition observed in the mutant compared to the WT under dark conditions (**Figure 2A**). Accordingly, HY5- and/or PIF-binding motifs were identified in the promoter regions of all five master regulators of ripening genes analyzed (**Supplementary Figure 4**).

Loss of *Sl-DET1/HP2* Function Alters Ethylene Biosynthesis, Signaling and Responsiveness During Tomato Ripening

To gain insight into the potential influence of light treatment and the loss of *Sl-DET1/HP2* function on fruit ethylene metabolism, we next monitored ethylene emission, 1-aminocyclopropane-1-carboxylic acid (ACC) content, ACC oxidase (ACO) activity and transcript abundance of ethylene biosynthetic genes in WT and *hp2* ripening fruits. In both genotypes and light conditions, the highest values of ethylene emission were detected from Bk to Bk3 (**Figure 3A**). Also, ACC accumulated at the end of the ripening (Bk12) in all conditions analyzed, which was associated with a drastic reduction in ACO activity from BK stage onward (**Figure 3A**). Compared to the WT, *hp2* fruits exhibited significantly lower ethylene emission rates, ACC content and ACO activity regardless of the light treatment. In both genotypes, climacteric ethylene emission was significantly lower under light than under dark conditions (**Figure 3A**).

To investigate whether these light-induced alterations in ethylene emission were associated with changes in the transcriptional profile of ethylene biosynthetic genes, the mRNA levels of all ACS- and ACO-encoding genes responsible for the climacteric ethylene burst in ripening tomato fruits were profiled. Overall, the influence of light exposure or the *hp2* mutation on the transcript abundance of these genes was highly variable, greatly varying depending on the gene analyzed or the ripening stage (**Figures 3B,C**). Therefore, no clear correlation was observed between the transcriptional regulation of tomato ACS- and ACO-encoding genes (**Figures 3B,C**) and the reduced ethylene biosynthesis (**Figure 3A**) observed in light compared to the dark treatment or in *hp2* compared to the WT genotype. Together, these findings indicate that light exposure and the *hp2* mutation, either combined or isolated, can cause an overall down-regulation in tomato ethylene biosynthesis, which is associated with complex changes in the transcriptional profile of *ACS* and *ACO* genes.

Based on these findings, we further investigated whether light hypersensitivity alters ethylene signaling in ripening tomato fruits. First, the ethylene signaling output was determined by monitoring the activity of the reporter protein GUS expressed under the control of the *EBS* ethylene-responsive promoter in *EBS::GUS* and *hp2-EBS::GUS* genotypes. Whether under light or dark conditions, the highest GUS activity values in both genotypes coincided with the climacteric burst of ethylene

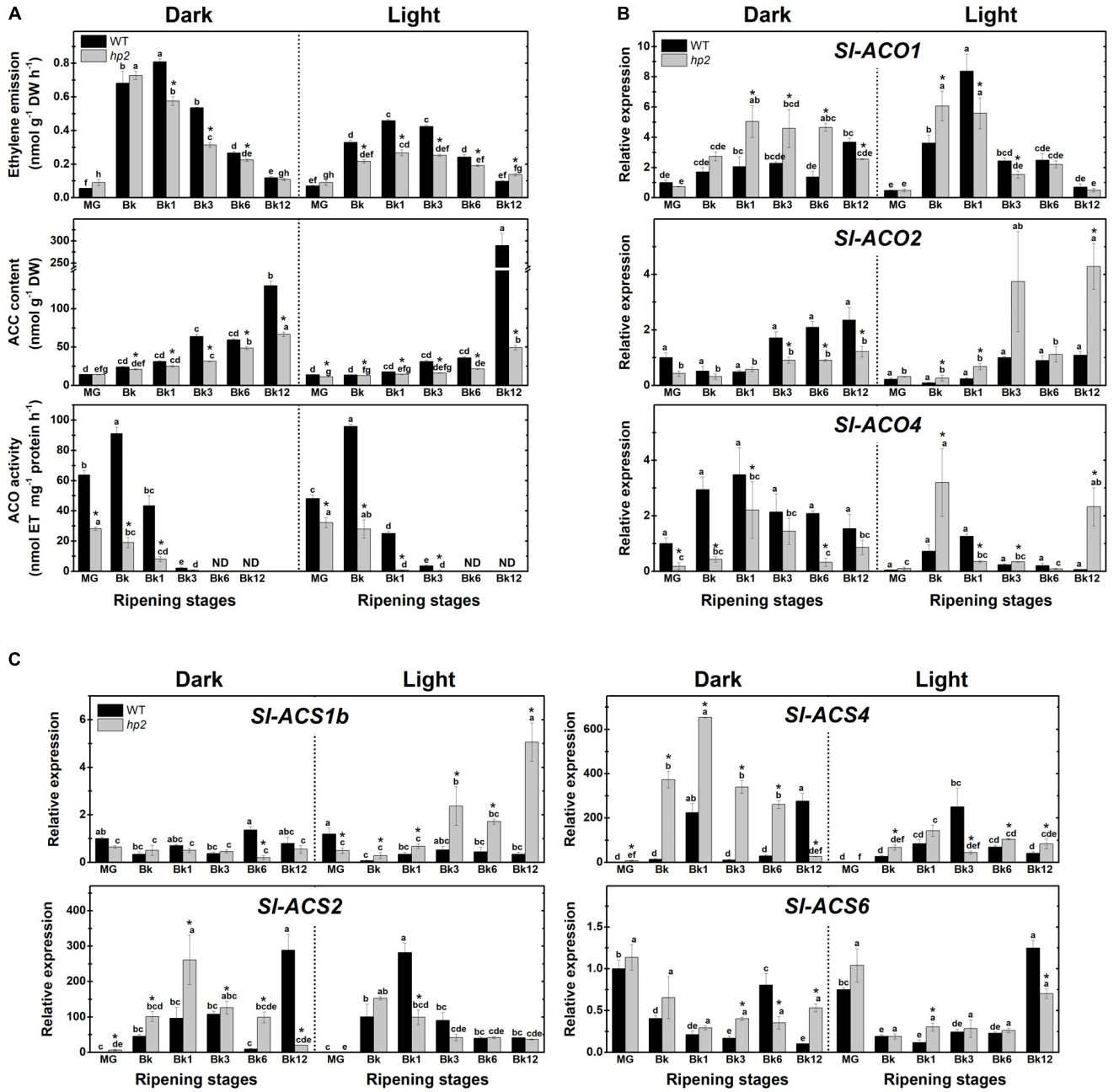

FIGURE 3 | Light-hypersensitivity represses ethylene metabolism in ripening tomato fruits. Treatment details as described in **Figure 1**. **(A)** Ethylene emission, 1-aminocyclopropane-1-carboxylic acid (ACC) content, *in vitro* ACC oxidase (ACO) activity. **(B)** Relative mRNA levels of tomato genes encoding ACO. **(C)** Relative mRNA levels of tomato genes encoding ACC synthase (ACS). Mean relative expression was normalized against wild-type (WT) samples at mature green (MG) stage under dark conditions. Data are means (±SE) of at least three biological replicates. Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$) within each genotype. Asterisks indicate statistically significant differences (Student's *t*-test, $p < 0.05$) between genotypes. *hp2*, *high pigment-2*; Bk, Breaker.

production (**Figure 4A**). However, the loss of *Sl-DET1/HP2* function resulted in higher *EBS* promoter activation, and this phenomenon was clearly intensified by the presence of light (**Figure 4A**).

The altered ethylene signaling output observed in *hp2* fruits was associated with marked differences in the transcript abundance of genes involved in ethylene perception and signaling (**Figure 4B** and **Supplementary Figure 5**). *ETHYLENE RESPONSE 3* (*Sl-ETR3*), one of the tomato ethylene receptor genes most highly expressed during ripening initiation (Liu M. et al., 2015), was strongly up-regulated in *hp2* compared to the WT regardless of the light conditions. To a certain extent, a similar trend was also observed for some other ETR genes, including *Sl-ETR4*, *Sl-ETR5* and *Sl-ETR6*.

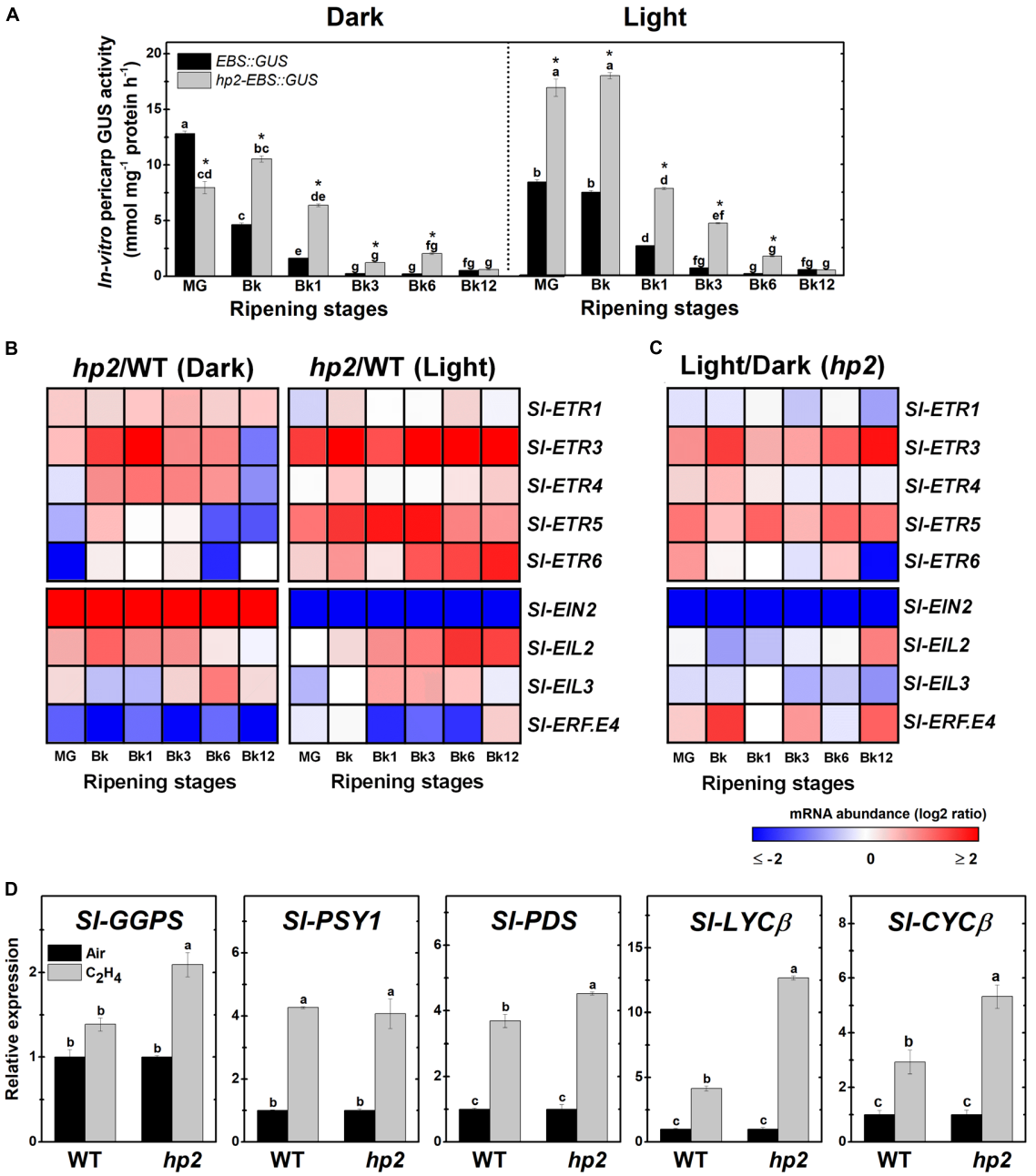

FIGURE 4 | Loss of *Sl-DET1/HP2* function promotes ethylene tissue sensitivity and signaling output. Treatment details as described in **Figure 1**. **(A)** *In vitro* GUS activity assayed in wild-type (WT) and *high pigment-2* (*hp2*) fruits carrying the ethylene-responsive promoter *EBS* fused to the GUS reporter protein (*EBS::GUS* and *hp2-EBS::GUS*). **(B)** Heatmap representation of the differences in relative mRNA levels of ethylene perception and signaling-related genes between the WT and *hp2* fruits ripened under light or dark conditions. **(C)** Heatmap representation of the differences in relative mRNA levels of ethylene perception and signaling-related genes between light and dark samples of *hp2* fruits at each sampling time. The relative transcript values are presented in **Supplementary Figure 5**. **(D)** Relative mRNA levels of tomato genes encoding carotenoid biosynthetic enzymes in WT and *hp2* fruits treated with 50 ppm ethylene for 6 h. Data are means (±SE) of at least three biological replicates. Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$) within each genotype (in **A**) or among all data (in **C**). In A, asterisks indicate statistically significant differences (Student's *t*-test, $p < 0.05$) between genotypes. MG, mature green; Bk, Breaker; ETR, ethylene response; EIN, ethylene insensitive; EIL, ethylene insensitive 3-like; ERF, ethylene response factor; GGPS, geranylgeranyl diphosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; LCYβ, chloroplast-specific β-lycopene cyclase; CYCβ, chromoplast-specific β-lycopene cyclase.

The mRNA levels of *ETHYLENE INSENSITIVE 2* (*Sl-EIN2*), which encodes a key component in the ethylene signaling cascade, was differentially affected by the *hp2* mutation depending on the light conditions, being more greatly expressed in *hp2* than in WT fruits in the dark and displaying the opposite pattern under light conditions

(**Figure 4B** and **Supplementary Figure 5**). Transcript levels of both primary (ETHYLENE INSENSITIVE 3-LIKE, EIL) and secondary (ETHYLENE RESPONSE FACTOR, ERF) ethylene-related transcription factors were also altered in *hp2* fruits compared to the WT. Both *Sl-EIL2* and *Sl-EIL3* were more abundantly expressed in *hp2* than in the WT fruits whereas the opposite was observed for the *Sl-ERF.E4*, which encodes a repressor of tomato carotenogenesis (Lee et al., 2012). The additive effect of light treatment and loss of *Sl-DET1/HP2* function was particularly observed for *Sl-ETR3*, *Sl-ETR5,* and *Sl-EIN2* (**Figure 4C**).

All tomato ethylene receptor and signaling-related genes analyzed, except *Sl-ETR3* and *Sl-EIN2*, displayed HY5 and PIF-binding motifs within their 3-kb promoter sequences (**Supplementary Figure 6**). Interestingly, *Sl-EIN2* was the only ethylene-related gene that was differentially affected by the loss of *Sl-DET1/HP2* function depending on the light conditions (**Figure 4B**).

To further investigate the relationship between ethylene responsiveness and carotenoid biosynthesis, carotenoid biosynthetic genes were profiled in both WT and *hp2* fruits at MG stage exposed to a short-term (6h) treatment with exogenous ethylene (**Figure 4D**). All genes profiled, except for *Sl-GGPS*, were significantly up-regulated in WT fruits, thereby validating the efficacy of the ethylene treatment and confirming the positive influence of this hormone on the transcriptional regulation of the carotenoid pathway in tomato fruits. Comparatively, the ethylene-induced up-regulation of genes such as *Sl-GGPS*, *Sl-PDS*, and particularly *Sl-LYC*β and *Sl-CYC*β, was significantly more pronounced in *hp2* than in the WT fruits, which corroborates the hypothesis that the increased responsiveness of *hp2* fruits to ethylene may be associated with the overaccumulation of carotenoids in this mutant.

Light-Hypersensitivity Promotes Auxin Responsiveness in Tomato Fruits

In concert with ethylene, auxin is also part of the regulatory network controlling tomato fruit ripening and carotenoid synthesis (Su et al., 2015). To evaluate whether the carotenoid overaccumulation and altered ethylene signaling observed in *hp2* fruits are associated with changes in auxin levels and signaling, we next compared the endogenous IAA content, *DR5* promoter activation and transcriptional profile of genes encoding auxin-related signaling elements in WT and *hp2* ripening fruits.

Endogenous IAA levels were remarkably similar in WT and *hp2* ripening fruits (**Figure 5A**). In contrast, the activity of the reporter protein GUS expressed under the control of the auxin-responsive *DR5* promoter was considerably higher in either light or dark-incubated fruits of *hp2-DR5::GUS* compared to the *DR5::GUS* (**Figure 5B**). In both genotypes, a progressive reduction in auxin signaling output, as indicated by the *DR5* promoter activation, was observed during fruit ripening. Auxin signaling output remained higher in *hp2-DR5::GUS* than in the *DR5::GUS* fruits from MG to Bk6 and from MG to Bk stage in dark- and light-incubated fruits, respectively.

As the higher auxin signaling output detected in *hp2* fruits were not associated with marked differences in endogenous IAA content between the genotypes (**Figures 5A,B**), it seems plausible to suggest that *hp2* fruits display increased sensitivity to this hormone compared to the WT. Corroborating these findings, the *hp2* mutation was found to trigger marked changes in the transcriptional profile of genes encoding auxin-associated signaling proteins such as Aux/IAA and ARFs (**Figure 5C**).

Among the five *Aux/IAA* tomato genes closely associated with fruit ripening – i.e., *Sl-IAA3, Sl-IAA4, Sl-IAA9, Sl-IAA15* and *Sl-IAA27* (Audran-Delalande et al., 2012) – a dramatic reduction in *Sl-IAA3*, *Sl-IAA4, Sl-IAA9* and *Sl-IAA27* mRNA levels in *hp2* compared to WT fruits was observed (**Figure 5C** and **Supplementary Figure 7**). *Sl-IAA15* mRNA levels were also reduced in *hp2* compared to the WT at certain ripening stages. Therefore, regardless of the light conditions, an overall down-regulation of *Sl-IAA* genes was observed in *hp2* fruits compared to the WT. The repressor role of light in the expression of these *Aux/IAA* genes was supported by the additive effect of light treatment and loss of *Sl-DET1/HP2* function on the mRNA levels of all *Aux/IAA* genes analyzed (**Figure 5D**).

The marked impact of loss of *Sl-DET1/HP2* function on auxin signaling output and *Aux/IAA* mRNA levels, prompted us to investigate whether changes in light signaling cause significant alterations in the transcript abundance of seven *ARF* genes highly expressed in ripening tomato fruits, i.e., *Sl-ARF2a*, *Sl-ARF2b*, *Sl-ARF3*, *Sl-ARF4*, *Sl-ARF5*, *Sl-ARF8a* and *Sl-ARF8b*. Data showed that transcript levels of *Sl-ARF2a* and *Sl-ARF2b*, which are considered key convergence points of auxin and ethylene signaling and important promoters of tomato fruit ripening and carotenoid biosynthesis (Hao et al., 2015; Breitel et al., 2016), were higher in *hp2* than in WT fruits (**Figure 5C** and **Supplementary Figure 8**). Similarly, mRNA levels of *Sl-ARF8b*, a known activator of auxin-dependent gene transcription (Kumar et al., 2014), were considerably higher in *hp2* than in WT fruits. Conversely, transcript abundance of *Sl-ARF3*, a well-established repressor of auxin-dependent gene transcription (Zouine et al., 2014), was dramatically reduced in *hp2* than in WT fruits regardless of the light treatment (**Figure 5C**). An overall reduction in *Sl-ARF8a* mRNA levels was also detected in *hp2* fruits compared to the WT, particularly under dark conditions. In contrast, the impacts of the loss of *Sl-DET1/HP2* function on *Sl-ARF4* and *Sl-ARF5* mRNA levels were considerably more variable as these genes were either up- or down-regulated in *hp2* compared to the WT depending on the ripening stage considered (**Supplementary Table 2**, i.e., non-significant influence of the genotype and the genotype × light treatment interaction). The combined effect of light exposure and the *hp2* mutation was clearly observed for all tomato ARF genes analyzed (**Figure 5D**). In summary, among all light-triggered alterations in the transcriptional profile of *Sl-ARF* genes, *Sl-ARF3* mRNA levels were down-regulated in response to both light exposure and the loss of *Sl-DET1/HP2* function, with the opposite being observed for *Sl-ARF2a*, *Sl-ARF2b,* and *Sl-ARF8b*.

Finally, the relationship between light and auxin responsiveness in *hp2* was also investigated by comparing the impacts of auxin treatment on the transcript abundance of the auxin-responsive genes *Sl-GH3*, *Sl-IAA4*, *Sl-IAA9* and

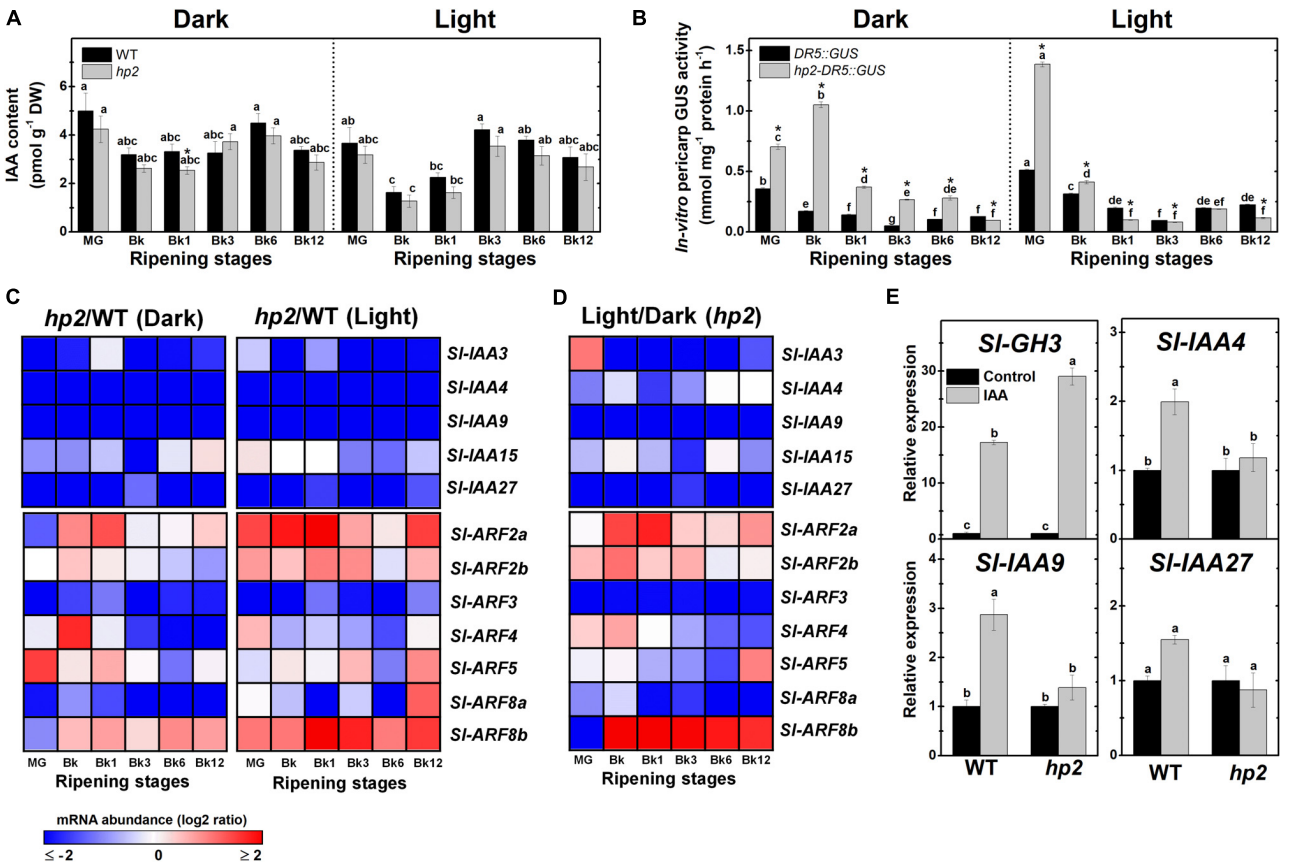

FIGURE 5 | Light-hypersensitivity promotes auxin sensitivity and signaling without altering endogenous IAA levels. Treatment details as described in **Figure 1**. **(A)** Endogenous indole-3-acetic acid (IAA) levels in wild-type (WT) and *high pigment-2* (*hp2*) fruits. **(B)** *In vitro* GUS activity assayed in WT and *hp2* fruits carrying the auxin-responsive promoter *DR5* fused to the GUS reporter protein (*DR5::GUS* and *hp2-DR5::GUS*). **(C)** Heatmap representation of the differences in mRNA levels of auxin signaling genes between the WT and *hp2* fruits ripened under light or dark conditions. **(D)** Heatmap representing differences in mRNA levels of auxin signaling genes between light and dark samples of *hp2* fruits at each sampling time. The relative transcript values are presented in **Supplementary Figures 7**, **8**. **(E)** Relative mRNA levels of auxin-responsive genes in WT and *hp2* fruits treated with 100 μM IAA for 6 h. Data are means (±SE) of at least three biological replicates. Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$) within each genotype (in **A,B**) or among all data (in **E**). In **(A,B)**, asterisks indicate statistically significant differences (Student's *t*-test, $p < 0.05$) between genotypes. MG, mature green; Bk, Breaker; Aux/IAA, auxin/indole-3-acetic acid; ARF, auxin response factor.

Sl-IAA27 (**Figure 5E**). Although *Sl-GH3* was clearly up-regulated in both WT and *hp2* fruits, the auxin-triggered accumulation of transcripts of this gene was significantly higher in the mutant, which further indicates increased auxin sensitivity in *hp2* compared to WT fruits. Auxin treatment promoted *Sl-IAA4*, *Sl-IAA9* and *Sl-IAA27* transcript accumulation in WT fruits but failed to alter the expression of these genes in *hp2* fruits (**Figure 5E**). These results are in line with the detection of lower *Sl-IAA* mRNA levels in the *hp2* compared to the WT, although both genotypes displayed similar endogenous IAA levels throughout the ripening phase (**Figures 5A,C**).

DISCUSSION

Assumptions that light-hormonal crosstalk may be involved in controlling tomato fruit ripening and carotenoid metabolism have been formulated for a long time in the literature (Lieberman, 1979; Yang and Hoffman, 1984), while unequivocal genetic or physiological evidence supporting this hypothesis remained lacking. As a major regulator of numerous ripening-associated processes, ethylene was one of the first hormones investigated as part of the regulatory mechanisms behind the light-dependent regulation of fruit carotenoid biosynthesis (Alba et al., 2000).

In vegetative tissues, ethylene biosynthesis is highly regulated by light quality, intensity and duration. Overall, light perception via photoreceptors, such as PHYs and CRYs, inhibits ethylene emission (Corbineau et al., 1995; Vandenbussche et al., 2003; Pierik, 2004; Giliberto et al., 2005; Melo et al., 2016), ACC accumulation (Jiao et al., 1987; Melo et al., 2016), ACO activity (Melo et al., 2016) and *ACS* transcript levels (Khanna et al., 2007). Our data revealed that the negative influence of light on ethylene metabolism typically found in vegetative tissues is also observed in ripening tomato fruits as indicated by the light-triggered reduction in ACC content, ACO activity and

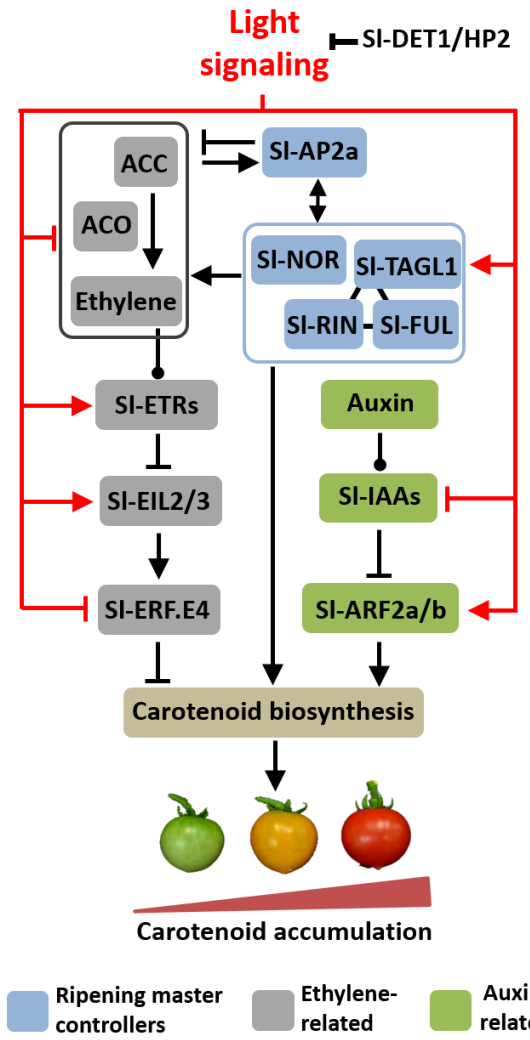

FIGURE 6 | Proposed model for light-hormonal interaction controlling tomato fruit carotenoid biosynthesis. Light signaling promotes master regulators of ripening, which positively regulate carotenoid biosynthetic genes. Light also down-regulates ethylene metabolism and emission and reduces the expression of *ETHYLENE RESPONSE FACTOR E4* (*Sl-ERF.E4*), a key repressor of tomato fruit carotenogenesis. Moreover, light represses *AUXIN/INDOLE-3-ACETIC ACID* (*Sl-IAAs*) genes and up-regulates both *AUXIN RESPONSE FACTOR 2* paralogues (*SlARF2a/b*), consequently promoting most components of the tomato carotenoid biosynthetic route. Additionally, light signaling up-regulates genes encoding ethylene receptor (ETRs) and intermediate components in ethylene signaling cascade (EILs). The arrow- and bar-headed lines indicate stimulatory and inhibitory effects, respectively. The lines terminated by a circle describe more complex interactions. RIN, ripening inhibitor; NOR, non-ripening; FUL, fruitfull; AP2a, apetala2a, TAGL1, tomato agamous-like1; ETR, ethylene response; EIL, ethylene insensitive 3-like.

ethylene emission, a response that was further intensified in fruits of the light-hyperresponsive *hp2* mutant. The main *ACS* and *ACO* family members expressed during ripening were either up- or down-regulated in response to light exposure or the loss of *Sl-DET1*/*HP2* function, suggesting that light-dependent down-regulation of the ethylene climacteric burst in tomato is linked to complex alterations in the transcript abundance of its biosynthetic genes. These findings contrast with the observation that PHY-mediated light perception in plant vegetative tissues is frequently associated with the modulation of ethylene biosynthesis via conspicuous changes in the *ACS* transcription (Rodrigues et al., 2014), as illustrated by the several 100-fold enhancements in *AtACS4* transcript abundance detected in *Arabidopsis* seedlings overexpressing *AtPIF5* (Khanna et al., 2007).

Ethylene biosynthesis in tomato fruits is tightly regulated by master regulators of ripening, stimulated by Sl-RIN, Sl-NOR, Sl-FUL, and Sl-TAGL1 (Itkin et al., 2009; Liu M. et al., 2015) and repressed by Sl-AP2a (Karlova et al., 2011). The up-regulation of all these ripening master regulators in *hp2* ripening fruits entails a complex interaction between the light signaling cascade and the regulatory cascade controlling ripening. Sl-AP2a acts as a negative regulator of tomato climacteric ethylene synthesis via a negative feedback loop (Karlova et al., 2011); therefore, the reduced ethylene production detected in *hp2* fruits may be associated with the up-regulation of *Sl-AP2a* in this mutant (**Figure 6**). In contrast, all ripening master regulators analyzed are well-established promoters of fruit carotenoid biosynthesis (Itkin et al., 2009; Martel et al., 2011; Liu L. et al., 2015); hence, their up-regulation in *hp2* ripening fruits is consistent with the over-accumulation of carotenoids in the mutant.

Besides altering ethylene biosynthesis, the loss of *Sl-DET1*/*HP2* function also impacted tomato fruit responsiveness to ethylene, a response associated with marked changes in ethylene receptors (ETRs) and downstream signaling transduction elements (EIN, EILs, ERFs). The receptor signaling model states that ETRs, including those involved in tomato ripening (i.e., Sl-ETR3 and Sl-ETR4), are negative regulators of ethylene responses (Kevany et al., 2007; Kamiyoshihara et al., 2012); consequently, reductions in the abundance of receptors promote tissue ethylene sensitivity (Tieman et al., 2000). However, information about the temporal fluctuations in ETR transcripts and protein levels during tomato ripening is controversial. Opposite temporal patterns between the mRNA and protein levels of Sl-ETR3 and Sl-ETR4 have been observed during tomato ripening, as the protein and transcript abundance of these receptors peak at the immature and ripening stages, respectively (Kevany et al., 2007). However, no significant alterations in ethylene receptor protein abundance were observed during tomato fruit ripening in a subsequent study (Kamiyoshihara et al., 2012). Therefore, on the one hand, the apparent contradiction between the up-regulation of *ETR* transcripts and the increased ethylene sensitivity detected in *hp2* fruits may be explained by the inverse pattern in ethylene receptor mRNA and protein levels already observed in tomato fruits (Kevany et al., 2007). On the other hand, if the *hp2*-triggered up-regulation of *Sl-ETR* expression results in increased receptor protein abundance, the increased ethylene sensitivity observed in the fruits of these mutants may be associated with a more complex alteration in ethylene perception and signaling cascade.

Acting downstream to ETR receptors, EIN2 plays a significant role in ethylene signaling by stabilizing EIN3/EIL transcription factors, which in turn will activate the transcription of

multiple ethylene-responsive genes, including secondary transcription factors (i.e., ERFs) (Alonso, 1999). Many of these downstream signaling transduction elements are involved in photomorphogenic responses, sometimes acting as integrators of light and ethylene signaling during vegetative plant development (Zhong et al., 2009). In tomato, *Sl-EIN2*, *Sl-EIL* or *Sl-ERF.E4* suppression disturbed fruit ripening and, consequently, altered carotenoid metabolism (Tieman et al., 2001; Fu et al., 2005; Lee et al., 2012). As these genes were differentially expressed in *hp2* fruits compared to the WT, it seems that disturbances in light signaling can affect multiple steps in the ethylene transduction cascade, which may contribute to explain the altered ethylene responsiveness detected in this mutant. In this context, it is also worth mentioning that *Sl-ERF.E4* mRNA levels were severely reduced in *hp2* fruits and this ERF has been proposed as a major repressor of carotenoid synthesis in tomato, as revealed by the over-accumulation of this class of isoprenoid in fruits of *Sl-ERF.E4*-knockdown transgenic lines (Lee et al., 2012). Therefore, it seems that the increased ethylene responsiveness of *hp2* fruits may compensate for its reduced ethylene biosynthesis, which is supported by the comparatively higher expression of carotenoid biosynthetic genes in the mutant when both WT and *hp2* fruits were supplemented with the same concentration of ethylene.

Tomato fruit carotenogenesis is undeniably regulated by ethylene-related signaling components, but other plant hormones have also been increasingly implicated in controlling this metabolic pathway (Kumar et al., 2014; Liu L. et al., 2015). Auxins, for instance, have been demonstrated to counteract the promotive influence of ethylene on tomato fruit ripening and carotenogenesis (Pirrello et al., 2012; Su et al., 2015). Here, we provide several lines of evidence indicating that the loss of *Sl-DET1*/*HP2* function promotes auxin responsiveness in fruit tissues via changes in the transcript abundance of auxin signaling-related genes. The increased activation of *DR5* promoter in *hp2* fruits was not associated with significant differences in the endogenous IAA levels between the mutant and WT genotypes but instead was accompanied by the down-regulation of the *Sl-IAA* genes most greatly expressed in tomato fruits (i.e., *Sl-IAA3*, *Sl-IAA4*, *Sl-IAA9,* and *Sl-IAA27*).

Accordingly, functional characterization studies have revealed that the down-regulation of *Sl-IAA3*, *Sl-IAA9* or *Sl-IAA27* disturbs auxin responsiveness in tomato plants. Whereas *Sl-IAA3* knockdown resulted in lower auxin sensitivity, *Sl-IAA9*- or *Sl-IAA27*-silenced lines exhibited increased auxin responsiveness (Wang et al., 2005; Chaabouni et al., 2009; Bassa et al., 2012). Therefore, the progressive reduction in *DR5* promoter activity observed from the MG to Bk12 stages in both dark- and light-incubated fruits may be linked to the gradual increment in transcripts of the repressor of auxin responsiveness *Sl-IAA3* (Chaabouni et al., 2009), and the progressive reduction in transcripts of *Sl-IAA9* and *Sl-IAA27*, two positive regulators of tissue responsivity to auxins (Wang et al., 2005; Bassa et al., 2012). Among these tomato *Aux/IAA* genes, *Sl-IAA3* has been suggested to represent a crossroad of auxin and ethylene signaling in tomato, being highly regulated by both these hormones. Recent findings also indicate that Sl-IAA3 mediates the interplay between light and ethylene signaling, since dark- and light-grown *Sl-IAA3*-knockdown tomato seedlings exhibited marked differences in ethylene sensitivity (Chaabouni et al., 2009) and this tomato *Aux/IAA* gene was particularly up-regulated in ripening fruits of PHY-deficient tomato plants (Bianchetti et al., 2017). Therefore, it seems tempting to speculate that the light-dependent down-regulation of Sl-IAA3 may be associated with the increased responsivity to ethylene observed in *hp2* fruits.

Aux/IAA proteins are known to inhibit the activity of ARF, and ARFs can either act as transcriptional repressors or activators of auxin-responsive genes (Zouine et al., 2014). Hence, changes in ARF abundance also significantly impact plant tissue responsiveness to auxins (Sagar et al., 2013; Zouine et al., 2014; Hao et al., 2015). Accordingly, the increased auxin responsiveness observed in *hp2* fruits was associated with a marked down- and up-regulation of *Sl-ARF3* and *Sl-ARF8b*, respectively, as the former is a repressor of auxin-dependent gene transcription whereas the latter is an activator of auxin responses. In both cases, the impact of the *hp2* mutation was intensified by light exposure, which suggests that the light-dependent transcriptional regulation of these two *ARF*s may be associated with the increased auxin responsiveness observed in the *hp2* fruits.

The up-regulation of *Sl-ARF2a* and *Sl-ARF2b* caused by the loss of *Sl-DET1*/*HP2* function is also consistent with the proposed role suggested for these two *ARF*s on tomato fruit ripening and carotenogenesis (Hao et al., 2015; Breitel et al., 2016). Both *Sl-ARF2* paralogs are known to cooperate in promoting the expression of master controllers of ripening, such as Sl-RIN and Sl-NOR, stimulating ethylene biosynthesis and signaling and inducing carotenoid biosynthesis (Hao et al., 2015; Breitel et al., 2016). Therefore, the up-regulation of both *Sl-ARF2a* and *Sl-ARF2b* genes observed in light-incubated *hp2* fruits agrees with the higher expression of genes encoding master controllers of ripening and carotenoid biosynthetic enzymes detected in this light-hyperresponsive mutant.

Here, we put forward the hypothesis that light-triggered changes in auxin and ethylene responsiveness and signaling are associated with the overaccumulation of carotenoids in *hp2* fruits. In the proposed working model of light-hormonal crosstalk controlling tomato carotenogenesis (**Figure 6**), the positive and negative influence of light on ethylene biosynthesis and signaling, respectively, are supported by both genetic (i.e., *hp2* mutation versus WT genotype) and physiological evidence (i.e., light versus dark treatment). The assumption that light modulates auxin signaling is corroborated by the marked down-regulation of *Aux/IAA* tomato genes and altered *ARF* expression profile in *hp2* fruits compared to the WT. The two *ARF* genes most closely associated with tomato fruit ripening and carotenogenesis (i.e., *Sl-ARF2a* and *Sl-ARF2b*) and the genes encoding the master regulators of ripening (e.g., Sl-RIN, Sl-NOR, Sl-FUL1, Sl-AP2a) were up-regulated, whereas *Sl-ERF.E4*, a repressor of tomato fruit carotenogenesis, was repressed in *hp2* fruits compared to WT counterparts. All these changes in the central ripening-related regulatory modules are consistent with the increased transcript abundance of carotenoid biosynthetic genes (e.g., *Sl-GGPS*, *Sl-PSY1*,

Sl-PDS, *Sl-LYC*β and Sl-*LYC*β) and the over-accumulation of carotenoids typically observed in the *hp2* mutant.

Although DET1 has long been identified as a major repressor of light signaling in plants (Chory et al., 1989), the molecular mechanisms responsible for its action on photomorphogenesis remain not yet fully characterized. However, accumulating evidence indicates that DET1 may interfere with multiple steps of the light signaling cascades. In Arabidopsis, DET1 interacts with DDB1 and COP10 to form the CDD complex, which physically associates with CUL4, giving rise to an E3 ligase that promotes the proteolytic degradation of photomorphogenesis-promoting factors, including HY5 (Yanagawa et al., 2004). DET1 has also been shown to positively and negatively regulate the accumulation of PIF and DELLA proteins, respectively (Dong et al., 2014; Li et al., 2015), which partially explains how DET1 represses *Arabidopsis* photomorphogenesis in darkness. Data also implicates DET1 action in chromatin remodeling (Benvenuto et al., 2002) and as a transcriptional co-repressor of key regulators of the circadian clock genes (Lau et al., 2011). Therefore, multiple mechanisms may be involved in the Sl-DET1/HP2-mediated regulation of ethylene and auxin pathways in ripening tomato fruits, including its influence on balancing HY5 and PIF protein abundance, possible global alterations in gene expression via chromatin remodeling, and its potential action as a transcriptional co-regulator. Hence, future work is needed to characterize the precise molecular mechanisms behind the Sl-DET1/HP2-mediated regulation of tomato fruit hormonal balance and physiology.

Although the exact mechanisms behind the light-triggered alterations in fruit hormone responsiveness are not yet clear, the data obtained in this study provide clear evidence that an intricate crosstalk between light, ethylene and auxin signaling may be involved in controlling tomato fruit carotenogenesis. Therefore, these findings open up a window of opportunity for further improvement in tomato fruit nutritional content through the combined manipulation of auxin, ethylene and light signaling-related genes.

AUTHOR CONTRIBUTIONS

LF, LP, MR, and AC designed the experiments. AC, RB, FA, and EP conducted the experiments and analyzed the results. AC, RB, and LF prepared 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.2018.01370/full#supplementary-material

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REVIEW
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Vitamin C Content in Fruits: Biosynthesis and Regulation

*Mario Fenech[1], Iraida Amaya[2], Victoriano Valpuesta[1] and Miguel A. Botella[1]**

[1] Departamento de Biología Molecular y Bioquímica, Instituto de Hortofruticultura Subtropical y Mediterránea (IHSM), Consejo Superior de Investigaciones Científicas, Universidad de Málaga, Málaga, Spain, [2] Instituto Andaluz de Investigación y Formación Agraria y Pesquera, Area de Genómica y Biotecnología, Centro de Málaga, Spain

Throughout evolution, a number of animals including humans have lost the ability to synthesize ascorbic acid (ascorbate, vitamin C), an essential molecule in the physiology of animals and plants. In addition to its main role as an antioxidant and cofactor in redox reactions, recent reports have shown an important role of ascorbate in the activation of epigenetic mechanisms controlling cell differentiation, dysregulation of which can lead to the development of certain types of cancer. Although fruits and vegetables constitute the main source of ascorbate in the human diet, rising its content has not been a major breeding goal, despite the large inter- and intraspecific variation in ascorbate content in fruit crops. Nowadays, there is an increasing interest to boost ascorbate content, not only to improve fruit quality but also to generate crops with elevated stress tolerance. Several attempts to increase ascorbate in fruits have achieved fairly good results but, in some cases, detrimental effects in fruit development also occur, likely due to the interaction between the biosynthesis of ascorbate and components of the cell wall. Plants synthesize ascorbate *de novo* mainly through the Smirnoff-Wheeler pathway, the dominant pathway in photosynthetic tissues. Two intermediates of the Smirnoff-Wheeler pathway, GDP-D-mannose and GDP-L-galactose, are also precursors of the non-cellulosic components of the plant cell wall. Therefore, a better understanding of ascorbate biosynthesis and regulation is essential for generation of improved fruits without developmental side effects. This is likely to involve a yet unknown tight regulation enabling plant growth and development, without impairing the cell redox state modulated by ascorbate pool. In certain fruits and developmental conditions, an alternative pathway from D-galacturonate might be also relevant. We here review the regulation of ascorbate synthesis, its close connection with the cell wall, as well as different strategies to increase its content in plants, with a special focus on fruits.

Keywords: ascorbic acid, vitamin C, cell wall, biosynthesis, fruit, regulation

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****Correspondence:***
Miguel A. Botella
mabotella@uma.es

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MULTIPLE ROLES OF VITAMIN C IN HUMANS

L-Ascorbic Acid (L-threo-hex-2-enono-1,4-lactone, ascorbate), also called vitamin C, is an essential antioxidant molecule in plant and animal metabolism and also functioning as a cofactor in many enzymes. While many animals are able to synthesize ascorbate in the liver or in the kidney, others, such as humans, non-human primates, guinea pigs, and certain groups of bats and birds have lost this ability due to the accumulation of mutations in the coding sequence of the last committed

enzyme of the pathway (L-gulono-1,4-lactone oxidase, GULO; Chatterjee, 1973; Nishikimi et al., 1994; Drouin et al., 2011). Dietary changes with the inclusion of abundant fruits and vegetables in the diet resulted in the loss of selective pressure to keep the pathway functional (Macknight et al., 2017). Thus, this molecule must be incorporated in the diet (hence classified as a vitamin), with vegetables and fruits as the major sources of ascorbate.

The role of ascorbate in mammals has extensively been studied throughout time, particularly since the 18th century with the discovery of its role in preventing scurvy (Lind, 1753; Baron, 2009). However, this was not obvious at the time because the lack of ascorbate in the diet takes about a month before the symptoms to occur. Thus, this disease was typically manifested during long sea travels with a diet scarce in fresh vegetables and fruits. In the earlies 1930's, Albert Szent-Györgyi identified and isolated the molecule responsible for this anti-scurvy activity. Thus, that molecule, previously called hexuronic acid, was renamed as ascorbic acid. One of the main symptoms in scurvy is skin impairment and injuries due to the involvement of ascorbate in the biosynthesis and stability of collagen. Ascorbate functions as a cofactor in the enzymatic hydroxylation catalyzed by Fe^{2+}/αKG-dependent dioxygenases prolyl 4-hydroxylase, prolyl 3-hydroxylase and lysyl hydroxylase (Myllylä et al., 1984; Peterkofsky, 1991; Pekkala et al., 2003; Padayatty and Levine, 2016) through the reduction of Fe^{3+} to the active Fe^{2+} (de Jong et al., 1982; Gorres and Raines, 2010). Prolyl hydroxylation is an essential post-translational modification that occurs in proline residues located at X and Y sites of procollagen Gly-X-Y tandem repeats during collagen biosynthesis. Whereas Prolyl 4-hydroxylases catalyze hydroxylation on Y locations, Prolyl 3-hydroxylases hydroxylate residues located at X sites, thus enabling the trimerization of collagen providing high thermal stability (Koide and Nagata, 2005). The hydroxylation catalyzed by these enzymes requires an Fe^{2+} ion located at the active center, which is oxidized to Fe^{3+} in the catalytic cycle and ascorbate is responsible of keeping the iron active by reducing it back to Fe^{2+}.

In addition to preventing scurvy, ascorbate is involved in many other processes which also require the action of other members of this family of mono- and dioxygenases. For these enzymes, ascorbate functions as a cofactor, maintaining activity of the metal ions located in the active centers. For example, ascorbate is important for the synthesis of carnitine, the lack of which is related to the common fatigue found in scorbutic patients. Trimethyllysine hydroxylase and γ-butyrobetaine hydroxylase require ascorbate to enhance their activity in the biosynthesis of carnitine (Rebouche, 1991). In addition, ascorbate is also known to act as a cofactor of dopamine β-monooxygenase (Rush and Geffen, 1980), and in peptide hormone metabolism, by acting as a cofactor of peptidylglycine α-amidating monooxygenase, involved in the C-terminal amidation of these regulatory molecules (Prigge et al., 1999). More recently, the activity of other key Fe^{2+}/αKG-dependent dioxygenases have been showed to be enhanced by ascorbate, as is the case of Ten-Eleven Translocations (TETs) enzymes. TETs are involved in DNA demethylation through an oxidation cascade from 5-methylcytosine to 5-hydroxymethylcytosine, 5-formylcytosine, 5-carboxylcytosine and, then, to cytosine by the Base Excision Repair (BER) mechanism (Blaschke et al., 2013; Minor et al., 2013; Hu et al., 2015). Importantly, ascorbate functions as a cofactor of histone demethylases harboring a Jumonji C (JmcC) domain (JHDMs), the same catalytic domain present in TETs (Young et al., 2015). Tri-, di- and monomethylated lysines in histones can be oxidized to hydroxymethyl lysines by JHDM and ascorbate in a similar way as occurring with DNA demethylation and TETs, with an spontaneous removal of this hydroxymethyl group (Young et al., 2015). All together, these findings show that ascorbate participates in the response to environmental stimuli, not only by buffering the cell redox state, but also by its involvement in the epigenetic control on gene expression. In addition, ascorbate enhances iron absorption (Hallberg et al., 1987, 1989), which is not only important to keep the Fe^{2+}/αKG-dependent dioxygenases active, but also for many other roles (Lieu et al., 2001; Muckenthaler et al., 2008).

MAJOR FRUIT SUPPLIES OF ASCORBATE IN HUMANS

Fresh fruits and vegetables are the major sources of this vitamin, therefore increasing its concentration will have an important impact in human nutrition. Ascorbate deficiency in developed countries has registered a decrease throughout time. At the end of last century. ascorbate deficiency in United States was around 13% of the population (Hampl et al., 2004), but it dropped to 7% in the last survey effectuated during 2003-2004 period (Schleicher et al., 2009). According to early experiments, a daily dose of less than 10 mg was found to prevent scurvy (Johnstone et al., 1946; Peters et al., 1953; Baker et al., 1969, 1971; Hodges et al., 1969, 1971). However, an Average Requirement (AR) of 90 mg/day for men and 80 mg/day for women, and a Population Reference Intake (PRI) of 110 mg/day for men and 95 mg/day for women, has been established by the European Food Safety Authority (EFSA Panel on Dietetic Products and Nutrition and Allergies [NDA], 2013). This is based on maintaining a plasma concentration around 50 μmol/L of ascorbate, indicative of an adequate status (Kallner et al., 1979). In United States and Canada, the Recommended Dietary Allowance (RDA) is 90 mg/day for men and 75 mg/day for women (Food and Nutrition Board Panel on Dietary Antioxidants and Related Compounds, 2000).

It is accepted that a diet rich in ascorbate has various health advantages (Wintergerst et al., 2006; Reczek and Chandel, 2015; Carr and Maggini, 2017; van Gorkom et al., 2018). Furthermore, in the last few years, ascorbate has been proposed as a treatment against different types of cancer through various mechanisms, such as increasing TET's activity, inducing oxidative stress in cancer cells or enhancing the activity of various chemical treatments (Ko et al., 2015; Yun et al., 2015; Agathocleous et al., 2017; Cimmino et al., 2017; Shenoy et al., 2017; Lu et al., 2018; Miura et al., 2018). Daily intake of ascorbate provided by fruits is dependent on several factors, but clearly the content

of ascorbate as well as the amount that is consumed are the most important factors. However, it is important to take into account the way it is consumed as this might have important consequences on ascorbate reduction and oxidation, and can also alter the bioavailability of ascorbate due to interactions with other phytochemicals such as Vitamin E or flavonoids (Packer et al., 1979; Tanaka et al., 1997; Carr and Vissers, 2013).

Ascorbate overall intake is dependent on the intrinsic amount of ascorbate of a specific fruit and its consumption (**Figure 1B**). According to FAOSTAT[1], tomato has been the most produced fruit in the world in the last 20 years, a trend that has increased during the last years (**Figure 1A**). The production has been 177 million tons in 2016, followed by banana (∼113 million tons), apple (∼89 million tons), cucumber (∼80 million tons) and grape (∼77 million tons). In the European Union in 2016, fruit production was dominated by grape (∼24 million tons), followed by tomato (∼18 million tons), apple (∼12.5 million tons) and orange (∼6.3 million tons) (Eurostat, 2017). However, a large proportion of tomato (61.5%), apple (26.8%) and grape (96.5%) is processed (Eurostat, 2017), leading both to a reduction of ascorbate content and a lower bioavailability of other nutrients that are ascorbate dependent (Hallberg et al., 1982, 1987). This is particularly evident in grape, with ∼90 % of the harvest destined to wine production (Eurostat, 2017), leading to negligible amounts of ascorbate (USDA Food Composition Databases[2]). Therefore, considering production along with consumption data (**Figure 1C**), ascorbate intake through orange surpasses that of grape. Tomato and apple fruits, although could be considered as moderate sources of ascorbate based on their relatively low content (Davey et al., 2000) are widely consumed and therefore provide important dietary sources of ascorbate. It is obvious that even a moderate increase in the content of ascorbate in these highly consumed fruits would rise their nutritional value. Therefore, the large consumption of tomato, its relatively low ascorbate and its high raw intake makes it an excellent target for increasing its ascorbate content from a nutritional point of view (**Figure 1**).

THE ROLE OF ASCORBATE IN PLANTS AND FRUITS

Ascorbate plays a plethora of roles in plant cells. Important properties of ascorbate are its antioxidant capacity and the finalization of oxidative chain reactions resulting in non-oxidative products such as dehydroascorbate (DHA) and 2,3-diketogulonic acid (Davey et al., 2000). The importance of ascorbate in scavenging ROS became evident when several of the genes involved in the ascorbate biosynthetic pathway were identified in genetic screenings searching for mutants hypersensitive to ozone, a powerful oxidant (Conklin et al., 1996). This screening resulted in the identification of five vitamin C-deficient (*vtc*) mutants, with four of those mutations affecting genes encoding enzymes of the Smirnoff-Wheeler pathway: *VTC1* (Lukowitz et al., 2001), *VTC2* and *VTC5* (Dowdle et al., 2007; Linster et al., 2007) and *VTC4* (Conklin et al., 2006).

Hydrogen peroxide (H_2O_2) plays essential roles in plants development and defense (Exposito-Rodriguez et al., 2017; Mittler, 2017; Mullineaux et al., 2018; Waszczak et al., 2018) and it can be found in different organelles within the plant cells (Exposito-rodriguez et al., 2013). However, H_2O_2 is also partly responsible for light-induced oxidative damage. Ascorbate is involved in the scavenging of the excess of H_2O_2 produced during the photosynthesis in high-irradiance conditions by the function of ascorbate peroxidases (APX), enzymes not present in animals (Wheeler et al., 2015). Together with APX, catalases also perform H_2O_2 scavenging (Mhamdi et al., 2010, 2012). However, plants lack catalases in chloroplasts, which experience a high production of H_2O_2 in thylakoids due to photosynthesis, as a consequence of the Mehler reaction. In these organelles, a thylakoidal APX (tAPX) catalyzes the reduction of H_2O_2 (Asada, 1999). Surprisingly, single and double mutants in chloroplastic APX (tAPX and stromal APX) are viable, suggesting alternative mechanisms for H_2O_2 detoxification (Giacomelli et al., 2007). 2-Cys peroxiredoxins (2-Cys PRX), localized in the chloroplast, reduce H_2O_2 and prevent oxidation of the thylakoidal membrane by reducing lipid hydroperoxide from thylakoid phospholipids (Baier and Dietz, 1997). Therefore, 2-Cys PRXs have been proposed as alternative H_2O_2 scavengers to APX in an alternative water-water cycle (Awad et al., 2015; Pérez-Ruiz et al., 2017) using glutathione, thioredoxin, glutaredoxin, cyclophilin, and/or tryparedoxin instead of ascorbate as cofactors (Stork et al., 2005). Together with APX and 2-Cys PRX, vitamin E (α-tocopherol) is a major lipophilic antioxidant also involved in preventing photodamage in the membrane of thylakoid lipids (Semchuk et al., 2009). Ascorbate also has a role in vitamin E function by the non-enzymatical reduction of α-tocopheryl radicals, hydroxyl radicals (·OH) and superoxide ions (O_2^-) (Asada, 1999; Davey et al., 2000; Mittler, 2017).

The use of ascorbate as a cofactor by other enzymes, such as the Fe^{2+}/α-KG-dependent dioxygenases and Cu^+-monooxygenases, is conserved among plants and animals. However, one of these common enzymes, a Fe^{2+}-dependent 4-hydroxyphenylpyruvate dioxygenase, has different functions in plants. Whereas in animals this enzyme is involved in tyrosine metabolism (Lindblad et al., 1970), in plants it is required for plastoquinone and tocopherols synthesis (Norris et al., 1998). Other light-responsive pigments that are very abundant in fruits, like anthocyanins, fail to accumulate in *vtc1* and *vtc2* mutant plants when exposed to high light. This finding, combined with the UV-B absorption by anthocyanin, suggests that ascorbate-mediated redox reactions act upstream of anthocyanin synthesis (Page et al., 2012).

Ascorbate was proposed to directly participate in photosynthesis as an electron carrier, although later a role as a photoprotectant was revealed (Smirnoff, 2000). The electron transfer from ascorbate to the primary oxidizing agent of photolysis was first coupled to the photophosphorylation reaction (Marrè et al., 1959). Then, the reduction of monodehydroascorbate (MDA) and DHA were suggested to rely on reductants formed in photosystem I (PSI). It is now

[1]http://www.fao.org/faostat/en/#data

[2]https://ndb.nal.usda.gov/ndb/search/list

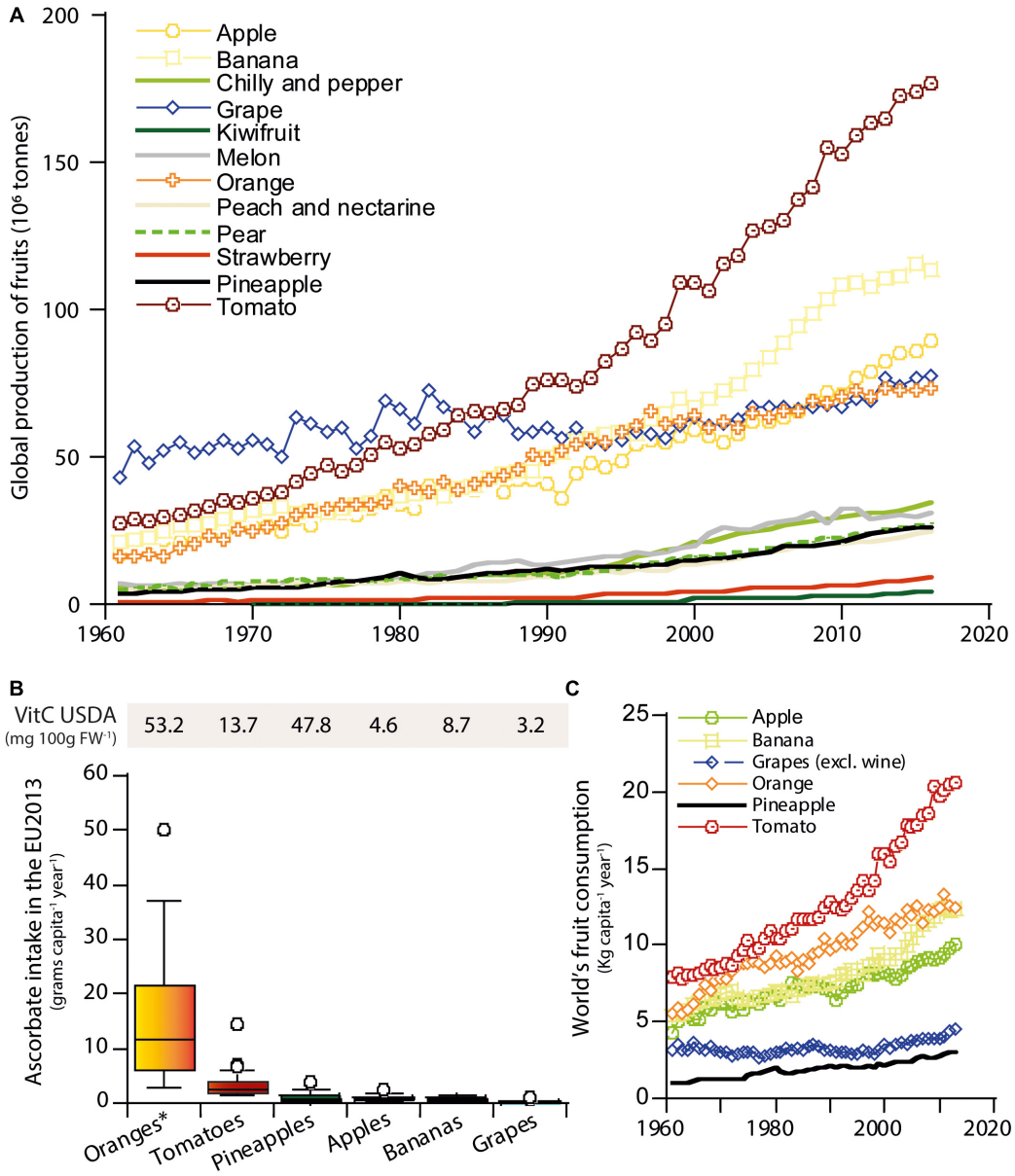


FIGURE 1 | Main fruit crops yield and consumption according to FAO. **(A)** Global fruit production, in million tons, and its evolution from 1961 to 2016. **(B)** Fruit ascorbate intake, in grams of ascorbate capita^{-1} year^{-1}, in the countries from the European Union in 2013. Data were generated considering ascorbate (VitC) levels of raw fruit available in USDA database (https://ndb.nal.usda.gov/ndb/search/list) and consumption data of each fruit (Kg capita^{-1} year^{-1}) from FAOSTAT. USDA IDs consulted: 9200 (Oranges *includes mandarins, raw, all commercial varieties), 11529 (Tomatoes, red, ripe, raw, year average), 9003 (Apples, raw with skin), 9132 [Grapes, red or green (European type, such as Thompson seedless), raw], 9266 (Pineapple, raw, all varieties), 9040 (Bananas, raw). Consumption data was obtained from Eurostat (http://ec.europa.eu/eurostat). **(C)** Evolution in the global consumption of fruits, in Kg capita^{-1} year^{-1}, from 1961 to 2013.

established that inside the thylakoid, luminal ascorbate acts as an electron donor of photosystem II (PSII) (Tóth et al., 2013) where the Oxygen-Evolving Complex is impaired (Katoh and San Pietro, 1967; Mano et al., 1997; Tóth et al., 2009), thus allowing the reduction of $NADP^+$ to NADPH by the electron-transport chain (Tóth et al., 2009, 2013). This is particularly important during abiotic stresses such as heat and high light that alter this complex by damaging the manganese cluster (Tyystjärvi, 2008). In addition, ascorbate can also dissipate energy from an excess of light irradiance acting as a cofactor of violaxanthin de-epoxidase, an enzyme involved in preventing photodamage by non-photochemical quenching (NPQ) (Yamamoto et al., 1972). When the irradiance is too high, the excess of energy normally transferred to chlorophyll *a* is used to de-epoxidize the carotenoid violaxanthin into zeaxanthin using the thylakoid luminal ascorbate as a cofactor in the xanthophyll cycle (Hieber et al., 2000). This has been supported experimentally by mutations in the enzyme's residues that bind ascorbate

(Saga et al., 2010) and by the analysis of Arabidopsis mutants with low ascorbate content (Müller-Moulé et al., 2002).

BIOSYNTHESIS AND METABOLISM OF ASCORBATE IN PLANTS

The predominant pathway through which ascorbate is synthesized in plants is the Smirnoff-Wheeler (SW) pathway (Wheeler et al., 1998). Contrary to the animal pathway, in the plant pathway there is no carbon inversion, as the carbon 1 in the D-glucose molecule remains as carbon 1 in ascorbate after conversion. In this pathway, a molecule of D-glucose-6-phosphate is transformed into D-fructose-6-phosphate by the action of phosphoglucose isomerase (PGI; **Figure 2**). Then, it is transformed into D-mannose-6-phosphate and D-mannose-1-phosphate by the action of phosphomannose isomerase (PMI; Maruta et al., 2008) and phosphomannomutase (PMM; Qian et al., 2007). Then, GDP-D-mannose pyrophosphorylase (GMP, encoded by *VTC1* in *Arabidopsis thaliana*) transfers guanosine monophosphate from GTP to form GDP-D-mannose (Conklin et al., 1996, 1997, 1999; Lukowitz et al., 2001). GDP-D-mannose is further transformed into GDP-L-galactose by the GDP-D-mannose-3′,5′-epimerase (GME), an enzyme that belongs to the extended short chain dehydratase/reductase (SDR) protein family, harboring a modified NAD^+ binding Rossman fold domain. Interestingly, GDP-L-galactose is not the only result of GME activity, since GDP-L-gulose can also be produced if GME catalyzes a 5′ epimerization instead of a 3′,5′ epimerization (Wolucka et al., 2001; Wolucka and Van Montagu, 2003; Major et al., 2005). Since GDP-L-gulose is a very rare sugar in plants with no structural function, it has been suggested that it is directly channeled to synthesize ascorbate. After GME releases GDP-L-galactose, this compound is then transformed into L-galactose-1-phosphate, L-galactose and L-galactono-1,4-lactone by GDP-L-galactose-phosphorylase (GGP, encoded by *VTC2* and *VTC5* in *A. thaliana*; Dowdle et al., 2007; Laing et al., 2007), L-galactose-1-phosphate phosphatase (GPP, encoded by *VTC4* in *A. thaliana*; Laing et al., 2004a; Conklin et al., 2006; Torabinejad et al., 2009; Nourbakhsh et al., 2015) and L-galactose dehydrogenase (L-GalDH; Gatzek et al., 2002; Laing et al., 2004b), respectively. Interestingly, for the final production of L-ascorbic acid, L-galactono-1,4-lactone must move from the cytosol to the intermembrane space of the mitochondria, where the active site of L-galactono-1,4-lactone dehydrogenase (GLDH) is located (Mapson and Breslow, 1958; Imai et al., 1998; Pineau et al., 2008; Schertl et al., 2012; Schimmeyer et al., 2016). The fact that the oxidation of L-galactono-1,4-lactone is carried out in plants by a dehydrogenase instead of an oxidase (GULO) as occurs in animals, is not trivial. Contrary to paradoxical GULO activity, GLDH does not release H_2O_2 and therefore the production of ascorbate in plants does not have side effects over the redox state of the cell (Wheeler et al., 2015). Although some data support the existence of a side branch of the pathway that converges with that of animals (Jain and Nessler, 2000; Radzio et al., 2003; Maruta et al., 2010), there is strong evidence that most of the ascorbate in plants is produced through GLDH (Pineau et al., 2008). A recent phylogenetic study on the origin of GLDH identified an ancient paralog arisen from the original GULO, followed by a loss of paralogs (Wheeler et al., 2015). Thus, in species with the SW pathway, GULO has been functionally replaced by GLDH following chloroplast acquisition in photosynthetic organisms, since the presence of both proteins seems mutually exclusive (Wheeler et al., 2015). Interestingly, L-gulose, a previously mentioned rare sugar in plants and also a product of GME activity, is proposed to be transformed into L-gulono-1,4-lactone by as yet unidentified enzymes (Wolucka and Van Montagu, 2003). Supporting the presence of GULO activity in plants are (1) external supplementation of L-gulono-1,4-lactone in the growth media increased ascorbate levels in WT tobacco leaves (Jain and Nessler, 2000) and (2) the synthesis rate of ascorbate can increase up to 15% when L-gulono-1,4-lactone is externally supplied in Arabidopsis cell culture (Davey et al., 1999). One possibility is that GLDH also uses L-gulono-1,4-lactone as substrate. However, this seems unlikely since GLDH is highly specific for L-galactono-1,4-lactone (Mapson and Breslow, 1958; Oba et al., 1995; Østergaard et al., 1997; Rodríguez-Ruiz et al., 2017). Transgenic tobacco BY2 cells overexpressing several Arabidopsis homologs of GULO from rat resulted in increased ascorbate content in lines overexpressing GULO2, GULO3 and GULO5 but only after external application of L-gulono-1,4-lactone (Maruta et al., 2010). However, GULO has lower substrate specificity than GLDH and can catalyze the oxidation of other aldono-lactones, including L-galactono-1,4-lactone (Davey et al., 2000). Interestingly, the overexpression of rat liver GULO increased ascorbate levels in tobacco leaves (Jain and Nessler, 2000) as well as in Arabidopsis leaves, and rescued the Arabidopsis *vtc1* mutant ascorbate levels to WT (Radzio et al., 2003).

Alternative ascorbate biosynthesis pathways have been proposed in plants. One is through myo-inositol, following a pathway similar to animals, since the oxidation of myo-inositol oxidation produces D-glucuronate by a *MYO-INOSITOL OXYGENASE* (*MIOX*). Arabidopsis plants overexpressing *MIOX4* showed a 2-3-fold ascorbate content (Lorence et al., 2004). However, based on early radiotracer experiments (Loewus, 1963) and more recent reports (Endres and Tenhaken, 2009, 2011; Ivanov Kavkova et al., 2018), its contribution to the ascorbate pool remains unclear. The second is through the D-galacturonate pathway. In this pathway, a D-galacturonate reductase (GalUR) uses D-galacturonate, to produce L-galactonic acid that is converted to L-galactono-1,4-lactone, the last intermediate within the SW pathway (Mapson and Isherwood, 1956; Shigeoka et al., 1979).

In addition to its biosynthesis, the ascorbate pool also depends on its recycling by the Foyer-Halliwell-Asada cycle (Foyer and Halliwell, 1976; Asada, 1999) and degradation (Loewus, 1999; Green and Fry, 2005). Although the biochemistry of biosynthesis and recycling of ascorbate is well established, its degradation is not clear and might not follow a single pathway. In the apoplast, it can be degraded through the conversion of ascorbate to 2-keto-L-gulonic acid that leads to L-tartaric acid formation in cytoplasm, a compound important for fruit quality particularly in the *Vitaceae* family (DeBolt et al., 2006). Ascorbate can also be degraded

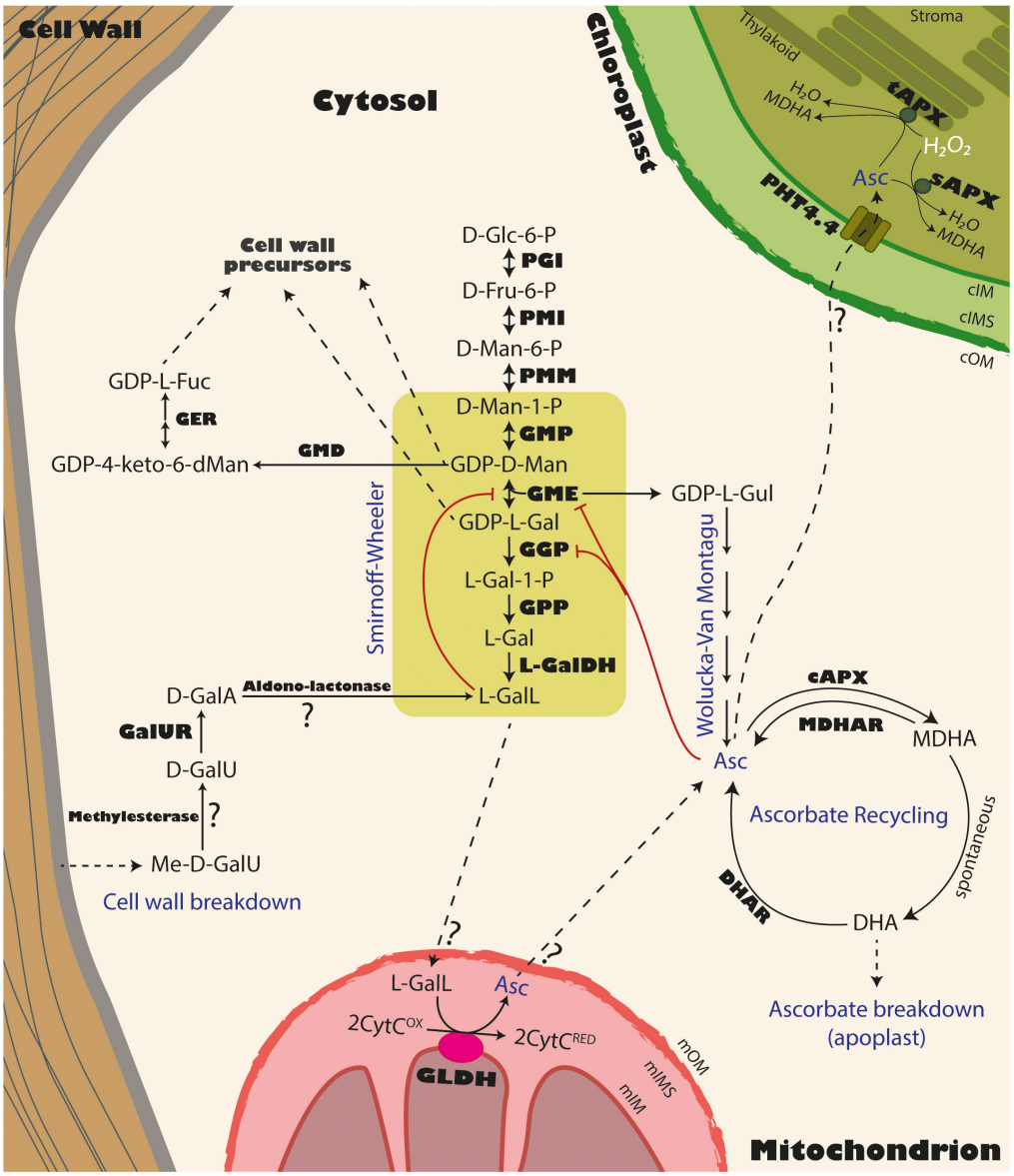


FIGURE 2 | Biosynthesis pathways of ascorbate in the plant cell. Solid lines represent the committed reactions within a pathway. Dashed lines represent the translocation of a molecule from a cellular compartment to another. Enzymes are displayed in bold: PGI, phosphoglucose isomerase; PMI, phosphomannose isomerase; PMM, phosphomannomutase; GMP, GDP-D-mannose pyrophosphorylase (Arabidopsis VTC1); GME, GDP-D-mannose-3′,5′-isomerase; GGP, GDP-L-galactose phosphorylase (Arabidopsis VTC2/VTC5); GPP, L-galactose-1-phosphate phosphatase (Arabidopsis VTC4); L-GalDH, L-galactose dehydrogenase; GLDH, L-galactono-1,4-lactone dehydrogenase; cAPX, cytosolic Ascorbate Peroxidase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; PHT4.4, inorganic phosphate transporter; sAPX, stromal ascorbate peroxidase; tAPX, thylakoidal ascorbate peroxidase; GMD, GDP-D-mannose-4,6-dehydratase (Arabidopsis MUR1/GMD1); GER, GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase (Arabidopsis GER1/GER2); GalUR, D-Galacturonate Reductase. Substrates and products are shown in regular shape: Glc, glucose; Fru, fructose; Man, mannose; Gal, galactose; Gul, gulose; GalU, Galacturonate; Me-D-GalU, methyl galacturonate; GalA, Galactonate; GalL, L-galactono-1,4-lactone; Asc, ascorbate; $CytC^{OX}$, oxidized cytochrome c; $CytC^{RED}$, reduced cytochrome c; MDHA, monodehydroascorbate; DHA, dehydroascorbate; GDP-α-keto-6-dMan, GDP-4-keto-6-deoxymannose; Fuc, fucose; mOM, mitochondrial outer membrane; mIMS, mitochondrial inter membrane space; mIM, mitochondrial inner membrane; cOM, chloroplastic outer membrane; cIMS, chloroplastic inter membrane space; cIM, chloroplastic inner membrane.

through the direct oxidation of DHA or through the oxidation of 4-O-oxalyl-L-threonic acid, leading to the production of both oxalic acid and L-threonic acid (Green and Fry, 2005). Additionally, it can also be degraded through the hydrolysis of DHA to 2,3-diketo-gulonic acid, and to oxalic acid and its esters, or to L-threonic acid under strong oxidative conditions (Parsons et al., 2011). In tomato, the main degradation products are oxalic acid, threonic acid and oxalyl threonic acid, but no tartaric acid has been detected (Truffault et al., 2017), suggesting that ascorbate degradation occurs mainly through DHA oxidation

rather than DHA hydrolysis, a pathway previously proposed in *Rosa sp.* cell cultures (Green and Fry, 2005). A broad perspective of ascorbate breakdown pathways in different species is provided by DeBolt et al. (2007).

BIOSYNTHESIS AND METABOLISM OF ASCORBATE IN FRUITS

Mutant analyses indicate that the SW pathway is the predominant if not the only pathway involved in ascorbate biosynthesis in green tissues (Dowdle et al., 2007; Lim B. et al., 2016). In heterotrophic tissues like fruits, the SW pathway is functional, as showed in several species including acerola, kiwi, strawberry, peach, tomato and apple (Badejo et al., 2009, 2012; Bulley et al., 2009; Imai et al., 2009; Ioannidi et al., 2009; Cruz-Rus et al., 2010; Mellidou et al., 2012a,b). However, depending on the fruit ripening stage, alternative pathways might become relevant, especially the D-galacturonate pathway (Mapson and Isherwood, 1956; Shigeoka et al., 1979), for which the degradation of cell wall pectin can provide abundant substrate (Agius et al., 2003; Cruz-Rus et al., 2010; Di Matteo et al., 2010; Badejo et al., 2012). Analyses of tomato introgression lines from a cross between *Solanum lycopersicum cv*. M82 and *S. pennellii* was used to find genetic elements associated with high ascorbate content in fruits. This was done through the identification of genes induced in the IL12-4 line, which contains 19.9 mg ascorbate/g FW, relative to *S. lycopersicum cv.* M82, which contains 12.2 mg ascorbate/g FW (Di Matteo et al., 2010). Interestingly, while genes of the SW pathway were not differentially expressed, a pectinesterase gene (TC177576) involved in breakdown of pectins was 4.4-fold more expressed in the IL12-4 line than in the parental M82. This result suggests that an additional supply of D-galacturonate due to cell wall degradation might be the cause of the ascorbate increase in this line. In addition, an ascorbate peroxidase (TC172881) was down-regulated in fruits of IL12-4 compared to M82 parental line, which may cause a higher ascorbate accumulation due to a lower degradation (Di Matteo et al., 2010). While the D-galacturonate pathway is more active as the fruit ripens, the SW pathway and ascorbate translocation from the leaves provide the bulk of ascorbate in fruits at immature green stage. The fact that the photosynthesis inhibitor DCMU diminished the pool of ascorbate only at green stage (Badejo et al., 2012) not only supports this, but also reinforces the tight relationship between the SW pathway and photosynthesis.

Considering the variety of functions that ascorbate exerts in plant cells and its tight regulation in green tissues, it is remarkable how variable the content of ascorbate can be among the fruits of different species (Davey et al., 2000) or even between varieties or cultivars from the same species (Cruz-Rus et al., 2011; Mellidou et al., 2012b). An obvious question is: what is the functional significance of this high variability in fruit ascorbate content? Fruit crops have different environmental requirements to optimize yield and, in addition, the pool of ascorbate is affected by abiotic factors such as light or temperature (Gautier et al., 2008; Zechmann et al., 2011; Suzuki et al., 2014), due to its role in the antioxidant cellular system (Jimenez et al., 2002; Massot et al., 2013). Therefore, small differences within species can depend on their cultivation requirements, harvest time or post-harvest conditions (Davey et al., 2007; Kevers et al., 2011; Oms-Oliu et al., 2011; Akhatou and Fernández-Recamales, 2014). However, the observed large differences in ascorbate content in closely related species likely have other causes. For example, several fold differences in ascorbate can be found between wild and cultivated tomato. Whereas domesticated tomato cultivars contain roughly 15 mg/100 g FW, wild varieties *S. pimpinellifolium* and *S. pennellii* contain around 40 mg/100 g FW (Lima-Silva et al., 2012) and up to 70 mg/100 g FW (Stevens et al., 2007), respectively. In fact, back-crosses with *S. peruvianum*, another wild species (Atherton and Rudich, 1986), have been shown to contain the highest amount of ascorbate in *Solanum* species, around 50 mg/100 g FW (Top et al., 2014). These wild tomato species grow naturally in Peru and Mexico, in coastal areas and river valleys less than 1000 m above sea level with abundant rainfalls. These two countries lie within the tropics of Capricorn and Cancer, respectively, with high irradiance and warm temperatures that may have favored the selection of individuals with high ascorbate content over time. Current evidence suggests that domestication of wild tomatoes by cross-breeding different species of *Solanum* started in these two countries (Esquinas-Alcazar, 1981) likely driven by the selection of higher fruit size and resistance to diseases like *Fusarium* wilt (Atherton and Rudich, 1986). However, the most important advances in tomato breeding have taken place during the last 200 years in Europe, mainly in France, Italy and England, with a strong participation of the United States since the early 1920s (Atherton and Rudich, 1986). It is likely that growing under more controlled and less harsh conditions has decreased the selective pressure to keep alleles conferring high ascorbate content, particularly because an apparent association between high ascorbate levels and low productivity has been reported in this species (Atherton and Rudich, 1986). However, in addition to the metabolic regulatory mechanisms that might explain these differences in ascorbate content, other factors such as water content must be considered. A known example is that of two tomato cultivars, Matador and Elin, subjected to salinity treatment. This increased their ascorbate content on fresh weight basis, but it was decreased on dry weight basis. In both cultivars, water and ascorbate content were reduced, but the loss of water was higher than that of ascorbate (Dumas et al., 2003). Fruit size and weight were directly related with water content, and they have been key traits selected during breeding programs, while this is not the case for ascorbate.

In most fruits, such as tomato, acidity decreases while sugar content increases during ripening (Gautier et al., 2008; Mellidou et al., 2012b). Major organic acids in tomato, contributing to fruit acidity, are malic and citric acids (Davies and Hobson, 1981). However, the change in ascorbate levels during fruit ripening is a trait dependent on the species. In tomato (Dumas et al., 2003; Gautier et al., 2008; Ioannidi et al., 2009; Badejo et al., 2012), grape (Cruz-Rus et al., 2010) and strawberry (Cruz-Rus et al., 2011), ascorbate content increases as the fruit ripens. This correlated with changes in the activity of enzymes affecting the redox state of the fruit during the breaker stage (Gautier et al.,

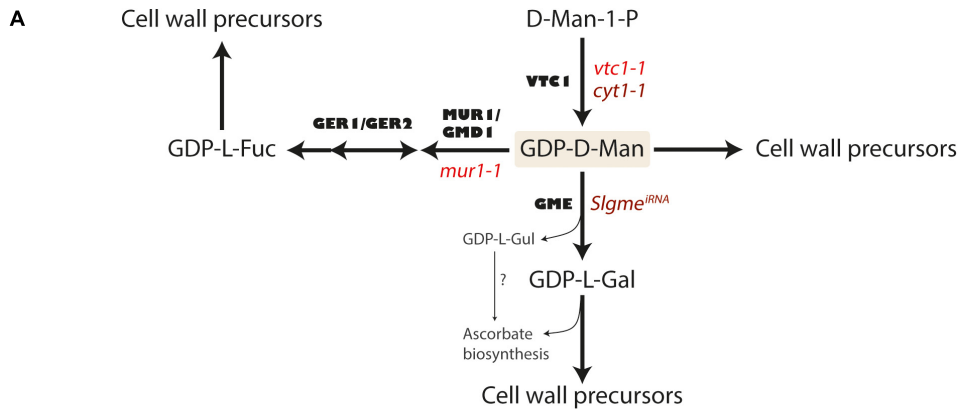
2008; Jimenez et al., 2002). Unlike tomato, grape and strawberry, kiwifruit showed a maximal ascorbate level at the immature green stage due to its high biosynthesis rate, it decreased as it ripened and then remained fairly stable until complete ripening (Li et al., 2010; Zhang J.Y. et al., 2018). In peach fruits, ascorbate content gradually decreased during ripening (Imai et al., 2009). In different studies, the pattern of ascorbate accumulation does not match the expression of a specific gene involved in ascorbate biosynthesis or recycling, and therefore there is no clear connection between the expression of biosynthetic genes and ascorbate content (Imai et al., 2009; Li et al., 2010; Lima-Silva et al., 2012). However, evidences gathered in these studies show that the overall size of the ascorbate pool correlated well with the oxidative status (i.e., activity of enzymes involved in redox state, H_2O_2 content) of the fruit, which is usually triggered at breaker stage (Jimenez et al., 2002; Gautier et al., 2008; Imai et al., 2009; Li et al., 2010).

RELATIONSHIP BETWEEN ASCORBATE AND CELL WALL BIOSYNTHESIS

A significant aspect of the ascorbate biosynthetic pathway is the intimate relationship shared with cell wall biosynthesis. Some of the early precursors of the SW pathway such GDP-D-mannose and GDP-L-galactose are among the non-cellulosic cell wall glycosyl residues forming pectins and hemicelluloses (**Figure 3**). For this reason, mutations or knock-downs in genes related to the early steps of the SW pathway lead to growth reduction or even arrest, due to impairment of cell wall formation during plant growth, including different stages of fruit development (Lukowitz et al., 2001; Hoeberichts et al., 2008; Mounet-Gilbert et al., 2016). Thus, knock-down mutants of the Arabidopsis *PMM* gene show between 20 and 50% of ascorbate levels relative to WT, altered protein N-glycosylation (specially a protein-disulfide isomerase post-translational modification, an abundant protein in the ER) and glycosylphosphatidylinositol (GPI) anchoring of proteins, leading to cell death after heat stress (Hoeberichts et al., 2008). Supplementation with L-galactono-1,4-lactone (Hoeberichts et al., 2008) or ascorbate (Cho et al., 2016) in the media recovered ascorbate levels but the mutants remained hypersensitive to heat. A null mutation in the Arabidopsis *GMP* gene (*cyt1* mutant) results in embryo arrest due to defects in *N*-glycosylation of proteins and altered composition of the cell wall (**Figure 3**; Lukowitz et al., 2001). The product of GMP activity, GDP-D-mannose, is used in the glycosylation of proteins, ascorbate biosynthesis and as a precursor of cell wall carbohydrates (Conklin et al., 1999). GDP-D-mannose is converted to GDP-L-galactose by the action of GME, but can also be converted to GDP-L-fucose by the sequential function of GDP-D-mannose-4,6-dehydratase (MUR1/GMD1; Bonin et al., 1997, 2003) and GDP-4-keto-6-deoxy-mannose-3,5-epimerase/4-reductase (GER1/GER2; Bonin and Reiter, 2000; Nakayama et al., 2003; **Figure 3**). All these three compounds, GDP-D-mannose, GDP-L-galactose and GDP-L-fucose are precursors of hemicelluloses and pectins (RG-II) when converted to D-mannosyl, L-galactosyl and L-fucosyl residues (Conklin et al., 1999; Reiter and Vanzin, 2001).

All the above evidences support the conclusion that a reduction in the production of GDP-D-mannose in the *cyt1* mutant is expected to have a significant impact on the structure of the cell wall. The importance of GDP-D-mannose in cell wall structure was further supported by the identification of the *mur1* mutant (Bonin et al., 1997). Mutations in *MUR1* produce a dwarf phenotype, mainly caused by a reduced content in fucose, since the supply of exogenous L-fucose reverted the dwarf phenotype (O'Neill et al., 2001). Interestingly, L-fucosyl residues in *mur1* cell wall xyloglucans are replaced by L-galactosyl residues (Reiter et al., 1993; Zablackis et al., 1996; Bonin et al., 1997). One possibility is that this substitution is the direct cause the dwarf phenotype of *mur1*. However, this does not seem to be the case since the Arabidopsis *mur2* mutant, affected in a xyloglucan-specific fucosyltransferase (*AtFUT1*), grows indistinguishably from WT despite having around 1% of the L-fucose content of the WT (Perrin et al., 1999; Vanzin et al., 2002). Moreover, the xyloglucans of jojoba seeds naturally contain L-galactosyl residues, and not fucosyl residues (Hantus et al., 1997; Pauly and Keegstra, 2016), suggesting that xyloglucan substitution of L-fucose by L-galactose residues is not the cause of growth impairment in *mur1*. In addition to this replacement of fucosyl by L-galactosyl residues in xyloglucan, the *mur1* mutant also has the same substitution in their RG-II fraction of pectins. In the RG-II structure, cross-linking mediated by boron is essential for a proper dimerization (O'Neill et al., 2001). Therefore, an alternative possibility was that changes in monosaccharide composition in the pectic RG-II *mur1* can impair this dimerization, which in turn would lead to dwarfism. In fact, the impaired dimerization in RG-II seems to be the cause of this dwarf phenotype because exogenous application of boron restituted the wild type phenotype (O'Neill et al., 2001). This is consistent with the finding that *mur2* mutants are neither affected in RG-II cross-linking nor L-fucose content (O'Neill et al., 2001). Furthermore, Arabidopsis *cgl* mutants, lacking the N-acetyl glucosaminyl transferase I in their Golgi apparatus (and hence, L-fucosylation), do not present altered growth (von Schaewen et al., 1993). Altogether, growth defects in *mur1* point to a structural defect which is due to impairment in RG-II dimerization, and not due to defects in protein fucosylation. However, defective interactions with different cell wall polymers cannot be completely ruled out, since the α-1,3-xylosyltransferase activity carried out by RGXT enzyme family, involved in RG-II synthesis, transfers D-xylose residues from UDP-xylose onto fucose (Egelund et al., 2006).

An additional link between ascorbate and cell wall biosynthesis comes from studies of tomato lines silencing *GME* (Gilbert et al., 2009; Mounet-Gilbert et al., 2016). Those lines with both copies of *GME* silenced showed reduced growth, higher fragility, lower fruit firmness and a 35–55% reduced ascorbate content in leaves and 20–40% of WT ascorbate levels in fruits (Gilbert et al., 2009). Consistent with the expected accumulation of GDP-D-mannose, the



B

	Cell Wall impairment	RG-II impairment (Boron rescue)	Asc (Asc rescue)
vtc1-1/cyt1-1	Defect in cellulose biosynthesis; reduced CW D-Man content/ desorganized CW	? (?)	25-30% (No) / ? (No)
mur1-1	Virtual lack of L-Fuc in non-cellulosic CW polymers	Impaired dimerization (Yes)	? (?)
Slgme^{iRNA}	Increase in mannan content	Impaired dimerization in gme2^{iRNA} and gme^{iRNA} lines, but not in gme1^{iRNA} lines (Yes)	Leaves: 40-60%, Fruits: 60-80%. gme1^{iRNA}and gme2^{iRNA} equal to WT (No)

FIGURE 3 | GDP-D-mannose and its biological relevance for ascorbate and cell wall biosynthesis in plants. **(A)** Reaction scheme for the novo synthesis of GDP-D-mannose in *Arabidopsis thaliana*. Mutants described for each step are indicated in lower case italic red letters. **(B)** Biological impairment over cell wall (RG-II, rhamnogalacturonate II) and ascorbate content in mutants of genes controlling the GDP-D-mannose pool. MUR1 and GMD1 encode two GDP-D-mannose-4,6-dehydratases. GER1 and GER2 encode two GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductases. The epimerase reaction is reversible whereas the reduction is not (Bonin et al., 1997). VTC1 encodes GMP, a GDP-D-mannose pyrophosphorylase, GME encodes a GDP-D-mannose-3′,5′-isomerase. D-Man-1-P, D-mannose-1-phosphate; GDP-D-Man, GDP-D-mannose; GDP-L-Gul, GDP-D-gulose; GDP-D-Gal, GDP-D-galactose; GDP-D-Fuc, GDP-D-fucose; Asc, Ascorbate.

silenced lines showed an increase in mannose-linked cell wall and defects in dimerization of RG-II by boron-mediated cross-linking, since phenotypic defects could be rescued by the application of external boron, but not with ascorbate (Gilbert et al., 2009; Voxeur et al., 2011; Mounet-Gilbert et al., 2016; Qi et al., 2017). All these results strongly suggest that this impairment has a cell wall structural basis rather than reduced ascorbate levels, similar to what was previously found in an Arabidopsis *mur1-1* mutant (O'Neill et al., 2001). Supporting this connection between ascorbate and cell wall biosynthesis at the GDP-D-mannose level, inactivation of GMP activity by knocking down Arabidopsis *KONJAC* genes involved in the activation of GMP resulted in reduced glucomannan content of cell walls and severe dwarfism (Sawake et al., 2015). The overexpression of *KONJAC1* caused a slight increase in ascorbate, whereas it resulted in a significant increase in the glucomannan content of plant cell walls, suggesting the presence of a mechanism that limits ascorbate accumulation.

This interaction between cell wall and ascorbate biosynthesis does not rely only on sharing common intermediates. As an enzyme cofactor, ascorbate is required for the activities of proline and lysine hydroxylases that, as previously mentioned, are involved in collagen biosynthesis in animals. In plants, proline hydroxylation is required for the production of hydroxyproline-rich glycoproteins (HRGP) such as arabinogalactans (AGPs) and extensins (EXTs). These proteins are part of cell wall structural glycoproteome acting as scaffolding components (Kishor et al., 2015; Marzol et al., 2018). AGPs are highly glycosylated HRGP proposed to function as cross-linkers of different cell wall polymers, thus conferring plasticity to the cell wall (Lamport et al., 2006). Recently, AGPs have been shown to be structural components of the cell wall by covalent attachment to pectins (rhamnogalacturonan I/homogalacturonate) and hemicelluloses (arabinoxylan), giving rise to the Arabinoxylan Pectin Arabinogalactan Protein complex APAP1 (Tan et al., 2013). In plants, EXTs have a role similar to that played by collagen in animals but contrary to collagen, EXTs can undergo *O*-glycosylation. This post-translational modification leads to oligo-arabinosylation of hydroxyproline residues that allow the formation of a three-dimensional network *in muro*, attaching to other cell wall components such as pectins (Hijazi et al., 2014; Kishor et al., 2015). Indeed, proline hydroxylation is the preceding step to *O*-glycosylation of extensins and arabinogalactans (Showalter and Basu, 2016). Overall, proline hydroxylase activity, promoted by ascorbate, is essential for cell wall assembly and stiffening. Conversely, ascorbate has been implicated

in fruit softening through non-enzymatic mechanisms, mainly by solubilizing pectins due to •OH radicals arising as a result of the Fenton reaction in the apoplast (Dumville and Fry, 2003). Because the architecture of pectins in the seed coat is important in interactions with other cell wall polymers (Turbant et al., 2016), this ascorbate-driven decrease in pectins might lead to seed abortion. These seemingly opposite effects of ascorbate in the cell wall can be explained by a fine-tuned regulation of the ascorbate content and its compartmentalization, aspects that are still poorly understood.

REGULATION OF ASCORBATE CONTENT

As an essential antioxidant, regulation of the ascorbate content is closely related with abiotic stresses that normally cause oxidative stress. High light in particular is translated into a ROS burst caused by an increased photoreduction and photorespiration. This, in turn, leads to increased ascorbate biosynthesis in order to detoxify these ROS (Asada, 1999). Low light, in contrast, causes a reduction of ascorbate. For example, *Arabidopsis* plants grown in continuous dark for 2 days only contained 20% of ascorbate relative to plants grown in light (Conklin et al., 2013).

Regulatory mechanisms that control ascorbate biosynthesis have been found at the level of transcription, translation, protein stability and activity for different components of the SW pathway. Light modulation of ascorbate content involves GMP stability (Wang et al., 2013), since GMP protein is degraded in the dark by the CONSTITUTIVE PHOTOMORPHOGENIC9-Signalosome subunit 5B (*CSN5B*; Wang et al., 2013). At the transcriptional level, low light decreases the expression of *GGP*, whereas high light causes its induction (Dowdle et al., 2007). Similarly, high light also induces the expression of *GLDH* in melon (Pateraki et al., 2004). NO treatment, which induces oxidative stress, causes an increase of *GLDH* mRNA levels in pepper (Rodríguez-Ruiz et al., 2017). At the activity level, *Arabidopsis* and barley plants exposed to high light showed an increment of GGP and GLDH activity (Smirnoff, 2000; Dowdle et al., 2007), A redox regulation has also been reported for the activities of L-GalDH in kiwifruit (Laing et al., 2004b), GME (Wolucka and Van Montagu, 2003) and GLDH (Leferink et al., 2009) in Arabidopsis. For GLDH in particular, Cys-340 has been identified as a redox-sensitive thiol residue required for an optimal conversion of L-galactono-1,4-lactone into ascorbate. This residue can be irreversibly oxidized by H_2O_2 unless it is previously S-glutathionylated (Leferink et al., 2009). This oxidation might be involved in the programmed cell death induced by some stresses like heat, since GLDH activity decreases during early stages of programmed cell death resulting in the inhibition of ascorbate biosynthesis (de Pinto et al., 2015). Therefore, the increased conversion of L-galactono-1,4-lactone to ascorbate under oxidative stress or high light might be an important control point of ascorbate biosynthesis (Smirnoff, 2000).

Probably, the best described regulatory control point of ascorbate biosynthesis is exerted by GGP (Laing et al., 2015). This study reports that the amount of GPP protein in Arabidopsis is controlled by a *cis*-acting upstream Open Reading Frame (uORF). Thus, at high ascorbate concentration there is a decrease of the translation of *GGP* mRNA, functioning as a negative feedback loop (Laing et al., 2015). More importantly, since this uORF has been identified in *GGP* genes from mosses to angiosperms, this ascorbate post-translational regulation is likely conserved throughout many plant species. Another possible control point exerted by ascorbate is L-GalDH, since the activity of this enzyme purified from spinach leaves is inhibited by ascorbate (Mieda et al., 2004). However, this is now under debate based on activity studies of the purified L-GalDH from kiwifruit (Laing et al., 2004b). The role of GLDH in ascorbate biosynthesis has also been studied during fruit development of tomato and pea. GLDH activity is inhibited by high ascorbate levels (Pallanca and Smirnoff, 2000; Mellidou et al., 2012b), a feedback control also found to affect GME activity in Arabidopsis (Wolucka and Van Montagu, 2003). Another link related with stress came with the finding that the activity of PMM is enhanced by a Ca^{2+}-dependent interaction with Calmodulin-Like 10 (CML10; Cho et al., 2016), of which the expression is boosted by H_2O_2 and biotic stress (Zimmermann et al., 2004). Accordingly, *Arabidopsis* transgenic lines expressing an artificial microRNA against CML10 fail to increase ascorbate levels under heat stress (Cho et al., 2016).

Other genes involved in the regulation of ascorbate levels are *AMR1* (Zhang et al., 2009) and *ERF98* (Zhang et al., 2012). AMR1 encodes an F-Box protein that represses the expression of virtually all the SW genes, particularly the expression of *GME* and *GGP*. Interestingly, this negative regulator of the pathway is barely expressed under high light conditions, pointing out the importance of the *de novo* biosynthesis of ascorbate in the response to light (Zhang et al., 2009). In contrast, *ERF98* is a positive regulator of the pathway since overexpression of this gene increase the content of ascorbate by enhancing the expression of genes of the SW pathway, in particular *GMP*, *GGP* and *L-GalDH*. Further analysis indicated that ERF98 can directly bind the promoter of the *GMP* gene (Zhang et al., 2012), supporting its regulatory role of the SW pathway.

An important aspect concerning ascorbate regulation is how it is distributed at the subcellular level. Cytohistochemical analysis, based on immunogold labeling and high-resolution immuno electron microscopy in tobacco and Arabidopsis leaves have shown that ascorbate is unevenly distributed at the subcellular levels (Zechmann et al., 2011). The estimated concentrations of ascorbate in Arabidopsis are: mitochondria (10.4 mM), chloroplasts (10.8 mM), peroxisomes (22.8 mM), nuclei (16.3 mM), vacuole (2.3 mM) and cytosol (21.7 mM) (Zechmann et al., 2011). In addition, low concentrations of ascorbate (0.002 mM) and DHA (0.36 mM) have been reported in the apoplast (Booker et al., 2012). These concentrations vary when plants are exposed to high light, which translates into an increase of ascorbate content in most cell compartments (Zechmann, 2011; Zechmann et al., 2011) with the exception of peroxisomes, whose content diminishes under high light. Interestingly, vacuolar ascorbate increases fourfold when

plants are exposed high light. This might be necessary to reduce the phenoxyl radicals that are oxidized by the high light associated-increase of H_2O_2 (Takahama, 2004). However, it is unknown whether the increase in ascorbate content in vacuole is due to the reduction of vacuolar MDHA through *trans*-membrane ascorbate-mediated electron transporters like cytochrome b561 (Griesen et al., 2004; Asard et al., 2013) or by direct transport of cytosolic ascorbate into the vacuole under high light using a transporter not identified yet.

Interestingly, despite the low concentration of ascorbate, the apoplast ratio of ascorbate/DHA ascorbate is important to determine the redox state of this compartment, which it turn controls redox-dependent signaling processes (Waszczak et al., 2018), such as stomata closure (Chen and Gallie, 2004) and chloroplast reprogramming leading to light acclimation (Karpinska et al., 2018). All these processes would be compromised if DHA and MDHA were not reduced back into ascorbate. Considering the little amount in the apoplast of glutathione and the enzymes in the Halliwell-Asada cycle other mechanisms must keep the redox homeostasis or the apoplast. First, apoplastic DHA produced by the spontaneous oxidation of MDHA enters the cytosol in exchange with ascorbate through facilitated diffusion using a yet-unknown protein (Horemans et al., 1996, 1997, 1998). Once in the cytosol, DHA is reduced to ascorbate by DHAR through the glutathione cycle. Second, MDHA is reduced to ascorbate in the apoplast by a cytochrome b-mediated *trans*-plasma membrane electron transport that uses cytosolic ascorbate as an electron donor (Horemans et al., 1994, 2000), which resembles the ascorbate restoration by electron transport across the tonoplast membrane (Asard et al., 2013), thus suggesting the involvement of cytochrome b561 in the reduction of apoplastic MDHA.

A similar question remains concerning MDHA and DHA reduction back to ascorbate in the thylakoid lumen. Taking into account the importance of luminal ascorbate in the maintenance of the functionality of the photosynthetic apparatus and energy dissipation (NPQ) commented above, MDHA and DHA must be reduced back to ascorbate. Since, to the best of our knowledge, there are no DHA reductases (DHAR) nor MDHA reductases (MDHAR) in the thylakoid lumen, other mechanisms should be involved. It has been shown that luminal DHA, produced by MDHA disproportionation in the lumen, crosses the thylakoidal membrane to the stroma (Mano et al., 1997), where it is reduced by the Halliwell–Asada cycle (Asada, 1999). The mechanism by which DHA crosses the thylakoidal membrane is not clear. Since no DHA transporter has been yet described in thylakoids (Foyer and Lelandais, 1996; Foyer and Noctor, 2011), the difference in DHA concentration between stroma and thylakoid lumen, and the lack of charge, would favor a high diffusion rate toward the stroma. On the other hand, ascorbate (newly synthetized and recycled from DHA) has to enter the lumen of the thylakoid. The diffusion hypothesis might also apply if the concentration of ascorbate in the stroma is much higher than that in the lumen, consistent with a non-active transport of ascorbate into the lumen previously reported (Foyer and Lelandais, 1996). However, unlike DHA, ascorbate has a negative charge making it a less suitable molecule to diffuse across the thylakoid membrane (Horemans et al., 2000). It has been recently reported that AtPHT4;4 transports ascorbate from the chloroplastic intermembrane space into the stroma (Miyaji et al., 2015). Interestingly, the homologous AtPHT4;1 is localized in the thylakoid membrane (Pavón et al., 2008) and its expression is modulated by light (Guo et al., 2008; Miyaji et al., 2015). Therefore, AtPHT4;1 is a good candidate to transport ascorbate across the thylakoid membrane.

APPROACHES TO INCREASE ASCORBATE IN FRUITS

Increasing ascorbate content in highly consumed fruits would clearly have an impact on human nutrition. A concomitant increase of ascorbate in tissues or organs that are submitted to oxidative stress, i.e., photosynthetic tissues, might have an additional beneficial effect on plant tolerance. However, whether or not ascorbate increases in fruit would have an effect on stress tolerance is not so clear, although is proposed that during fruit development and ripening oxidative stress might occur (Brennan and Frenkel, 1977; Rogiers et al., 1998; Jimenez et al., 2002; Huan et al., 2016). Most of the attempts used to increase ascorbate levels are based on biotechnology and basically consist in the overexpression of genes involved in different aspects of ascorbate metabolism (biosynthesis, recycling, or regulation). A second approach to increase the content of ascorbate would be through the selection of specific genomic regions that determine high ascorbate from a donor cultivar (or related species) and introgression into the cultivar of interest using molecular-assisted breeding (Singh and Singh, 2015). While in the first approach it is possible to use genes from different species and promoters that drive high or specific expression in desired tissues (Amaya et al., 2015), as far as the target species is amenable of transformation, the second approach relies in the identification of natural variants that can be used to inter-cross with these lines of interest. Although to date there are limited reports using this approach, the clear advantage is that these lines can be directly put into production because it does not involve transgenesis and therefore are not subjected to GMO regulation (Huang et al., 2016).

Biotechnological Approaches

There are abundant reports in the literature showing an increase of ascorbate in plants using biotechnological approaches (Valpuesta and Botella, 2004; Macknight et al., 2017; Mellidou and Kanellis, 2017). However, most of the studies have been performed in plants that do not produce edible fruits such as Arabidopsis, tobacco or rice and thus, most of the analyses were focused on vegetative tissues. Within fruits, tomato has been the preferred model due to its adoption as a model of fleshy fruits, its commercial value and the availability of efficient transformation protocols. The highest increase of ascorbate in tomato fruits reported so far has been about sixfold and was achieved by ectopically expressing *GGP* from kiwi (Bulley et al., 2012). Interestingly, the transgenic tomato lines with the highest increase of ascorbate showed fruits with developmental defects

and did not produce seeds (Bulley et al., 2012). A possible explanation is that an increase of metabolic flux to the synthesis of ascorbate had the effect of draining metabolites that are required for cell wall biosynthesis, particularly during seed development. Alternatively, this sharp increase of ascorbate might cause an increase in pectin solubilization (Dumville and Fry, 2003), which might provoke defects in seed development. Interestingly, in the same study, overexpression of *GGP* also caused an increase in ascorbate content of approximately twofold in strawberry without obvious defects during seed formation. There can be several explanations for these differences in fruit development, first the ascorbate increase in strawberry fruit is smaller, thus not being enough to solubilize pectins, second strawberry is a false fruit with the real fruits (the achenes) located outside the fleshy part, and third the composition of the cell wall surrounding the fruits might be different in terms of pectin composition.

Genes involved in ascorbate biosynthesis from alternative pathways have also been used to increase ascorbate content in tomato fruit. Three different studies in tomato have been published expressing the *D-Galacturonate Reductase* (*GalUR*) gene from strawberry (Agius et al., 2003). In two reports, overexpression of *GalUR* caused an increase between 2 and 2.5-fold, which resulted in enhanced tolerance to various abiotic stresses (Cai et al., 2015; Lim M.Y. et al., 2016). In the third study, *GalUR* is driven by the constitutive 35S promoter or the tomato fruit-specific *polygalacturonase* (*PG*) promoters (Amaya et al., 2015). In both cases, transgenic lines showed a modest (1.3-fold) increase of ascorbate content. However, a comprehensive metabolomic analysis indicated complex changes in metabolites as well as concomitant increase of total antioxidant capacity in transgenic tomato fruits, suggesting that the increase of ascorbate is associated with a tight regulation of the cellular redox state of fruits.

Other approaches have employed genes involved in ascorbate recycling or transcription factors involved in the regulation of genes of the SW pathway. Overexpression of the cytosolic *DHAR1* gene from potato increased the ascorbate content by 1.9-fold in transgenic tomato fruits (Li et al., 2012). Two additional reports using regulatory factors also show a modest increase of ascorbate in fruits. Identification and overexpression of SlHZ24, a transcription factor that binds the promoter of the tomato *SlGMP3* gene (Hu et al., 2016), caused a 1.6-fold increase of total ascorbate in tomato fruits at the breaker stage. Further analysis indicated that SlHZ24 also can bind *in vitro SlGME2* and *SlGGP* promoters, suggesting that this transcription factor can target multiple genes involved in ascorbate biosynthesis. The tomato *SlDof22* negatively regulates ascorbate accumulation in tomato, and reduction of the endogenous expression of this gene by RNAi increased the levels of ascorbate 1.3-1.6-fold in mature fruits. Transcriptomic analysis indicated that the *SlDof22* silenced lines had increased expression of several genes involved in the SW pathway and recycling of ascorbate (Cai et al., 2016). Further, the authors showed that *SlDof22* can bind the promoter of the tomato *SOS1*, a Na^+/H^+ antiporter involved in Na^+ homeostasis and essential for salt tolerance (Zhu, 2002). However, how the *SOS* pathway and the ascorbate biosynthetic pathway are connected remains elusive.

From a breeding perspective, the increases of ascorbate between 1.5 and 2-fold using biotechnological approaches in tomato here reported might not seem outstanding (Amaya et al., 2015; Cai et al., 2015; Lim M.Y. et al., 2016). However, considering the large consumption of tomato, its relatively low ascorbate and its high raw intake, we believe that the reported increments would have a positive impact from a nutritional point of view, more so considering the recent reports on the health beneficial effects of a rich ascorbate diet. The sixfold ascorbate increase reported by Bulley and coworkers (Bulley et al., 2012) would have a tremendous impact on ascorbate intake. Although the reported developmental defects make it unviable for agricultural use, from a scientific perspective it might be a useful model to investigate the role that high ascorbate has in tomato physiology.

Molecular Breeding and Genome Selection for Ascorbate Improvement

Improving fruit ascorbate content using marker-assisted selection requires prior identification of the genetic basis for natural variation of ascorbate. This can be achieved by genetic mapping and quantitative trait loci (QTL) analysis or genome-wide association studies (GWAS) in a developed mapping population, or alternatively in a diverse set of genotypes within the species, that are genotyped and phenotyped to determine molecular markers associated to specific traits (Mackay et al., 2009; Singh and Singh, 2015). Next, identified markers need to be validated for their application to select new cultivars with increased ascorbate content.

Several studies have shown that ascorbate content in fruits exhibit a quantitative inheritance, with several loci involved in ascorbate variation (Stevens et al., 2007; Zorrilla-Fontanesi et al., 2011). These studies have rarely identified the genes controlling the variation in ascorbate content, but they mark the genomic regions, and associated markers, and provide relevant information about the genetic architecture of the trait (how many loci and their quantitative contribution), as well as environmental effects. In some studies, candidate genes in those regions have been identified, with examples described below in apple, strawberry and tomato.

Apple

In this species (*Malus domestica*), a population derived from the cultivars "Telamon" and "Braeburn" was used to identify several QTLs for ascorbate content in fruit skin and flesh on linkage groups (LG) 6, 10, and 11 in the apple genome (Davey, 2006). The QTL identified on LG10 collocates with a major QTL controlling flesh browning (Sun et al., 2014). Four regions on LG 10, 11, 16 and 17 controlling ascorbate were detected over different years in another study using the same population (Mellidou et al., 2012a). Collocations between *GGP*, *DHAR* and a nucleobase-ascorbate transporter and some of the QTL were identified. In the case of *GGP*, allelic variations in two different *GGP* genes (*MdGGP1* and *MdGGP3*) were associated with ascorbate content (QTL on LG 11 and LG 10) both in the population and across commercial apple cultivars (Mellidou

et al., 2012a). In particular, differences in the expression of *MdGGP1* between fruits from high- and low-ascorbate cultivars indicate a key role for *MdGGP1* in the regulation of fruit ascorbate content (Mellidou et al., 2012a). An allele-specific SNP in this gene represents a promising tool for molecular breeding for enhanced fruit ascorbate content in apple. In the same study, the gene *MdDHAR3-3* was associated with a stable QTL for flesh browning on LG 17, suggesting that regulation of redox status of the ascorbate pool via DHAR is important for post-harvest fruit quality traits in apple. In agreement with this, transcriptomic studies revealed that prolonged post-harvest storage downregulated *DHAR* expression, resulting in the oxidation of ascorbate and thus enabling browning to occur (Mellidou et al., 2014). Therefore, besides the nutritional relevance of increasing ascorbate content in fruits, it has been shown that increased ascorbate is associated with improved post-harvest quality in fruits such as pear and apple (Davey, 2006; Mellidou et al., 2012a). For example, increased flesh browning in apple fruits is associated with the presence of a less reduced ascorbic acid pool (Davey, 2006).

Strawberry

Strawberry (*Fragaria* × *ananassa*) is the fruit with the highest global production among berries, reaching a value of over nine million tons (FAOSTAT see text footnote[1]), and it typically contains high ascorbate. However, ascorbate content varies widely between strawberry cultivars and *Fragaria* species, ranging from 10 to 80 mg/100 g FW (Cruz-Rus et al., 2011; Mezzetti et al., 2016). Using a biparental population of 95 F1 progenies derived from two strawberry breeding lines, three QTL explaining a total of 45% variation were identified on LG IV-2, LG V-1 y LG VII-1 (Zorrilla-Fontanesi et al., 2011). Two of the detected QTLs were stable in different years and candidate genes were identified based in orthologous positions in the diploid *F. vesca* reference genome. The gene *FaGalUR* collocated with the position of the stable QTL on LG IV-2 and a gene encoding a myoinositol oxygenase (*FaMIOX*) was located within the stable QTL on LG V-1 (Zorrilla-Fontanesi et al., 2011), although the role of this pathway remains controversial.

FaDHAR and *FaGMP* collocated with the QTL detected only 1 year on LG VII-1. Recently, a transcriptomic analysis by RNA-seq in pools of progeny lines contrasting in ascorbate content derived from the same population identified differential expression of gene *MANNOSE-6-PHOSPHATE ISOMERASE 1* (*FaM6PI1*) while *FaMIOX* was not differentially expressed (Vallarino et al., 2019). The *FaM6PI1* gene was also located within the confidence interval of the major QTL detected on LG V-I, and it is highly similar to the Arabidopsis *PMI* gene that encodes the first enzyme in the SW pathway (Maruta et al., 2008). Therefore, gene *FaM6PI1* was proposed as a candidate gene contributing to the natural variation in ascorbate content in strawberry.

Tomato

Several loci controlling ascorbate content have been detected using different populations derived from crosses between cultivated varieties (*S. lycopersicum*) and related wild *Solanum* species. Common genomic regions controlling ascorbate content have been identified on chromosomes 2, 8, 9, 10, and 12 (Stevens et al., 2007). In general, wild alleles increased ascorbate content and QTL were relatively stable across years or environments. The tomato gene *GME2* lies within the QTL interval on chromosome 9 (bin 9-J) and other candidate genes localized within QTL intervals were *MDHAR3* in bin 9-D, *GMP2* in bin 9-E, and *GLDH* in bin 10-E (Stevens et al., 2007). Further studies confirmed that this MDHAR activity was linked to ascorbate content in tomato fruits, which was found beneficial for an extended shelf life after chilling (Stevens et al., 2007). The role of MDHAR in governing ascorbate pool size was demonstrated through assessing expression and activity profiles throughout fruit ripening (Mellidou et al., 2012b). In an independent report, 163 tomato accessions were analyzed for several traits including ascorbate content by a GWAS approach and, again, significant SNPs associated to *MDHAR* were identified (Sauvage et al., 2014). All together, these reports indicate a relevant role of MDHAR in governing natural variation in ascorbate content in tomato.

Using transcriptomic analysis, a QTL detected in three trials on introgression line IL12-4 (*S. pennellii* in a *S. lycopersicum* background) was associated with up-regulation of genes involved in pectin degradation (Di Matteo et al., 2010). Further analyses of mutant variants and expression studies in introgression sublines from IL12-4 supported that pectinesterases might have a crucial role in determining ascorbate content in fruits of IL12-4 (Ruggieri et al., 2015). These studies suggested that ascorbate accumulation in IL12-4 fruits was achieved by increasing flux through the D-galacturonate pathway, as indicated above.

Recombinant Inbred Lines (RIL) have also been used to identify QTL/candidate genes linked to ascorbate content in tomato fruits. Thus, transcriptomic analyses in fruits of two groups of contrasting RILs suggested that ascorbate content co-regulates with genes involved in hormone signaling, and that they are dependent on the oxidative status of the fruit (Lima-Silva et al., 2012). Another study in tomato using the same RIL population, derived from the wild-relative *S. pimpinellifolium* TO-937, detected four QTL with a joint contribution of 42.1% to the variation of ascorbate content (Capel et al., 2015).

Melon

A limited number of genetic studies on ascorbate have been conducted in melon, although this fruit serves a significant source of this vitamin. There is considerable variation within the species. Ascorbate content in different varieties of the most widely consumed Cantaloupe and Honeydew melons range from about 10 to 29 mg/100 g FW, with the former types having higher content than the latter (Laur and Tian, 2011). This crop has a high global production (∼31 million tons; FAOSTAT) and it is also amongst the highest productions in the European Union (∼3 million tons; Eurostat, 2017). A single QTL for ascorbate has been mapped on LG 5 using different populations (Sinclair et al., 2006; Park et al., 2009). However, low reproducible RAPD markers were used in these studies, hampering their application in breeding programs.

Overall, the number of studies identifying QTLs affecting fruit ascorbate content is still rather limited to draw conclusions on common loci across different species. In order to effectively

introduce QTLs using marker-assisted selection in order to develop new fruit varieties with increased ascorbate content, loci must be validated in independent studies. Also, it is important to use additional populations and to perform the QTL analysis in different locations in order to determine QTL stability. To date, only natural variation in *GGP* and *MDHAR* alleles have been shown in independent studies to be useful in increasing ascorbate in apple and tomato, respectively (Stevens et al., 2008; Mellidou et al., 2012a). Pyramiding QTLs has the potential to increase ascorbate content, particularly in those cases when an individual QTL has a limited effect. There are already reports in which *S. lycopersicum* lines containing two chromosomal fragments from *S. pennellii* double the ascorbate content in ripe tomato fruit (Sacco et al., 2013; Rigano et al., 2014). Furthermore, with the recent establishment of high-throughput genotyping platforms, the selection of lines that include only specific genomic regions of interest will now be performed in a very efficient manner (Crossa et al., 2017).

CONCLUSION AND FUTURE PERSPECTIVES

The importance of ascorbate for humans has been recently highlighted through the characterization of its role in the activity of TETs and histone demethylases. Therefore, it is important to understand the mechanisms that determine the levels of ascorbate in fruits, a major source for ascorbate in human diet. An essential role for ascorbate in plants and animals is to maintain the oxidative status in the active center of several enzymes. It is also essential for scavenging ROS produced during photosynthesis. The identification of *vtc* mutants clearly highlighted an essential role of ascorbate in oxidative stress tolerance (Conklin et al., 1996). Ascorbate has additional roles during plant growth since early reports indicated that external application of ascorbate caused a significant increase in seedling growth and effects on cell division (Hausen, 1935; Havas, 1935), although the molecular mechanisms are not completely understood.

Most of the molecular studies have been performed in the model plant Arabidopsis *thaliana*, allowing the identification of all the catalytic steps of the SW pathway. However, with the exception of the established role of GGP as a key biosynthetic control step, very little is known about the factors that determine the final content of ascorbate in different tissues.

In fruits from different species or even within the same species large differences can be observed, with fruits that show extremely high content of ascorbate such as camu (Castro et al., 2015) and acerola (Badejo et al., 2009). How these fruits can accumulate such large amounts, or what is the advantage of having such a high content of ascorbate in these fruits is not known. As previously indicated, an important aspect of ascorbate is the close interconnection between its biosynthesis and that of the non-cellulosic cell wall components, which might hamper a proper understanding of the regulation of ascorbate biosynthesis. Since degradation of the cell wall is a common process during fruit ripening, alternative pathways such as that using D-galacturonate may have an important role in the final accumulation of ascorbate in this organ. Considering all this, it is important to extend the research to ascorbate-rich fruits to identifying regulators that determine high-ascorbate accumulation. An advantage is that the high conservation of proteins of the SW pathway among plant species makes it relatively easy to identify the orthologous genes. With the current genomic tools and high throughput sequencing technology, GWAS could be a good approach to identify these components. The use of segregating populations using contrasting parental lines can also be a good choice, considering the expedition of gene identification through combination of bulk segregant analysis (BSA), high-throughput next-generation sequencing, efficient SNP arrays, mapping by sequencing approaches (Takagi et al., 2013), or global gene expression studies (Amaya et al., 2016).

The CRISPR/Cas9 technology has greatly improved our capacity to engineer targeted mutations in eukaryotic genomes (Doudna and Charpentier, 2014). In tomato, CRISPR/Cas9 has been recently used to modify quantitative trait variation in some key agronomical traits such as fruit size, inflorescence number and plant size in tomato (Rodríguez-Leal et al., 2017). In a recent report, genome editing of the uORF of *GGP* in lettuce increased the ascorbate content by 1.5-fold, leading to oxidative stress tolerance (Zhang H. et al., 2018). A similar edition of tomato *GGP1* also led to an ascorbate increase of ∼1.5-fold in leaves (Li et al., 2018). Thus, a future trend will be to use genome editing to target gene determinants in either the *cis*-regulatory elements to modify their gene expression, substrate affinity, catalytic efficiency, generation of specific alleles or targeting interacting partners to modulate the ascorbate content in fruits. All this will be further facilitated by increasing sequence replacements via homologous recombination as has been already reported in Arabidopsis through CRISPR/Cas9 (Miki et al., 2018).

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REVIEW
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Modifications in Organic Acid Profiles During Fruit Development and Ripening: Correlation or Causation?

Willian Batista-Silva[1,2†], Vitor L. Nascimento[1,2†], David B. Medeiros[1,2], Adriano Nunes-Nesi[1], Dimas M. Ribeiro[1], Agustín Zsögön[1] and Wagner L. Araújo[1,2]**

[1] Departamento de Biologia Vegetal, Universidade Federal de Viçosa, Viçosa, Brazil, [2] Max-Planck Partner Group at the Departamento de Biologia Vegetal, Universidade Federal de Viçosa, Viçosa, Brazil

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National Engineering Research Center for Vegetables, Beijing Academy of Agriculture and Forestry Sciences, China

***Correspondence:**
Agustín Zsögön
agustin.zsogon@ufv.br
Wagner L. Araújo
wlaraujo@ufv.br

[†] These authors have contributed equally to this work

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The pivotal role of phytohormones during fruit development and ripening is considered established knowledge in plant biology. Perhaps less well-known is the growing body of evidence suggesting that organic acids play a key function in plant development and, in particular, in fruit development, maturation and ripening. Here, we critically review the connection between organic acids and the development of both climacteric and non-climacteric fruits. By analyzing the metabolic content of different fruits during their ontogenetic trajectory, we noticed that the content of organic acids in the early stages of fruit development is directly related to the supply of substrates for respiratory processes. Although different organic acid species can be found during fruit development in general, it appears that citrate and malate play major roles in this process, as they accumulate on a broad range of climacteric and non-climacteric fruits. We further highlight the functional significance of changes in organic acid profile in fruits due to either the manipulation of fruit-specific genes or the use of fruit-specific promoters. Despite the complexity behind the fluctuation in organic acid content during fruit development and ripening, we extend our understanding on the importance of organic acids on fruit metabolism and the need to further boost future research. We suggest that engineering organic acid metabolism could improve both qualitative and quantitative traits of crop fruits.

Keywords: carbon metabolism, development, fruit, metabolism, organic acids, primary metabolism, ripening

INTRODUCTION

True fruits are specialized plant organs found solely in angiosperms (i.e., flowering plants), and these unique organs are believed to have evolved to improve seed dispersal and protection (Karlova et al., 2014). The natural diversity of angiosperms, ranging from small herbs to massive trees, coupled with their extraordinary ability to grow in a wide variety of habitats, has resulted in their intimate association with humans. This is particularly true considering the human connection with the fruits and seeds of flowering plants and their economic and nutritional value. Although fruits are usually characterized as derived from a mature ovary containing seeds, many structures frequently called 'fruit' are, in fact, composed of a variety of other flower tissues types (Seymour et al., 1993, 2013).

The countless types of fruits present in angiosperms can be operationally organized within a few broad categories by using combinations of traits such as: (i) dehiscence or indehiscence; (ii) fleshy or dry exterior; and free (apocarpous) or fused (syncarpous) carpels (Seymour et al., 2013). These variations are further exemplified, for instance, by fleshy fruits, which have evolved by an enlargement of seed-surrounding tissues to create attractive flesh for seed-dispersing animals. Dry fruits, on the other hand, have a dry mesocarp that normally needs to open in order to release the seeds inside via mainly abiotic dispersal mechanisms (Fuentes and Vivian-Smith, 2009). It is tempting to suggest that this high diversity in fruit types is adaptive and associated to specific dispersers. This fact apart, the existence of significant correlations between fruit type and habitat conditions in angiosperms indicates that the evolution of fruit fleshiness is more likely associated with changes in vegetation habitats than in dispersers itself (Bolmgren and Eriksson, 2005). Both explanations are not mutually exclusive. In any case, fleshy fruit evolution is an important and continually recurring theme in the study of flowering plant evolution. However, caution should be exercised when making assumptions with respect to the adaptive value of particular fruit traits (Niklas, 2016).

Developmental stages of fruits can be divided in: (i) fruit set; (ii) growth; (iii) maturation; and (iv) ripening. Fruit set occurs during and after fertilization, which can be defined as the transition of a quiescent ovary to a rapidly growing young fruit and depends on the successful completion of pollination and fertilization (Hamamura et al., 2012). Additionally, in the absence of pollination and successful fertilization, levels of hormones such as auxins and gibberellins drop and the flower begins a terminal phase of senescence, ending in floral abscission – an effect that was closely associated with cellular pH in the abscission zone cells (Sundaresan et al., 2014). Parthenocarpy, another physiological event occurring in the absence of pollination, is characterized by intensive alterations of phytohormones such as auxin, gibberellin (GA), cytokinin or combinations thereof during fruit set (McAtee et al., 2013). In fact, exogenous application of these phytohormones alone can trigger fruit development including fruit set and fruit growth, to a certain extent, and their combinations would induce a normal fruit growth in the absence of fertilization (Srivastava and Handa, 2005; Mignolli et al., 2018). Accordingly, increased GA content or perception are associated with parthenocarpic fruits in tomato (*Solanum lycopersicum* L.) mutants such as *pat* (Mazzucato et al., 1998), *pat-2* (Fos et al., 2003), *pat-3/4* (Fos et al., 2001), whereas facultative parthenocarpic fruits are observed in the *procera* mutant (Carrera et al., 2012). Not only gibberellin but also auxin has been determinant in parthenocarpy in tomato fruits as shown in the mutants *pin4* (Mounet et al., 2012) *arf7* (De Jong et al., 2009), *arf8* (Goetz et al., 2007), *iaa9* (*entire*) (Wang et al., 2005). On the other hand, when pollination and fertilization take place, a cascade of events is triggered, leading to development of seeds and fruit growth.

During fruit growth, a signal, most likely derived from seeds (sources and sinks for cytokinin and auxin), induces neighboring tissues to expand, by both cell division and expansion, with a positive correlation between seed number and fruit size (Bohner and Bangerth, 1988). This fact apart, polyploidy, which is associated with cell expansion, is another important feature involved in the determination of fruit weight and size in tomato (Cheniclet et al., 2005). Additionally, there is a concomitant accumulation of storage products and sugars (Carrari and Fernie, 2006). Fruit maturation begins when growth stops, reaching the competence to ripen, but the ripening process itself is a subsequent step. Ripening is a complex process whereby several metabolic changes related to softening and flavor characteristics as well as organoleptic traits take place (Lira et al., 2016). The precise transition between all the stages of fruit development, including maturation and ripening, requires a high amount of energy. This energetic demand is provided by metabolic adjustments on the abundance of different classes of carbon compounds (e.g., organic acids, amino acids, and sugars) during development (Osorio et al., 2013). These metabolic changes from normal development toward fruit ripening are coupled with a generally brief stage of accelerated ripening that is normally associated with enhanced respiration (Osorio et al., 2013; Cosme Silva et al., 2017).

Fleshy fruits are characterized by a broad range of sizes, shapes, and colors. Moreover, different species presents unique flavor characteristics that are of pivotal importance in several processes. Such aspects are attractive to frugivorous animals, enhancing seed dispersal, and furthermore have become an indispensable part of the human diet (Barry and Giovannoni, 2007; Karlova et al., 2014). Fleshy fruits are quite diverse, ranging from grapes (*Vitis vinifera* L.) and tomatoes, which are derived from the ovary, (the so-called true fruits), through apples (*Malus domestica* L. Borkh) and pineapples (*Ananas comosus* L. Merril), to strawberries (*Fragaria x ananassa* Duch.), which are derived from the receptacle tissues or from expansion of the sepals (called pseudo- or accessory fruits) (Barry and Giovannoni, 2007).

Fleshy fruits have traditionally been classified as climacteric or non-climacteric, based on physiological differences observed within their respiratory pattern and reliance on ethylene biosynthesis during ripening. Climacteric fruits, such as apple, banana (*Musa paradisiaca* L.), papaya (*Carica papaya* L), and tomato (further details in **Table 1**) show an increase in respiration and ethylene production at the onset of the ripening process (Cherian et al., 2014; Karlova et al., 2014). On the other hand, non-climacteric fruits, such as citrus (*Citrus* spp.), grapes, melon (*Cucumis melo* L.), and strawberries (*Fragaria* spp.) do not show the respiratory burst and ethylene production remains at a basal level during the whole fruit development including maturation and ripening (Giovannoni, 2004; Cherian et al., 2014). During maturation, fruits go through dramatic transformations in color, aroma, nutrient composition, flavor, and firmness. Additionally, during this process, the production of reactive oxygen species plays an important role, for instance, in the biosynthesis of carotenoids and in the transformations of chloroplasts to chromoplasts (Li and Yuan, 2013). Barsan et al. (2012) have shown an intriguing metabolic shift coupled with disrupted thylakoid biogenesis machinery and elevated energy production during tomato fruit ripening. These authors have also shown a strong decrease in the abundance of proteins of light reactions (photosynthesis, Calvin cycle, and photorespiration)

TABLE 1 | Main sugars and organic acid found in both climacteric and non-climacteric ripe fruits.

Fruits	Main sugar	Main organic acid	Reference
Climacteric			
Apple	Fructose	Malate	Wu et al., 2007; Zhang et al., 2010
Apricot	Glucose/Fructose	Malate/Citrate	Gurrieri et al., 2001; Fan et al., 2017
Atemoya	Fructose/Glucose	Fumarate/Malate	Alique and Oliveira, 1994; Anaya-Esparza et al., 2017
Banana	Fructose	Malate	Morvai and Molnár-Perl, 1992
Blueberry	Glucose/Fructose	Citrate	Ayaz et al., 2001; Perini et al., 2018
Guava	Fructose	Citrate	Bashir and Abu-Goukh, 2003
Mango	Fructose	Citrate/Malate	Medlicott and Thompson, 1985; Cosme Silva et al., 2017
Papaya	Glucose	Citrate	Selvaraj et al., 1982; Souza et al., 2014
Peach	Glucose/Fructose	Malate/Citrate	Morvai and Molnár-Perl, 1992; Cirilli et al., 2016
Pear	Fructose/Sorbitol	Malate/Citrate	Zhen et al., 2016
Non-climacteric			
Blackberry	Fructose	Isocitrate	Fan-Chiang and Wrolstad, 2010
Grape	Glucose	Malate	Martínez-Esteso et al., 2011
Lemon	Fructose	Citrate	Asencio et al., 2018
Lima	Fructose	Citrate	Albertini et al., 2006; Asencio et al., 2018
Lychee	Sucrose/Glucose	Tartaric/Malate	Harvey et al., 1975
Longan	Sucrose/Fructose	Malate/Oxalate	Yang et al., 2009
Orange	Fructose	Citrate	Albertini et al., 2006
Pineapple	Sucrose/Fructose	Citrate	Luengwilai et al., 2018
Ponkan	Sucrose/Fructose	Citrate/Quinate	Albertini et al., 2006; Lin et al., 2015
Strawberry	Fructose/Glucose	Citrate	Lee et al., 2018

and carbohydrate metabolism (starch synthesis/degradation), mostly between breaker (∼35 days after anthesis) and red stages (55 days after anthesis), as well as an increase in terpenoid biosynthesis (including carotenoids) and stress-response proteins (ascorbate-glutathione cycle, abiotic stress, redox, and heat shock). All these transformations are the result of complex and dynamic processes that involve a series of molecular and biochemical changes under genetic regulation and/or in response to environmental perturbations (Osorio et al., 2013).

Due to their economic importance, organoleptic traits are recurrent object of investigations seeking to improve fruit quality (Chen et al., 2012; Tieman et al., 2017). Among the several characteristics that are clearly important for fruit quality, such as nutritional and sensorial quality (e.g., visual aspect, firmness, and taste), palatability is assumedly of major metabolic significance, once this trait is mainly dependent on the balance between organic acids (acidity) and sugar (sweetness) levels (Kader, 2008; Brasil and Siddiqui, 2018). These two classes of metabolites are directly connected to central carbon metabolism, where they are also involved in the biosynthetic route of diverse compounds such as amino acids, vitamins, and terpenic aroma volatiles, which influence fruit aroma (Lin et al., 2015; Beauvoit et al., 2018). The biochemical changes underlying fruit ripening and its regulation have been extensively studied in different fruit types (Giovannoni, 2001, 2004; Barry and Giovannoni, 2007; Osorio et al., 2013; Giovannoni et al., 2017). However, the role that organic acids play during this process is currently not fully understood. Are the complex organic acid profile changes over the course of fruit development simply a consequence of the process or do they play an active role in the sequence of events leading to fruit maturation? Here, we provide an overview of the latest discoveries and suggest future directions regarding organic acids metabolism during fruit development and ripening. We first discuss the general roles of organic acids during fruit maturation, we then focus on the metabolic behavior of those compounds and their relationship with both sugars and hormones during fruit development. Finally, we highlight the importance in studying organic acid metabolism during both fruit development and fruit ripening on different fruits and outline strategies to improve both qualitative and quantitative traits of crop fruits.

THE FUNCTIONAL DIVERSITY OF ORGANIC ACIDS: MORE THAN SIMPLE INTERMEDIARIES?

During fruit development, organic acids levels are usually inversely related to sugar levels. As such, during maturation and ripening, sugars accumulate, mainly due to sugar import or from starch degradation, whereas organic acids that accumulated in young fruits strongly decrease (Carrari et al., 2006; Carrari and Fernie, 2006; Fait et al., 2008; Mounet et al., 2009; Beauvoit et al., 2018). Malate and citrate are considered the most abundant organic acids present in both climacteric and non-climacteric ripe fruits (**Figure 1**). Particularly, malate accumulation and degradation occur differently in climacteric and non-climacteric fruits (**Figure 1**). Whilst some climacteric fruits use malate as a substrate during the respiratory burst, non-climacteric continue accumulating malate throughout ripening (Cherian et al., 2014). Interestingly, citrate levels are largely decreased during the

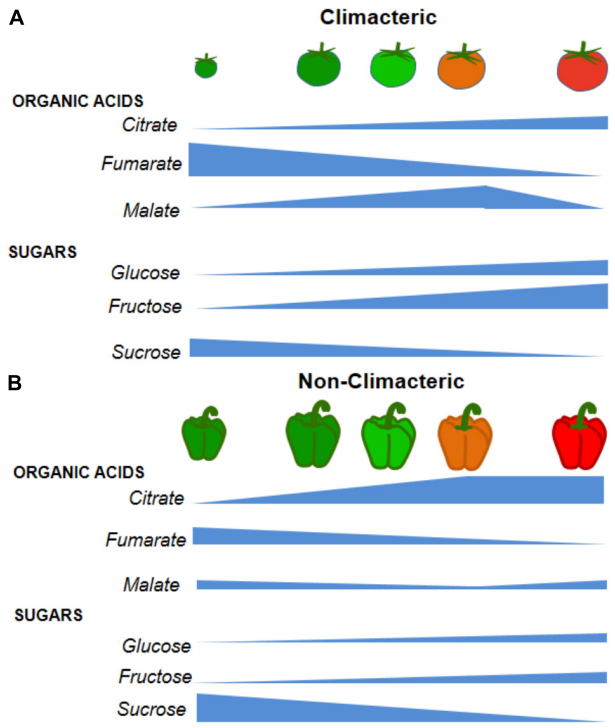

FIGURE 1 | Comparative metabolite accumulation during fruit growth in two significant examples of climacteric (tomato, *Solanum lycopersicum*) and non-climacteric (pepper, *Capsicum* spp.) species. **(A)** Organic acids and sugars changes that occur in climacteric fruits during different stages of development and ripening using tomato fruit as a model of climacteric fruit. The concentration of each metabolite is dependent on the metabolic intensity and the time of development of the fruit with major changes during the climacteric peak phase (approximately 35 days after anthesis). **(B)** Organic acids and sugar changes that occur in non-climacteric fruit during different stages of development and ripening using pepper as a model. Although lacking a climacteric peak, fruits considered non-climacteric present metabolite profile alterations during maturation, but with lower intensities than in climacteric fruits.

ripening process followed by decreases in malate as a respiratory substrate after the climacteric peak in papaya fruits (Manrique and Lajolo, 2004; Cosme Silva et al., 2017) (**Table 2**). Equally, during ripening of the non-climacteric orange and lemon fruits there is a decline in titratable acidity, mostly due to the catabolism of citrate (Iglesias et al., 2007; Agrumes et al., 2018) (**Table 2**). In fact, the metabolism and accumulation of organic acids in fruits are under both genetic and environmental control (Etienne et al., 2013). Moreover, through principal component analysis, the existence of a highly conserved change in the dynamics of metabolic processes during fruit development and ripening across species belonging to climacteric and non-climacteric groups has been recently demonstrated (Klie et al., 2014). Therefore, enhancing our current understanding of these factors and their interactions is of pivotal importance for fruit quality improvement.

The last decade has witnessed an intensive effort to enhance our understanding of the alternative functions of tricarboxylic acid (TCA) cycle components in addition to their recognized role as energetic intermediaries in plants (Millar et al., 2011). Most studies using transgenic approaches to investigate the role of TCA cycle intermediates, however, have been performed on vegetative organs, such as leaves and roots (Fernie and Martinoia, 2009; Araujo et al., 2012; Zhang and Fernie, 2018). The accumulation of TCA cycle intermediates is highly variable depending on plant tissues, developmental stages and environmental factors, most likely due to its direct link to organic acids export and photosynthesis regulation. However, the complex pathways through which organic acids are metabolized and precise details of how they are regulated *in vivo* remains, to date, insufficiently understood (Sweetlove et al., 2007; Fernie and Martinoia, 2009).

Organic acids can support numerous and diverse functions in plants. For instance, the C3 species Arabidopsis (*Arabidopsis thaliana*), soybean (*Glycine max*) and sunflower (*Helianthus annuus*) can accumulate high levels of fumarate (Fernie et al., 2004; Finkemeier et al., 2013). Higher levels of fumarate have been associated with the supply of carbon skeletons to support growth (Zell et al., 2010). Similarly, malate has not only an important role during photosynthesis in CAM and C4 plants (Fernie and Martinoia, 2009; Zell et al., 2010), but has also been associated with stomata regulation (Medeiros et al., 2016, 2017). Remarkably, malate and fumarate levels show similar diurnal changes to those of carbohydrates in some C3 plants, wherein they increase during the day and decrease during the night, suggesting that these organic acids can also function as transient carbon storage molecules (Fahnenstich et al., 2007). This fact apart, the contribution of organic acids to metabolic processes affecting fruit development and fruit quality remains to be elucidated. Dissecting these mechanisms is required to fully understand the key components underlying organic acid metabolism on energetic processes in fruit growth and development.

The function of TCA cycle intermediates have been extensively demonstrated in diverse aspects of plant growth (Nunes-Nesi et al., 2008; Araújo et al., 2014b) and in response to stress conditions (Sweetlove et al., 2010; Nunes-Nesi et al., 2014). Additionally, signaling functions have also been recently demonstrated for different TCA cycle intermediates from human (Yang et al., 2012) to plant (Finkemeier et al., 2013). Thus, citrate (Wellen et al., 2009), fumarate (Yang et al., 2012) and succinate are all involved in signaling in animal cells whereas also citrate (Gray et al., 2004) malate (Gray et al., 2004; Geigenberger and Fernie, 2014) and 2-oxoglutarate (Huergo and Dixon, 2015) were all recognized to play signaling functions in plants. Remarkably, the mode of action of these metabolites within the signaling network in which they are involved is variable (Wellen et al., 2009). For instance, it has been demonstrated that citrate regulates the expression levels of genes related to alternative respiratory pathways in both tobacco (*Nicotiana tabacum*) and Arabidopsis (Gray et al., 2004; Clifton et al., 2005). In addition, citrate, malate, and 2-oxoglutarate can affect nitrogen assimilation by controlling the abundance of Nitrate Reductase (NR) transcripts in tobacco (Müller et al., 2001). It is reasonable to assume, therefore, that TCA cycle intermediates are good candidates to play signaling roles in angiosperm fruit development as well as during fruit maturation and ripening.

TABLE 2 | Metabolic behavior of different sugars and organic acids present in the mesocarp of different fruit during growth and ripening under optimal growth conditions.

	Sugars			Organic Acids			
Fruits	**Glucose**	**Fructose**	**Sucrose**	**Citrate**	**Malate**	**Fumarate**	**Reference**
Apple	Increase	Increase	Increase	–	Increase	–	Ackermann et al., 1992; Wu et al., 2007; Zhang et al., 2010
Banana	Increase	Increase	Decrease	–	Increase	–	Morvai and Molnár-Perl, 1992
Grape	–	–	–	–	Increase	–	Sweetman et al., 2009
Guava	Increase	No change	Traces	Increase	Traces	–	Bashir and Abu-Goukh, 2003; Jain et al., 2003; Batista Silva et al., 2018
Kiwifruit	–	–	–	–	Increase	–	Walton and Jong, 1990; Cui-Cui et al., 2018
Lemon	Increase	Increase	Decrease	Increase	No change	–	Albertini et al., 2006
Lime	No change	Increase	increase	Increase	Increase	–	Albertini et al., 2006
Melons	Increase	Increase	Increase	–	–	–	Seymour and McGlasson, 1993; Karaman et al., 2018
Orange	Increase	Increase	Increase	Increase	No change	–	Albertini et al., 2006; Guo et al., 2016; Zhou et al., 2018
Papaya	No change	No change	Traces	Decrease	–	–	González-Aguilar et al., 2003; Souza et al., 2014
Peach	Increase	Increase	Decrease	Decrease	Increase	–	Cirilli et al., 2016
Pineapple	Increase	Increase	Few changes	Increase	Few changes	–	Saradhuldhat and Paull, 2007; Luengwilai et al., 2018
Plum	No change	Increase	Decrease	Increase	Increase	Increase	Famiani et al., 2012
Strawberry	Increase	Increase	Increase	Increase	Increase	–	Rafeii, 2017; Shanmugam et al., 2017
Tomato	Increase	Increase	No change	–	Increase	Decrease	Carrari and Fernie, 2006; Centeno et al., 2011; Osorio et al., 2012, 2013
Watermelon	Increase	Increase	Increase	–	–	–	Gao et al., 2018

Transgenic tomato plants with differential expression of all genes encoding TCA cycle enzymes have been generated and characterized (Nunes-Nesi et al., 2013). Collectively, this has allowed the generation of a thorough set of plant lines in which the activity of enzymes in the pathway are progressively decreased. The characterization of these plants has provided advances in our knowledge regarding the TCA cycle metabolic connections with other metabolic pathways (Fernie et al., 2004; Sweetlove et al., 2010). Indeed, these studies provided compelling evidence of the distribution of control in the plant TCA cycle. Moreover, they have also demonstrated that organic acids play important functions in the control of several important processes in connection with mitochondrial metabolism, including photosynthesis (Nunes-Nesi et al., 2005), carbon to nitrogen metabolism (Araujo et al., 2008), and redox balance (Igamberdiev and Bykova, 2018).

The signaling importance of TCA cycle intermediates might also rely on how exactly plant metabolism is reprogrammed following changes in their levels. For instance, reductions on the expression of aconitase (ACO) (Carrari et al., 2003) and malate dehydrogenase (MDH) resulted in reduction in both fruit size and yield. Years later, Nunes-Nesi et al. (2007) used transgenic tomato plants deficient in the mitochondrial fumarase activity (FUM) to show a strong effect on photosynthesis caused by impairments in the stomatal function followed by a subsequently reduced TCA cycle flux, affecting carbohydrate and organic acid oxidation at the whole plant level. Tomato plants with reduced expression of citrate synthase (CS) (Sienkiewicz-Porzucek et al., 2008), NAD-dependent isocitrate dehydrogenase (NAD-ICDH) (Sienkiewicz-Porzucek et al., 2010), cytosolic NADP-dependent Isocitrate dehydrogenase (NADP-ICDH) (Sulpice et al., 2010) and 2-oxoglutarate dehydrogenase (OGDH) (Araújo et al., 2012) showed no differences in either carbon assimilation or fruit yield. In contrast, a mild reduction in mitochondrial NAD-ICDH, as well as NADP-ICDH activity in antisense transgenic lines resulted in altered nitrate assimilation and pigmentation and amino acids contents, coupled with reduced fruit diameter and fresh weight, probably associated to source:sink alterations (Sienkiewicz-Porzucek et al., 2010; Sulpice et al., 2010). Additionally, changes in OGDH resulted in early senescence, coupled with significant alterations in metabolites pattern during fruit development (Araújo et al., 2012). Moreover, antisense inhibition of the Iron-Sulfur Subunit of Succinate dehydrogenase (SDH) was associated with enhanced fruit yield (Araújo et al., 2011) whereas SDH and MDH mutant plants were characterized by bigger fruits affecting fruit quality (Nunes-Nesi et al., 2005; Araújo et al., 2011; Centeno et al., 2011).

Collectively, these data provide compelling evidence that the metabolic connections associated with TCA cycle-related organic acids are responsible, at least partially, for such changes and

therefore an extensive metabolic reprogramming also occurs in fruits when changes in TCA cycle take place. It seems therefore tempting to speculate that the enzymes of the TCA cycle as potential target to further improve fruit quality. However, further demonstration of the importance of changes in organic acid levels in fruits are required to obtain a full comprehension of this process. This will most likely occur by the genetic manipulation of fruit-specific genes using fruit-specific promoters (Fernandez et al., 2009) to understand fruit maturation effects.

THE METABOLIC BEHAVIOR OF ORGANIC ACIDS DURING FRUIT DEVELOPMENT AND RIPENING

Fleshy fruit ripening is often characterized by a breakdown of stored carbohydrates to sugars coupled with reductions in acidity alongside with increases in flavor and aroma volatiles (Klee and Giovannoni, 2011; Cherian et al., 2014). It is accepted that organic acids are important in the control of fruit growth via cell expansion through water uptake (Liu et al., 2007). Accordingly, organic acids accumulation during the early stages of fruit development is directly related to the supply of substrates for the maintenance of respiration processes during the development (Seymour et al., 2013). Remarkably, different species including apples, berries, citrus, grape, kiwifruit (*Actinidia deliciosa*), peach, pepper, and tomato, present a highly similar metabolic pattern in which higher organic acids concentration are observed in the first stages of fruit development followed by clear reductions in their levels as maturation progresses (Nardozza et al., 2013; Osorio et al., 2013; Lin et al., 2015). Additionally, quantitative and qualitative variations of organic acids and sugars are usually observed in relation not only to cultivars and genotypes but also during maturation stages, affecting flavor without changes in fruit development and ripening (Xi et al., 2017). Accordingly, as in many other fleshy fruits, malate and citrate are the predominant organic acids identified in ripe peach (*Prunus persica* (L.) Batsch). The accumulation of organic acids is seemingly well regulated during fruit development and is differentially controlled during growth stages (Masia et al., 1992; Moing et al., 2000). By using six peach cDNAs encoding key proteins involved in organic acid metabolism and solute accumulation, Etienne et al. (2002) demonstrated that genes involved in organic acid showed a stronger expression during fruit ripening than during the earlier phases of development. Remarkably, their expression patterns were not necessarily correlated with the changes in organic acid contents (Etienne et al., 2002). The content of organic acids and soluble sugars was evaluated in apricot (*P. armeniaca* L. cv. Harcot), plumcot (plum-apricot hybrid, *P. salicina* × *P. armeniaca* L. cv. Harmony), plum (*P. salicina Lindl.* cv. Formosa), and peach (*P. persica* L. Batsch cv. Jinmi) (Haejin et al., 2014). Notably, organic acids increased mostly during the early stages of fruit growth and decreased until fruits were fully ripen, whereas sucrose, fructose, and glucose, but not sorbitol, increased during fruit development (Haejin et al., 2014).

By investigating a number of grapefruit (*Citrus paradisi*) cultivars produced in Turkey, Kelebek (2010) showed that, in most cases, sucrose was the predominant sugar, followed by fructose and glucose, while citrate was the most abundant organic acid, followed by malate, and that their content increased with ripening. The changes in sugars and organic acid concentrations in six different citrus cultivars Ponkan' (*C. reticulate*) and 'Satsuma' (*C. unshiu*), sweet orange 'Newhall' (*C. sinensis*) and 'Early Gold' (*C. sinensis*), pummelo 'HB' (*C. grandis*) and grapefruit 'Flame' (*C. paradise*) was recently analyzed (Zhou et al. (2018). This study has shown that the variations in sugar concentrations of this six citrus cultivars were relatively similar with sucrose as the major sugar component at every stage. Similar results were also previously found (Lin et al., 2015). Kafkas et al. (2007) additionally investigated different strawberry genotypes and found that fructose, the main sugar, increased during ripening. By contrast, the concentration of citrate is variable between genotypes, while the concentration of malate in all genotypes does not change during fruit ripening (Basson et al., 2010). Additionally, a wide comparison of sugars and organic acids content in different genotypes of strawberry, sweet cherry, long mulberry and small mulberry demonstrated that fructose and glucose were the major sugars found in the fruits, while citrate and ascorbate were the predominant organic acids in strawberry and mulberry, and tartaric acid in sweet cherry (Mahmood et al., 2012). In papaya, a climacteric fruit, four cultivars, namely Coorg Honey Dew, Pink Flesh Sweet, Sunrise, and Washington, were characterized according to their chemical composition and sucrose was the predominant sugar in all cultivars (Selvaraj et al., 1982). However, in cv. Washington an increased glucose content was observed 140 days after anthesis in comparison with the others cultivars. Additionally, no changes were observed in organic acids concentration in the different papaya cultivars (Selvaraj et al., 1982). Conversely, seven tomato cultivars were studied in relation to their compositional changes during different ripening stages. It was demonstrated that the sugar content was differentially modified according to the cultivars under different ripening stages with increases in all those seven cultivars most likely due to starch conversion to sugars, whilst fruit acidity was slightly increased in all cultivars (Kaur et al., 2006). Altogether, these studies suggest that sugars and organic acid levels can in fact be highly variable without impacting normal fruit development and ripening.

Over the past decade, much research effort has been devoted to understanding the metabolic behavior of several fleshy fruits ranging from physiology and biochemistry to broad molecular and genetics approaches. Thus, over the following sections we provide a detailed discussion about the organic acids behavior during fruit development as well as its relationship with other important metabolites, paying particular attention to sugars and hormones.

Organic Acids Versus Sugar Metabolism

The ripening process of fleshy fruits is characterized by coordinated changes in whereby fruit biochemistry and physiology are both drastically altered (Brady, 1987; Giovannoni, 2004; Giovannoni et al., 2017). These changes during ripening are typically variable according to the species and maturation stages as well as in response to stress conditions, often due to

changes in secondary metabolism, thereby potentially increasing plant defenses and the concentrations of compounds involved in plant protection (Miller et al., 1998; Giovannoni, 2004; Giovannoni et al., 2017). Nevertheless, the main modifications observed during ripening are associated with color and textural alterations coupled with modifications of sugars, organic acids, and volatile compounds (Giovannoni, 2004). Altogether, such modifications contribute to fruit flavor, especially by adjusting the balance between sugar and organic acids (Chaimanee and Suntornwat, 1994). Accordingly, the major respiratory substrates present in most fruits are carbohydrates and organic acids and both their nature and concentration largely affect organoleptic quality as taste, sight, and smell (Seymour et al., 2013).

Sugar accumulation has been intensely investigated during fruit development in different species under diverse conditions. Throughout ripening the vast majority of fleshy fruits are characterized by increases in sugar contents whereas organic acids decrease (Giovannoni et al., 2017). Citrus species are the exception to this metabolic 'rule,' especially at the peak of maturity or ripening. Global transcriptome analysis has suggested that during middle and later stages of citrus fruit development both carbohydrate synthesis and catabolism are mostly down-regulated while sugar transport appears to be rather operative. This can be deduced from the up-regulation of sucrose phosphate synthase (SPS), which in turn is correlated with total soluble solids (TSS) and the up-regulation of citrate synthase (CS) (Cercós et al., 2006; Wang et al., 2017). In parallel with fruit growth, sugars and organic acids are accumulated but in different stages of development. Glucose, fructose and sucrose increase in an exponential manner during cell division phase, reaching stable levels during final growth and ripening process. The content of citrate, the main organic acid found in citric fruits, increases upon cell division stage reaching higher levels in the middle of stage II and decreasing, mostly due to its catabolism, during ripening (Iglesias et al., 2007; Hussain et al., 2017). Both metabolic patterns and concentration are highly variable according to the species. In this vein, accumulation of sucrose, glucose, and fructose during ripening are especially observed in sweet fruits such as apples (Jakopic et al., 2018; Williams and Benkeblia, 2018), litchi (Yang Z. et al., 2013), melons (Burger et al., 2000; Huang et al., 2017), peach (Cirilli et al., 2016), strawberries (Shanmugam et al., 2017), mango (Cosme Silva et al., 2017), papaya (Paull et al., 1999) and watermelons (Liu et al., 2013) (**Table 2**). In general, sugar accumulation in fruit is directly controlled by increasing the activities of sucrose synthase (Suzy) and SPS (Chen et al., 2004).

Sucrose, glucose, and fructose are the most abundant carbohydrates and widely distributed food components present in plants. Their ratios vary considerably between fruits and, to a lower extent, in the same fruit according to maturation stage (Arena et al., 2013). Notably, the oxidation of such carbohydrates via glycolysis provides substrates for the TCA cycle during cell respiratory processes, contributing not only to the generation of intermediates such as organic acids, but also contributing to cellular energy supply (Osorio et al., 2013). Remarkably, during the climacteric stage, there is a large increase in the rate of substrate oxidation, mediated mainly by mitochondrial oxidases and, as result, there is an increased glycolytic flux. Interestingly, this enhanced flux has been associated with a close relationship between the activities of key glycolytic enzymes such as pyruvate kinase and phosphofructokinase in different fruits including apple, avocado, banana, and tomato (Rhodes and Wooltorton, 1967; Bennett et al., 1987; Beauvoit et al., 2018).

Similarly to sugars, organic acids are also able to support several facets of plant metabolism. Thus, the accumulation of organic acids in plant cells is highly correlated with other metabolic pathways and appears to be under the control of many factors (Lin et al., 2016). Both the organic acid type and its levels are extremely dependent of species, development stages, and the tissue analyzed. Although changes in content of organic acids are strongly fruit-dependent the most abundant organic acids in several fruits are citrate and malate (Romero Rodriguez et al., 1992), both being variable over different stages of fruit development (**Table 2**). Unlike soluble carbohydrates, which are imported into the fruit as photosynthate, the majority of the organic acids present in fleshy fruits are not imported but rather synthesized *in situ*, mostly from imported sugars from glycolysis mediating starch and cell wall degradation (Etienne et al., 2013). This is in good agreement with findings showing that starch accumulation plays an important role in determining the soluble solids content (°Brix index) of mature fruit, which is directly influenced by the activity of invertases, such as tomato LIN5 (Schaffer and Petreikov, 1997; Vallarino et al., 2017). Therefore, organic acids appear as highly valuable metabolites from a metabolic engineering perspective, once the organic acid-to-sugar ratio defines a range of quality parameters at harvest time in fruits.

By using integrative analyses of metabolomics and transcriptome during fruit ripening in ponkan (*Citrus reticulata*) fruits, it was showed that increases in sugars content are followed by considerable reductions in the content of organic acids (Lin et al., 2015). Perhaps more importantly, it was demonstrated that such behavior might be driven by SPS, asparagine transferases (AST), ATP-citrate lyase and glutamate decarboxylase (GAD) mediating shifts in sucrose metabolism from synthesis to degradation, which was regulated by the balance between SPS and SuSy activity (Lin et al., 2015, 2016). In addition, increased enzyme activity from both glycolysis and the TCA cycle during later maturation were observed, indicating that the flux is somehow changing from sucrose metabolism to organic acid metabolism, with citrate degradation occurring mainly through the gamma-aminobutyric acid (GABA) and acetyl-CoA pathways (Lin et al., 2015, 2016). It was further demonstrated that ponkan fruits under hot air treatment could activate citrate degradation via the GABA shunt especially by modulating aconitase, isocitrate dehydrogenase and glutamate decarboxylase cascade, but not the glycolytic pathway (Chen et al., 2012).

Positive correlation between malate levels and the expression of genes involved in starch synthesis has been observed in pepper (*Capsicum* spp.) fruits, meaning that malate metabolism most likely regulates transitory starch metabolism and that this process is probably conserved between climacteric and non-climacteric fruits (Osorio et al., 2012). Indeed, reduction of the activities of either the mitochondrial malate dehydrogenase (mMDH) or fumarase (FUM) in tomato fruits via targeted antisense

approaches have demonstrated the physiological importance of malate metabolism in the activation state of ADP-glucose pyrophosphorylase (AGPase), which is correlated with the accumulation of transitory starch and soluble solids at harvest (Centeno et al., 2011). However, due to the limited amount of information available on the connection between other organic acids metabolism and fruit quality, we cannot rule out a similar role for them during fruit development.

For certain fruits, citrate can be found in considerable concentrations. It is present from 8 to 15% (dry weight basis) in fruits such as strawberries and lemons (*Citrus lemon*). In both lemon and lime (*Citrus aurantifolia*) citrate accumulation can be as high as 8% of the fruit dry weight (Müller et al., 1996). The process involved in the metabolism and accumulation of citrate in mesocarp cells of fruits is under both genetic and environmental control. Several studies using different approaches as transcriptomics (Christelle et al., 2002), proteomics (Katz et al., 2011; Molassiotis et al., 2013) and metabolomics (Guo et al., 2016) have aided in the understanding of the different mechanisms involved in the control of the acidity and the quality of fruits. In addition, different agricultural practices including irrigation, nutrition (Kumar and Kumar, 2007) as well as controlled temperature (Burdon et al., 2007) can also impact the levels of fruit metabolites and as such the ratio between sweetness and acidity. However, there are no clear explanations for the changes observed in both malate and citrate in the cell.

To better understand how citrate metabolism is affected in Ponkan fruits, plants grown at low temperature and water stress in an open field experiment were compared to plants grown in optimal greenhouse conditions (Lin et al., 2016). It was observed that the expression levels of phosphoenolpyruvate carboxylase (PEPC), CS, ACO, and GAD were increased in response to low temperature, but not in water stressed plants compared to control conditions. These results, coupled with the changes in citrate levels under such conditions, indicated that low temperature may be a major factor influencing citrate metabolism during maturation in ponkan fruits. Similarly, it was observed that in sweet orange (*Citrus sinensis*), a non-climacteric fruit, the activities of the enzymes involved in organic acid metabolism including malic enzyme (ME), ICDH, ACO, and alcohol dehydrogenase increased during the first 3 weeks of post-harvest storage. Concomitantly, increased activity of enzymes involved in sugar catabolism such as hexokinase (HXK), Susy, UDPG pyrophosphorylase and PPi-dependent phosphofructokinase was also observed (Echeverria and Valich, 1989). Notably, these enzymes are necessary for organic acid usage and the subsequent oxidization of sugars in harvested sweet oranges (Echeverria and Valich, 1989). Pineapple is characterized by high contents of organic acids (**Tables 1**, **2**), primarily controlled by the activity of key enzymes such as CS, ACO, PEPC, MDH, and ME. In particular, ACO seems to play a major role in modifying the acidity in pineapple (Saradhuldhat and Paull, 2007). Altogether, it seems reasonable to assume that a very tight connection between sugar and organic acid metabolism occurs during fruit development. However, exactly how this metabolic regulation occurs still remains to be elucidated.

Studies with papaya (*Carica papaya*), a typical climacteric fruit, have revealed that the accumulation of sugar, especially sucrose, occurs between 20 and 30 days before physiological maturation. During this stage, there is a significant increase in acid invertase (AI) activity with lower SPS and Susy activities in papaya mesocarp (Zhou and Paull, 2001). It has been also demonstrated that after harvesting there was still sucrose synthesis, and more importantly that the SPS activity is highly correlated with the sucrose content, indicating the importance of this enzyme during the ripening in papaya (Gomez et al., 2002). Although in papaya fruits the main organic acids are citrate, malate, and ascorbate, their accumulation occurs to relatively low concentrations (de Oliveira and Vitória, 2011). Particularly, citrate and malate contents are reduced over the course of ripening (for details see **Table 2**) (Brekke et al., 1971; Souza et al., 2014; Silva et al., 2015).

Analysis of sugars and organic acids content during the development of peach (*Prunus persica*) fruits showed that malate, quinate and shikimate concentrations were high at the beginning but declined at the end of fruit development (Wu et al., 2005). Thus, citrate concentration was maximal in immature fruits, whereas increased sugars concentration, mainly sucrose, occurred in mature fruits. Interestingly, during peach ripening, sucrose degradation was accompanied by an increase of glucose and fructose levels coupled with distinct regulation of transcripts encoding neutral invertases (NI), indicating differential or non-redundant functions of each putative NI isoform in peach (Borsani et al., 2009). Enzymes such as NI and PEPC were identified as important components of the carbon metabolism operating during peach post-harvest ripening (Borsani et al., 2009).

Malate is the predominant organic acid in many fruits, including both climacteric and non-climacteric fruits such as plum (*Prunus salicina*), banana, tomato, grape and apple (Morvai and Molnár-Perl, 1992; Sweetman et al., 2009; Centeno et al., 2011). Citrate is the predominant organic acid in citric fruits like oranges, lime and lemon (Albertini et al., 2006). Notably, citrate is also found as the main organic acid in grape, guava, papaya, pineapple and strawberry as show in **Table 1** (Clements, 1964; Brekke et al., 1971; Jain et al., 2003; Batista Silva et al., 2018). Nevertheless, the accumulation and degradation of organic acids are not directly associated with respiratory and climacteric characteristics of the fruit. Thus, it is known that some climacteric fruits such as tomato appear to utilize malate during the respiratory burst (Goodenough et al., 1985). By contrast, banana and mango (*Mangifera indica*) continue to accumulate malate throughout ripening, even at the climacteric stage (Selvaraj and Kumar, 1989), whilst non-climacteric fruits also display widely varying malate accumulation and degradation (Sweetman et al., 2009). In addition, the accumulation of malate and citrate is seemingly a result of close interaction between metabolism and vacuolar storage and is also controlled by several environmental factors that affect the acidity of fleshy fruit by acting on various cellular mechanisms (Etienne et al. (2013). Taken together, the close relationship between organic acids and sugars metabolism during ripening seems to be a broadly connected factor that contribute in every way to the improvement of quality and

flavor during fruit ripening. For this reason, it seems clear that a more in-depth understanding of the assessment of sugars and organic acids content and the use of genetic and agricultural tools capable of changing this relationship is of pivotal interest to food experts and researchers. Collectively, these results indicate that alterations in genes involved in the organic acids metabolism can determine the quality and extension of shelf-life of non-climacteric fruits, which have their physiological changes reduced after being detached from the plant.

Organic Acids Versus Hormonal Control of Fruit Development and Ripening

Although hormones have been extensively studied as signaling molecules involved in different aspects of plant life cycle, it has been only recently that research has focused on understanding of their action during fruit development as well as in controlling sugars and organic acids metabolism in fruits during the ripening process (**Figure 2**) (McAtee et al., 2013; Karlova et al., 2014; Kumar et al., 2014). Accordingly, it has been demonstrated that hormones affect sugars and starch metabolism and that they can extend post-harvest life (Sagar et al., 2013a; Bastías et al., 2014; Karlova et al., 2014). However, there is a scarce literature available on the hormonal control of starch hydrolysis and the resulting sugar accumulation coupled with mitochondrial respiration (McAtee et al., 2013; Kumar et al., 2014). This fact apart, both the maturation and ripening have been associated in a number of studies to metabolic alterations involved with multiple genetic and biochemical pathways (Osorio et al., 2013). Although these changes have been observed in the context of responses to hormones (e.g., ethylene and ABA), the link between hormonal control and metabolite accumulation remains rather limited (Giovannoni, 2004; McAtee et al., 2013; Giovannoni et al., 2017).

The advent of 'omics' approaches has enabled significant progress in the characterization of hormone responses in fruits in general (Osorio et al., 2011). In addition, one important aspect of fruit development is the modulation of its metabolism, mainly driven by changes in sugars, organic acids and secondary metabolites immediately after fruit setting and partially recovering during or after ripening (Carrari et al., 2006). According to (Bapat et al., 2010), in climacteric fruit such as tomatoes, papaya, peaches, banana, apples, melon and other, ethylene synthesis plays a predominant role during ripening, and this still remains as one of the most studied hormones (**Figure 2A**). On the other hand, in non-climacteric fruits the respiratory burst and rise in ethylene production are not evident. It is widely accepted that although no single 'master controller' is able to control the ripening in non-climacteric fruits. Increased levels of different hormones like ethylene, abscisic acid (ABA), and brassinosteroids (BRs) have been suggested to promote ripening through complex interactions, whereas auxin delays some ripening associated process in those fruits (**Figure 2B**) (Fortes et al., 2015). Thus, we will now briefly discuss how changes in the main hormones involved in the process of formation and ripening of fruits impact organic acid metabolism, controlling fruit composition via crosstalk with other hormones or by themselves.

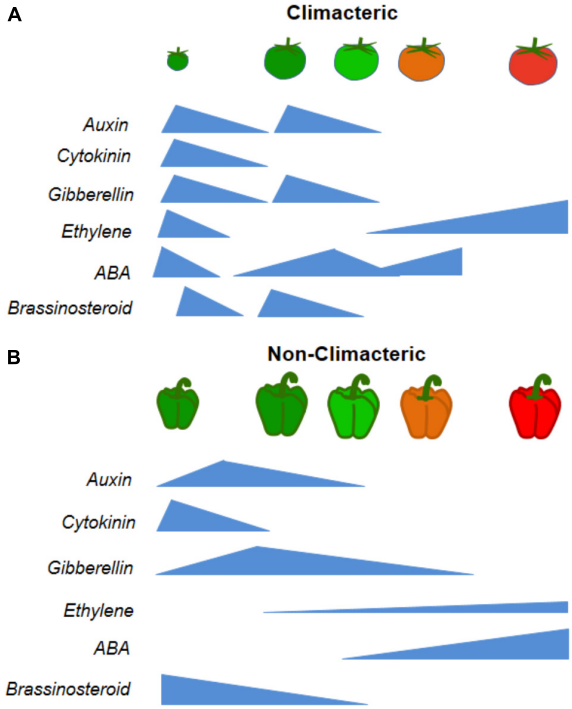

FIGURE 2 | Hormonal pattern changes of two significant examples of climacteric *(Solanum lycopersicum)* or non-climacteric (*Capsicum* spp.) during fruit development and ripening. **(A)** Differential hormones concentrations in tomato fruit during development: increases in auxin, cytokinin, gibberellin, and brassinosteroids at fruit set, followed by increases in auxin, cytokinin and gibberellin at fruit growth until maturation, which have their levels drastically reduced, with increases in ethylene and abscisic acid (ABA). **(B)** Differential hormonal patterns in non-climacteric fruit during development show increases in auxin, cytokinin and gibberellin during fruit set and growth, followed by reductions along the maturation phase, with increases in ABA and brassinosteroids, and few changes in ethylene.

Ethylene has been shown to control many ripening-associated metabolic pathways. It is involved not only in the expression of senescence associated genes and defense signaling, but also in fruit ripening, where the autocatalytic ethylene production leads to changes in cell wall metabolism, carotenoid accumulation, chlorophyll degradation, synthesis of volatiles compounds, and modulation of sugars and acids contents (Giovannoni, 2001; Alexander and Grierson, 2002; Osorio et al., 2013; Farcuh et al., 2018).

The importance of ethylene in the production of aroma volatiles has been also genetically demonstrated by the antisense suppression of ethylene production, which resulted in strong inhibition of aroma in melon (*Cucumis melo* L.) fruits (Ayub et al., 1996). The ripening of the climacteric fruit peach is largely controlled by ethylene and thus increase ethylene production leads to enhanced respiration coupled with changes in both chemical composition and physical characteristics of the fruit (Paul et al., 2012). Furthermore, enhanced ethylene biosynthesis are accompanied by increased levels of citrate, malate and glucose and fructose but decreased sorbitol and sucrose levels following harvesting (Borsani et al., 2009).

It has been also shown that ethylene is involved in an organ-specific manner in strawberry fruit ripening by differentially controlling the levels of amino acids, glucose, and fructose, as well as citrate and malate in the achene and the receptacle (Merchante et al., 2013). Strawberry plants with altered sensitivity to ethylene were used to unravel its role during fruit ripening process, as well as to further enhance our understanding of the modulation of metabolic pathways (Merchante et al., 2013). Similarly, it has been demonstrated that in grape, a non-climacteric fruit, ethylene seems to be also required for berry development and ripening (Chervin et al., 2004). Indeed, it has been suggested that ethylene could be triggering the onset of ripening. In fact, Ethylene Responsive Factor (*VviERF045)* from grape affects a range of different processes including, photosynthetic capacity, secondary metabolism, expression of key genes related to changes in epidermis and cuticle of the berry, cell expansion, as well as activation of several defense related genes (Leida et al., 2016).

Recent examples of cross-talk among different hormones have revealed a highly complex interplay of signals during grape development and ripening (Fortes et al., 2015). Accordingly, in non-climacteric fruits the responses of ethylene seem to be associated, via highly specific cross-talk, with other hormones such as ABA, auxin (Davies et al., 1997; McAtee et al., 2013) and BRs (Symons et al., 2006), all of which are known to play a functional role in grape berry ripening. Non-climacteric fruits may also display climacteric-like behavior following harvest (Katz et al., 2004). Similarly, differential expression of component of the ethylene-signaling pathway have been also observed in several non-climacteric fruits including citrus and grape (Tesniere et al., 2004; Trainotti et al., 2005).

The participation of ethylene in ripening has been extensively investigated in tomato fruits using several mutant that drastically affect the ripening processes of tomatoes fruits. For instance, *ripening inhibitor* (*rin*), *non-ripening* (*nor*), *green ripe* (*Gr*), *green-flesh* (*gf*), *colorless non-ripening* (*Cnr*), *never ripe* (*Nr*), *high pigment 1 (hp1*), *high pigment 2* (*hp2*), and *dark green* (*dg*) have been investigated in the context of ripening (Lanahan et al., 1994; Mustilli et al., 1999; Vrebalov et al., 2002; Levin et al., 2003).

Although the use of such mutants has clearly provided significant insights on the respective functional roles and also hierarchical regulation based on each gene (Giovannoni, 2004), the complete understanding of the ripening regulatory network remains rather fragmented. Nevertheless, by analyzing three dominant ripening mutants of tomato, *nor, rin, and Nr*, along the developmental and ripening periods it was possible to identify very strong correlations between ripening-associated transcripts and specific metabolite groups, such as organic acids from the TCA cycle, sugars, and cell wall-related metabolites, such as lipoxygenase, pectate lyase and poligalacturonase (PG) underlining the importance of these metabolic pathways during fruit ripening (Carrari et al., 2006; Centeno et al., 2011; Osorio et al., 2011). Organic acids, including the two TCA cycle intermediates malate and citrate, were strongly affected across ripening, suggesting that organic acids are regulated at the transcriptional level in climacteric fruit. Importantly, malate plays a crucial role in transitory starch metabolism in normal tomato fruit development and ripening and it seems that its regulation is also conserved in non-climacteric fruits (Centeno et al., 2011; Osorio et al., 2011, 2012, 2013). Additionally, in strawberry fruit organic acids including succinate, fumarate and 2-oxoglutarate displayed substantial changes during ripening, associated with a heavy demand for carbon skeleton components (Fait et al., 2008). In pepper fruit, citrate, dehydroascorbate, and malate are highly correlated to genes associated with starch and cell wall pathways as well as protein degradation, suggesting the importance of these organic acids during pepper fruit development and ripening (Osorio et al., 2012). Altogether, these results underlie the pivotal significance of the metabolic pathways associated with sugars and organic acids, revealing also multiple ethylene-associated events that occurs during climacteric and non-climacteric fruit ripening. It is important to mention that ethylene does not act regulating ripening alone but it rather works likely in conjunction with others phytohormones such as auxin, ABA, and cytokinin (McAtee et al., 2013; Kumar et al., 2014).

Future studies should therefore explore the hormone signaling network by combining model plant based knowledge on the molecular mechanisms involved in hormone signaling and the association with available genome information of other plant species. In apple fruit, the main organic acid is malate (**Table 1**). By using the 1-methylcyclopropene (1-MCP) (inhibitor of ethylene perception) it has recently been shown that ethylene is involved in the regulation of the levels of organic acid once this compound delayed the reduction of malate and citrate content during ripening (Lu et al., 2013).

In fruits with lower ethylene requirement to ripen, ABA appears to have a crucial role given its increase following ripening process (Setha, 2012). In strawberry, a non-climacteric fruit model, the effect of ABA has been investigated (Jiang and Joyce, 2003) demonstrating that endogenous ABA may play a role in changes of fruit color during ripening via an up-regulation of both ethylene production and phenylalanine ammonia-lyase (PAL) activity. In good agreement, compelling evidence suggest that exogenous ABA can significantly accelerate strawberry fruit ripening, most likely by the down regulation of 9-*cis*-epoxycarotenoid dioxygenase gene (*FaNCED1*) as demonstrated by virus-induced gene silencing (VIGS) leading to decreased content of ABA that can significantly retard the ripening (Jia et al., 2011). The *FaNCED1* is a predominant contributor to ABA accumulation during fruit ripening and it has been also evidenced that soluble sugars, especially sucrose, may act as a promoter to trigger ABA accumulation (Jia et al., 2011). The interaction between sugar and ABA has been recently reviewed by (Li et al., 2011), suggesting a core mechanism involved in the regulation of non-climacteric fruit ripening. Remarkable, reduced expression of *NCED* in strawberry resulted in delayed fruit maturation with changes in several metabolites such as organic acids and sugars implicating ABA in the control of fruit quality.

In climacteric fruits such as tomato and banana the levels of ABA increased before an increase in ethylene (McAtee et al., 2013). Remarkably, ABA signaling may also impact different aspects of fruit maturation (Sun et al., 2012). Exogenous ABA treatment consequently increase the ABA

content in both flesh fruits and seeds, triggering ethylene biosynthesis by the up regulation of ACS and ACO expression and therefore inducing fruit ripening (Zhang et al., 2009). It has been also suggested that *LeNCED1*, which initiates ABA biosynthesis at the onset of fruit ripening, can be the original inductor of ABA accumulation and might play a key role not only in the ripening process but also during the senescence of tomato fruits (Zhang et al., 2009). The suppression of the gene encoding *NCED1* resulted in a down regulation of several other genes involved in ripening process including the cell wall related PG and pectinmethylesterase (PME), which can also contribute to changes in TCA cycle intermediates (Sun et al., 2012). Although those studies collectively provided evidence that ABA is involved in fruit maturation, it still remains unclear whether ABA acts directly or via altering ethylene levels, given the already well established cross-talk between those hormones.

Overexpression of ABA-responsive elements *SlAREB1* in tomato, resulted in increased content of organic acids (e.g., citrate and malate), hexoses, hexose-phosphates, and amino acids in immature green, mature green, and red ripe fruits (Bastías et al., 2014). These modifications correlated with an up-regulation of genes encoding enzymes involved in carbohydrate and amino acid metabolism suggesting a possible role for this transcription factor in the regulation of fruit organoleptic properties (Bastías et al., 2014). Whether modification of the expression of other enzymes of the TCA cycle involved in the synthesis of organic acids and amino acids are affected by *SlAREB1* remains to be determined. In tomato fruits, organic acids are a crucial quality determinant during ripening process and flavor, and correlate with the expression of genes associated with ethylene and cell wall metabolism-related pathways (Carrari et al., 2006; Carrari and Fernie, 2006; Osorio et al., 2012). Further studies are clearly required to elucidate the real mechanism connecting ABA and fruit ripening as well as metabolites changes and fruit quality.

It is currently accepted that auxin participates in various processes ranging from fruit formation to ripening, mainly via a crosstalk between gibberellins and ethylene (McAtee et al., 2013). In fact, auxin coordinates the ethylene synthesis consequently the ripening process (Li et al., 2016). Accordingly, genes related to carotenoid metabolism, cell degradation, and energy metabolism were strongly down-regulated by exogenous auxin further impacting tomato ripening (Su et al., 2015). Recently, RNA-Seq analysis of tomato fruit following exogenous auxin application has shown that several genes involved in the TCA cycle and oxidative phosphorylation pathway were significantly down-regulated indicating that auxin affects fruit ripening by impacting mainly fruit respiration rate (Li et al., 2016). Moreover, auxin-treated fruits were characterized by increased levels of citrate, succinate and malate which indicate that auxin application seems to enhance fruit acidity (Li et al., 2017). Furthermore, exogenous auxin altered the expression patterns of ethylene and auxin signaling-related genes during ripening, suggesting a significant crosstalk between these two hormones during tomato ripening (Li et al., 2016). Recently, an important role for auxin during ripening as a modulator of the levels of sugar and organic acids has been demonstrated in tomato fruits (Sagar et al., 2013a; Hao et al., 2016; Li et al., 2017).

Recent studies have also demonstrated that loss or gain of function of several auxin response genes, such as *SlARF4*, *SlARF2a*, *SlIAA17* and *SlIAA27*, leads to conspicuous changes in fruit pigment accumulation, sugar content, starch accumulation, phenylpropanoids component, organic acids contents and other fruit quality attributes (Bassa et al., 2013; Sagar et al., 2013a,b; Hao et al., 2015; Su et al., 2015). By using a transcriptome analysis approach Li et al. (2016) have suggested that exogenous auxin retards tomato ripening process and interferes on the normal expression patterns of many genes involved in metabolic pathways. More recently, Li et al. (2017) analyzed the metabolic changes following exogenous auxin showing that besides metabolites such as sugars and amino acids, a total of nine organic acids were detected in tomato fruits under different developmental stages. Briefly, higher contents of succinate and ascorbate when compared with control samples were observed 10 days after auxin treatment. Notably, auxin seems to affect citrate levels keeping it higher than in control fruits at the end of ripening, indicating that auxin application might increase fruit acidity, affecting sour taste of fruit (Li et al., 2017).

Over the last decades have witnessed the characterization of numerous mutants for synthesis or signaling of several hormones in different model species such as Arabidopsis (Gazzarrini and McCourt, 2003), tomato (Carvalho et al., 2011), and rice (Yang D.-L. et al., 2013). This resource, coupled with the integration of transcriptomics and metabolomics approaches, has greatly enhanced our understanding of the molecular and biochemical events associated with ripening in both climacteric and non-climacteric fruits. However, despite our current understanding of how organic acid metabolism is associated with hormones metabolism, the exact mechanisms underlying their interaction during fruit ripening clearly require further elucidation.

The role of GA is well established during fruit-set and fruit development, controlling the cell expansion and it has been revisited recently (McAtee et al., 2013; Obroucheva, 2014). However, there is evidence that GA can delay tomato fruit ripening by preventing some of the changes triggered by ethylene. Unfortunately, relatively little work is current available directly connecting gibberellins and metabolic changes in fruits. Nevertheless, some advances were observed on this theme. Accordingly, the impacts of GAs on primary metabolism have been also previously demonstrated in tomato plants with reduced levels of the TCA cycle enzyme 2-OGDH (Araújo et al., 2014a). In the same vein, it has been observed that gibberellic acid (GA3) causes ripening delay in citrus (Biale, 1978) and mango fruits, reducing the ascorbate content (Kader, 2008). In strawberry, GA3 showed an inhibitory effect on fruit ripening, evidenced by a decrease in the respiratory activity (Martínez et al., 1994). GAs seems to affect the primary metabolism mediated by changes in 2-oxoglutarate, thus linking TCA cycle function with amino acid, glucosinolate, flavonoid, alkaloid, and gibberellin biosynthesis (Araújo et al., 2014a). Defining the precise nature of the interaction between organic acids coupled with the GA-mediated regulation of fruit clearly remains an exciting topic for future research.

Due to the multifunctionality of BRs, more attention has been given to their association with fruit ripening recently. BRs plays an important regulatory role in various physiological processes, including growth, seed germination, flowering, changes in enzymatic activities, and fruit set (Clouse and Sasse, 1998; Khripach et al., 1998; Sánchez-Rodríguez et al., 2017) and has been recently associated with fruit ripening. Exogenous BRs analogs on endive (*Cichorium endivia* L.) play an important role in increasing the contents of the organic acids such as citrate, oxaloacetate and succinate (Mario et al., 2013). Mazorra Morales et al. (2014) showed the interconnection between BRs and ethylene in the regulation of the mitochondrial electron transport chain in post-harvest of papaya fruits. The authors showed that exogenous BRs application affect the AOX-dependent electron transport, which is antagonized by ethylene, suggesting that, BRs and ethylene act antagonistically regulating the AOX capacity during papaya ripening. The role of BR application has been also investigated in strawberry, suggesting it as an important molecule to improve qualities traits, mainly by increasing soluble solid contents, inducing sugar and organic acids content, as well as the production of secondary metabolites such as anthocyanin and phenolic compounds (Rafeii, 2017). The role of BRs during fruit ripening has been also investigated in various fruits such as tomato (Vidya Vardhini and Rao, 2002), grapes (Symons et al., 2006), papaya (Mazorra et al., 2013), strawberry (Mohammadrezakhani et al., 2016), and mango (Zaharah et al., 2012). Notably, it is directly related with an extensive crosstalk with ethylene levels, affecting numerous processes. However, relatively few studies have clearly demonstrated the impacts of this phytohormone on primary metabolism and specifically at the organic acids, although it is possible to observe that their content usually increase in presence of exogenous BRs, which can be an interesting avenue for research.

Polyamines (PAs), another group of signaling molecules, has been extensively studied in recent years. PAs are small aliphatic amines with an important role in plant growth process including fruit ripening and (Walden et al., 1997; Guo et al., 2018; Wuddineh et al., 2018). In plants, PAs are initially converted from glutamate, a key amino acid involved in N assimilation, to putrescine (Put), then converted to spermidine (Spd) and, in the end, to spermine (Spm) by the action of Spd synthase (SPDS) and Spm synthase (SPMS) being further decarboxylated to *S*-adenosyl-L-methionine (dcSAM) generating SAM as a reaction product which is catalyzed by SAM decarboxylase (SAMDC) (Wen-Wei et al., 2006; Michael, 2016; Guo et al., 2018). SAM is a common precursor for both PA and ethylene biosynthesis but their physiological functions are distinct at times and can be antagonist mainly during senescence (Pandey et al., 2000).

Accordingly, Gupta et al. (2013) showed that silencing of 1-aminopropane-1-carboxylate synthase gene (*SlACS*) delays ripening simultaneously improving fruit quality in tomato and increasing the PAs levels associated with down-regulation of cell wall hydrolyses. Notably, still in tomato fruit, PAs has been identified as a great contributor of fruit ripening mainly associated with the activity of both ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) (Rastogi and Davies, 1990, 1991). In this vein, Pandey et al. (2015) demonstrated that the overexpression of ODC triggers the biosynthesis of Put, Spd and SPM which, in its turn, inhibits ethylene production delaying fruit ripening, but enhances tomato fruit quality traits. Additionally, overexpression of *SPDS* in tomato promote fruit ripening, increasing sugars content, as well as lycopene coupled with ethylene production (Neily et al., 2011). In grape fruits, PAs also have an important role in the aroma development (Fortes et al., 2015), while in peach it plays a key role in fruit firmness and soluble sugar content followed by an abrupt decreased in Put during post-harvest (Liu et al., 2006). Application of exogenous Spr in peach fruits reduced ripening by impacting ethylene and auxin metabolism and signaling (Patrizia et al., 2012). Remarkably, polyamines are reported to be important molecules involved in strawberry ripening (Tilak and Raymond, 1996). Recently, demonstrated that PAs, especially spermine (Spm), regulate strawberry fruit ripening in an ABA-dominated, IAA-participating and ethylene-coordinated manner controlling several physiological parameters, including firmness, and the content of anthocyanin, sugar, polyamine, auxin (IAA), abscisic acid (ABA), as well as ethylene emission. Notably, these changes are coupled with alterations in *FaSAMDC* expression which can promote and inhibit ripening (Guo et al., 2018). PAs play also important functions in several others fruits in a manner which may generate controversial conclusions, thus it is important to mention that, more studies are required to further understand the significance and roles of PAs in dry and fleshy fruit development.

Lastly, but not least important, very few studies have demonstrated the association between salicylic acid (SA) and the maintenance of fruit quality during post-harvest. Sweet cherry treated with exogenous SA revealed an effective and environmentally friendly tool to maintain fruit quality during storage associated with the maintenance of the sugar and organic acid content in the fruit as well as with enhancements of both the concentration of bioactive compounds concentration and the antioxidant activity (Giménez et al., 2016). Interestingly, SA also culminated with delays on ripening process in banana (Srivastava and Dwivedi, 2000), sweet cherry (Yao and Tian, 2005), and kiwifruit. In all the above mentioned results, SA seems to act inhibiting fruit ripening, mainly by reducing not only the respiratory rate but also sugar and total acid content. It seems reasonable to assume that coupling the application of such hormones or chemical compounds with both molecular and metabolic analysis in order to provide information concerning the role of hormones in the regulation of fruit taste should greatly facilitate advances in our understanding of the metabolic control mediated by hormones in fruits.

FUTURE AVENUES FOR UNVEILING THE ROLE OF ORGANIC ACIDS METABOLISM DURING FRUIT DEVELOPMENT AND RIPENING

Although changes in the levels of organic acids are unequivocally important during fruit ripening, it seems necessary to study post-harvest physiology in more realistic environments, which

means creating links with companies involved in fruit storage and transport and breaking down the variables that affect organic acid content and other important traits. The understanding of the primary metabolism in fruit is directly connected with fruit quality and seems to be an obvious target for future improvement, however, the complicating factor in this approach is that the metabolism is very dynamic over fruit development and changes are considerable throughout the fruit growth until ripening with many signaling process. Nevertheless, emerging tools can nowadays provide the opportunity to turn this information into a mechanistic understanding of fruit quality, and ultimately to design better fruits in which studding primary metabolism alongside with modeling tools can proved novel information into a mechanistic understanding to mainly develop better fruits (Beauvoit et al., 2018). Additionally, the attention has turned to synthetic biology approaches, mainly by multigene engineering toward multi-gene interventions as recently reviewed elsewhere (Kopka and Fernie, 2018). In parallel, the adoption of synthetic biology may directly provide more effective connections that would circumvent problems associated with feedback regulation of the plants native enzymes and the interactions between TCA cycle and many other processes in the plant could be further expanded as the previous demonstration of signaling function (Gilliham and Tyerman, 2016).

Genetic engineering technologies such as CRISPR/Cas9 could be used to specifically edit the sequence (Čermák et al., 2017; Zsögön et al., 2017) or alter the transcriptional rates (Lowder et al., 2018) of specific genes. Multiplex approaches, targeting various genes simultaneously, are ideally suitable to better understand genetic networks and their interactions (Lowder et al., 2015; Jin et al., 2016). The suitability of this approach has recently been demonstrated in fleshy fruit species such as tomato (Hashimoto et al., 2018) and kiwifruit (Zupeng et al., 2018). The fast pace of advance and improvement in genome engineering techniques, such as the recent introduction of improved endonucleases (Moreno-Mateos et al., 2017; Li et al., 2018) or even single-nucleotide base editing without DNA cleavage (Komor et al., 2016; Gaudelli et al., 2017) suggest that highly efficient genome manipulation tools will soon be available to dissect the complex genetic network involved in fruit maturation control.

Finally, to increase our understanding of the quality and how specific compounds can be changed to improve the ratio between acidity and sugar in fleshy fruits, it seems that coupling integrative approaches (omics) with systems biology is necessary. This would allow the generation of plants, or better fruits, more adapted to stress conditions. Importantly, it is a general opinion that a fruit is a reflection of the conditions to which the plant has been exposed during its development (Poiroux-Gonord et al., 2010). In summary, to increase our knowledge on metabolism during fruit development and the pivotal importance that organic acid metabolism plays on it further research is clearly required.

Considering the importance of amino acids profile during fruit ripening, such as glutamate, the major amino acid of ripe fruits, the usage of such tools could facilitate investigation and simultaneously increasing quality or even extending fruit shelf life, mainly with higher reparatory rate and reduced postharvest time. Teasing out the connections of organic acid metabolism with a hormones may help us understand the real contribution of each hormone on central metabolism. Moreover, rational bioengineering of plants with modified levels of organic acid would also benefit from an increased knowledge of the biochemical regulations and connections inherent to the metabolism of organic acids. The development of plants with altered organic acid composition in fruits should also take into consideration that this pathway is tightly connected with several other aspects of plant metabolism. As such, changes in organic acid metabolism within fruits may not always be beneficial, especially for plants growing under sub-optimal environmental conditions. Different lines of evidence have pointed out that changes in organic acid content might greatly improve fruit organoleptic characteristics. It is important to mention, however, that the majority of those advances have been made in model organisms as well as in some plants of agricultural relevance. To successfully transfer these advances to major food crops, which are generally more recalcitrant to genetic manipulation, still remains a great challenge. To further increase our understanding concerning how organic acids affect fruit metabolism we suggest two complementary approaches: (a) it is possible that the usage of introgression Lines (ILs), as the ones developed in tomato (Eshed and Zamir, 1995) could allow us to identify phenotypes with alterations in the levels of TCA cycle intermediates to analyze the relationship between developmental process and primary metabolism; and (b) to genetically engineer fruit-specific inhibitions within TCA cycle enzymes and/or organic acid metabolism/transport to further analyze the metabolic behavior connecting it with fruit development and ripening. Although both approaches have been successfully used already, it seems clear that they also open the opportunity to greatly accelerate the improvement of crops that have clearly lacked the attention they deserve. The adaptation of high-throughput phenotyping alongside more sensitive flux profiling methodologies, is likely to enable us to pursue new avenues of research to increase our understanding of the complex networks governing organic acid function and hormone metabolism in general during fruit ripening.

CONCLUSION AND FUTURE PERSPECTIVES

Although the summary presented here provides a scaffold for understanding the connections between organic acid and hormone metabolism in fruit development, we posit that it is of pivotal importance that these emerging studies should be expanded. More fundamental knowledge is still required to identify further strategies for manipulation that would improve fruit quality and consequently fruit metabolism. It seems reasonable to anticipate that approaches such as genomics, transcriptome, proteomics and metabolomics coupled with genome editing can present itself as an important data generator that would allow the production of a mechanistic map of fruits in general and their association with phytohormones and fruit developmental changes.

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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Down-Regulation of *PpBGAL10* and *PpBGAL16* Delays Fruit Softening in Peach by Reducing Polygalacturonase and Pectin Methylesterase Activity

Hangkong Liu[1†], Ming Qian[2†], Chunhui Song[1], Jinjin Li[1], Caiping Zhao[1], Guofang Li[1], Anzhu Wang[1] and Mingyu Han[1]*

[1] College of Horticulture, Northwest A&F University, Yangling, China, [2] Centre of Pear Engineering Technology Research, State Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, Nanjing, China

β-galactosidases are cell wall hydrolases that play an important role in fruit softening. However, *PpBGAL*s mechanism impacting on ethylene-dependent peach fruit softening was still unclear. In this study, we found that *PpBGAL4, -6, -8, -10, -16,* and *-17* may be required for ethylene-dependent peach softening and *PpBGAL10*, *-16* may make a main contribution to it among 17 *PpBGALs*. Utilization of virus-induced gene silencing (VIGS) showed that fruits were firmer than those of the control at 4 and 6 days after harvest (DAH) when *PpBGAL10* and *PpBGAL16* expression was down-regulated. Suppression of *PpBGAL10* and *PpBGAL16* expression also reduced *PpPG21* and *PpPME3* transcription, and polygalacturonase (PG) and pectinmethylesterases (PME) activity. Overall, total cell wall material and protopectin slowly declined, water-soluble pectin slowly increased, and cellulose and hemicellulose was altered significantly at 4 DAH, relative to control fruit. In addition, *PpACO1* expression and ethylene production were also suppressed at 4 DAH because of inhibiting *PpBGAL10* and *PpBGAL16* expression. These results suggested that down-regulation of *PpBGAL10* and *PpBGAL16* expression delays peach fruit softening by decreasing PG and PME activity, which inhibits cell wall degradation and ethylene production.

Keywords: peach, β-galactosidases, virus-induced gene silencing (VIGS), softening, polygalacturonase, pectin methylesterase

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Universidade de São Paulo, Brazil

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Fabrizio Costa,
Fondazione Edmund Mach, Italy
Antonio Ferrante,
Università degli Studi di Milano, Italy

***Correspondence:**
Mingyu Han
hanmy@nwsuaf.edu.cn

[†]These authors have contributed equally to this work.

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INTRODUCTION

Peach (*Prunus persica* [L.] Batsch) is a typical climacteric fruit that readily softens after harvest (Yoshioka et al., 2010). The short shelf-life of peaches decreases their market value and represents a major factor limiting the expansion of the fresh market peach industry. Fruit ripening and softening is a complex and coordinated process which is usually accompanied by changes in firmness, color, and flavor (Osorio et al., 2013). Many studies have reported that the process of fruit softening is related to cell wall modifications involving depolymerization of pectins and matrix glycans, solubilization of pectin polymers, and the loss of neutral sugars from pectin side chains (Ruiz May and Rose, 2013; Tucker, 2014; Paniagua et al., 2016). Enzymes related to cell wall modifications that potentially play a role in fruit softening include polygalacturonase (PG; EC3.2.1.15), pectin

methylesterases (PME; EC3.1.1.11), β-galactosidase (β-gal; EC3.2.1.23), cellulase (EC3.2.1.4), and xyloglucan endotransglycosylase (EC2.4.1.207) (Hinton and Pressey, 1974; Lazan et al., 2004; Belleau-Deytieux et al., 2009; Qian et al., 2016). β-Gal increases cell wall porosity by depolymerizing galactose side chains of xyloglucan, rhamnogalacturonan I, and hemicelluloses, which allows binding of PG, PME, or other cell wall hydrolases to pectin; consequently accelerating fruit softening (Brummell and Harpster, 2001; Gerardi et al., 2012; Pose et al., 2013).

In plants, β-gals belong to the glycoside hydrolase 35 family. β-*gal* genes have been identified in *Arabidopsis thaliana* (Ahn et al., 2007), tomato (Smith and Gross, 2000), Japanese pear (Tateishi et al., 2005), *Brassica campestris* (Liu et al., 2013), and peach (Guo et al., 2018). More specifically, the transcript abundance of 17 Arabidopsis β-*gal* genes was measured by q-PCR in five tissues: leaves, roots, flowers, green seedlings, and etiolated seedlings (Ahn et al., 2007). In tomato, seven *TBGs* were found to be expressed in fruits, four in leaves and flowers, five in roots, and six in stems (Smith and Gross, 2000). Similar observations have been reported in Japanese pear (Tateishi et al., 2005) and *B. campestris* (Liu et al., 2013). These studies have described the tissue-specific expression of plant β-*gal*s and their extensive functional divergence. Previous studies have also indicated that β-*gals* contribute to a variety of biological processes, including fruit softening (Pressey, 1983; Carey et al., 1995; Smith et al., 2002), flower senescence (Raghothama et al., 1991), fruit abscission (Wu and Burns, 2004), cell wall loosening (Dopico et al., 1989), galactolipid turnover (Bhalla and Dalling, 1984), and xyloglucan mobilization (de Alcântara et al., 1999).

Several studies have specifically focused on the role of β-*gals* during fruit softening. *Fa*β*gal1* in strawberry (*Fragaria* × *ananassa*) displayed a softening-associated expression pattern with peak transcript levels in red fruit (Trainotti et al., 2001). In another study, inhibition of *Fa*β*Gal4*, which is expressed mainly in receptacles during strawberry fruit ripening, resulted in silencing of *Fa*β*Gal1*, which resulted in an increase in the amount of covalently bound pectin and fruit that was 30% firmer than control fruit (Paniagua et al., 2016). Smith et al. (2002) found that four of six antisense lines with down-regulated *TBG4* produced significantly firmer tomato fruit than control fruit. One line had lower *TBG4* mRNA levels and exo-β-gal activity and higher galactosyl content, suggesting that *TBG4* is involved in cell wall modifications associated with fruit softening (Smith et al., 2002). Similar results have been reported for *pPGBII* in papaya (Othman et al., 2011) and *MA-Gal* in banana (Zhuang et al., 2006).

As a plant hormone, ethylene plays a significant role in fruit softening (Hayama et al., 2006; Khan and Singh, 2009; Harb et al., 2012; Bu et al., 2013; Tatsuki et al., 2013). Many studies about β-*gal* genes mainly focus on the ethylene-dependent fruit softening. *PpGAL1* and *PpGAL4* may play a crucial role in 'LaFrance' pear softening, and their expression was up-regulated by exogenous ethylene or down-regulated by 1-MCP (1-Methylcyclopropene) (Mwaniki et al., 2005). In antisense-ACO melon, ethylene was found to be suppressed to less than 0.5% of the level in control fruit, with a concomitant decrease in β-*gal* gene expression (Nishiyama et al., 2007). Ban et al. (2016) also found that *Dk*GAL1 in persimmon participating in fruit softening could be regulated by ethylene. In addition, investigations of β-*Gal* in apple, *TBG4* in wild-type tomato, two ripening-impaired tomato mutants (rin and Nr), and *AV-GAL1* in avocado, have all strongly suggest that a regulative mechanism exists between ethylene and β-*gals* during ethylene-dependent fruit softening (Moctezuma et al., 2003b; Tateishi et al., 2007; Wei et al., 2012). However, the regulative mechanism between ethylene and β-*gal* genes during ethylene-dependent fruit softening was still unclear.

Rapid fruit softening in peach is a significant problem that affects fresh-market production. The molecular regulation of softening in peach, however, is still unclear. Although the importance of β-*gals* in fruit ripening and softening has been documented in many previous studies, the study about *PpBGALs* in peach is limited in the report which 17 *PpBGALs* (*PpBGAL1-17*) were only be identified by bioinformatics methods and displayed divergent expression during softening of four different peach cultivars (Guo et al., 2018). However, little is known about the roles of *PpBGALs* in ethylene-dependent peach softening. This includes characterizing which ones exhibit softening-associated expression patterns and how they may be involved in the regulation of fruit softening in peach. In the present study, we profiled the expression of 17 *PpBGALs* coming from the study of Guo et al. (2018) in response to propylene and 1-MCP treatments during peach fruit softening. *PpBGALs* exhibiting consistent softening-associated expression patterns were identified, and the function of *PpBGAL10* and *PpBGAL16* in peach fruit softening was explored using virus-induced gene silencing (VIGS). The overall objective was to develop a better understanding of the molecular mechanisms by which *PpBGALs* regulate ethylene-dependent peach fruit softening.

MATERIALS AND METHODS

Plant Material and Treatments

'Qian jian bai' (QJB) peach trees, grown at the Experimental Station of the College of Horticulture, Northwest Agriculture and Forestry University, Yangling, Shaanxi, China were used in this study. Fruits were harvested at commercial maturity (exhibiting partially red, light-green skin and slightly firm flesh; Qian et al., 2016) and transported to the laboratory. Undamaged fruits were selected and divided randomly into three groups, each containing 150 fruits. Each group was then sub-divided into three additional groups. Fruits in the first and second group were placed in hermetic containers and treated for 24 h with 500 μL L^{-1} propylene or 5 μL L^{-1} 1-MCP, respectively. Propylene treatment can eliminate interference of exogenous ethylene when endogenous ethylene production of peach fruit is measured by gas chromatography (Trace GC Ultra, Thermo Fisher, New York, NY, United States). The third group of fruit was sealed in a hermetic container with air for 24 h as control. Following treatment, fruits from each of the groups were stored at 25°C and 75% relative humidity. Fruit samples were taken every other day until they were fully softened and at each sampling the fruit were frozen rapidly in liquid nitrogen and stored at −80°C until further analysis.

Determination of Fruit Firmness, Ethylene Production, and Enzyme Activity

Fruit firmness of five randomly selected fruits from each sub-group receiving each treatment was measured using a GY-4 firmness meter (Top Instrument Co., Hangzhou, China) equipped with a 7.9-mm probe. The skin of the peel was removed from a section of the fruit surface and a probe was inserted and the pressure it required to penetrate the flesh of the fruit was recorded. Ethylene production was analyzed as described by Liguori et al. (2004). Briefly, nine fruits from each sub-group in each treatment were sealed in a jar for 60 min, and a 1-mL air sample was analyzed by gas chromatography (Trace GC Ultra, Thermo Fisher, New York, NY, United States). The enzyme activity of β-gal, PG, and PME in 1 kg fresh weight (FW) peach flesh was determined as reported by Gross (1982), Lazan et al. (1989), and Hagerman and Austin (1986), respectively. One unit (U) of β-gal and PG enzyme activity was defined as the amount of hydrolyzed enzyme producing 1 mol p-nitrophenol and galacturonic acid per minute, respectively. One unit of PME enzyme activity was defined as the amount of enzyme producing 1 μmol CH_3O^- by de-methylesterification per minute. Separation and measurement of cell wall materials (dry mass) was performed as described by Santiago-Domenech et al. (2008). Each experiment was carried out in three replicates.

Cloning of *PpBGAL10* and *PpBGAL16* and Virus Induced Silencing (VIGS)

PpBGAL10 and *PpBGAL16* came from previous report (Guo et al., 2018), gene-specific primers used to clone their coding sequences were designed using Primer Premier 6.0 (Supplementary Table S1). Restriction enzyme cutting sites and protective bases were added to the forward and reverse primers. Each 50-μL PCR amplification mixture contained 1 μL high-fidelity DNA polymerase (Vazyme, Nanjing, China), 10 μL buffer, 1 μL dNTPs, 5 μL cDNA template, 3 μL each of the forward and reverse primers, and 27 μL sterilized double-distilled H_2O. Amplifications were performed on a GeneAmp PCR System 9700 (ABI, Waltham, MA, United States) using the following cycling conditions: 2 min at 95°C, followed by 40 cycles of 10 s at 95°C, 30 s at the selected annealing temperature, and 15 s at 72°C, with a final extension of 10 min at 72°C. The PCR products were subjected to electrophoresis on 1% agarose gels and then inserted in a pMD18-T vector (Takara, Dalian, China) for sequencing. After verifying the coding sequence, the target gene was cloned into a pTRV2 vector. The two recombinant plasmids (pTRV2-*PpBGAL10* and pTRV2-*PpBGAL16*), as well as a control (a pTRV2 empty plasmid) were separately introduced into *Agrobacterium tumefaciens* GV3101 using a freeze-thaw method (Fire et al., 1998). Individual colonies were subsequently incubated overnight at 28°C in 1 mL LB medium containing 50 mg mL^{-1} kanamycin, 50 mg mL^{-1} gentamicin, 50 mg mL^{-1} rifampicin, 20 mM acetosyringone, and 10 mM MES. An aliquot of each culture was then inoculated into 100 mL of the same antibiotic LB medium and incubated to an A_{600} of 1.0–2.0 at 28°C. *Agrobacterium* infection was performed according to the method of Jia et al. (2011). Cells were collected by centrifugation at 5000 × *g* and 25°C for 5 min and then resuspended in an equal volume of infiltration buffer containing 10 mM $MgCl_2$, 200 μM acetosyringone, and 10 mM MES (pH 5.6) and incubated at 25°C for 3 h. Finally, 1 mL of a 1:1 (v/v) mixture of induced *Agrobacterium* harboring pTRV2, and *Agrobacterium* with either pTRV2-*PpBGAL10* or pTRV2-*PpBGAL16*, was infiltrated into fruit using a 1-mL syringe. Fruit were infiltrated at nightfall when the bacterial culture was at the end of the second exponential growth phase. Infiltrated peach fruit of three constructs was picked at 1 week after infiltration and stored at 25°C and 75% relative humidity, respectively (Li et al., 2017). Each construct contains 150 fruits and then divided equally into three sub-groups. Fruit samples of each sub-groups were taken every other day until control fruit fully softening, and stored at −80°C after freezing quickly in liquid nitrogen. The ethylene production of infiltrated fruit and in other experiments (including fruit firmness, gene expression, enzyme activity, and cell wall components) at the infected position were performed using the above-mentioned methods.

RNA Extraction and Reverse Transcription

Total RNA was extracted as described by Lester et al. (1994). RNA quality and integrity were determined using 1% agarose gel electrophoresis and ultraviolet spectrophotometry (Thermo NanoDrop 2000, Wilmington, DE, United States). Reverse transcription was conducted using a Prime Script RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China).

Reverse Transcription-Quantitative PCR (RT-qPCR)

Specific primers for 17 *PpBGALs* coming from previous report (Guo et al., 2018), *PpPG21*, *PpPME3*, *PpACS2*, and *PpACO1* were designed using Primer Premier 6.0 (Qian et al., 2016; Li et al., 2017) (Supplementary Table S1). RT-qPCR analyses were conducted using an iQ5 real-time PCR system (Bio-Rad, Plano, TX, United States). A 10-μL reaction volume was used for each sample comprising 1 μL cDNA, 1 μL of each primer, 2 μL ddH_2O, and 5 μL of 2× SYBR Premix Ex *Taq* II (TaKaRa, Dalian, China). The PCR protocol specified in the SYBR Premix Ex *Taq* kit manual was as follows: 1 min at 95°C, followed by 40 cycles of 15 s at 95°C, 20 s at the selected annealing temperature, and 20 s at 72°C, followed by 10 s at 95°C, and finally 39 cycles to construct a melting curve. The peach 18S ribosomal RNA (rRNA) gene was used as a reference gene and for normalization of the data. Relative expression levels for each of the analyzed genes were calculated as described by Livak and Schmittgen (2001). Each sample was composed of three biological replicates.

Statistical Analysis

Microsoft Excel 2010 and IBM SPSS Statistics 22 were used for data processing and to determine significant statistical differences between sample representing different time points and treatments using *post hoc* Tukey's test of One-way ANOVA

($p < 0.05$) for differences. Figures were generated and combined using Sigma Plot 10.0.

RESULTS

Fruit Firmness, Ethylene Production, and β-Gal Activity During Peach Fruit Softening

Fruit firmness in QJB control fruit decreased slowly over the first 2 days after harvest (DAH), declined rapidly from 2 to 4 DAH, and then decreased slowly (**Figure 1A**). Ethylene production increased slowly during the first 2 DAH, increased significantly from 2 to 4 DAH, and then rapidly decreased in subsequent DAH (**Figure 1B**). Changes in β-gal activity exhibited a similar trend after harvest to ethylene production, with maximum β-gal activity observed at 4 DAH (**Figure 1C**).

Identification of *PpBGALs* With a Ripening-Associated Pattern of Expression

RT-qPCR was used to analyze the expression profiles of 17 *PpBGALs* during QJB fruit softening to provide information on the potential role of *PpBGALs*. Among the *PpBGALs* examined, *PpBGAL2, -4, -6, -8, -9, -10, -16*, and *-17* were up-regulated and exhibited their maximum expression level at 4 DAH, with the exception of *PpBGAL17* which exhibited peak transcript levels at 6 DAH (**Figure 2**). However, *PpBGAL15* exhibited a tendency to be down-regulated, and *PpBGAL12* firstly decreased in the peach fruit and then increased (**Figure 2**). *PpBGAL3* and *-7* were up-regulated during the first 2 DAH and then down-regulated; *PpBGAL1, -5, -11*, and *-13* were barely detected while *PpBGAL14* expression was not detected during QJB softening (**Figure 2**).

Propylene and 1-MCP Treatments Alter β-Gal Expressions

The QJB peach fruits were treated with propylene and 1-MCP to determine the potential role of *PpBGAL* family members during ethylene-dependent fruit softening. Fruit firmness decreased markedly at 2 DAH in response to the propylene treatment (**Figure 1A**). Correspondingly, ethylene production and PpBGAL activity increased rapidly during the first 2 DAH (**Figures 1B,C**). *PpBGAL4, -6, -8, -10,* and *-16* expression was significantly up-regulated and peaked at 2 DAH in propylene-treated fruit; similarly, *PpBGAL1*, *-17* and *PpBGAL7* expression also increased significantly but peaked at 4 or 6 DAH, respectively (**Figure 2**). Transcript levels of *PpBGAL2, -3, -5, -9, -11, -12, -13*, and *-15* were not significantly affected by the propylene treatment (**Figure 2**).

Fruits treated with 1-MCP softened more slowly relative to non-treated control fruit (**Figure 1A**). Ethylene production was also lower relative to the control fruit at 4 DAH, with peak ethylene levels exhibited at 6 DAH (**Figure 1B**); β-gal activity was significantly inhibited at same time, with maximum activity exhibited at 8 DAH (**Figure 1C**); Expression of *PpBGAL 2, -3, -4, -5, -6, -8, -10, -11, -16,* and *-17* was inhibited, while transcript levels of *PpBGAL1, -7, -9, -12, -13*, and *-15* were barely affected (**Figure 2**).

VIGS of *PpBGAL10* and *PpBGAL16*

Virus-induced gene silencing technology was used to suppress the expression of *PpBGAL10* and *PpBGAL16* (RNAi-10 and RNAi-16, respectively) in fruit tissues to confirm the roles of these genes in peach fruit softening. The infiltrated surfaces of control fruits developed a typical red flush, whereas little or no red color was evident at the areas of fruit infiltrated with RNAi-10 and RNAi-16 (**Figure 3**). Expression of *PpBGAL10* and *PpBGAL16* was significantly decreased at 4 DAH in RNAi fruit (**Figure 4A**). The fruits infiltrated with the two RNAi constructs softened more slowly, as measured by changes in fruit firmness, during the period of 2–6 DAH than control fruit infiltrated with an empty vector construct (**Figure 4B**). Total β-gal activity, however, was not significantly different between the fruit infiltrated with the RNAi constructs and the control fruit from 0 to 4 DAH (**Figure 4C**).

The amounts of various cell wall components (cell wall material, protopectin, water-soluble pectin, hemicellulose, and cellulose) were different in the RNAi constructs fruit than in the

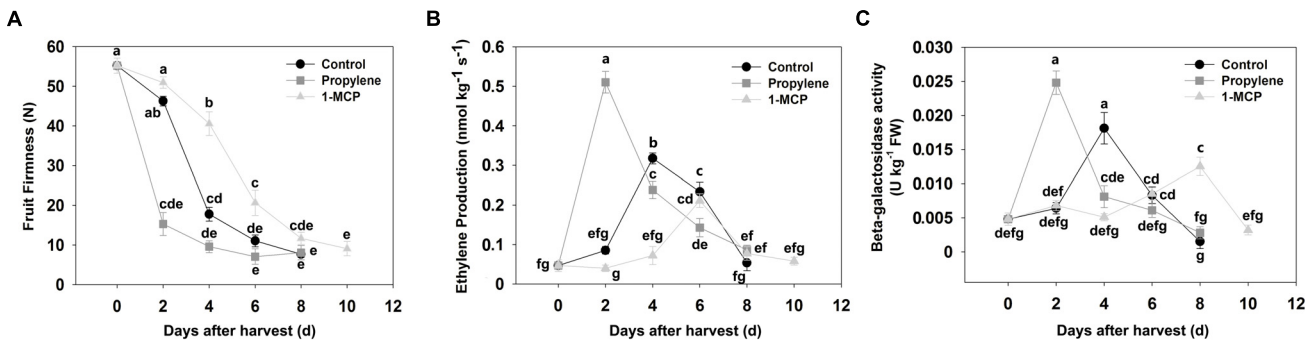

FIGURE 1 | Fruit firmness, ethylene production, and β-galactosidase activity in control, propylene-treated, and 1-MCP-treated 'Qian Jian Bai' peach fruit during storage. **(A)** Fruit firmness; **(B)** ethylene production; **(C)** β-gal activity. Each experiment was repeated three times. Data represent the mean ± SE ($n = 3$). Significant differences ($p < 0.05$) between means are indicated by different letters.

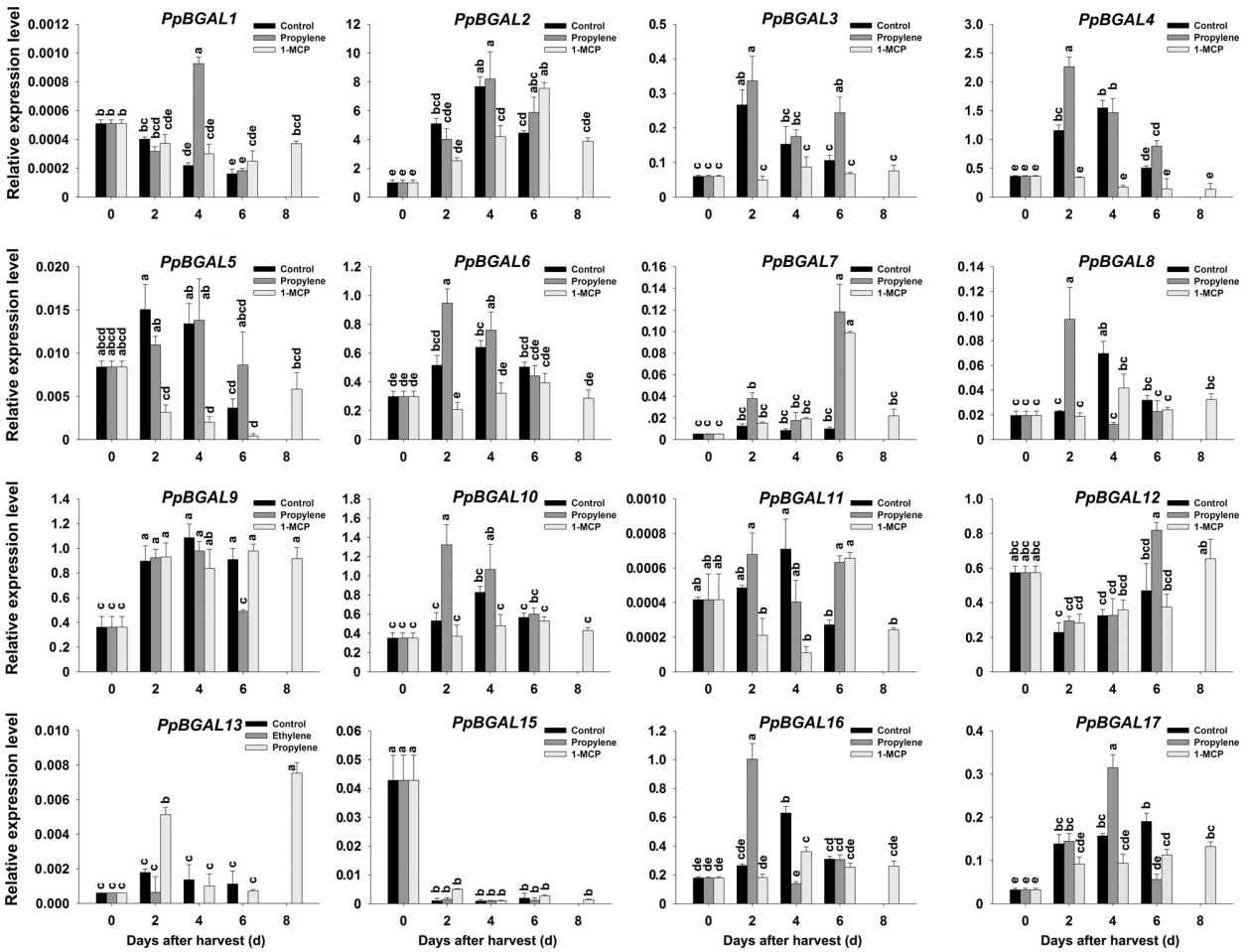

FIGURE 2 | Transcript levels of 17 *PpBGALs* in control, ethylene-treated, and 1-MCP-treated 'Qian Jian Bai' peach fruit during storage. The peach 18S rRNA gene was used as a reference. The experiment was repeated three times. Data represent the mean ± SE ($n = 3$). Significant differences ($p < 0.05$) between means are indicated by different letters.

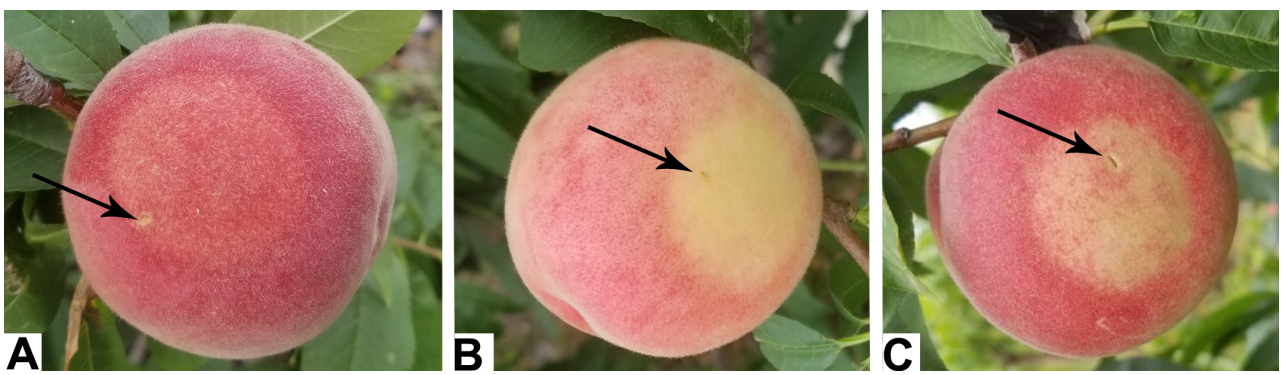

FIGURE 3 | Phenotypes of infiltrated fruits. **(A)** TRV2 (control); **(B)** TRV2-*PpBGAL10* (RNAi-10); **(C)** TRV2-*PpBGAL16* (RNAi-16). Black arrows indicate the injection site.

control fruit (**Figure 5**). In RNAi constructs fruit, the amount of cell wall material (dry mass), protopectin, and cellulose were greater at 4 DAH (**Figures 5A,B,D**). Although water-soluble pectin content increased from 2 to 6 DAH in both the control and RNAi, the increase was greater in the control (**Figure 5C**). Interestingly, hemicellulose content was higher in the control

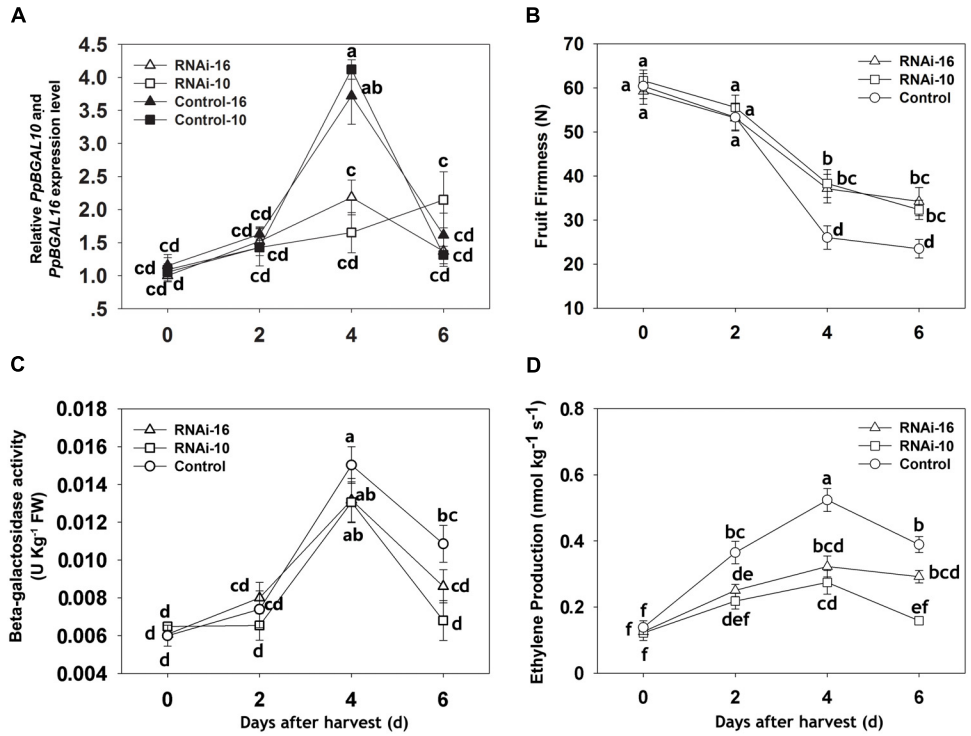

FIGURE 4 | Changes in the expression level of *PpBGAL10* and *PpBGAL16*, fruit firmness, β-gal activity, and ethylene production during storage of control (TRV2), RNAi-10 (TRV2-*PpBGAL10*), and RNAi-16 (TRV2-*PpBGAL16*) fruit. **(A)** Relative transcript abundance of *PpBGAL10* and *PpBGAL16*, RNAi-10, RNAi-16 and Control-10, Control-16 represents expression of *PpBGAL10* and *PpBGAL16* in RNAi and Control fruit, respectively, **(B)** fruit firmness, **(C)** β-gal activity, and **(D)** ethylene production RT-qPCR expression levels were normalized using the cycle threshold value of the peach 18S rRNA gene. Data represent the mean ± SE ($n = 3$). Significant differences ($p < 0.05$) between means are indicated by different letters.

fruit than in RNAi fruit from 2 to 4 DAH and then decreased sharply in all three groups (**Figure 5E**).

Transcript levels of softening-related genes (*PpPG21* and *PpPME3*) and the enzyme activity of cell wall hydrolases (PG and PME) were measured in control and RNAi fruits from 0 to 6 DAH (**Figure 6**). Expression of *PpPG21* and *PME3* reached their maximum at 4 DAH in control fruit and was significantly higher than in RNAi fruit, but no significant differences were observed in the expression of these genes between RNAi-10 and RNAi-16 fruit from 0 to 6 DAH (**Figures 6A,B**). PG maximum activity was higher in the control fruit though peaked at 4 DAH in both control and RNAi fruit (**Figure 6D**). PME activity, which peaked at 2 DAH in RNAi fruit, increased slowly in control fruit from 2 to 4 DAH and was higher at 4 and 6 DAH than in the RNAi fruit (**Figure 6E**).

Down-Regulation of *PpBGAL10* and *PpBGAL16* Affects Ethylene Production and Ethylene-Related Gene Expression

The contribution of ethylene to the softening of respiratory climacteric fruit is well known. In the present study, ethylene production and transcript levels of ethylene-related genes (*ACO1* and *ACS2*) were analyzed in RNAi and control fruits from 0 to 6 DAH. As illustrated in **Figure 4D**, ethylene production at 4 DAH was significantly lower in RNAi fruit than in control fruit, however, *PpACS2* transcript levels were higher in RNAi fruit from 0 to 6 DAH. In addition, *PpACS2* expression was similar in both types of RNAi fruit (**Figure 6C**). Interestingly, *ACO1* expression level was significantly higher at 4 DAH than in either of the two different RNAi fruit that exhibited similar levels of expression to each other (**Figure 6F**).

DISCUSSION

Possible Role of *PpBGAL* Family Members in Fruit Softening

Several studies have focused on the possible role of β-gals in fruit ripening and softening (Smith et al., 2002; Lazan et al., 2004; Yoshioka et al., 2011; Paniagua et al., 2016). Guo et al. (2018) reported three *PpBGAL* genes (*PpBGAL2, -8* and *-16*) in 'Hu Jing Mi Lu' and five *PpBGAL* genes (*PpBGAL1, -2, -9, -12,* and *-16*) in 'Xia Hui 8' peach fruit were up-regulated during storage. However, results of the present study indicate that *PpBGAL2, -4, -6, -8, -9, -10, -16*, and *-17* may participate in QJB fruit softening due to exhibit softening-associated patterns of expression, with transcript levels being up-regulated during the process of fruit softening in QJB peach fruit (**Figure 2**). Therefore, it appears

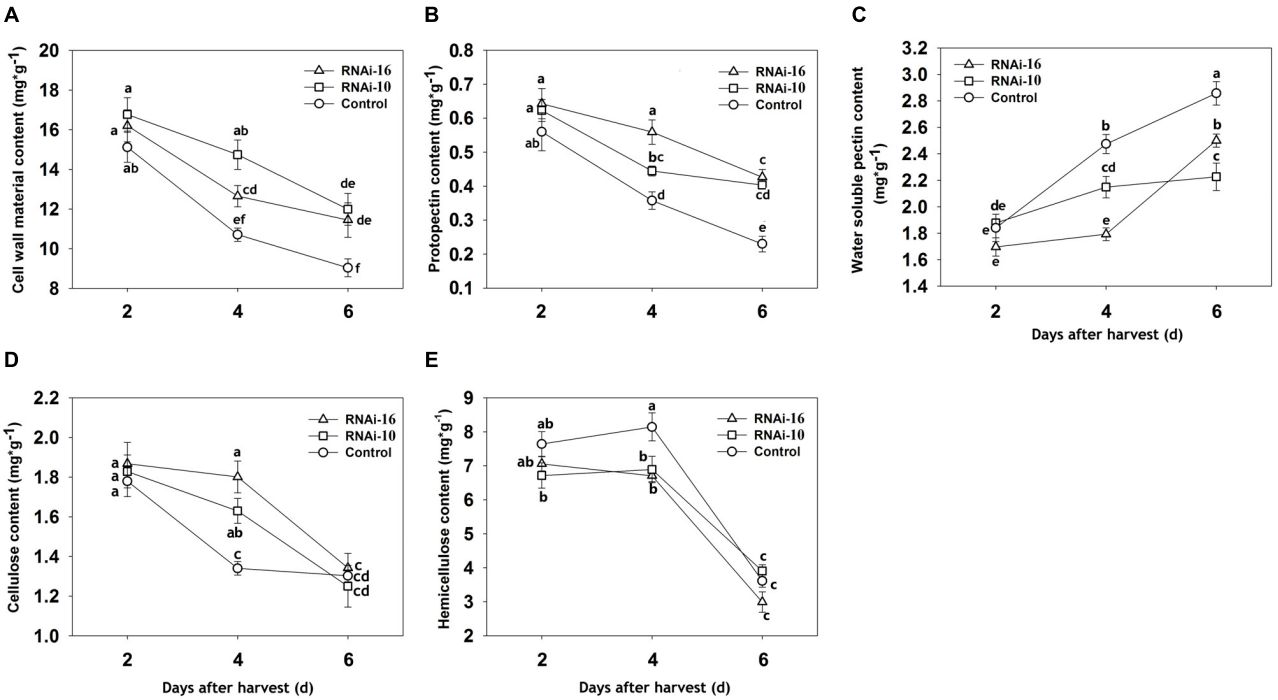

FIGURE 5 | Changes in the level of cell wall components in control (TRV2), RNAi-10 (TRV2-*PpBGAL10*), and RNAi-16 (TRV2-*PpBGAL16*) fruit. **(A)** Cell wall material (dry mass), **(B)** protopectin, **(C)** water-soluble pectin, **(D)** cellulose, and **(E)** hemicelluloses in RNAi and control fruit during storage. Each experiment was repeated three times. Bars represent the mean ± SE (n = 3). Significant differences ($p < 0.05$) between means are indicated by different letters.

that several *PpBGALs* could involve in peach fruit softening while their expression can vary between different peach cultivars.

Our results also indicate that, *PpBGAL4, -6, -8, -10, -16*, and *-17* can be induced by endogenous ethylene (**Figure 2**), which has been reported to be increased by propylene treatment (Ban et al., 2016). Therefore, the six *PpBGALs* may play an important role in ethylene-dependent QJB fruit softening. In addition, *PpBGAL16* exhibited the same expression pattern in three different peach cultivars ('Hu Jing Mi Lu,' 'Xia Hui 8,' and QJB), and exhibits a low level of expression during the storage of 'Yumyeoung' and 'XiaCui.' Notably, both of these latter cultivars maintain fruit firmness for a longer period of time than the former three cultivars and barely synthesize any ethylene during storage (Guo et al., 2018). Therefore, it appears that *PpBGAL16* may play a pivotal role in ethylene-dependent peach fruit softening. *PpBGAL10* exhibited the pattern of expression as well as *PpBGAL16* in propylene-treated and control fruit (**Figure 2**). Meanwhile, it may be an ortholog of PpGAL3 has been reported to play a role in cell wall disassembly in ripening Japanese pear (Tateishi et al., 2005). Therefore, *PpBGAL10* may also play an important role in line with *PpBGAL16* during peach softening.

PpBGAL2 and *PpBGAL9* may participate in QJB fruit softening in an ethylene-independent manner. The expression of *PpBGAL1, -5, -11, -13*, and *-14* were very low or undetectable in naturally softened QJB peach fruit, while *PpBGAL3, -7, -12*, and *-15* exhibited hardly showed soften-related expression patterns (**Figure 2**) and were only slightly induced by exogenous propylene; suggesting that they have negligible roles in ethylene-dependent peach fruit softening.

Down-Regulation of *PpBGAL10* and *PpBGAL16* Delays Peach Fruit Softening

To further elucidate the functional role of *PpBGALs* in ethylene-dependent peach fruit softening, VIGS technology was utilized to suppress the expression of two principle *PpBGALs* (*PpBGAL10* and *PpBGAL16*) in fruit infiltrated with RNAi constructs. Results indicated that fruit softening was delayed in fruit infiltrated with both RNAi constructs (**Figure 4B**), however, β-gal activity was only slightly lower in the RNAi fruit (**Figure 4C**) when the expression of *PpBGAL10* and *PpBGAL16* was significantly down-regulated (**Figure 4A**). These results are consistent with studies in strawberry which found that the down-regulation of *Faβ Gal4* resulted in delayed fruit softening but no significant change in total β-Gal enzyme activity (Paniagua et al., 2016). Similar results have also been reported by Carey et al. (2001) and Smith et al. (2002) in tomato. We suggest that down-regulation of *PpBGAL10* and *PpBGAL16* may lead to reduced exo-β-galactanase activity, a change that would have a negligible effect on total β-Gal enzyme activity (Moctezuma et al., 2003a; Paniagua et al., 2016).

PG can depolymerize cell wall due to mediate homogalacturonan depolymerization requiring to be de-methylesterified by PME (Brummell and Harpster, 2001). Thus, PG and PME had been abundantly reported to contribute to

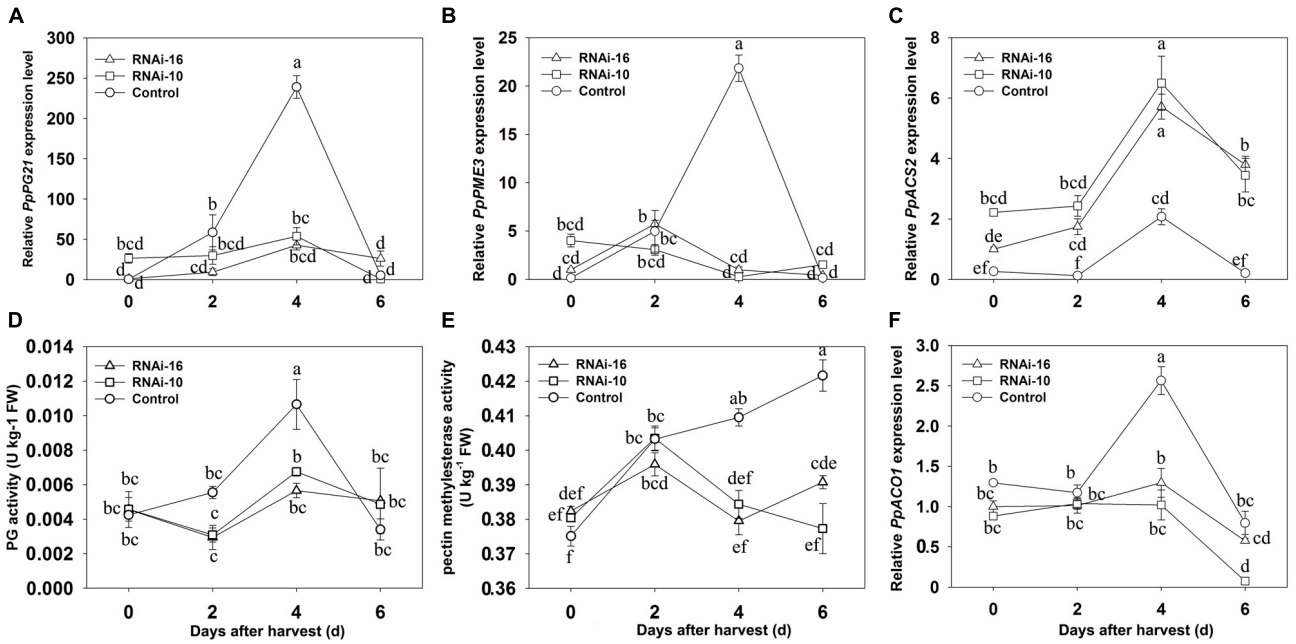

FIGURE 6 | Changes in softening-related gene expression and cell wall hydrolase activity in control, RNAi-10, and RNAi-16 fruit during storage. **(A)** Relative expression of *PpPG21*; **(B)** relative expression of *PpPME3*; **(C)** relative expression of *PpACS2*; **(D)** polygalacturonase activity; **(E)** pectin methylesterase activity; and **(F)** relative expression of *PpACO1*. Each experiment was repeated three times. Data represent the mean ± SE (n = 3). Significant differences ($p < 0.05$) between means are indicated by different letters.

fruit softening because of involving a role in cell wall metabolism (Micheli, 2001; Smith et al., 2002; Jayani et al., 2005; Payasi et al., 2009; Pose et al., 2013). β-gal increases cell wall porosity by depolymerizing the galactose side chains of xyloglucan, rhamnogalacturonan I, and hemicelluloses, which then allows the binding of PG, PME, or other cell wall hydrolases to pectin; thus accelerating fruit softening (Brummell and Harpster, 2001; Gerardi et al., 2012; Pose et al., 2013). Therefore, the activity of PG and PME in RNAi fruit might be affected by down-regulating expression of *PpBGAL10* and *PpBGAL16*. Our results indicated PpPG21 and PpPME3, two key genes encoding PG and PME, respectively, have significant lower expression in RNAi fruit than control fruit at 4 DAH, resulting in the reduction of PG and PME enzyme activity (**Figures 6A,B,D,E**). It is consistent with a viewpoint that β-galactosidase and ripening-related expansins may regulate other cell wall modify-related enzyme activities (Brummell and Harpster, 2001). These results suggest that the down-regulation expression of *PpBGAL10* and *PpBGAL16* delays peach fruit softening due to reduce PG and PME activity rather than β-gal activity.

Down Regulation of *PpBGAL10* and *PpBGAL16* Impacts Cell Wall Components

Accompanied by rapid declining of fruit firmness, water-soluble pectin contents could dramatically increase during melting peach fruit softening (Murayama et al., 2009). A slower rate of increase in water-soluble pectin was observed in RNAi-10 and RNAi-16 fruit where PG and PME activity was inhibited (**Figure 5C**). This result is consistent with results reported in strawberry after the down-regulation of *FaPG1*, *PL*, and *Faβ-Gal4* genes (Santiago-Domenech et al., 2008; Pose et al., 2013; Paniagua et al., 2016). In addition, A decrease of protopectin content was occurred during 'Okubo' peach softening (Li et al., 2009). Our results displayed its levels in RNAi-10 and RNAi-16 fruit were higher (**Figure 5B**). These data suggested that softening of RNAi-10 and RNAi-16 fruit was delayed because of suppressing pectin metabolism. Therefore, it was indicated that the amount of ionically and covalently bound pectin was potentially higher in RNAi-10 and RNAi-16 fruit than in control fruit. Yoshioka et al. (2011) found that bound pectin (ionically and covalently) content was the higher in non-softening peach fruit than in softening at different storage time. Santiago-Domenech et al. (2008) and Figueroa et al. (2010) have also confirmed the depolymerization of bound pectin may be due in part to the solubilization of pectin. Moreover, fruit softening in peach is associated with pectin solubilization and depolymerization (Yoshioka et al., 2011). Therefore, the present results suggest that the inhibition of *PpBGAL10* and *PpBGAL16* transcription helps to reduce bound pectin solubilization and depolymerization by suppressing PG and PME activity, thereby delaying peach softening. In addition, changes of cellulose and hemicellulose level indicate cellulase and hemicellulase may be also influenced in RNAi-10 and RNAi-16 fruit, suggesting delaying fruit softening is likely a cooperative process which many cell wall modified enzymes engage together, but this mechanism is unclear and still required to further study.

Suppression of *PpBGAL10* and *PpBGAL16* Reduces Ethylene Production

Ethylene is a hormone that plays an essential role in fruit softening through its ability to regulate several cell wall hydrolysis-related genes (Hayama et al., 2006; Tatsuki et al., 2013). Therefore, a reduction in ethylene production may greatly delay fruit softening. Ethylene production was significantly reduced in the present study when the expression of *PpBGAL10* and *PpBGAL16* was down-regulated. We propose three hypotheses to explain the reduction in ethylene production. First, the level of cell wall galactose in RNAi-10 and RNAi-16 fruit was likely reduced due to the observed inhibition of PG and PME activity, delaying pectin solubilization and depolymerization. Galactose, as a signaling molecule, has been confirmed to stimulate ethylene production in tomato fruits and tobacco leaf disks (Kim et al., 1987; Philosoph-Hadas and Aharoni, 1987). Therefore, a reduction in galactose content may reduce ethylene production by suppressing the transcription of *PpACO1*. Second, specific wall fragments, oligogalacturonides (OGAs) which are short breakdown products of homogalacturonan consisting of 9–15 GalA residues, have been suggested to induce ethylene release during pectin solubilization and depolymerization (Simpson et al., 1998; Wolf et al., 2012), and PME-dependent demethylation-esterification of OGAs is essential to this process (Osorio et al., 2008). In the present study, the amount and demethylation of OGAs are thus probably reduced in RNAi-10 and RNAi-16 fruit where PME and PG activity is reduced. This scenario would also result in a reduction in ethylene production. A third hypothesis, that cell wall damage acts as a signal has been supported by experiments involving various cell wall-related mutants (Seifert and Blaukopf, 2010). Interestingly, 1-aminocyclopropane-1-carboxylic acid (ACC), a direct precursor in ethylene synthesis, responds to cell wall damage (De Cnodder et al., 2005; Tsang et al., 2011). Thus, we suggest that the signal derived from cell wall damage is weak in RNAi-10 and RNAi-16 fruit where softening is delayed, however, due to the higher level of cell wall integrity in the RNAi fruit. This would result in a lower level of ACC content relative to control fruit. *PpACO1* expression in RNAi-10 and RNAi-16 fruit was also inhibited (**Figure 6F**). Therefore, ethylene production was lower in these fruit, relative to the control fruit. Although all three hypotheses can explain the reduction in ethylene production observed in the RNAi-10 and RNAi-16 fruit, some unresolved issues remain, such as direct proof of the involvement of changes in galactose, OGA, and ACC contents in RNAi fruit and the identification of specific receptors of galactose and OGAs in cytomembranes. Confirmation of these hypotheses will thus require further complex experiments.

A reduction in ethylene production may delay peach fruit softening when the expression of *PpBGAL10* and *PpBGAL16* is down-regulated. The reduction in ethylene production, however, hardly affected β-gal activity in RNAi-10 and RNAi-16 fruit. These observations may suggest the existence of an indirect method of regulation between ethylene and *PpBGALs*. Ethylene can also regulate anthocyanin synthesis (El Kereamy et al., 2003; Cheng et al., 2016). Consequently, the inhibition of ethylene production may prevent anthocyanin synthesis and explain the lack of color change in the RNAi fruit where *PpBGAL10* and *PpBGAL16* are down-regulated (**Figure 3**).

CONCLUSION

Our study demonstrated that *PpBGAL10* and *-16* are the main β-gal genes contributing to ethylene-dependent peach fruit softening. VIGS-induced down-regulation of *PpBGAL10* and *PpBGAL16* expression delays peach fruit softening by reducing PG and PME activity, which inhibits cell wall degradation and reduces ethylene production. The present study has provided strong evidence that β-gals play an important role in peach fruit softening.

AUTHOR CONTRIBUTIONS

MH and AW designed the experiments. HL, GL, and CS performed all plant physiological and molecular experiments. MQ and JL analyzed the data. HL and MQ wrote the manuscript. HL and CZ revised 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.2018.01015/full#supplementary-material

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Regulation of Fig (*Ficus carica* L.) Fruit Color: Metabolomic and Transcriptomic Analyses of the Flavonoid Biosynthetic Pathway

Ziran Wang[1], *Yuanyuan Cui*[1], *Alexander Vainstein*[2], *Shangwu Chen*[3] *and Huiqin Ma*[1]*

[1] *Department of Fruit Tree Sciences, College of Horticulture, China Agricultural University, Beijing, China,* [2] *The Robert H. Smith Faculty of Agriculture, Food and Environment, Institute of Plant Sciences and Genetics in Agriculture, The Hebrew University of Jerusalem, Rehovot, Israel,* [3] *College of Food Science and Nutrition Engineering, China Agricultural University, Beijing, China*

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***Correspondence:**
Huiqin Ma
hqma@cau.edu.cn

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Combined metabolomic and transcriptomic analyses were carried out with fig cultivar Green Peel and its color mutant "Purple Peel." Five and twenty-two metabolites were identified as having significantly different contents between fruit peels of the two cultivars at young and mature stages, respectively. Cyanidin O-malonylhexoside demonstrated a 3,992-fold increase in the mature purple peel, the first identification of a major cyanidin in fig fruit; cyanidin 3-O-glucoside, cyanidin O-malonylhexoside O-hexoside and cyanidin-3,5-O-diglucoside were upregulated 100-fold, revealing the anthocyanins underlying the purple mutation. Beyond the visible differences, there was very significant accumulation of the colorless flavonoids procyanidin B1, luteolin-3′,7-di-O-glucoside, epicatechin and quercetin-3-O-rhamnoside in the mature "Purple Peel" compared to "Green Peel." At the young stage, only cyanidin O-malonylhexoside, cyanidin O-malonylhexoside O-hexoside and esculetin were upregulated a few fold in the mutant. Transcriptome analysis revealed a downregulated expression trend of genes encoding phenylpropanoid and flavonoid biosynthetic pathway enzyme in the young "Purple Peel" compared to the young "Green Peel," whereas significant and simultaneous upregulation was revealed in almost all of the flavonoid and anthocyanin pathway components and relevant transcription factors in the mature-stage mutant. The role of R2R3-MYB transcription factors in the color morph mutation and its possible relation to the activity of retrotransposons are discussed. Moreover, large-scale upregulation of small heat-shock protein genes was found in the mature mutant. This is the first work to reveal comprehensive metabolome and transcriptome network changes underlying a fig mutation in a single horticultural attribute, and its profound effects on fruit nutrition and quality.

Keywords: fig (*Ficus carica* L.), anthocyanin, flavonoid, peel color mutation, transcriptome, metabolome

INTRODUCTION

The fruit peel is in essence the fruit boundary; it maintains fruit integrity and protects it from the external environment. Secondary metabolites in the peel, such as pigments, tannins and aroma compounds, affect fruit appearance, quality and storage (Li et al., 2013). Anthocyanin pigments—pelargonidin, cyanidin, delphinidin, peonidin, petudinin and malvidin, often in their glycosylated form—are commonly identified in pink, red, purple and other deep-colored fruit (Jaakola, 2013).

Anthocyanin metabolism is catalyzed by a number of enzymes from the phenylpropanoid and flavonoid biosynthetic pathways (Bilyk and Sapers, 1986; Pelletier et al., 1997; Falcone Ferreyra et al., 2012). As an initial precursor of anthocyanins and other flavonoids, phenylalanine produces colorless secondary intermediate metabolites that are sequentially catalyzed by phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase, 4-coumarate:coenzyme A ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavanone 3′-hydroxylase (F3′H), flavonoid 3′5′-hydroxylase, and dihydroflavonol 4-reductase (DFR); unstable colored anthocyanins are then synthesized from the colorless anthocyanins by anthocyanidin synthase (ANS) (Boss et al., 1996; Falcone Ferreyra et al., 2012) via the full metabolic pathway. Finally, the unstable colored anthocyanins are transformed into blue–violet, brick-red or magenta glycosides by UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT) (Pelletier et al., 1997; Dick et al., 2011; Saito et al., 2013), resulting in different types and numbers of substituents in the B ring of the anthocyanin, which determine the color hue and chromaticity of the anthocyanidins in specific tissues and cellular environments (Espley et al., 2007). Brightly colored fruit commonly show high gene expression of the key downstream enzymes of the anthocyanin biosynthetic pathway, such as those encoding DFR, ANS, and UFGT (Han et al., 2012). Sharply upregulated *FcANS1* expression was revealed in the peel of a dark-colored fig during fruit ripening (Cao et al., 2016), whereas *UFGT* was identified as the critical gene for anthocyanin biosynthesis in grape and strawberry (Kobayashi et al., 2001; Griesser et al., 2008). In recent years, combined high-throughput methods have been used to study color development. Integrated metabolomic and transcriptomic network analyses in fruit and flowers have elucidated a series of secondary metabolites with changes in content, and the corresponding differentially expressed genes (Lou et al., 2014; Matus, 2016), broadening the global view of plant color regulation.

Color mutations are frequently observed in flowers and fruit. The color change is usually produced by single-gene mutations, as in grape (Kobayashi et al., 2004; Hichri et al., 2011a), apple (Xie et al., 2012), pear (Li et al., 2013), and blood orange (Rodrigo et al., 2003). Studies on color mutations have revealed that in addition to the aforementioned structural biosynthetic genes, transcription factors play important roles in modulating anthocyanin biosynthetic pathway activity and color changes (Lepiniec et al., 2006; Saito et al., 2013). The MBW complex [MYB transcription factor in a complex with basic helix-loop-helix (bHLH) and WD40 proteins] has been shown to regulate the expression of anthocyanin genes (Ramsay and Glover, 2005; Petroni and Tonelli, 2011). In the model plant *Arabidopsis*, MYB transcription factors TT2, PAP1/PAP2, MYB75, MYB90, MYB113, and MYB114, bHLH transcription factors TT8, GL3, and EGL3 and the WD40 repeat protein TTG1 regulate the expression of *DFR*, *ANS*, *UFGT* and other downstream genes, affecting anthocyanin biosynthesis (Gonzalez et al., 2008; Saito et al., 2013). Recently, NAC (NAM, ATAF1,2, CUC2) transcription factors have also been reported to affect anthocyanin biosynthesis in blood-fleshed peaches (Zhou et al., 2015).

As one of the world's earliest cultivated fruit trees, more than 600 fig (*Ficus carica* L.) cultivars have been described (Flaishman et al., 2008). The fruit are termed syconia and demonstrate a typical double-sigmoid growth curve, including two rapid size-increment phases (phase I and III) and a slow growth phase between them (phase II) (Crane and Baker, 1953; Kislev and Bar-Yosef, 2006). When the fruit matures (in phase III), its colors are diverse; depending on the cultivar, the peel color can be green, yellow–green, yellow, red, purple, or violet–black. Fig peel color is primarily due to the accumulation of anthocyanins, with anthocyanin type and content differing among the different cultivars (Dueñas et al., 2008). Four anthocyanins have been reported in purple fig cultivars, namely cyanidin-3-O-glucoside, cyanidin-3-rutinoside, pelargonidin-3-glucoside and cyanidin-3,5-diglucoside. Yellow fig cultivars accumulate carotenoids such as lutein, zeaxanthin, β-cryptoxanthin and β-carotene (Yemiş et al., 2012). Cyanidin-3-O-glucoside chloride has been reported as the predominant anthocyanin in the peel of cvs. Black Mission and Brown Turkey (Solomon et al., 2006; Ercisli et al., 2012). As these trees rely mainly on vegetative propagation, mutation is an important agent of change in fig cultivar development.

"Green Peel" ("Qingpi") is a major fig cultivar in China with green-colored fruit; "Purple Peel" ("Zibao") is a bud mutation of "Green Peel," with fruit that turn an appealing dark purple in phase III. In this study, targeted metabolome and transcriptome comparisons were carried out using young and mature fruit of "Green Peel" and "Purple Peel" fig. Beyond identifying specific anthocyanins in the mutant, we reveal very significant accumulation of a set of flavonoids and procyanidin B1, together with systematic transcriptional changes for structural genes, transcription factors and other regulators of the phenylpropanoid and flavonoid biosynthetic pathways, providing valuable information on fruit color and its complex effect on fruit quality components.

Abbreviations: 4CL, 4-Coumarate:coenzyme A ligase; ANS, Anthocyanidin synthase; bHLH, Basic helix-loop-helix; CHI, Chalcone isomerase; CHS, Chalcone synthase; COG, Clusters of orthologous groups of proteins database; DEG, Differentially expressed gene; DFR, Dihydroflavonol 4-reductase; F3H, Flavanone 3-hydroxylase; F3′H, Flavanone 3′-hydroxylase; LAR, Leucoanthocyanidin reductase; PAL, Phenylalanine ammonia-lyase; UFGT, UDP-glucose: flavonoid 3-O-glucosyltransferase.

MATERIALS AND METHODS

Plant Materials and Treatments

The common fig cultivar Green Peel and its bud mutation cv. Purple Peel were cultivated in Weihai City (37°5′ N, 122°1′ W), Shandong Province in China. The soil type is sandy loam. The sampled fig orchard is 1 km from the sea and managed in the same way as the other orchards in this major fig-growing region in China. There were no significant or remarkable differences in 63 tested/observed morphological/horticultural items listed by the UPOV (International Union for the Protection of New Varieties of Plants, Geneva, Switzerland, http://upov.int) [UPOV TG/265/1 (E)] between "Green Peel" and its purple mutant, except for fruit color at ripening (Xu et al., 2016). The main crop fruit used for the metabolome study and RNA-sequencing (RNA-Seq) were collected on 18 Oct 2015, and fruit samples used for RT-qPCR validation were collected on 25 Oct 2016. The fig has a continuous fruiting characteristic, with different development stages of the main crop fruit growing along the shoots. Fruits in the late stage of phase II and in the middle of phase III were sampled from the two cultivars and termed "Green Peel" young fruit (GY), "Purple Peel" young fruit (PY), "Green Peel" mature fruit (GM) and "Purple Peel" mature fruit (PM), respectively. Three biological replicates were collected per sample, each with 20 fruits randomly collected from 15 fig trees in the same plot of the commercial orchard. We took the figs back to the laboratory, and the peel (about 2 mm thick) was carefully excised with a razor blade, collected, frozen in liquid nitrogen, roughly ground and kept at −80°C for further use.

Extraction and Separation of Polyphenol Secondary Metabolites

Fig peel samples were further ground to a fine powder in liquid nitrogen and thoroughly mixed, then a ca. 3-g sample was freeze-dried and crushed using a mixer mill (MM 400, Retsch) with zirconia beads for 1.5 min at 30 Hz. Sample (100 mg) was extracted with 1 mL 70% methanol containing 0.1 mg/L lidocaine as an internal control for 12 h on a rotating wheel at 4°C in the dark. After 10,000 g centrifugation for 10 min at 4°C, the extracts were absorbed (CNWBOND Carbon-GCB SPE Cartridge, 250 mg, 3 mL; ANPEL, Shanghai, China, www.anpel.com.cn) and filtered (SCAA-104, 0.22-μm pore size; ANPEL) before LC–MS analysis. A quality-control sample was prepared by equal blending of all samples; during the assay, the quality control sample was run every 10 injections to monitor the stability of the analytical conditions.

Samples (5 μL) were injected into a HPLC system (Shim-pack UFLC SHIMADZU CBM30A) equipped with a C18 column (Waters ACQUITY UPLC HSS T3, 1.8 μm, 2.1 mm × 100 mm). The binary solvent system was ultra-pure water containing 0.04% acetic acid as mobile phase A and acetonitrile containing 0.04% acetic acid as mobile phase B. The A:B (v/v) gradient was 95:5 at 0 min, 5:95 at 11.0 min, 5:95 at 12.0 min, 95:5 at 12.1 min, 95:5 at 15.0 min. The flow rate was kept at 0.40 mL/min, and the column temperature was maintained at 40°C.

Metabolite Identification and Quantification

The HPLC effluent was connected to an electrospray ionization (ESI)-triple quadrupole-linear ion trap–MS/MS system (Applied Biosystems 4500 Q TRAP). Metabolite identification and quantification were carried out following Chen et al. (2013). In brief, the inspected mass spectra were 50–1,000 m/z. Nitrogen was used as both the nebulizer/auxiliary and collision gas. The ESI source was set to positive ionization mode, the source temperature was held at 550°C; the capillary voltage was 5.5 kV. The monitoring mode was set to multiple-reaction monitoring (MRM).

Metabolite identification was based on the primary and secondary spectral data annotated against public databases, namely MassBank (http://www.massbank.jp/), KNAPSAcK (http://kanaya.naist.jp/KNApSAcK/), HMDB (http://www.hmdb.ca/), MoToDB (http://www.ab.wur.nl/moto/), and METLIN (http://metlin.scripps.edu/index.php), following the standard metabolic operating procedures. Metabolite quantification was carried out using MRM. Partial least squares discriminant analysis (PLS–DA) was carried out with the identified metabolites. Metabolites with significant differences in content were set with thresholds of variable importance in projection (VIP) ≥ 1 and fold change ≥ 2 or ≤ 0.5.

RNA-Seq and Annotation

RNA isolation and purification, and cDNA library construction and sequencing were as performed previously (Chai et al., 2017). In brief, fig samples' total RNA was extracted by the CTAB method (Cao et al., 2016). RNA quantity and quality were determined by NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, USA), respectively. RNA integrity was determined by 1% agarose gel electrophoresis, and the RNA concentration was adjusted for uniformity. mRNA was isolated from total RNA using magnetic beads with oligo (dT); cDNA was synthesized using a cDNA Synthesis Kit (TaKaRa) and linking the sequencing adapter to both ends (Chai et al., 2014). The library preparations were sequenced on an Illumina HiSeq 4000 platform and the unigene sequences obtained from our laboratory transcriptome database by RSEM software were integrated for annotation (Chai et al., 2017). The whole set of annotated genes can be found in the National Center for Biotechnology Information (NCBI) SRA database (accession number SRP114533).

Analysis of Differentially Expressed Genes (DEGs)

For gene-expression analysis, counts were mapped to the reading of each gene by HTSeq v0.5.4p3 and then normalized to FPKM (fragments per kilobase of transcript per million mapped reads) following Mao et al. (2005). DEGs were recruited by $\log_2$ (fold change) ≥ 1 and corrected $P \leq 0.005$. All DEGs were analyzed by gene ontology (GO) enrichment using GOseq (1.10.0) (Götz et al., 2008) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment using KOBAS software (Mortazavi et al., 2008).

Real-Time Quantitative PCR (RT-qPCR) Validation

RNA extraction and quality detection were carried out by RNA-Seq. Reverse transcription was performed using HiFi-MMLV cDNA First-Strand Synthesis Kit (Invitrogen). Twenty color-related genes were selected for RT-qPCR with specific primers designed by Primer Premier 5 software (Supplementary Table 1). The RT-qPCR was performed with an ABI 7500 Fast Real-Time Detection System (Applied Biosystems) using the Ultra SYBR Mix kit (CWBIO, Beijing, China). The amplification system consisted of 10.4 μL Ultra SYBR Premix System II, 0.8 μL of 10 μmol/L upstream primer, 0.8 μL of 10 μmol/L downstream primer, 2 μL template, and sterile distilled water to a total volume of 20 μL. The amplification program was 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 55°C for 1 min. Relative quantitative analysis of data was performed by the $2^{-\Delta\Delta CT}$ method with reference genes *β-actin* and *18S-RNA*. Three technical replicates were carried out for each sample to ensure reproducibility and reliability. Statistical analysis of variance (ANOVA) followed by Duncan's new multiple range test were performed with SPSS Version 16.0 (Chicago, IL, USA). The significance level was set to $P < 0.05$.

RESULTS

Phenotype of "Green Peel" and Its Mutant "Purple Peel"

No morphological differences were detected between the fruit of "Green Peel" and its purple mutant, except for fruit color at ripening. The young fruit used in the present study were harvested in the late stage of fig development phase II, when both "Green Peel" and its purple mutant have a deep green appearance, with a very slight copper hue on the surface of the purple mutant. When the fruits were halved, the texture was hard, and the internal female flowers were a pink–garnet color (**Figures 1A,B**).

Fig development is very rapid in phase III. The fruit quickly enlarge, reaching their final size and harvest quality in 5–7 days. "Green Peel" fruit turned yellow–green in appearance, whereas the mutant developed a dark purple peel. Mature fruit were soft and succulent, and female flowers of both cultivars were deep red inside the fruit (**Figures 1C,D**). As a measure of fruit quality, "Green Peel" and its purple mutant had an average fruit weight of 33.9 ± 2.66 g and 33.4 ± 2.4 g, 18.51 ± 1.03 and 18.34 ± 1.15 °Brix in soluble solids, and peel thickness of 2.14 ± 0.32 and 2.16 ± 0.24 mm, respectively, with no significant differences in the assayed horticultural attributes.

Targeted Secondary Metabolite Assay

The general secondary metabolite profiles of "Green Peel" and "Purple Peel" fig fruit showed marked differences (Supplementary Figures 1, 2). A total of 101 metabolites were identified from GY, PY, GM, and PM samples, each with three biological replicates: 18 phenylpropanoids, 40 flavones, 12 flavonols, 16 flavonoids, 8 anthocyanins, 5 proanthocyanidins, and 2 catechin derivatives (**Table 1**). Setting VIP ≥ 1.0 together with fold change ≥ 2 or ≤ 0.5 as thresholds for significant differences, the contents of 5 and 22 metabolites

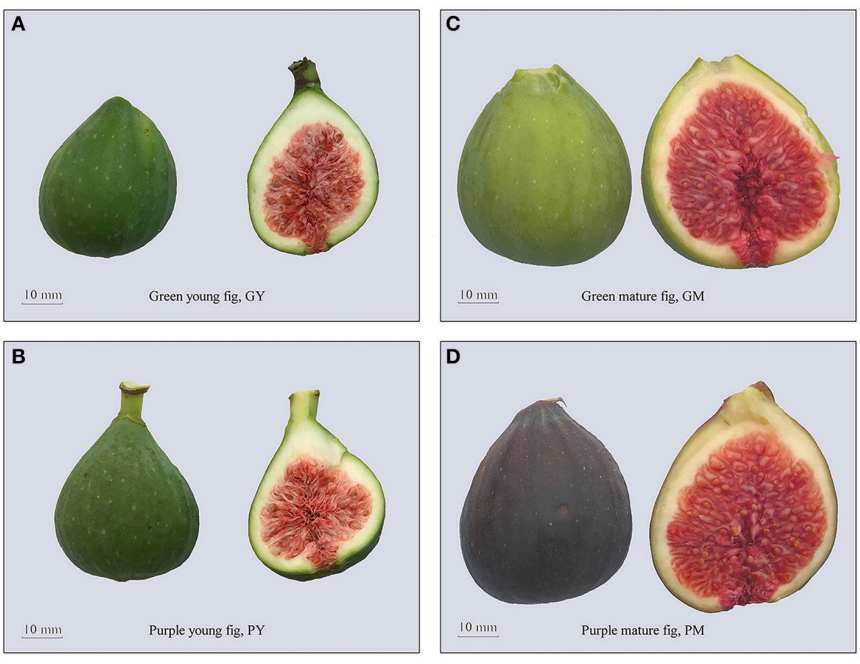

FIGURE 1 | The phenotype of fig (*Ficus carica* L.) cv. Green Peel and its mutation cv. Purple Peel at young and mature stages. **(A)** "Green Peel" young fruit. **(B)** "Purple Peel" young fruit. **(C)** "Green Peel" mature fruit. **(D)** "Purple Peel" mature fruit. GY, "Green Peel" young fruit; GM, "Green Peel" mature fruit; PY, "Purple Peel" young fruit; PM, "Purple Peel" mature fruit.

TABLE 1 | Differentially accumulated phenolic compounds in the peel of "Green Peel" and "Purple Peel" fruit.

Component name	Metabolite name	Content		Fold change (PY/GY; PM/GM)	VIP
		Green fig	Purple fig		
ANTHOCYANIN					
GY vs. PY	Cyanidin O-malonylhexoside	3.81E + 03	2.68E + 04	7.03	3.00541
	Cyanidin O-malonylhexoside O-hexoside	6.37E+03	1.85E+04	2.90	2.21738
GM vs. PM	Cyanidin O-malonylhexoside	1.93E+03	7.69E+06	3992.21	3.42056
	Cyanidin 3-O-glucoside	1.38E+05	6.37E+07	461.40	2.96158
	Cyanidin O-malonylhexoside O-hexoside	5.85E+03	2.44E+06	416.60	2.91667
	Cyanidin-3,5-O-diglucoside (cyanin)	4.62E+05	5.26E+07	113.87	2.58938
PROCYANIDIN					
GM vs. PM	Procyanidin B1	2.79E+04	8.98E+06	322.26	2.84928
	Procyanidin B2	3.64E+04	1.67E+05	4.58	1.44844
	Procyanidin B	1.30E+04	4.69E+04	4.02	1.29685
	Procyanidin B3	3.03E+03	1.22E+04	3.60	1.32665
	Procyanidin A	8.23E+03	1.77E+04	2.15	1.01483
FLAVONE					
GY vs. PY	Apigenin	5.67E+04	2.37E+04	0.42	1.90703
GM vs. PM	Luteolin-3′,7-di-O-glucoside	1.98E+05	1.11E+07	56.06	2.38774
	3′,6-Dimethylflavone	7.24E+03	1.77E+04	2.44	1.02284
	Chrysin	1.22E+05	5.19E+04	0.43	1.09238
	Tangeretin	4.13E+04	1.28E+04	0.31	1.28301
FLAVONOIDS					
GY vs. PY	7-O-Methyleriodictyol	1.91E+04	6.37E+03	0.33	2.18819
GM vs. PM	Epicatechin (EC)	6.22E+04	8.15E+05	13.09	1.87203
	Catechin (C)	5.95E+04	3.32E+05	5.57	1.52977
	Hesperetin 5-O-glucoside	2.32E+06	5.91E+06	2.55	1.13184
	7-O-Methyleriodictyol	8.73E+03	1.84E+04	2.10	1.02489
FLAVONOL					
GM vs. PM	Quercetin-3-O-α-arabinofuranoside (Avicularin)	2.60E+04	9.77E+04	3.76	1.32249
	Quercetin-3-O-glucoside (isoquercitrin)	6.44E+06	1.50E+07	2.33	1.08046
PHENYLPROPANOIDS					
GY vs. PY	Esculetin	6.67E+03	1.37E+04	2.05	1.75354
GM vs. PM	Quinic acid	5.90E+03	2.55E+04	4.32	1.39172
	Cinnamic acid	2.09E+05	6.99E+04	0.34	1.19368
	Esculetin	1.16E+04	2.40E+03	0.21	1.4841

GY, "Green Peel" young fruit; PY, "Purple Peel" young fruit; GM, "Green Peel" mature fruit; PM, "Purple Peel" mature fruit. Metabolite fold changes, value >1.0 represents increase; value <1.0 represents decrease. Differentially accumulated phenolic compounds were identified by threshold VIP (variable importance in projection) ≥1, and fold change ≥2 (upregulation) or ≤0.5 (downregulation).

were significantly different between "Green Peel" and its purple mutant at the young and mature stage, respectively.

Anthocyanins

Four kinds of cyanidin glycosides, delphinidin O-hexoside, malvidin-3-O-galactoside and rosinidin O-hexoside were identified in all samples. In the PY peel, cyanidin O-malonylhexoside and cyanidin O-malonylhexoside O-hexoside were found with 7.03- and 2.9-fold increments compared to GY, which could explain the slight hue on the PY peel. At the mature stage, cyanidin glucoside pigments were responsible for the mutant purple color: cyanidin O-malonylhexoside was increased 3,992.21-fold in the PM vs. GM samples, whereas cyanidin 3-O-glucoside, cyanidin O-malonylhexoside O-hexoside and cyanidin-3,5-O-diglucoside increased 461.4-, 416.6-, and 113.87-fold, respectively (**Table 1**).

Flavonoids, Flavones, and Flavonols

Among the monomeric flavonoids, epicatechin, catechin, hesperetin 5-O-glucoside, and 7-O-methyleriodictyol demonstrated significantly higher contents in the PM; epicatechin was 13.09-fold its content in GM. In young fig fruit samples, apigenin and flavanone 7-O-methyleriodictyol showed 1.2- and 1.6-fold decreases in GY vs. PY (**Table 1**), but no other differences met the criteria.

The A- and B-type procyanidins are dimer flavonoids; their contents only differed in the mature fruit group. The content of procyanidin B1 [epicatechin-(4β → 8)-catechin] was 322.26-fold

higher in the GM vs. PM fruit. Procyanidins B2 [(−)-Epicatechin-(4β → 8)-(−)-epicatechin], B3 [catechin-(4α → 8)-catechin], A1 [epicatechin-(2β → 7,4β → 8)-catechin] and A2 [epicatechin-(2β → 7,4β → 8)-epicatechin] were 2- to 4.5-fold higher in the GM vs. PM (**Table 1**), which were much less than that of procyanidin B1 in the fruit.

For the flavones, luteolin-3′,7-di-O-glucoside and 3′6-dimethylfavone contents were 56.06- and 2.44-fold higher, respectively, in the GM vs. PM. Chrysin and tangeretin revealed significant decreases in the PM, whereas apigenin, the upstream substrate of luteolin, was remarkably lower in the GY vs. PY. A significant increase was found for two quercetin glycosides in the GY vs. PY with a moderate fold change (**Table 1**).

Phenylpropanoids

The phenylpropanoid biosynthetic pathway is upstream of the anthocyanin and flavonoid biosynthetic pathways. We identified 18 general metabolites of phenylpropanoids. Esculetin and quinic acid contents were 2.05- and 4.33-fold higher in the PY and PM peels, respectively, whereas cinnamic acid and esculetin contents in the PM were less than half that in the GM (**Table 1**).

Transcriptome Analysis

RNA-Seq produced 31,591,009, 25,146,641, 32,429,280 and 27,147,120 clean reads from GY, PY, GM and PM libraries, respectively. Clean data from the 12 libraries of 4 samples (3 replicates for each samples), were averaged to 96,158 transcripts of 796.42 bp in length, and 79,355 unigenes were obtained using Trinity software (Supplementary Table 2). The N50 value was 1236 bp, and the average length of the unigenes was 683.07 bp.

There were 2,385, 1,087, 3,911, and 5,413 DEGs in the four comparison groups: GY vs. PY, GM vs. PM, GY vs. GM, and PY vs. PM, respectively. Comparing the two cultivars, 1,009 and 616 genes were upregulated, and 1,376 and 471 genes were downregulated in GY vs. PY and GM vs. PM, respectively (**Figure 2A**). Venn diagram analysis showed 51 DEGs that were common to all four comparison groups (**Figure 2B**). GO analysis assigned 46,748, 34,527 and 22,307 unigenes to the biological process, cell component and molecular functional class, respectively (Supplementary Figure 3). The clusters of orthologous groups of proteins database (COG) annotation allocated 15,726 unigenes into 25 COG categories (Supplementary Figure 4); the general functional cluster prediction (2,115 unigenes, 13.45%) was the largest group, followed by signaling mechanism (1,897 unigenes, 12.06%), posttranslational modification and protein turnover (1,572 unigenes, 10.00%).

KEGG analysis revealed plant hormone signal transduction, starch and sucrose, phenylpropanoid biosynthesis and alpha-linolenic acid metabolism as the significantly changed pathways in GY vs. PY. Plant hormone signal transduction, phenylpropanoid and flavonoid biosynthetic pathways were significantly changed in GY vs. GM and GM vs. PM (**Table 2**).

Phenylpropanoid, Flavonoid, and Anthocyanidin Biosynthetic Pathways

At maturity, most of the secondary metabolite pathways were strengthened by gene-expression upregulation in the "Purple Peel" mutant fruit, except for the DEGs *PAL* and *4CL*. Two *PAL* genes (*c388_g1* and *c388_g2*) were downregulated (-1.14- and−1.02-fold) and five *4CL* unigenes were downregulated, in line with the decreased cinnamic acid content in the PM peel. Simultaneous large-scale upregulation of structural genes of the phenylpropanoid, flavonoid and anthocyanin biosynthetic pathways, including *CHS*, *CHI*, and *flavonol synthase* (2 DEGs each), *UFGT* (4 DEGs), and other genes (1 DEG) dominated secondary metabolite synthesis modulation

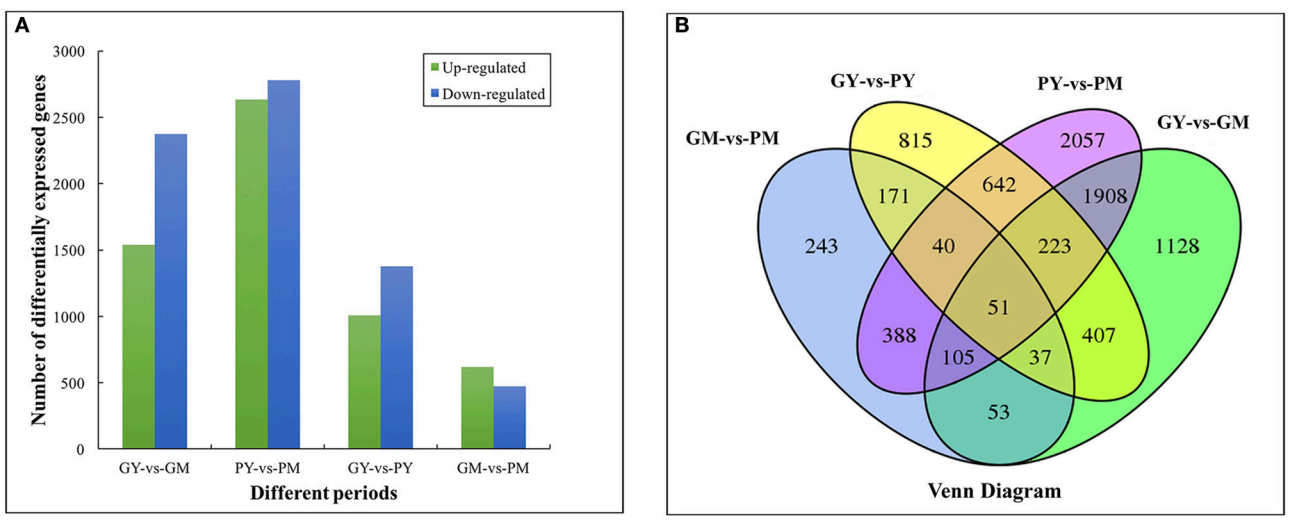

FIGURE 2 | Functional annotation and classification of differentially expressed genes between young and mature stages of "Green Peel" and "Purple Peel." **(A)** Numbers of differentially expressed genes. **(B)** Venn diagram. GY, "Green Peel" young fruit; GM, "Green Peel" mature fruit; PY, "Purple Peel" young fruit; PM, "Purple Peel" mature fruit.

TABLE 2 | Significantly enriched KEGG pathways between "Purple Peel" and "Green Peel" figs.

No.	Pathway	DEGs with pathway annotation	All genes with pathway annotation	*P*-value	Corrected *P*-value	Pathway ID
GY vs. PY						
1	Plant hormone signal transduction	34	227	1.96E-11	6.13E-09	ko04075
2	Starch and sucrose metabolism	22	281	5.22E-07	8.17E-05	ko00500
3	Phenylpropanoid biosynthesis	3	182	3.38E-05	0.003522746	ko00940
4	Alpha-linolenic acid metabolism	14	66	8.31E-05	0.006499291	ko00592
GM vs. PM						
1	Flavonoid biosynthesis	17	52	4.02E-08	1.06E-05	ko00941
2	Protein processing in endoplasmic reticulum	32	318	1.34E-06	0.000176186	ko04141
3	Estrogen signaling pathway	16	92	3.99E-06	0.000349801	ko04915
GY vs. GM						
1	Plant hormone signal transduction	58	227	9.51E-16	3.03E-13	ko04075
2	Phenylpropanoid biosynthesis	10	182	8.45E-09	1.35E-06	ko00940
3	Flavonoid biosynthesis	15	52	3.71E-07	3.95E-05	ko00941
PY vs. PM						
1	Plant hormone signal transduction	62	227	1.36E-10	4.45E-08	ko04075
2	Flavonoid biosynthesis	23	52	4.44E-07	7.26E-05	ko00941
3	Phenylpropanoid biosynthesis	11	182	1.39E-05	0.001513077	ko00940

GY, "Green Peel" young fruit; PY, "Purple Peel" young fruit; GM, "Green Peel" mature fruit; PM, "Purple Peel" mature fruit. Significant pathways were identified by corrected P ≤ 0.01.

in GM vs. PM (**Figure 3**). High fold upregulation and high RPKM (reads per kilobase of transcript per million mapped reads) enhanced the flux in the flavonoid and anthocyanidin biosynthetic pathways. Structural genes *CHS* (*c46769_g2*) and *CHI* (*c658_g1*) showed 3.38- and 4.27-fold increments, *F3H* (*c72067_g1*) 5.47-fold upregulation, *F3′H* (*c42263_g3*) 2.65-fold upregulation, together with 2 flavonol synthase genes that not only catalyze the conversion from kaempferol to quercetin (Pelletier et al., 1997), but also from apigenin to luteolin (Martens et al., 2003; Jaakola, 2013); this could largely explain the high accumulation of luteolin-3′,7-di-O-glucoside in the PM (**Table 1**). Catechin is produced from leucocyanidin catalyzed by leucoanthocyanidin reductase (LAR) (*c31753_g1*, 3.95-fold upregulation); the enzyme also catalyzes leucodelphinidin and leucopelargonidin to gallocatechin and afzelechin, respectively, neither of which demonstrated significant content differences between the cultivars, corresponding to the lower change in content of A-type procyanidin (**Table 1**, **Figure 3**).

LAR expression (*c31753_g1*) was upregulated 3.95-fold. *ANS* (*c59676_g1*) was one of the most significantly DEGs in the GM vs. PM group, increasing 10.67-fold, followed by two *UFGT* genes (*c78174_g2* and *c45009_g5*) which showed 9.98-fold and 5.84-fold upregulation in the PM fruit (**Figure 3**). Anthocyanidin 3-O-glucosyltransferase 2 (*c45009_g5*, 5.84-fold upregulation) catalyzes cyanidin to cyanidin-3-O-glucoside. Cyanidin-3,5-O-diglucoside can be glycosylated from cyanidin-3-O-glucoside or cyanidin-5-O-glucoside; UDP-glycosyltransferase 75D1 (*c43420_g2*, 1.58-fold upregulation) catalyzes cyanidin-3-O-glucoside to cyanidin-3,5-O-diglucoside, which also supports the high measured accumulation of the two cyanidin mono- and di-glucosides, flavonoids and procyanidins in the "Purple Peel" fig (**Table 1**, **Figure 3**).

Transcription Factors

There were 74 and 45 differentially expressed transcription factor genes identified in GY vs. PY and GM vs. PM, respectively, whereas from young fruit to mature fruit, 140 and 141 DEGs were identified as transcription factors in GY vs. GM and PY vs. PM, respectively (**Table 3**, Supplementary Table 3). The differentially expressed transcription factors were annotated as encoding MYB, bHLH, AP2/ERF, WRKY, HD-ZIP, heat-shock transcription factor (HSF), NF-Y, DIVARICATA, and MADS-box (**Table 3**).

Almost all of the *MYB* DEGs could be further assigned to the *R2R3 MYB* family, which is closely associated with anthocyanin biosynthesis in fruit trees (Allan et al., 2008; Liu et al., 2016). Nineteen and nine *R2R3-MYB*s were differentially expressed in the young fruit (GY vs. PY) and mature fruit (GM vs. PM), respectively. Among the *MYB* DEGs in young fruit, 6 genes were found upregulated and 13 downregulated in PY (**Figure 4A**). In mature fruit, there were 9 recognized *MYB* DEGs: 5 more highly expressed *MYB*s in PM, and 4 more highly expressed *MYB*s in GM, but all with low FPKM values (**Figure 4B**). Along fig fruit development, 33 *MYB* DEGs (7 upregulated and 26 downregulated) were illustrated in GY vs. GM, 29 *MYB* DEGs (18 upregulated and 11 downregulated) in PY vs. PM (**Table 3**). Nine *MYB*s had significantly increased transcripts in both PY vs. PM and GM vs. PM, 4 of them also showing upregulation in GY vs. GM.

We further recruited five *R2R3-MYB*s—unigenes c*31006*_g1, *c39054_g1, c37406_g4, c38737_g1,* and *c43569_g2*—which showed high fold change in expression between the two cultivars and/or developmental stages (**Figures 4A,B**). The expression of *c43569_g1* and *c31006_g1* was specifically increased in PM. Protein sequence comparison revealed that c43569_g1 is highly

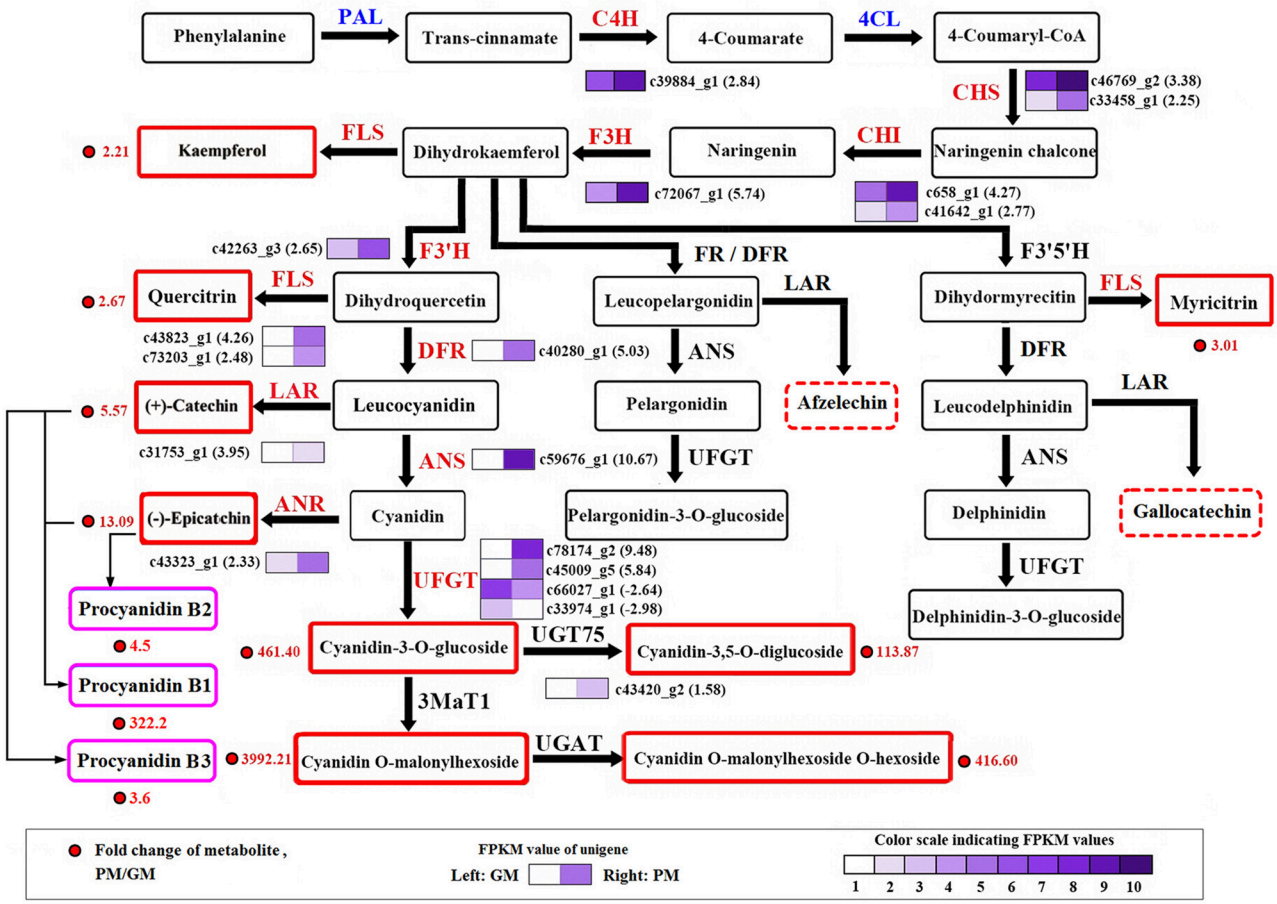

FIGURE 3 | Transcript profiling of genes in the phenylpropanoid and flavonoid biosynthetic pathways in cv. "Green Peel" and "Purple Peel" at mature stages. GM, "Green Peel" mature fruit; PM, "Purple Peel" mature fruit. Grids with color-scale from light to dark represent RPKM values 0–10, 10–20, 20–40, 40–80, 80–160, 160–320, 320–640, 640–1,280, 1,280–2,560, and over 2,560, respectively. PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavanoid 3′-hydroxylase; DFR, dihydroflavonol 4-reductase; FR, flavanone 4-reductase; ANS/LDOX, anthocyanidin synthase/leucocyanidin oxygenase; UFGT, UDP glucose-flavonoid 3-O-glcosyl-transferase; FLS, flavonol synthesis; LAR, leucocyanidin reductase; ANR, anthocyanin reductase.

homologous (72%) to MdMYB110a of apple (**Figure 4C**), which plays a key role in the red flesh apple phenotype (Chagné et al., 2013). The unigene c31006_g1 clustered with AtMYB123 of *Arabidopsis* and PpMYB9 of *Prunus persica*, which regulates anthocyanin accumulation in different plant tissues (Zhou et al., 2016). The highly expressed MYB c39054_g1 in PY was closely related to the flavonoid MYB repressor PpMYB20 (**Figure 4C**; Zhou et al., 2016), whereas c37406_g4 and c38737_g1 clustered with the anthocyanin activator groups, with high similarity to AtMYB44, VvMYBPA1 and VvMYBPA2, which regulate anthocyanin biosynthesis in *Arabidopsis* and grape (Terrier et al., 2009; Jung et al., 2010; Zhou et al., 2016). **Figure 4D** illustrates the fig R2R3-MYBs' highly homologous R2 and R3 DNA-binding domains at the N-terminus (Espley et al., 2007), and highly variable truncated C-terminal region, which might relate to fig color morph regulation.

Thirteen *bHLH* DEGs were found in young fruit (GY vs. PY); 2 were highly expressed in PY, and 11 were downregulated. Eight *bHLH* revealed differences in mature-stage fruit (GM vs. PM): 2 *bHLH* were highly expressed in PM (**Table 3**). During fruit development, 29 *bHLH* DEGs were screened in the Green Peel cultivar, including 4 upregulated and 25 downregulated from young to mature fruit, whereas among 26 *bHLH* of the Purple Peel cultivar, 7 contigs or transcripts were upregulated and 19 were downregulated from young to mature fruit (**Table 3**). We found 2 *bHLH* DEGs (FPKM $\geq$ 300)—*c21697_g1* and *c43844_g1*—expressed at very high levels in the PY, that decreased rapidly at the mature stage of "Purple Peel," and their expression levels were very low in GY and GM (Supplementary Figure 5).

Heat-Shock Proteins (HSPs)

HSPs are involved in protein synthesis, folding, cell localization and protein degradation; they also play a role in maintaining intercellular environmental stability (Wang et al., 2004; Waters, 2013). In the mature fig fruit, 15 small HSP family DEGs were identified, including 9 *HSP20*, 3 *HSP90*, 2 *HSP70*, and 1

TABLE 3 | Differentially expressed transcription factors in the peel of young and mature fruit of "Green Peel" and "Purple Peel" fig.

Comparison group	Gene name	Number of DEGs	Upreg-ulated DEGs	Downreg-ulated DEGs	Description	Biological functions
GY vs. PY	MYB	19	6	13	MYB TFs	Cell development and anthocyanin pathway
	AP2/ERF	21	6	15	Ethylene-responsive TF	Plant development and stress response
	bHLH	13	2	11	Basic helix-loop-helix protein	Plant development and substance metabolism
	Other TFs	21	13	8		
	In total	74	27	47		
GM vs. PM	MYB	9	5	4	MYB TFs	Cell development and anthocyanin pathway
	AP2/ERF	10	10	0	Ethylene-responsive TF	Plant development and stress response
	bHLH	8	2	6	Basic helix-loop-helix protein	Plant development and substance metabolism
	Other TFs	18	6	7		
	In total	45	23	17		
GY vs. GM	bHLH	29	4	25	Basic helix-loop-helix protein	Plant development and substance metabolism
	MYB	33	7	26	MYB TFs	Cell development and anthocyanin pathway
	AP2/ERF	22	11	11	Ethylene-responsive TF	Plant development and stress response
	WRKY	18	9	9	WRKY DNA-binding protein	Defense responses and plant development
	HD-ZIP	8	3	5	Homeobox-leucine zipper protein	Photomorphogenesis and fruit ripening
	MADS-box	5	0	5	MADS-box TFs	Fruit development and ripening
	Other TFs	25	1	24		
	In total	140	35	105		
PY vs. PM	MYB	29	18	11	MYB TFs	Cell development and anthocyanin pathway
	bHLH	26	7	19	Basic helix-loop-helix protein	Plant development and substance metabolism
	AP2/ERF	19	12	7	Ethylene-responsive TF	Plant development and stress response
	WRKY	15	11	4	WRKY DNA-binding protein	Defense responses and plant development
	HD-ZIP	10	0	10	Homeobox-leucine zipper protein	Photomorphogenesis and fruit ripening
	HSF	8	7	1	Ethylene-responsive TF	Plant growth, development and stress response
	HAP	4	0	4	Nuclear TF Y subunit A	Embryonic development and chloroplast biogenesis
	Other TFs	30	11	19		
	In total	141	66	75		

GY, "Green Peel" young fruit; PY, "Purple Peel" young fruit; GM, "Green Peel" mature fruit; PM, "Purple Peel" mature fruit. Differentially expressed genes were identified by FDR ≤ 0.001 and absolute value of log_2 ratio ≥ 2.

HSP40, all of which showed significantly higher expression in the GM vs. PM (**Table 4**); moreover, 3 genes encoding heat-shock transcription factors (HSFs) (*c45384_g1*, *c26517_g2,* and *c43194_g3*) showed a significant expression increment in the PM (**Table 4**). HSFs bind to the heat shock element of the HSP gene promoter to form transcription complexes, which promote HSP gene expression (Scharf et al., 2012). HSPs are molecular chaperones, also known as stress-induced proteins, which function in protein folding and assembly, protect enzymes from denaturation and cellular degeneration with pigment and flavonoid accumulation, responding to stress and maturation in fig (Sun et al., 2002; Neta-Sharir et al., 2005).

RT-qPCR Validation of the Transcriptomic Data

To validate the key RNA-Seq results, we selected 20 DEGs (4 transcription factor genes, 4 phenylpropanoid biosynthetic pathway genes, and 12 flavonoid biosynthetic pathway genes) (Supplementary Figure 6) and analyzed their expression levels in PY, GY, PM, and GM using RT-qPCR. The expression patterns of these genes were very similar to the RNA-Seq results, with correlation coefficients (R^2) > 0.83 (**Figure 5**). The results validated the relevance of the RNA-Seq data and RT-qPCR showed good consistency for both up- and downregulated gene expression.

DISCUSSION

Natural mutations have been, and still are, observed, deliberately selected for and used in fruit crop production. However, the resultant differences in gene structure and expression regulation in the mutants has only recently begun to be revealed. A combined metabolome and transcriptome study can provide us with new, large-scale information on the shifted secondary metabolic product profiles and the underlying modifications in gene-expression networks.

Large-Scale Secondary Metabolite and Pathway Regulation

Color mutants are widely used in horticultural and other crops, especially those that are commonly propagated vegetatively, such as most fruit trees. The color mutants are usually promoted and regarded as presenting a single-attribute difference. Herein, we identified 4 cyanidin glycosides in "Purple Peel" fig fruit,

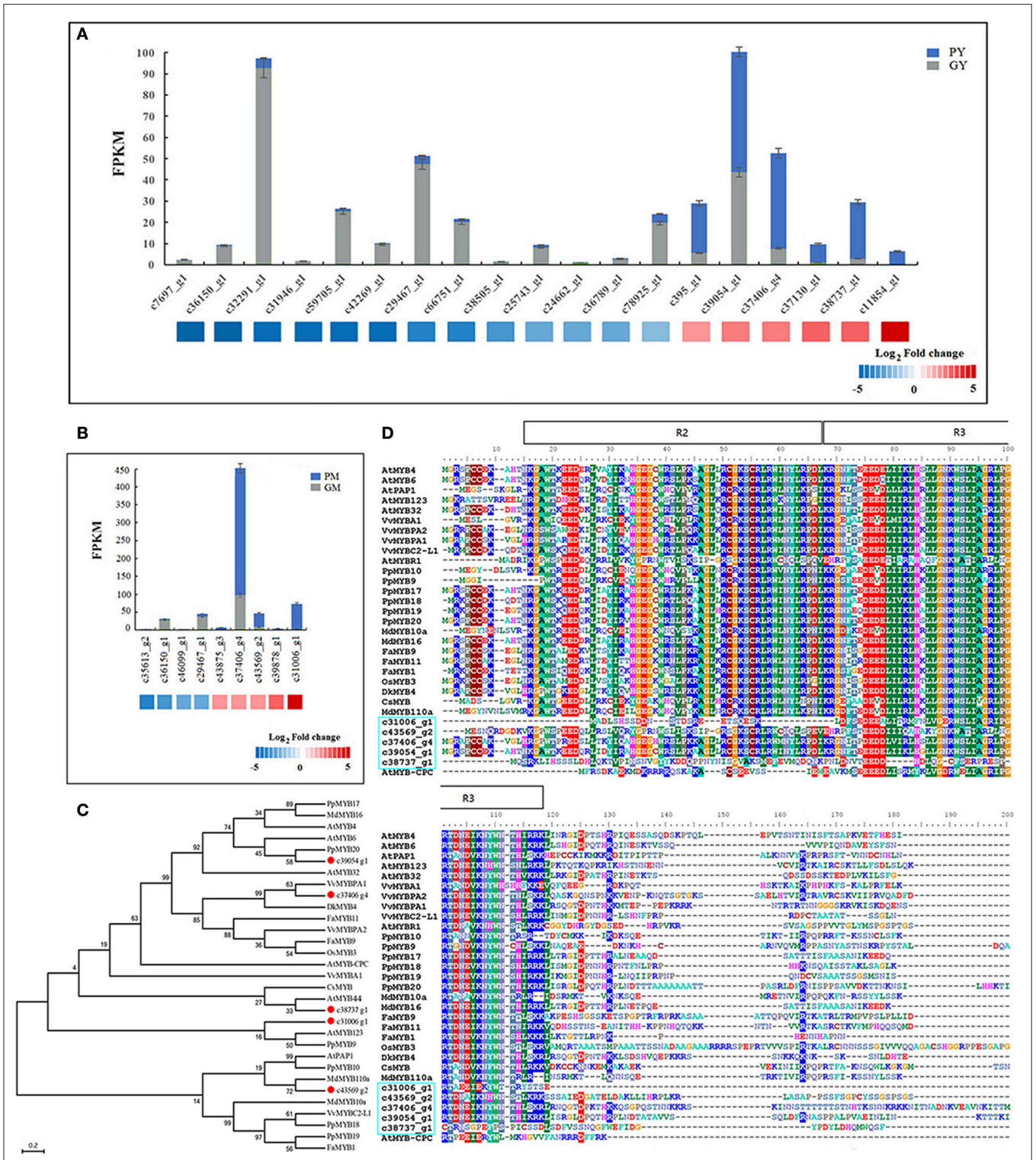

FIGURE 4 | Differentially expressed *MYB* genes between "Green Peel" and "Purple Peel" fruit at young and mature stages. **(A)** Differentially expressed *MYB* genes between the two cultivars' young fruit. **(B)** Differentially expressed *MYB* genes between the two cultivars' mature fruit. **(C)** Phylogenetic analysis of five fig *MYBs* recruited by high fold expression change. **(D)** R2R3-MYB protein sequence alignment of five fig *MYBs* recruited by high fold expression change; R2R3 motif is indicated at the top.

TABLE 4 | Differentially expressed heat-shock protein (HSP) and heat-shock transcription factor (HSF) genes in the mature stage of "Purple Peel" and "Green Peel" fig.

Gene name	Seq_ID	Log_2 FC (PM/GM)	*P*-value	GM_ FPKM	PM_ FPKM	Regulated
HSP20	c44815_g2	5	1.03E-06	1.92	64.35	Up
	c46276_g2	4.39	1.01E-15	33.21	698.96	Up
	c32064_g1	4.22	5.79E-38	16.06	301.27	Up
	c46276_g3	4.08	1.99E-33	34.29	582.76	Up
	c46276_g1	3.08	2.27E-32	52.55	445.82	Up
	c44815_g1	3.07	1.52E-25	26.18	220.94	Up
	c22071_g1	2.71	6.01E-07	3.02	20.29	Up
	c46998_g1	2.69	2.08E-15	5.43	35.67	Up
	c25561_g1	2.65	3.11E-12	4.63	29.67	Up
HSP70	c43747_g1	7.19	5.85E-06	0	14.5	Up
	c46871_g7	6.83	1.25E-32	0	11.25	Up
	c45569_g1	3.57	9.76E-30	2.11	26.18	Up
HSP90A	c39629_g1	4.69	3.12E-10	0	2.47	Up
	c31839_g1	3.99	8.83E-08	6.36	102.47	Up
HSP40	c39984_g1	3.83	8.29E-43	14.91	213.78	Up
HSF	c43194_g3	3.75	3.96E-41	6.35	86.71	Up
	c26517_g2	3.05	2.30E-04	1.69	14.73	Up
	c45384_g1	2.2	4.22E-12	7.3	33.91	Up

Differentially expressed genes were identified by FDR ≤ 0.001 and absolute value of log_2 ratio ≥ 2 (2-fold). FC, fold change.

determined the substance responsible for the mutated purple color, and more importantly, revealed highly significant accumulation of colorless procyanidin B1, luteolin-3′,7-di-O-glucoside, epicatechin and other important secondary metabolites in the phenylpropanoid and flavonoid biosynthetic pathways. These findings illustrate, for the first time, a panorama of the large-scale secondary metabolite changes for a color mutation in the ancient fruit crop *Ficus carica*. The cyanidin glucosides in PM differed from those in other dark-colored fig cultivars, such as cyanidin-3-O-rhamnoglucoside (cyanidin-3-O-rutinoside), reported as the main anthocyanin in the peel of "Black Mission," "Bursa," and "Brown Turkey" figs (Solomon et al., 2006; Ercisli et al., 2012). Acyl-modified anthocyanins are common in *Arabidopsis* (D'Auria et al., 2007), and increased cyanidin 3-O-(malonyl)-glucoside has been reported in the cool-cultivated red lettuce to be the only pigment responding to temperature (Becker et al., 2014). A comparison of different cranberry cultivars indicated that highly pigmented berries also have higher contents of colorless flavonol (Bilyk and Sapers, 1986). Anthocyanins and flavonoids affect fruit color and taste; their antioxidant and nutraceutical capacities confer healthful properties, reducing the risk of cardiovascular morbidity and mortality (Holt et al., 2002; Wu et al., 2012).

The large-scale transcription expression increments in phenylpropanoid and flavonoid biosynthetic pathway genes in "Purple Peel" fig, revealed by RNA-Seq, strongly supported our metabolome results. Similarly, most of the structural genes in the anthocyanin biosynthetic pathway are upregulated during fruit development of red vs. green color mutations of pear (Yang et al., 2013). Coordinated expression changes of *F3H*, *F3′H*, *DFR1*, *ANS,* and *UFGT* have also been demonstrated in differently colored Chinese bayberries (Niu et al., 2010), grapes (Boss et al., 1996), *Arabidopsis* (Pelletier et al., 1997; Saito et al., 2013) and other plants (Quattrocchio et al., 1993).

The mutated color attribute is observed late in fruit development. However, significant changes in phenylpropanoid biosynthesis were found between the young fruit of the two cultivars, indicating that the mutation-induced change in expression could occur far earlier than the emergence of the phenotype. Anthocyanins are end products of the flavonoid biosynthetic pathway; our finding of upregulation of almost all of this pathway's genes, from the upstream *CHS* to the end gene *UFGT,* during "Purple Peel" fruit ripening suggests that fundamental transcriptional regulation of the flavonoid and pigment biosynthetic pathways could be a major factor in the mutation, coordinating gene expression, fruit coloration, and the accumulation of flavonoid intermediates and procyanidins. In crabapple cultivars with dark red, pink and white petal colors, *CHS* has been found responsible for the red coloration (Tai et al., 2014). Upstream pathway expression regulation has also been reported in arctic mustard flowers, which have a broad range of purple to white petal color polymorphisms; in the white-flowered individuals, *CHS* was significantly repressed, whereas the expression of other structural genes in the anthocyanin biosynthetic pathway was similar to that in the colored individuals (Dick et al., 2011). The enzymes DFR and LAR are shared by the anthocyanin and flavanone biosynthetic pathways. DFR from different plants has specific substrate biases for dihydroquercetin, dihydrokaempferol and dihydromyricetin (Hua et al., 2013; Saito et al., 2013). LAR belongs to the reductase–epimerase–dehydrogenase family and the short-chain dehydrogenase/reductase superfamily, and each of the LARs has a specific C-terminal domain which may have different substrate specificity (Tanner et al., 2003). From our metabolome and transcriptome data, it seems that fig DFRs and LARs favor dihydroquercetin to produce leucocyanidin and catechin, rather than afzelechin and gallocatechin synthesis (**Figure 3**); thus, only cyanidin glycosides were the dominant anthocyanins, as with the B-type procyanidin in fig fruit (**Table 1**).

Transcription Factors in Fruit Color Formation and Ripening

Our finding of upregulation of most or all of the biosynthesis genes in the mutant fruit suggests mutation of a transcription factor. MYBs play a critical role as key transcription factors for all of the anthocyanin biosynthetic pathway genes or for the regulation of single key genes in fruit and flower color formation (Kobayashi et al., 2004; Espley et al., 2007; Tai et al., 2014). In apple, *CHS* is positively regulated by *MYB4* and *MYB5* expression (Clark and Verwoerd, 2011), whereas strawberry *FcMYB1* switches anthocyanins and flavonoid-derived compound accumulation on and off (Salvatierra et al., 2013). Loss of the MYB cis-element in the *CHS* promotor leads to white crabapple morphs (Dick et al., 2011). In our study, differentially expressed *MYB*s were recruited in the "Purple Peel" fig (**Table 3**, **Figure 4**), indicating that MYBs in the MBW

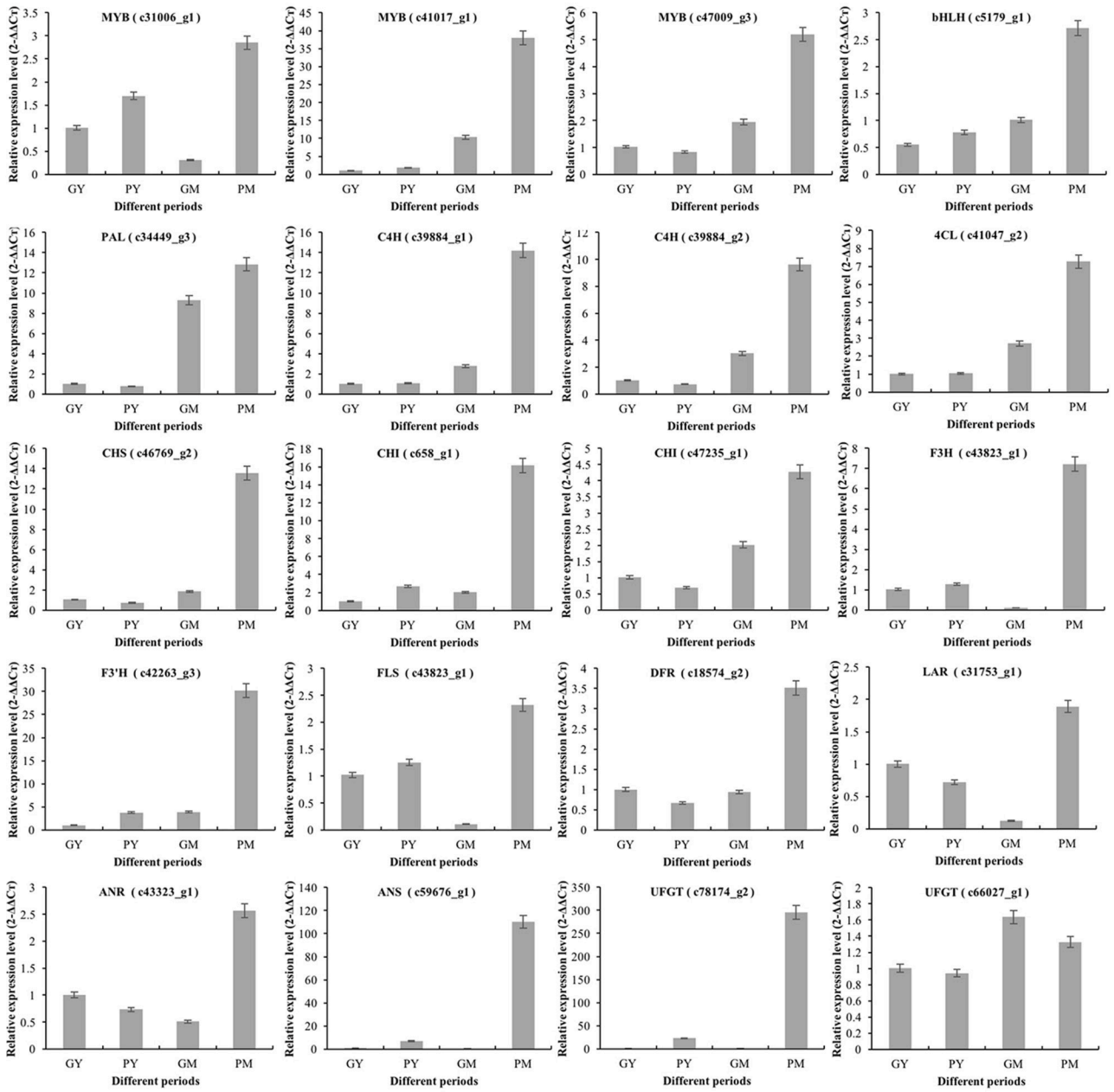

FIGURE 5 | Expression of representative genes in young and mature stages of "Purple Peel" and "Green Peel" fig fruit validated by qRT-PCR. GY, "Green Peel" young fruit; GM, "Green Peel" mature fruit; PY, "Purple Peel" young fruit; PM, "Purple Peel" mature fruit.

complex are key regulators of the pathway of anthocyanin and flavonoid biosynthesis in fig.

Hypothesized Nature of the Fig Purple Mutation

Red and black dominate the color spectrum of bird-dispersed fruit worldwide (Willson and Whelan, 1990). Anthocyanin synthesis and pigmentation can be regarded as the wild type for the fruit color trait. In grapes, white cultivars are thought to be mutations of red cultivars (Boss et al., 1996; Kobayashi et al., 2004; Hichri et al., 2011b), and all of the green grape cultivars have a common origin (Walker et al., 2007). The small seeds contained inside the fig syconia are dispersed by birds. We therefore assume that figs with a dark peel are the wild type, those with a green peel represent a color mutation, and the "Purple Peel" mutant of "Green Peel" can be regarded as a reverse mutation, regaining the wild-type trait.

Understanding the nature of the green-color fruit as a mutant of the wild type could facilitate analysis of the mechanism underlying the reverse mutation. Any functional loss of key

enzymes in the anthocyanin biosynthetic pathway could lead to a green or white mutation, such as via insertion in the structural genes, and turned off or repressed structural gene expression by MYB transcription factors associated with the components of the MBW complex (Feller et al., 2011; Petroni and Tonelli, 2011; Tai et al., 2014). A large number of publications have demonstrated MYB family transcription factors as key regulators in phenylpropanoid, flavonoid, anthocyanin and proanthocyanidin biosynthesis (Falcone Ferreyra et al., 2012; Verdier et al., 2012; Liu et al., 2015; Xu et al., 2015). Moreover, studies with different fruit have revealed conserved components of the regulatory complex controlling anthocyanin biosynthesis in all higher plants, including conserved cis-regulation elements in promotors of key genes of the pathways (Quattrocchio et al., 1993; Koch et al., 2001; Stracke et al., 2007; Dick et al., 2011). The function and expression level of MYBs could be significantly affected by different types of mutations. A single amino-acid substitution in the R2 domain of *VvMYB5b* was found to affect the protein's ability to activate the transcription of flavonoid genes (Hichri et al., 2011b). A retrotransposon insertion in grape *mybA1* blocks the gene's expression, leading to loss of pigmentation in white grape cultivars (Kobayashi et al., 2004). In our study, differential expression of both transposons and retrotransposons was recorded, and a significant upregulation trend in a large number of reverse transcriptase, integrase and gag sequences was revealed in the "Green Peel" as compared to its purple mutant (Supplementary Table 4), suggesting that "Green Peel" is a retrotransposon insertion mutation.

In grapes, *VvMYBA1* and *VvMYBA2* have different on/off switch mechanisms: Gret1 retrotransposon insertion in the promoter of *VvMybA1* switches off *VvMybA1* expression, whereas a non-synonymous single-nucleotide polymorphism present in the coding region switches off the function of *VvMybA2* and leads to white grape berries (Kobayashi et al., 2004; Walker et al., 2007). In our case, *MYB*s, together with the changes in transposon and retrotransposon activation, could be candidates for the "Purple Peel" fig mutation from its "Green Peel" progenitor (Ramsay et al., 2003).

In summary, this combined metabolome and transcriptome study gives us a picture of modulated anthocyanin and flavanoid expression in the "Purple Peel" fig mutant, revealing the large-scale changes in nutritionally important compounds and gene expression in a horticultural mutation with a single phenotypic attribute. Our results provide new information on the anthocyanidin, flavonol and procyanidin metabolites of fig and the global transcriptional changes in fig color regulation, secondary metabolism pathways and regulators in fruit ripening and quality formation.

ETHICS STATEMENT

The study was approved by fig cooperatives in Weihai City, Shandong Province in China.

AUTHOR CONTRIBUTIONS

HM and SC designed the experiments. ZW and YC conducted the experiments and analyzed the results. ZW, YC, AV, SC, and HM prepared the manuscript. All authors have read and approved the manuscript 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.2017.01990/full#supplementary-material

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Metagenomic and Metatranscriptomic Analyses of Diverse Watermelon Cultivars Reveal the Role of Fruit Associated Microbiome in Carbohydrate Metabolism and Ripening of Mature Fruits

Thangasamy Saminathan[1†], Marleny García[2†], Bandana Ghimire[1], Carlos Lopez[1,2], Abiodun Bodunrin[1], Padma Nimmakayala[1], Venkata L. Abburi[1], Amnon Levi[3], Nagamani Balagurusamy[2] and Umesh K. Reddy[1*]*

[1] Gus R. Douglass Institute and Department of Biology, West Virginia State University, Institute, WV, United States, [2] Laboratorio de Biorremediación, Facultad de Ciencias Biológicas, Universidad Autónoma de Coahuila, Torreón, Mexico, [3] U.S. Vegetable Laboratory, Agricultural Research Service, United States Department of Agriculture, Charleston, SC, United States

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***Correspondence:**
Umesh K. Reddy
ureddy@wvstateu.edu
Nagamani Balagurusamy
bnagamani@uadec.edu.mx

[†] These authors have contributed equally to this work.

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The plant microbiome is a key determinant of plant health and productivity, and changes in the plant microbiome can alter the tolerance to biotic and abiotic stresses and the quality of end produce. Little is known about the microbial diversity and its effect on carbohydrate metabolism in ripe fruits. In this study, we aimed to understand the diversity and function of microorganisms in relation to carbohydrate metabolism of ripe watermelon fruits. We used 16S metagenomics and RNAseq metatranscriptomics for analysis of red (PI459074, Congo, and SDRose) and yellow fruit-flesh cultivars (PI227202, PI435990, and JBush) of geographically and metabolically diverse watermelon cultivars. Metagenomics data showed that Proteobacteria were abundant in SDRose and PI227202, whereas Cyanobacteria were most abundant in Congo and PI4559074. In the case of metatranscriptome data, Proteobacteria was the most abundant in all cultivars. High expression of genes linked to infectious diseases and the expression of peptidoglycan hydrolases associated to pathogenicity of eukaryotic hosts was observed in SDRose, which could have resulted in low microbial diversity in this cultivar. The presence of GH28, associated with polygalacturonase activity in JBush and SDRose could be related to cell wall modifications including de-esterification and depolymerization, and consequent loss of galacturonic acid and neutral sugars. Moreover, based on the KEGG annotation of the expressed genes, nine α-galactosidase genes involved in key processes of galactosyl oligosaccharide metabolism, such as raffinose family were identified and galactose metabolism pathway was reconstructed. Results of this study underline the links between the host and

fruit-associated microbiome in carbohydrate metabolism of the ripe fruits. The cultivar difference in watermelon reflects the quantum and diversity of the microbiome, which would benefit watermelon and other plant breeders aiming at the holobiont concept to incorporate associated microbiomes in breeding programs.

Keywords: watermelon, microbiome, ripe fruits, metagenomics, metatranscriptomics

INTRODUCTION

Watermelon (*Citrullus lanatus*) is a major cucurbit crop grown in tropical and subtropical regions of the world (Chomicki and Renner, 2015). Because of its nutritional properties, watermelon represents ∼7% of the world area of vegetable cultivation[1]. The watermelon fruit is rich in water (90%), sugar, fiber, vitamins, amino acids, minerals and carotenoids, especially lycopene, flavonoids, and triterpenoids. Nutritional composition of plants is mediated by the different stages of development. A complex and highly coordinated developmental phase of fruit ontogeny is ripe stage, where several physiological changes occur (Gapper et al., 2013). Recently, plants have been considered a holobiont, a unit encompassing both the host and its associated microbiome (Vandenkoornhuyse et al., 2015). The microbiome is associated in the form of colonization outside the plant as well as inside, such as vascular bundles, roots, and leaves (Berendsen et al., 2012). Most microorganisms, particularly those colonizing roots and stems, seem to originate from the rhizosphere and colonize plant organs as part of their life cycle. Some microorganisms are able to move systemically within the plant (Hallmann and Berg, 2006; Rosenblueth and Martínez-Romero, 2006), whereas others are restricted to below-ground parts of plants (Hallman et al., 2001; Compant et al., 2011). This plant-associated microbiome is highly diverse and comprises a range of different taxa (James et al., 1994; Rosenblueth and Martínez-Romero, 2006). Distinct microbial communities in low density have been reported in flowers, seeds, and fruits (Compant et al., 2010).

Interactions between plant tissues and microbiota can be beneficial, including mutualistic interactions that promote plant health and productivity and can have adverse or no effects on the plant phenotype (Müller et al., 2016). The beneficial effect of direct plant growth promotion by microbes is based on improved nutrient acquisition and hormonal stimulation. The presence of neutral and mutualistic microorganisms prevent the colonization of pathogenic microorganisms, thus protecting plants against infectious diseases (Andreote et al., 2014; Vandenkoornhuyse et al., 2015). The reduction in *Fusarium* wilt infection in watermelon has long been observed in soil containing non-pathogenic *Fusarium oxysporum*, *Pseudomonas fluorescens* and several archaea (Alabouvette and Couteaudier, 1992). One of the mechanisms for disease suppression in plants could be competition for nutrients and colonizing sites (Shimotsuma et al., 1972; Alabouvette and Couteaudier, 1992). However, the taxonomy and metabolism of the plant-associated microbiome can be directly related to the nutrient components present in a specific part of the plant. In addition, microbial community shifts can occur due to environmental factors and plant developmental activity, thereby producing a dynamic process in which the microbial community and the relations between microbe–microbe and microbe–plant (fruit) may strongly vary (Müller et al., 2016).

Traditional studies on plant microbiota have focused on culturable bacterial groups, but they do not give a clear idea of the plant–microbe interactions because of limitations because of unculturable microorganisms. Next-generation sequencing (NGS) technologies have allowed for studying this hidden microbial diversity in terms of different environmental parameters. Several studies have been used NGS to elucidate microbiomes associated with barley (Bulgarelli et al., 2015), corn (Peiffer et al., 2013), lettuce (Rastogi et al., 2012), potato (İnceoğlu et al., 2011), and rice (Edwards et al., 2015) for different developmental aspects. Ripening changes in tomato were found regulated at multiple levels (DNA, RNA, and protein) and dependent on the coordinated activity of multiple plant hormones (Zhong et al., 2013). The modifications during ripe stage include the accumulation of pigments and sugars and the production of aromatic compounds and flesh softening (Gapper et al., 2013). While glucose and fructose are main sugars during the initial phase of watermelon fruit development, sucrose is more than 70% during the ripe stage (Yativ et al., 2010). Textural changes in ripe fruits are highly associated with carbohydrate metabolism. These changes are mainly due to the dissolution of the middle lamella, the reduction of cell-to-cell adhesion and the weakening of parenchyma cell walls as a result of the action of cell wall-modifying enzymes. Pectins, major components of fruit cell walls that contain α-1, 4-linked D-galacturonic acid, are extensively modified in ripe fruits by their involvement in cell wall extension and fruit softening (Jacob et al., 2008). In apple and strawberry, softening was reduced due to downregulation of polygalacturonase genes (Paniagua et al., 2014). In addition, cell wall and middle lamella modifications are accomplished by many ripe stage related genes encoding polygalacturonase, pectin methylesterase, pectate lyase, β-galactosidase, and cellulase (Brummell and Harpster, 2001; Mercado et al., 2011). The endophytes *Bacillus* and *Kocuria* isolated from papaya fruits could produce extracellular enzymes such as amylase, cellulase, pectinase, and xylanase to act on carbohydrate metabolism toward fruit nutrient composition (Krishnan et al., 2012).

Apart from the above-mentioned factors, hormones also regulate ripe stage and pigmentation process. Transcriptional regulation of ripe stage of fruits coincides with the exposition to the growth hormone ethylene (Wechter et al., 2008). It has been reported that watermelon is sensitive to ethylene and under it, this fruit exhibits acute symptoms of softening by

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

the alteration of polygalacturonase, pectinmethylesterase, and α- and β-galactosidase enzymes (Karakurt and Huber, 2002). Ethylene has also been correlated with carotenoid biosynthesis of watermelon at ripe stage (Grassi et al., 2013), and microbial production of ethylene and carotenoids have been reported previously (Tian and Hua, 2010; Jasim et al., 2015).

While, Berendsen et al. (2012) reported that microbiota might play a fundamental role in the regulation of plant development and affect fruit quality and yield. Little information is available on the role of the microbiome in the ripe fruits of watermelon. In this study, we aimed to analyze the microbiome of ripe fruits of watermelon cultivars of yellow and red flesh by employing both 16S metagenomics and metatranscriptomics to understand and predict their role in ripe fruits.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Watermelon fruits from cultivars with red flesh [PI459074, Congo, and Sweet Dakota Rose (SDRose)] and yellow flesh [PI227202, PI435990, and Jubillee Bush (JBush)] were selected based on fruit flesh color. Selfed seeds of selected cultivars were obtained from the germplasm resources information network (GRIN[2]) and were grown in an experimental field at West Virginia State University for two seasons (summer 2015 and 2016). The soil bed was covered with polyethylene mulch and the plants were irrigated daily at regular intervals with a drip system. All agronomic practices including fertilization and insecticide application followed regular agronomic practices.

Preparation of Fruit-Flesh for DNA and RNA Extraction

Three replications of mature fruits from each cultivar grown in summer 2016 were collected at ripe stage from the field. Ripe fruits were selected based on the following observations: (a) appearance of yellow color of the fruit in the spot touching the ground; (b) the presence of a dried-up stalk attached to the fruit; (c) slightly rough, ridged, and a dull-opaque appearance of rind; and (d) giving a hollow sound when you thump it with your knuckles. Fruit flesh was collected aseptically from all genotypes. The external surface of the watermelon was rinsed with running water, dried and surface-sterilized with 70% ethanol to avoid the interference of epiphytic bacteria contamination. The cutting utensils (knife, spatula) and board were also surface-sterilized with 70% ethanol. Fruits were cut vertically and the middle flesh was scraped out with a sterile spatula. Samples were flash-frozen in liquid nitrogen and stored at −80°C.

Genomic DNA Isolation

Genomic DNA was isolated from frozen flesh by using a power food-microbial DNA isolation kit (MO BIO Laboratories, United States). An amount of 500 mg fruit flesh was homogenized in phosphate buffer saline solution. The microbial cells were lysed by microbeads with the lysis buffer provided in the kit. DNA quality and quantity were analyzed by use of the Nanodrop spectrophotometer 1100 (Nanodrop, Wilmington, DE, United States). Isolated DNA was stored at −20°C and diluted to 1 ng/μL with sterile water for 16S metagenomic analysis.

[2]https://www.ars-grin.gov/

16S rRNA Library Construction and Sequencing

The 16S rRNA V4 region was amplified with the bacterial primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) and archaeal primers U519F (5′-CAGYMGCCRCGGKAAHACC-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) with a unique barcode. All PCR reactions involved the Phusion High-Fidelity PCR Master Mix (New England Biolabs, United States). Quantification and purification of PCR products involved a standard procedure (Novogene Bioinformatics Technology, Beijing). Sequencing libraries were generated by using the TruSeq DNA PCR-free sample preparation kit (Illumina, United States) as instructed. The library quality was assessed with the Qubit 2.0 Fluorometer (Thermo Scientific) and the library was sequenced on an Illumina HiSeq2500 platform to generate 250 bp paired-end reads.

Data Analysis of 16S Amplicons

After truncating the barcode and primer sequences, paired-end reads were merged by using FLASH (Magoč and Salzberg, 2011) to obtain raw reads (Supplementary Table S2). Quality filtering on the raw reads involved specific filtering conditions to obtain high-quality clean reads (Bokulich et al., 2013) according to the QIIME quality control process (Caporaso et al., 2010b). The tags were further compared with the reference database (Gold database) by using the UCHIME algorithm (Edgar et al., 2011) to remove chimera sequences and to obtain effective tags. Sequence analysis involved use of Uparse (Edgar, 2013), and sequences with ≥97% similarity were assigned to the same operational taxonomic units (OTUs). A representative sequence for each OTU was screened for species annotation with the GreenGene Database (DeSantis et al., 2006) based on RDP Classifier (Wang et al., 2007). The phylogenetic relationship of different OTUs, differences among dominant species in samples (groups), and multiple sequence alignment were analyzed by using PyNAST v1.2 (Caporaso et al., 2010a) against the "Core Set" dataset in the GreenGene database.

RNA Extraction for Metatranscriptome Analyses

RNA was extracted from frozen flesh by the TRIzol method (Life Technologies, Carlsbad, CA, United States). Cell lysis involved grinding flesh in liquid nitrogen and further homogenization with TRIzol reagent as suggested by the manufacturer. The extracted total RNA was purified by using the Zymo research purification kit (Zymo Research, Irvine, CA, United States) as described. RNA quality and quantity were analyzed by using agarose gel electrophoresis and the Agilent 2100 Bio-analyzer (Agilent Technologies, Santa Clara, CA, United States); the extracted RNA was stored at −80°C.

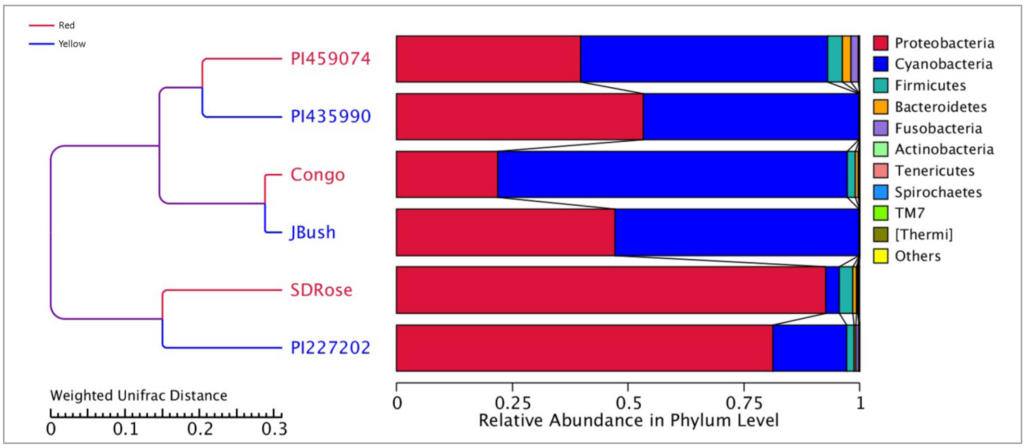

FIGURE 1 | Bacterial diversity at phylum level in ripe fruits of six watermelon cultivars based on 16S rRNA analysis.

Library Preparation and Sequencing for Metatranscriptome

Ribosomal RNA was removed from total RNA and the mRNA obtained was fragmented randomly in fragmentation buffer before cDNA synthesis. The final cDNA library was ready after purification, terminal repair, A-tailing, ligation of sequencing adapters, size selection and PCR enrichment. The library concentration was quantified by using the Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, United States), adjusted to 2 ng/μl before checking the insert size on an Agilent 2100 Bio-analyzer, and quantified to a greater accuracy by quantitative PCR. Finally, the libraries were sequenced with an Illumina HiSeq2500 platform. The Illumina reads for 16S and metatranscriptome were deposited with the Sequence Reads Archive (NBCI) under the following accession numbers (SAMN08118885, SAMN08118886, SAMN08118887, SAMN08118888, SAMN08118889, SAMN08118890, SAMN 08118891).

Removal of Ribosomal RNA Sequences

Raw RNA-Seq reads were first processed to eliminate adapter and low-quality sequences by using the FastQC program[3]. Removal of the rRNA sequences from the dataset involved use of the SortMeRNA software with the default rRNA database included in the software package, which includes 16S, 23S, 18S, and 28S rRNAs (Kopylova et al., 2012; Leimena et al., 2013). The watermelon genome database is a relatively complete one with annotations, ESTs, transcriptome, etc. from cultivars 97103 and Charleston Gray[4]. The sequences obtained in our transcriptomics study were matched against the watermelon database to eliminate all genes that matched the watermelon genome. Blastn was performed on the remaining reads with a minimum alignment bit score of 54 by using a filtering database consisting of complete ribosomal RNA loci and tRNA sequences of bacteria, archaea, and eukaryote taken from the NCBI and SILVA databases (Pruesse et al., 2007). Thus, filtered sequence reads that passed the rRNA/tRNA filter were reconstructed by using Trinity (version r20140413pl); all samples were then integrated before removing redundant ones with CD-HIT-EST (identity threshold set to 0.95) to obtain unigenes.

Taxonomic Annotation

For taxonomic identity and functional assignment of unigenes, filtered reads were aligned to the NCBI NR database (e-value $\leq$ 1e-5) by using Blastn. From earlier work, minimum bit score thresholds of 148, 110, and 74 can be used for phylogenetic and functional assignments at genus level (with >80% confidence level), phylogenetic and functional assignment at the family level (with >80% confidence level) and for a reliable function (COG) assignment (with >95% confidence level), respectively (Leimena et al., 2013). The phylogenetic profiling based on mRNA reads at the phylum level involved reads containing minimum bit alignment score of 148 and the highest rank was selected for the species annotation by using the LCA algorithm (applied in MEGAN software system) to ensure its biological significance (Huson et al., 2011). The top 35 phyla in each sample were selected from the results of species annotation and abundance information, and then clustered by their taxonomy information and the inter-sample differences among samples, to obtain a Species Abundance Heat-map.

Functional Annotation of KEGG, eggNOG, and CAZy

The unigenes were functionally annotated by mapping to different functional protein databases with BLAST software. Because of more than one result for each mapping unigene, the comparison was done to ensure biological significance, and the BLAST Coverage Ratio (BCR) of reference and Query genes were calculated to ensure a BCR (Ref) and BCR (Que) > 40%, then the corresponding functional annotation information was finally summarized for each watermelon cultivar. Predicted

[3]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
[4]http://cucurbitgenomics.org/

unigenes were assigned to COGs (Tatusov et al., 2000) by blast searches against the COG database (NCBI[5]) with e-value $< 10^{-6}$ for COG assignments. The Kyoto Encyclopedia of Genes and Genomes (KEGG) functional annotation (Kanehisa et al., 2008) of identified proteins involved use of the KEGG Automatic Annotation Server (KAAS[6]) (Moriya et al., 2007) based on a bidirectional best hit assignment method.

Gene Expression and Comparative Analysis

The unigenes were used as a reference to align with RNA-Seq by Expectation-Maximization (RSEM) (Li and Dewey, 2011). Following the alignments, the number of reads mapped to each watermelon cultivar unigene was derived, then normalized to reads per kilobase of exon model per million mapped reads (RPKM). Relative gene expression was determined by counting the number of unigenes assigned to a particular protein-encoding gene. Normalization was obtained by dividing each gene count by the total mRNA read count of each dataset and multiplying by the average of the total mRNA read count across all datasets (Dillies et al., 2013). Metabolic mapping of the metatranscriptome profiles was performed quantitatively by mapping the KEGG annotation of the identified protein sequences into metabolic pathway maps by using the iPath v2 module[7]. Gene expression of the metabolic pathways was indicated by the line width, determined from the $\log_2$ values of the read count of KEGG-annotated proteins. Reads with alignment bit-scores ≥ 74 were used to create the global metabolic activity pathways.

RESULTS AND DISCUSSION

Distribution of Bacterial Communities in Watermelon Fruits Based on 16S rRNA Analysis

We analyzed 16S rRNA to study the bacterial communities associated with red- and yellow-flesh cultivars of watermelon at ripe stage. The cultivar details including total soluble solids and citrulline contents are given in Supplementary Table S1. The red- and yellow-flesh cultivars are from Africa, Asia, and North America. 16S metagenomics sequence data revealed nearly 200,000 raw and clean reads for each cultivar, with average read length of 250 nt (Supplementary Table S2). Proteobacteria was the most abundant phylum in the ripe fruits of watermelon cultivars SDRose, PI227202, and PI435990 (**Figure 1** and Supplementary Table S3). Firmicutes and Bacteroidetes were in less abundance in almost all cultivars tested, and Fusobacteria was recorded highly in PI459074. Proteobacteria represent various taxonomic groups and different ecological statuses, such as endophytes/symbionts (asymptomatic, endophytic bacteria possibly in symbiotic interaction) and saprophytes (bacteria from various environments including soil). Their dominant presence

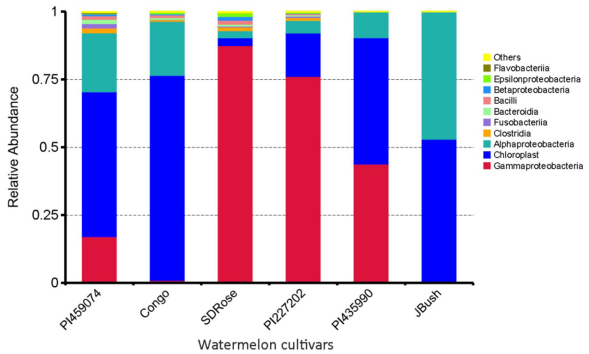

FIGURE 2 | Bacterial community composition by class in ripe fruits of watermelon cultivars based on 16S rRNA analysis.

in fruits of watermelon could be attributed to the fruit's ability to use a wide variety of carbon sources such as carbohydrates, amino acids, and lipids, which could help resist different environmental changes that occur during fruit development (Peighamy-Ashnaei et al., 2006; Kazakov et al., 2009; Xia et al., 2015).

Earlier, Glassner et al. (2015) reported that Firmicutes, Actinobacteria, β-proteobacteria, and γ-proteobacteria are of major abundance in the flesh of melon fruit, *Cucumis melo* L., another member of the Cucurbitaceae family. Similarly, Proteobacteria, Acidobacteria, Bacteroidetes, and Firmicutes were found the most abundant phyla in grapes (Zarraonaindia et al., 2015). A great percentage of Cyanobacteria was observed in PI459074, Congo, and JBush. They presented a "chloroplast bacterial genome" as a major abundant bacterial class (**Figure 2**). Earlier, plastids were found to have a cyanobacterial ancestor (Douglas and Turner, 1991), and a key role for plastids, specifically chromoplasts, in ripe fruits has been mentioned (Kang et al., 2010). Another important bacterial class in PI459074, Congo and JBush was α-proteobacteria, but in the remaining cultivars, γ-Proteobacteria was the most abundant. The class Bacilli was present in PI459074, Congo, and SDRose; members of this class, such as the *Bacillus* genus, was found predominant in papaya, along with *Kocuria*, *Acinetobacter,* and *Enterobacter* species (Shi et al., 2010; Krishnan et al., 2012). Antagonistic activity of *Bacillus subtilis* toward fungal and bacterial pathogens of cucurbits is also well-documented (Zeriouh et al., 2014).

The relative abundance of bacterial families differed among all tested cultivars, nevertheless very low bacterial diversity was observed in all cultivars. Genes involved in defense response and resistance may undergo differential expression during development and ripe stage of watermelon fruits to control pathogens and consequently could also restrict the establishment of non-pathogenic bacteria, which could explain the reduced bacterial diversity in watermelon fruits (**Figure 3**). Except for SDRose, PI227202 and PI435990, other cultivars showed major abundance among "other" bacterial types. Families belonging to the γ-Proteobacteria class presented the highest abundance. In SDRose, Enterobacteriaceae was the most abundant family (**Figure 3**). Members of Enterobacteriaceae include important

[5]ftp://ftp.ncbi.nih.gov/pub/COG/COG

[6]http://www.genome.jp/tools/kaas/

[7]https://pathways.embl.de/

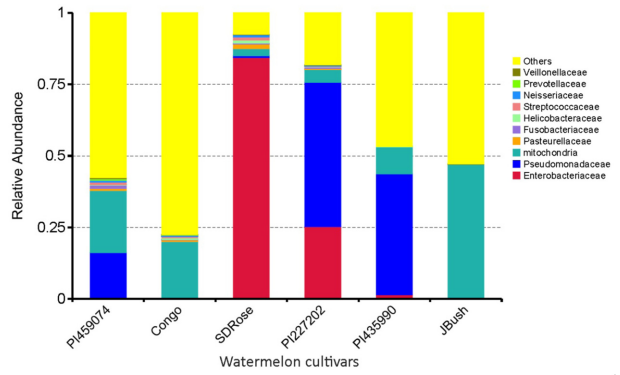

FIGURE 3 | Relative abundance in terms of families of bacteria in ripe fruits of watermelon cultivars based on 16S rRNA analysis.

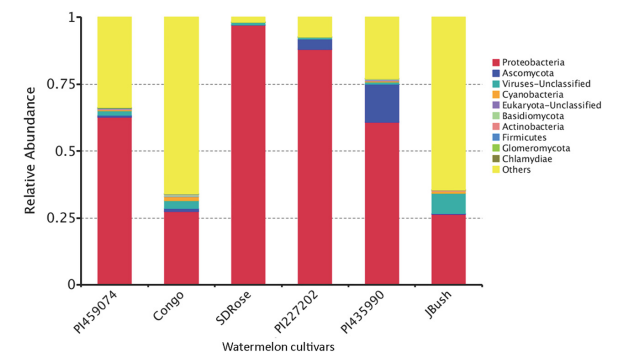

FIGURE 4 | Relative abundance of the transcriptionally active microbiome at phylum level in ripe fruits of watermelon cultivars.

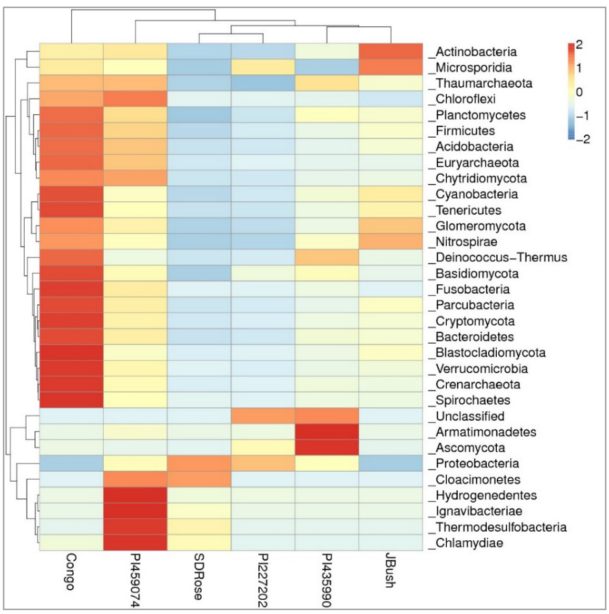

FIGURE 5 | Heatmap of watermelon cultivars based on a comparison of the transcriptionally active microbiome in ripe fruits. Plotted cultivar name on the *X*-axis and selected phyla on the *Y*-axis. The absolute value of "*Z*" represents the distance between the raw score and the population mean in units of the standard deviation. "*Z*" is negative when the raw score is below the mean, positive when above.

pathogens for humans, such as *Salmonella* and *Escherichia coli* O157, even though bacteria belonging to this family were previously isolated from plant tissues, exhibiting antibiotic resistance (Markova et al., 2005). For PI227202 and PI435990, Pseudomonadaceae was the most abundant family. Members of this family have been found as endophytic microorganisms in watermelon. Compant et al. (2011) also observed that *Pseudomonas* was among the predominant bacterial isolates from the interior of flowers, fruits, and seeds of grapevine.

Active Microbiome Associated with Watermelon Cultivars

We used metatranscriptomic analysis for in-depth study of the active microbiome and gene expression and associated metabolic pathways in ripe fruits of six cultivars of watermelon fruits. HiSeq2500 generated ∼64, 41, 53, 68, 61, and 33 million paired-end reads for PI459074, Congo, SDRose, PI227202, PI435990, and JBush, respectively. Data on sequenced reads and mapped reads for all six cultivars is in Supplementary Table S4. Bacteria, archaea, fungi, eukaryote, and viruses were found in the ripe fruits of all six watermelon cultivars. Five bacterial phyla (Proteobacteria, Cyanobacteria, Actinobacteria, Firmicutes, and Chlamydiae), three fungal phyla (Ascomycota, Basidiomycota, and Glomeromycota), unclassified eukaryote and unclassified viruses were the top 10 phyla in all samples (**Figure 4**).

Proteobacteria was the most transcriptionally active phyla in all samples. Ascomycota, a fungal group composed of plant and human pathogens and organisms of biotechnological importance was observed along with other fungal phyla, the large fruit-body producer Basidiomycota and the arbuscular mycorrhizal Glomeryomycota (Berbee, 2001). The other bacterial phyla were Firmicutes and Actinobacteria, a well-known secondary metabolites producer, although they were not predominant in all watermelon fruits tested. The obligate intracellular pathogen bacterium Chlamydiae, unclassified eukaryotes, unclassified viruses, and Cyanobacteria showed minor abundance (**Figure 4**). Members of Cyanobacteria have been reported to produce carotenoids, which could contribute to the carotenoid accumulation driven by the plant in the ripe fruits (Lv et al., 2015). Carotenoid production in fruits produce changes in fruit color and also contributes to the biosynthesis of aroma components because carotenoids serve as substrates for the production of norisoprene and monoterpene aroma volatiles of the fruits (Lewinsohn et al., 2005). This capability has been reported in cyanobacterial members such as *Microcystis aeruginosa* (Jüttner, 1976). Apart from their role in carotenoid production, cyanobacteria could also contribute to the aroma and flavor of watermelon fruit. Fungi phyla such as Basidiomycota and Ascomycota present in our findings have been reported to degrade carotenes, resulting in the production of volatile aroma compounds (Zorn et al., 2003).

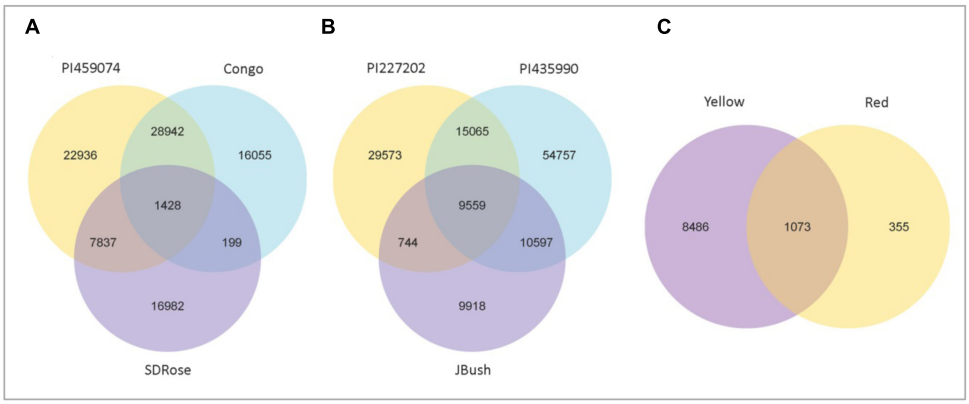

FIGURE 6 | Venn diagram showing the number of shared and unique genes among red types and yellow types and between red and yellow types of watermelon. Gene number among samples of red flesh group **(A)**, yellow flesh group **(B)**, and between red and yellow flesh groups **(C)**. 1428 common genes among red types, 9559 genes were common among yellow types, and 1073 genes were common between yellow and red flesh types.

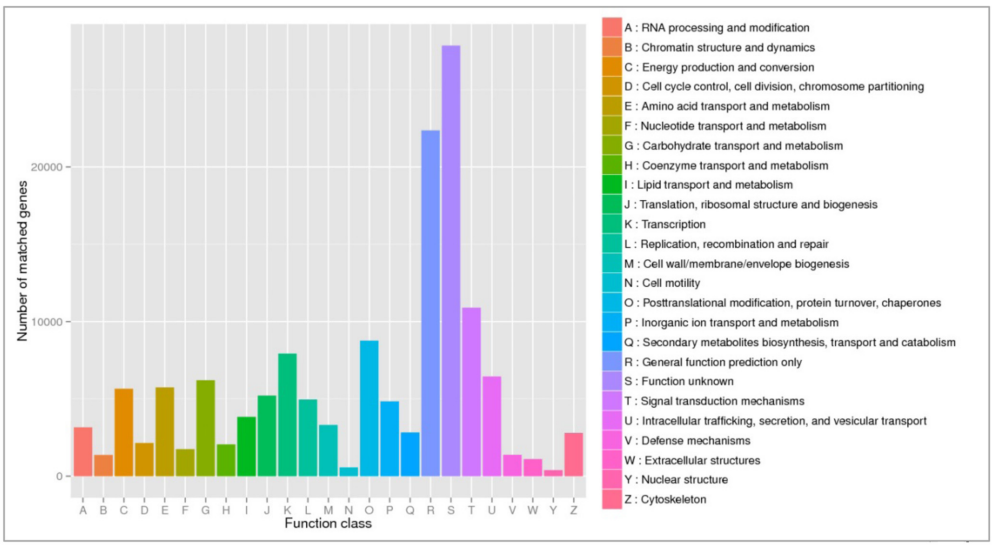

FIGURE 7 | eggNOG functional annotation of orthologous groups among ripe fruits of all watermelon cultivars of this study based on metatranscriptomic data.

In the cultivars PI435990 and PI227202, the most abundant active microbiome was Proteobacteria, Ascomycota, and unclassified viruses. Nevertheless, PI435990 showed Cyanobacteria, unclassified eukaryotes, Basidiomycota, and Chlamydiae in minor abundance. In PI459074 and Congo, Proteobacteria was a dominant phylum, followed by unclassified viruses. Other phyla present in these two varieties were Ascomycota, Actinobacteria, Cyanobacteria, Basidiomycota, and Glomeromycota. SDRose presented less active microbiome diversity, which could be due to the relative predominance of Proteobacteria. Although Proteobacteria were also predominant in JBush, a high abundance of unclassified viruses was also observed, followed by Cyanobacteria, Actinobacteria, and Ascomycota (**Figure 4**). A heatmap is presented to compare the inter-sample differences among the six cultivars of the dominant 35 phyla. Ascomycota and Armatimonadetes phyla showed the highest abundance in the PI435990 cultivar as compared with the other cultivars (**Figure 5**). Armatimonadetes is a recently defined bacterial phylum and is phylogenetically related to Chloroflexi, Actinobacteria, Firmicutes, Deinococcus–Thermus, and Cyanobacteria. Deinococcus–Thermus and Thaumarchaeota, observed in considerably in Congo cultivar, are extremophiles that tolerate oxidation, desiccation, radiation conditions, and biosynthesize carotenoids as a defense mechanism (Tian and Hua, 2010). The presence of Deinococcus–Thermus was reported in apple flower microbiota (Shade et al., 2013) and on the surface of tomato (Telias et al., 2011).

A high abundance of Actinobacteria was observed in the JBush cultivar. Actinobacteria are known for synthesis of secondary metabolites, many of which are found in plants and used as bioactive compounds because of their antimicrobial activity (Fiedler et al., 2008). Furthermore, their diverse metabolism

allows them to participate in the metabolism of carbohydrates, including polysaccharides (Pasti and Belli, 1985; Schäfer et al., 1996; Watanabe et al., 2003), which is important in ripe stage of watermelon fruit. The presence of the phylum Glomeromycota in Congo and JBush is significant because the phylum comprises arbuscular mycorrhizal (AM) fungi, which play an important role in plant development and diversity by their phosphate mobilization and nutrient uptake (Bucher, 2007), control of pests and fungal pathogens. The predominance of the Microsporidia phylum in JBush is interesting because it is a known parasite of higher eukaryotes (Keeling and Fast, 2002).

It has been reported in previous studies that the relation between plant-associated microbiome and plant hormones that promote the ripening of fruits (Zouari et al., 2014; Gamalero and Glick, 2015). It is well-known that endogenous and exogenous ethylene triggers in ripe fruits of watermelon through cell wall-degrading enzymes and pectin solubility (Huber et al., 2001; Karakurt and Huber, 2002, 2004). Ethylene forming enzyme 2-oxoglutarate oxygenase/decarboxylase (EFE) of microbial origin produces gaseous ethylene, which subsequently permeates across the bacterial membrane in inducing ripe stage of fruit (Digiacomo et al., 2014). They engineered *E. coli* to synthetize ethylene by the insertion of EFE from *Pseudomonas syringae* to induce the ripe stage in tomato, kiwifruit and apples. In another study, 11 endophytic Proteobacteria belonging to *Pantoea* sp., *Polaromonas* sp., *Pseudomonas* sp., and *Ralstonia* sp. showing 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity were isolated from the fruit tissue of *Elettaria cardamomum* (Jasim et al., 2015). Expression of the ACC deaminase gene has been previously related to ripe tomato (Sheehy et al., 1993). Higher abundance of Proteobacteria described in this

TABLE 1 | Carbohydrate-active enzymes (CAZyme) distribution in watermelon varieties.

CAZyme family	Activity	Associated metabolism	Variety
AA3	Integral component of membrane	Membrane transport	JBush
GH3	β-1,4-Glucosidase, β-1,4-xylosidase, β-1,3-glucosidase, β-L-arabinofuranosidase, others	Carbohydrate and energy metabolism	SDRose
GH13	α-Amylase, catalytic domain, and related enzymes	Carbohydrate and energy metabolism	SDRose
GH16	β-1,3(4)-Endoglucanase, others	Energy metabolism	PI435990
GH17	Glucan endo-1,3-β-D-glucosidase glucan 1,3-β-glucosidase, others	Carbohydrate metabolism	Congo
GH18	Chitinase, endo-β-*N*-acetylglucosaminidase, non-catalytic Proteins	Aminoacid metabolism	SDRose
GH23	G-Type lysozyme, peptidoglycan lytic transglycosylase	Membrane transport	SDRose
GH27	α-Galactosidase, α-*N*-acetylgalactosaminidase, Isomalto-dextranase	Carbohydrate and energy metabolism	Congo
GH28	Polygalacturonase, rhamnogalacturonase Others	Carbohydrate metabolism	JBush SDRose
GH32	Invertase, others	Carbohydrate metabolism	PI435990
GH36	α-Galactosidase, α-*N*-acetylgalactosaminidase	Carbohydrate metabolism	Congo JBush
GH103	Peptidoglycan lytic transglycosylase	Membrane transport	SDRose
GT1	UDP-Glucuronosyltransferase 1-β-Galactosyltransferase	Carbohydrate metabolism	JBush
GT2	Cellulose synthase Chitin synthase	Carbohydrate metabolism	PI459074
GT8	Lipopolysaccharide α-1,3-galactosyltransferase	Lipid metabolism	SDRose
GT19	Lipid-A-disaccharide synthase	Membrane transport	SDRose
GT28	1,2-Diacylglycerol 3-β-Galactosyltransferase	Carbohydrate metabolism	PI227202
GT48	1,3-β-Glucan synthase	Carbohydrate metabolism	Congo
GT51	Murein polymerase	Membrane support	JBush
CE4	Acetyl xylan esterase Chitin deacetylase	Carbohydrate metabolism	PI227202
CE8	Pectin methylesterase	Carbohydrate metabolism	Congo
CE11	UDP-3-0-Acyl-*N*-acetylglucosamine deacetylase	Carbohydrate metabolism	PI227202
CE14	*N*-Acetyl-1-D-myo-inosityl-2-amino-2-deoxy-α-D-glucopyranoside deacetylase	Carbohydrate metabolism	PI227202
CBM2	Cellulose-binding domain	Carbohydrate metabolism	Congo
CBM3	Cellulose-binding domain	Carbohydrate metabolism	PI435990
CBM10	Cellulose-binding domain (aerobic bacteria) and dockerin (anaerobic fungi)	Carbohydrate and energy metabolism	Congo
CBM13	Mannose- and xylan-binding domain	Carbohydrate metabolism	SDRose
CBM14	Chitin-binding domain	Structure and Carbohydrate metabolism	PI227202
CBM18	Chitin-binding domain (eukaryotic only)	Structure and Carbohydrate metabolism	Congo
CBM19	Chitin-binding domain (eukaryotic only)	Structure and Carbohydrate metabolism	JBush
CBM20	Starch-binding domain	Carbohydrate metabolism	Congo JBush
CBM48	Glycogen-binding domain	Carbohydrate metabolism	PI227202
CBM50	Peptidoglycan metabolic process	Membrane metabolism	SDRose
CBM57	Quinoprotein amine dehydrogenase, β-chain-like	Signal transduction	Congo

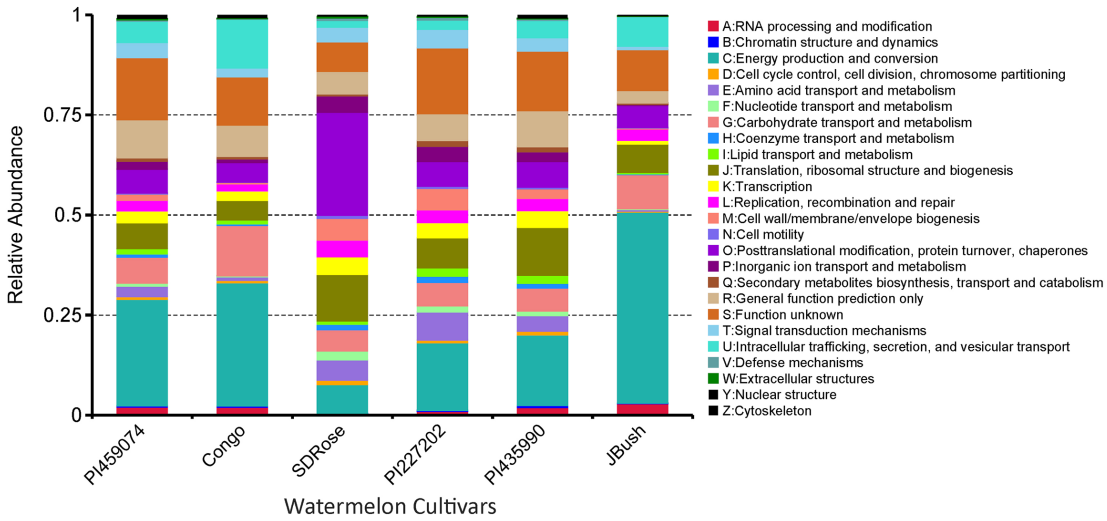

FIGURE 8 | eggNOG function comparison of ripe fruits of watermelon cultivars. Relative abundance of different functional annotation groups was constructed by using metatranscriptome data from the six watermelon cultivars.

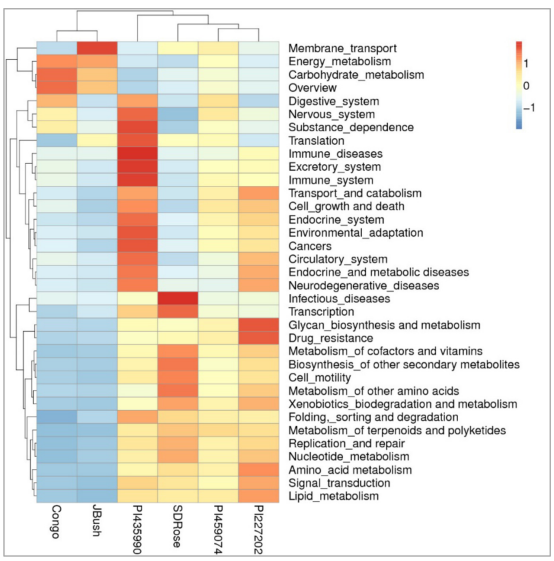

FIGURE 9 | KEGG function comparison of ripe fruits of watermelon cultivars by heatmap analysis. Plotted cultivar name on *X*-axis and KEGG pathways on *Y*-axis. The absolute value of "*Z*" represents the distance between the raw score and the population mean in units of the standard deviation. "*Z*" is negative when the raw score is below the mean, positive when above.

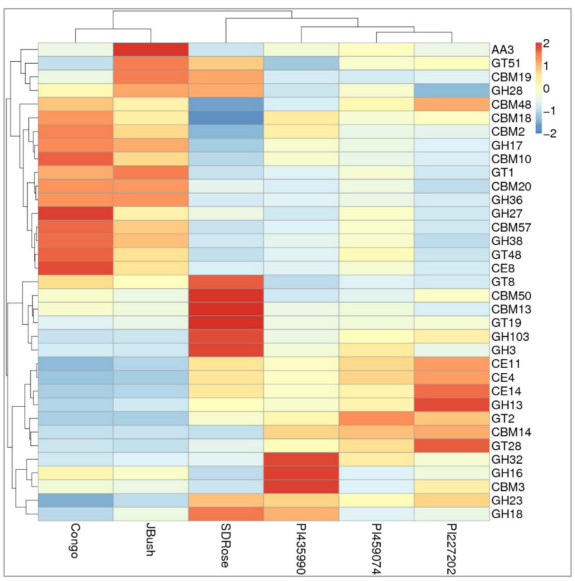

FIGURE 10 | Heatmap of the carbohydrate-active enzymes in watermelon cultivars. Different families of structurally related catalytic and carbohydrate-binding modules of enzymes that degrade, modify, or create glycosidic bonds were found. Within this class of Enzyme Classes were Glycoside hydrolases (GHs), Glycosyltransferases (GTs), Carbohydrate esterases (CEs), Auxiliary activities (AAs), and Carbohydrate-binding modules (CBMs). Plotted cultivar name on *X*-axis and predicted enzymes on *Y*-axis. The absolute value of "*Z*" represents the distance between the raw score and the population mean in units of the standard deviation. "*Z*" is negative when the raw score is below the mean, positive when above.

present study could play a major role in ripe watermelon fruits.

Functional Profile of Active Microbiomes of Watermelon Cultivars

The primary cell wall of the fruit contains approximately 35% pectin, 25% cellulose, 20% hemicellulose, and 10% proteins (Brownleader et al., 1999). At ripe stage, the cell wall undergoes different modifications including de-esterification and depolymerization, and consequently loss of galacturonic acid and neutral sugars followed by solubilization of oligosaccharides and remaining sugar residues (Zandleven et al., 2005). An active

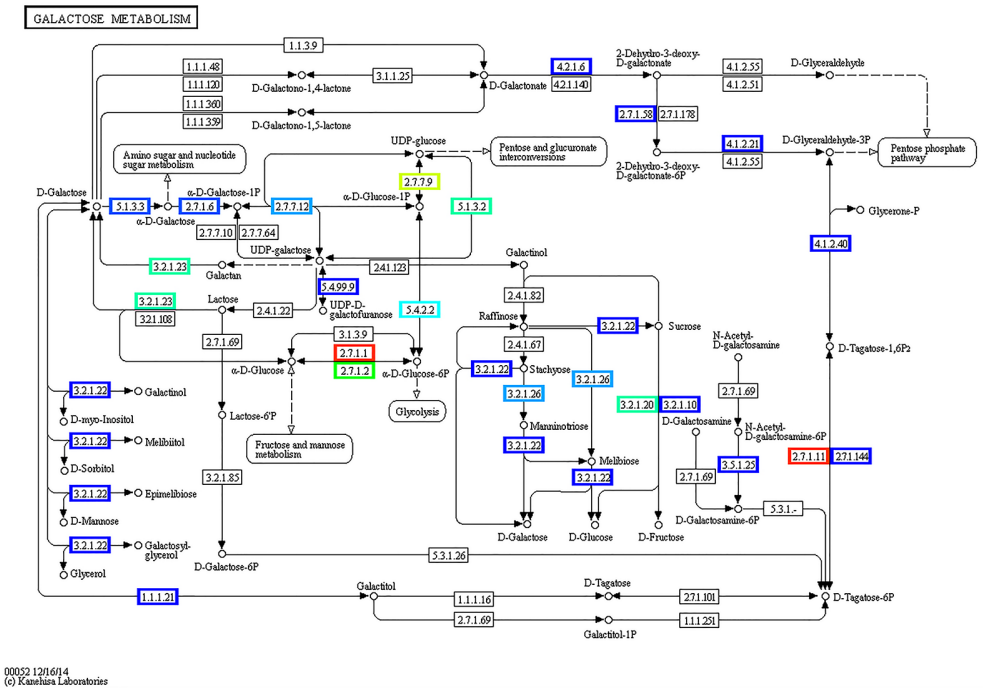

FIGURE 11 | Reconstructed pathway of galactose metabolism based on metatranscriptomic data by KEGG annotation. Predicted enzymes from metatranscriptomic data were associated with galactose metabolism, displayed in this figure.

microbiome could play a major role in this process with its hydrolytic enzymes, in addition to the corresponding enzymes of the host.

To obtain general insights into microbial metabolism, transcripts were compared in the following databases: evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Carbohydrate-Active Enzymes Database (CAZy). Based on the analyses of unique and shared genes (**Figure 6**), it was observed that 22,936, 16,055, and 16,982 unique genes were expressed in red flesh cultivars PI459074, Congo, and SDRose, respectively. Similarly, 29,573, 54,757, and 9,918 unique genes were expressed in yellow flesh cultivars PI227202, PI435990, and JBush, respectively. A great sharing of genes was surprisingly observed in yellow and red cultivars, so despite being different cultivars, environmental conditions facilitate the development of relatively similar microbial composition in both types. Genes involved in signal transduction, post-translational modification, transcription, carbohydrate metabolism, intracellular trafficking and amino acid and energy metabolism were the most abundant in all analyzed cultivars (**Figure 7**). The high abundance of these genes could be related to the maintenance of basic cellular machinery, which allowed for growth and cell maintenance of microbial communities during the changes occurring in ripe watermelon. We observed different families of structurally related catalytic and carbohydrate-binding modules (or functional domains) of enzymes that degrade, modify, or create glycosidic bonds. Within this class of enzymes were glycoside hydrolases (GHs), glycosyl transferases (GTs), carbohydrate esterases (CEs), auxiliary activities (AAs), and carbohydrate-binding modules (CBMs) (**Table 1**). Although the microbial diversity of JBush cultivar is low, genes related to energy and carbohydrate metabolism were highly expressed in this cultivar (**Figures 8**, **9**). The availability of sugars in ripe watermelon promotes the expression of genes related to carbohydrate metabolism, such as glucosidases, galactosidases, cellulose binding module, and starch binding module (**Figure 10**).

However, in the case of SDRose, despite low expression of genes related to energy metabolism (<10%), high expression of post-translational modification genes (∼25%) was observed (**Figure 8**). In addition, the expression of genes related to infectious diseases was observed (**Figure 9**), which could explain the low microbiome diversity expressed in this cultivar. Many bacterial genera of the most abundant Proteobacteria phylum in this cultivar, such as *Xanthomonas*, *Erwinia* and *Pseudomonas*, are known pathogens (Kersters et al., 2006). Moreover, fruits at ripe stage are more vulnerable to pathogen attack and environmental stress (Levi et al., 2006). Among the different enzymes expressed in this cultivar, peptidoglycan hydrolases were present. Bacteria use this enzyme for cell wall assembly and disassembly during growth and division; nonetheless, pathogenic bacterial species also use them to cause pathogenicity in eukaryotic hosts (Humann and Lenz, 2009).

On eggNOG annotation, PI227202 and PI435990 cultivars showed a similar pattern of gene expression (**Figure 8**). Regardless, these cultivars presented differences in the expression of genes encoding the CAZ. GH32, associated with hydrolysis of fructans; GH16, associated with the hydrolysis of

galactose-containing polysaccharides and galactose monomers; and CBM3, linked with cellulose and chitin binding were observed in major abundance in PI435990 as compared with the other cultivars (**Figure 10**). CEs (CE11, CE4, CE14), associated with polysaccharide deacetylation, glycosyltransferase protein-associated GT28, amylase activity-associated GH13 and glycogen-binding protein-associated CBM48 were predominant in Pl227202. These results suggest the role of microbiome-associated gene expression of carbohydrate metabolism in ripe watermelon fruits.

As mentioned above, cell wall modifications include de-esterification and depolymerization, and consequent loss of galacturonic acid and neutral sugars followed by solubilization of oligosaccharides and remaining sugar residues. The presence of GH28, associated with polygalacturonase activity in JBush and SDRose could be related to cell wall modifications (Zandleven et al., 2005). Further, alkaline pectinases also have been correlated in softening of ripe fruits. *Bacillus*, belonging to Firmicutes (Kapoor et al., 2001) *Pseudomonas* sp. of Proteobacteria (Hayashi et al., 1997) and actinomycetes (Beg et al., 2000a,b) have been reported for their alkaline pectinase activity.

Predicted Pathways of Carbohydrate Metabolism

KEGG annotation of the expressed genes revealed nine α-galactosidase genes involved in key processes of galactosyl oligosaccharide metabolism, such as genes belonged to raffinose family oligosaccharides. Based on these genes, the pathway of galactose metabolism was reconstructed (**Figure 11**). In watermelon fruits, the main free sugars in tissues are sucrose and hexoses (Yativ et al., 2010). In the pathway of sucrose formation, stachyose is converted into sucrose via a sequential action of various enzymes (Dai et al., 2006; Yativ et al., 2010) such as α-galactosidase, which converts stachyose to raffinose and galactose, followed by galactokinase conversion of galactose to galactose-1-phosphate, which is converted to UDP-galactose by UDP-galactose pyrophosphorylase. Later, UDP-galactose-4-epimerase acts on UDP-galactose to form UDP glucose, which is converted to sucrose by sucrose synthase activity combined with fructose. Reconstruction of the galactose metabolism pathway based on our results clearly demonstrated the role of the active microbiome and the gene expression of carbohydrate-active enzymes of ripe watermelon fruits.

Results of this study showed the presence of different phyla of microbiome in ripe watermelon suggested the important role of different classes of bacteria in ripe stage through their metabolic activity and gene expression in carbohydrate metabolism. Moreover, it can be assumed that new phenotypes, without altering the plant genomic information can be developed because of the dynamic interactions between plants and their associated microbiome. The role of plant-associated microbiome in hormonal control during different developmental stages of fruits is a meaningful aspect to understand and requires further studies.

AUTHOR CONTRIBUTIONS

UR, PN, TS, and NB designed the plan of study. VA and AL maintained the research materials and recorded the phenotypic data. TS, BG, and AB extracted genomic DNA and RNA from watermelon fruit flesh. NB, UR, TS, MG, and CL analyzed the data using the bioinformatic pipeline. TS, MG, BG, CL, PN, NB, and UR wrote and approved the final version of the manuscript. All authors agree to account for the accuracy and integrity of the paper.

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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.2018.00004/full#supplementary-material

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Histological Features of the Olive Seed and Presence of 7S-Type Seed Storage Proteins as Hallmarks of the Olive Fruit Development

*Adoración Zafra[1], Mohammed M'rani-Alaoui[2], Elena Lima[1], Jose Carlos Jimenez-Lopez[1] and Juan de Dios Alché[1]**

[1] Plant Reproductive Biology Laboratory, Department of Biochemistry, Cell and Molecular Biology of Plants, Estación Experimental del Zaidín, Granada, Spain, [2] Département de Biologie, Université Abdelmalek Essaadi, Tétouan, Morocco

The production of olive oil is an important economic engine in the Mediterranean area. Nowadays, olive oil is obtained mainly by mechanical processes, by using the whole fruit as the primary raw material. Although the mesocarp is the main source of lipids contributing to olive oil formation, the seed also contributes to the olive oil composition and attributes. The olive seed is also becoming an interesting emerging material itself when obtained after alternative processing of the olive fruit. Such seed is used for the production of differential oil and a unique flour among other bioactive products, with increasing uses and applications in cosmetics, nutrition, and health. However, olive seed histology has been poorly studied to date. A complete description of its anatomy is described for the first time in the present study by using the 'Picual' cultivar as a model to study the development of the different tissues of the olive seed from 60 to 210 days after anthesis. A deep analysis of the seed coats, endosperm storage tissue and the embryo during their development has been performed. Moreover, a panel of other olive cultivars has been used to compare the weight contribution of the different tissues to the seed, seed weight variability and the number of seeds per fruit. In addition to the histological features, accumulation of seed storage proteins of the 7S-type (β-conglutins) in the seed tissues has been assessed by both biochemical and immunocytochemical methods. These hallmarks will help to settle the basis for future studies related to the location of different metabolites along the olive seed and mesocarp development, and therefore helping to assess the appropriate ripening stage for different commercial and industrial purposes.

Keywords: β-conglutins, cotyledon, development, endosperm, olive, radicle, seed, seed storage proteins

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****Correspondence:***
Juan de Dios Alché
juandedios.alche@eez.csic.es

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INTRODUCTION

Alimentary industries based in the preparation of table olives and olive oil are of paramount importance for the economy of Mediterranean countries and some areas of America and Australia. The very well valued Extra Virgin Olive Oil (EVOO) is produced exclusively by mechanical processes where the whole fruit is used. Other olive oil qualities [Virgin Olive Oil (VOO) and

Ordinary Virgin Oil (OVO)] are also produced mainly from whole fruits as the primary raw material. Thus, the obtained juices also contain components from the seed, which contribute to olive oil aroma and other potential properties like peroxidase activity (Luaces et al., 2003, 2007). The olive seed itself is also becoming an interesting material with multiple uses beginning to emerge (Rodríguez et al., 2008; Matos et al., 2010; Pattara et al., 2010; Naghmouchi et al., 2015). Morphological characteristics of the olive pit have been used as descriptors of pomological interest for varietal characterization in the olive tree (Barranco and Rallo, 1984). However, differences between the seed have not been reported in detail to our knowledge.

The histology of the different tissues of the seeds have been described in a variety of species other than olive (*Olea europaea* L.). In these studies, the structure of the seed coat was one of the most widely topics described in the literature. Early in the thirties, a deep study on the almond seed surface was performed aimed to easily distinguishing the different varieties of almonds, hence helping identifying misrepresentation or adulteration (Pease, 1930). Examination of *Arabidopsis* seed coat development showed major morphological changes associated with the transition of the integuments into the mature seed coat (Beeckman et al., 2000). Analysis of the seed coat histological distribution has also been performed in *Cucurbita pepo* L. to examine mutations concerning the lignification of the testa (Zraidi et al., 2003). Similarly, the seed coat of *Chenopodium quinoa* was histologically studied aimed to assess and improve quality of the seeds for human and animal consumption (Raamsdonk et al., 2010). The seed coat form of other species such as *Passiflora ligularis* Juss or *Strychnos potatorum* L. has also been analyzed (Cárdenas-Hernández et al., 2011; Mishra and Vijayakumar, 2015). Regarding endosperm anatomy, a new approach in the disclosure of the history of flowering plants has been provided after comparison of the patterns of endosperm development as well as analysis of phylogenetic and ontogenic evolution of this tissue using several basal flowering plants (Floyd and Friedman, 2000). The histology of the seeds from plants like *Vitis vinifera* L., *Paronychia*, *Theobroma cacao* L., *Annona squamosa* L., and *Medicago truncatula* has been described (Cadot et al., 2006; Kaplan et al., 2009; Rangel-Fajardo et al., 2012; Martínez et al., 2013; Verdier et al., 2013). The structure and storage content of *Arabidopsis* and *Cuphea glutinosa* endosperms has also been scrutinized (Li et al., 2006; Di Santo et al., 2012). Finally, the anatomy of the cotyledons has been particularly studied in *Theobroma cacao* L. and *Eurycoma longifolia* seeds (Elwers et al., 2010; Danial et al., 2011), where descriptions of the pattern of distribution of the polyphenolic compounds and the development of the vascular system have been provided. By means of non-destructive techniques, the structure of whole seeds has been also examined. As result, valuable information about the transport system for gas exchange in embryos of the *Arabidopsis* seed has been provided (Cloetens et al., 2006). Similarly, a 3D reconstruction of the compartments present in the maize seed have been performed (embryo, endosperm, nucellus, and pericarp) from 7 to 21 days after pollination (Rousseau et al., 2015).

The number of studies focused on the olive seed histology is still reduced. Initial studies dealt with the description of morphological, histological, and ultrastructural changes in the olive pistil during flowering (Suárez et al., 2012), and the localization of seed storage proteins (SSPs) in the olive seed. SSPs are synthesized in abundance in the developing seeds and are accumulated primarily in the protein storage vacuoles (PSVs) of terminally differentiated cells of embryo and endosperm (Herman and Larkins, 1999). Previous reports indicate that mature olive seeds contains very similar subcellular structure in both the embryo and endosperm tissues, essentially with electro-dense protein bodies (PBs) surrounded by lipid bodies with diameters ranging from 0.5–2.0/μm (Ross et al., 1993; Zienkiewicz et al., 2014). The endosperm and the cotyledon are considered storage tissues, where members of the 11S protein family are the most abundant from the total of seed proteins (Alché et al., 2006). However, asynchrony exists in the formation of PBs between both tissues (Jiménez-López et al., 2015). The analysis of the protein synthesis along the seed formation has determined three periods: (I) a period of early synthesis (before 105 days after anthesis, DAA), (II) a rapid and massive period of synthesis (105–130 DAA), and (III) a period characterized by slow synthesis (from 130 DAA until full ripening) (Wang et al., 2001).

Authors have also fixed their attention to describe the intracellular events occurring during the first hours of the *in vitro* germination process (Zienkiewicz et al., 2011; Jiménez-López and Hernández-Soriano, 2013), drawing their attention particularly to PBs. Zienkiewicz et al. (2011) also revealed that the cellular organization of the olive leaf is achieved after 26 days of germination.

β-Conglutins, vicilins of 7S globulins are also major SSPs in different plants, particularly legumes. Among them, they have been particularly studied in Lupinus species (Jimenez-Lopez et al., 2015, 2016, 2018; Lima-Cabello et al., 2017a,b, 2018). They belong to the Cupin superfamily, and mainly associate (as storage protein function) with plant physiological processes through the supply of amino acids during seedling germination (Monteiro et al., 2010). Primary evidence of the presence of β-conglutins in the olive arise from transcriptomic analyses, as the presence of 7S globulins transcript sequences have been detected in the olive seed (unpublished results). However, direct evidence of the presence and distribution of β-conglutins in the olive seed has not been provided to date.

In spite of these pioneer studies, an overall histological description of the olive seed is yet missing to date. Here we perform a report of the different tissues of the olive seed throughout its development and we use a new molecular tool: the 7S SSPs (β-conglutins) recently described in the olive seed, and a specific antibody developed to evidence the presence of these proteins and their changes as markers along tissue development. The results shown here may serve as a hallmark for analyzing seed (and fruit) maturity and to monitor the presence of these proteins in future biotechnological and alimentary uses due to their increasing interest. Finally, cell localization of these proteins is also reported.

MATERIALS AND METHODS

Plant Material

Seeds used for microscopy analysis were collected from olive trees (*Olea europaea* L. cv. 'Picual') cultivated at the Estación Experimental del Zaidín (Granada). Four stages were considered: (0) small developing fruit, (I) green fruit, (II) fruit at veraison, and (III) mature fruit. The collection took place 60, 105, 130, and 210 DAA, respectively. Seeds from different cultivars were kindly provided by the Protected Certificate of Origin "Poniente de Granada." The cultivars studied were 'Ombliguillo,' 'Llorón,' '3,' 'Lechín,' 'Hojiblanca,' 'Picual,' 'Lucio,' 'Alameño,' 'Nevadillo,' 'Loaime,' 'Azul,' and 'Gordal de Alhama.' Twenty fruits per cultivar were dissected by using a knife, a de-stoning commercial device, and a scalpel to dissect the pulp (mesocarp + epicarp), stones and the seed tissues respectively. Weight measurements were performed individually using 20 samples of the complete mature fruit (210 DAA) and each one of the dissected tissues [mesocarp + epicarp, whole endocarp (stone), testa, endosperm, and embryo]. The number of seeds obtained from each fruit was also counted.

Preparation of Samples for Microscopy

Seeds from olive fruits at four developmental stages were collected. The mesocarps + epicarps (pulp) and the endocarps (stones) were removed with a knife and a de-stoning device, respectively. At the stage 0, the complete seed was used. In the rest of the stages the obtained seeds were carefully dissected into two parts: on the one hand the coat and the endosperm were treated together, on the other hand the embryo was carefully excised. Once the embryo was obtained, the apical part (radicle) and the middle part (cotyledons) were treated separately (**Figure 1**). The plant materials were fixed with 4% (w/v) paraformaldehyde and 0.2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 h at 4°C with points of vacuum treatment to improve penetration of the fixative. Samples were dehydrated in ethanol series and embedded in Unicryl resin at −20°C using ultraviolet light. Semithin sections were obtained with a Reichert-Jung Ultracut E microtome using a glass knife. Sections were placed on Biobond-coated slides and used for cytochemical staining.

Histological Study

For histological observations, sections were stained with a mixture of basic dyes [0.05% (w/v) methylene blue and 0.05% (w/v) toluidine blue] aimed to stain the carboxyl groups of proteins, which reveal the presence of such components. Most non-stained structures correspond to lipids. Stained samples were observed in a LM Zeiss Axioplan (Carl Zeiss, Oberkochen, Germany). Photomicrographs were obtained with a ProgRes MF Cool Digital Camera, by using the ProgRes CapturePro 2.6 software (Jenoptik, LaserOptic System).

Development of an Anti-β-conglutin Antibody

Olive transcriptomic information together with sequence information of β-conglutins from different species was used to define potential cross-reactive epitopes of these proteins present in these species (Jimenez-Lopez et al., 2015). The peptide RLENLQNYRIVEFQS was selected as a cross-reactive component on this basis and was synthesized and used to immunize rabbits by Agrisera (Sweden) (Prod. No. AS15 2892). The resulting sera were affinity-purified with the synthetic peptide, and their specificity assessed by Western blotting and ELISA (not shown).

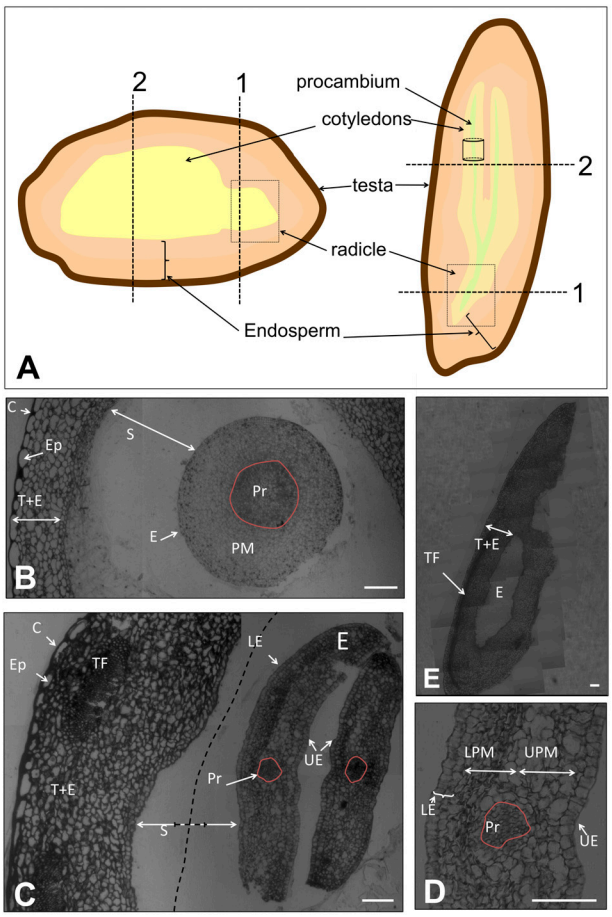

FIGURE 1 | (A) Representation of the tissues of the olive seed in longitudinal- versus cross-sections. Dotted lines/boxes show the orientation of the samples for microscopy analysis. **(B)** Cross section of the complete seed at the radicle site (pointed out with "1" in **A**) at 60 DAA. **(C)** Cross section of the complete seed at the cotyledon site (pointed out with "2" in **A**) at 60 DAA. Dotted lines indicate the shortening of the space between endosperm-embryo. **(D)** Detail of the cotyledon observed in panel **(C)**. **(E)** Longitudinal section of the complete seed at 60 DAA. Pr, procambium; PM, premesophyll; E, embryo; S, space between endosperm-embryo; C, cuticle; Ep, epidermis; T+E, testa and endosperm; TF, transversal fibers; LE, lower epidermis; UE, upper epidermis; UPM, upper premesophyll cells; LPM, lower premesophyll cells. Scale bars: 100 μm.

Protein Extraction and Western Blotting Analysis

Plant material (as described) was used to prepare protein extracts by grinding with liquid nitrogen. Proteins were extracted with

40 mM Tris–HCl pH: 7.0, 2% Triton X-100, 60 mM DTT and 10 μl/sample of protease inhibitor cocktail (Sigma). Samples were denatured with Laemmli sample buffer at 95°C for 5 min and separated on 4–20% TGX precast SDS-PAGE mini-gels (Bio-Rad). Protein profiles were determined by means of Stain-free technology using a Gel Doc™ EZ System (Bio-Rad), and normalized for total protein (30 μg/lane). Gels were blotted to supported nitrocellulose using a Trans-Blot Turbo (Bio-Rad) semi-dry device and blocked with 5% skimmed milk in TBS plus 0.05% Tween-20 for 1 h at room temperature (RT) with agitation. Blot was incubated in the anti-β-conglutin primary antibody at a dilution of 1:1000 for 8 h at 4°C with agitation in TBS-T plus 5% skimmed milk. The antibody solution was decanted and the blot was rinsed briefly twice, then washed once for 15 min and 3 times for 5 min in TBS-T at RT with agitation. Blot was incubated in secondary antibody [anti-rabbit IgG horseradish peroxidase conjugated, from Sigma (A-0545)] diluted to 1:2000 in for 1h at RT with agitation. The blot was washed as above and developed for 3 min with Clarity Western ECL substrate (Bio-Rad). Exposure time was 6–12 min in a C-Digit scanner (LI-COR Biotechnology, United States). The intensity of the reacting bands and their approximate Mw was determined with the Image Studio™ software (LI-COR Biotechnology, United States) as the average ± SD of three experiments.

TEM Immunolocalization of Olive 7S SSPs (β-Conglutins)

Ultrathin sections (70 nm) were obtained using a Reichert-Jung ultramicrotome and picked up using 200 mesh nickel grids coated with formvar. The grids were then sequentially treated with a blocking solution [5% (w/v) bovine serum albumin, 0.1% (v/v) Tween 20 in phosphate-buffered saline], a diluted (1:100) solution of the anti-7S antiserum in blocking solution, a 1:1000 solution of the secondary antibody (goat anti-rabbit IgG: 30 nm gold, BB International), and finally contrasted using a 5% (w/v) uranyl acetate alternative solution (Ted Pella Inc., CA, United States) and observed in a JEM-1011 (Jeol) transmission electron microscope (TEM). Negative control sections were treated as above but using preimmune serum instead of the anti-conglutin antiserum. Morphometric measurements were performed using the UTHSCSA ImageTool (version 3.00 for Windows) software.

Statistical Analysis

The Kolmogorov–Smirnov test was used to test the normality of all weight parameters. The Pearson test was performed aimed to determine whether whole fruit and mesocarp weight were correlated. For Western blotting and immunocytochemical analysis, values expressed as mean ± SEM of individual experiments were assessed for statistical significance of the data by analysis of variance followed by Dunnett's analysis. *P*-values $\leq$0.001 were considered statistically significant. All analyses were performed using IBM SPSS statistics v.24 software.

RESULTS

Olive Seed Anatomy at Early Stages of Fruit Development

The complete seed was processed 60 DAA to visualize general structure at a very early stage of development. At this moment, dissection of the seed into its tissues was not achievable without tissue damage due to the small size of the seed and the high compaction of the tissues. In **Figure 1A**, a schematic draw of the different tissues of the olive seed is displayed, as well as the positions selected for longitudinal- and cross-sections performed in this study.

A cross section of the complete seed at the radicle level showed that the testa and the endosperm tissues were immature, without appreciable differentiation among these two tissues (**Figure 1B**). The cells appeared unstained, indicating no clear accumulation of storage material neither in the endosperm nor in the embryo, as previously described (Jiménez-López et al., 2015).

No presence of the aleurone layer was detected. However, the presence of the cuticle and the pro-epidermal layer cells from the testa was visible. The cuticle was evidenced by an intense staining with methylene blue at the outermost site. The pro-epidermal layer of cells was placed under the cuticle, composed of long-shaped cells. Regarding the embryo, isodiametric cells were observed with slight differences among them. In the center of the embryo, the cells appeared intensely stained, this central structure corresponding to the precambium. The premesophyll cells were located surrounding those of the precambium. The pre-dermal cells appeared in the outer part, characterized by the presence of notorious nuclei. The embryo and the endosperm were separated by an ample space that remained unstained (**Figure 1B**). Similarly, a cross section of the embryo at the cotyledon level showed that the testa and the endosperm appeared undifferentiated. However, in this area, the presence of transversal fibers was patent. The thickness of the precursor of the testa and endosperm at the cotyledon level was approximately twofold that at the radicle level. The width increment was due to both, the presence of transversal fibers, and the rise in the number of cells (**Figure 1C**). The embryo cross-section at the level of the cotyledon showed cells with a quite marked differentiation (**Figure 1E**). Four types of cells were observed: those forming the procambium, the upper epidermis, the lower epidermis, and the premesophyll (**Figures 1C,E**). It was observed that the upper and lower epidermis contained one and two layers of cells, respectively, in both cases with a cubic shape. On the other hand, the premesophyll contained non-stained cells with variable shape and size. The presence of the procambium cells was evidenced as a group of small and densely packed cells among the premesophyll. A longitudinal section of the complete seed showed the position of the embryo within the seed as well as the disposition of the transversal fibers (**Figure 1D**).

The Formation of the Seed Coat Throughout Olive Fruit Development

After fertilization, the integuments of the ovule normally develop into the seed coat or testa. The histological analysis of this tissue

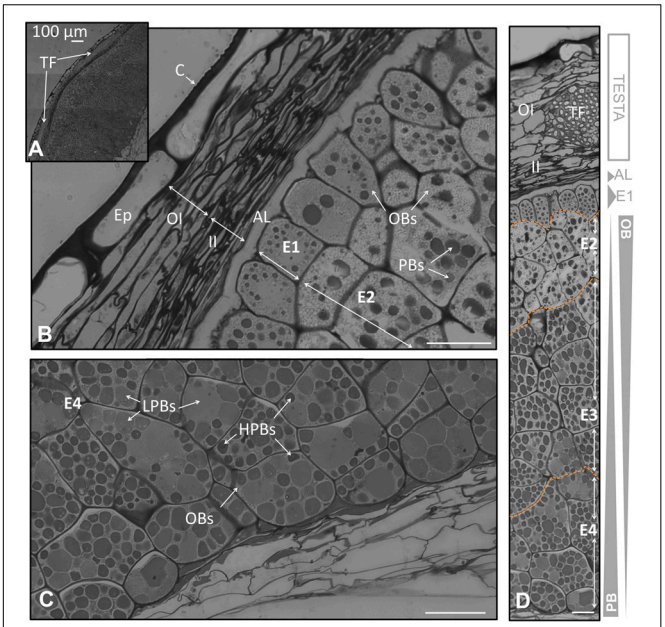

FIGURE 2 | Anatomical structure of the coats and the endosperm of the olive seed 105 DAA, corresponding to green fruit. **(A)** Cross section of the testa and the endosperm. **(B)** Testa and adjacent cells corresponding to the endosperm. **(C)** Cell of the endosperm corresponding to the part in contact with the embryo. **(D)** Testa and endosperm cross section showing the differential content in lipids and proteins along it. Orange dotted lines demarcate the different layers of the endosperm. OBs, oil bodies; PBs, protein bodies; C, cuticle; Ep, epidermis; OI, outer integument; II, inner integument; TF, transversal fibers; AL, aleurone layer; E1, endosperm layer 1; E2, endosperm layer 2; E3, endosperm layer 3; E4, endosperm layer 4.

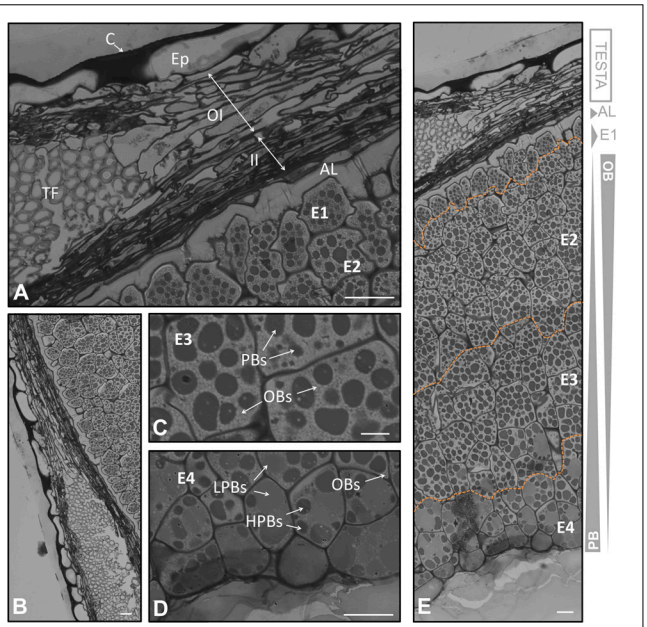

FIGURE 3 | Anatomical structure of the coats and the endosperm of the olive seed 130 DAA, which corresponds to the fruit at the veraison stage. **(A,B)** Cross section of the testa and cells adjacent to the endosperm. **(C)** Cells in the middle part of the endosperm. **(D)** Cells corresponding to the part in contact to the embryo. **(E)** Testa and endosperm showing differential protein/lipid content. Orange dotted lines demarcate the different layers of the endosperm. OBs, oil bodies; PBs, protein bodies; C, cuticle; Ep, epidermis; OI, outer integument; II, inner integument; TF, transversal fibers; AL, aleurone layer; E1, endosperm layer 1; E2, endosperm layer 2; E3, endosperm layer 3; E4, endosperm layer 4.

along three stages of the seed development has revealed that three layers can be distinguished: (i) mucilage or cuticle, (ii) epidermis, (iii) integument (**Figures 2–4**).

At the green fruit stage (105 DAA), the cuticle appeared strongly stained, forming a layer that covered evenly the non-stained and long shaped cells from the epidermis. Underneath appeared the integument composed by 8–10 well packed cells in a longitudinal orientation. The integument was divided into two parts: the outer and the inner integument; each one formed by 4–5 layers of cells. In the inner integument the cells displayed a more-flatted form, with minor intracellular spaces in comparison to the outer integument (**Figure 2B**). A cross section of the coat showed the presence of transversal fibers. These fibers crossed the integuments at the line of separation between both integuments causing a prominence of the coat (**Figures 2A,D**). This prominence causes the typical ornamentation of the olive seed that can be macroscopically distinguished.

At the veraison stage (130 DAA), a conspicuous loss of thickness of the cuticle in certain areas was detected. The cells from the epidermis appeared slightly distorted when compared to those at the green fruit stage. Besides, the start of a laxation in the cells from the outer integuments was noticed, whilst in the inner integument the cells appeared more densely packed. The transversal cells crossing the integument were observed to suffer also a light loosening, which also contributed to a progressive loss of compaction of the seed coat (**Figures 3A,B**).

At fruit maturity (210 DAA), the seed coat was characterized by the structure disorganization of the different layers. The cuticle was irregularly disposed over the epidermal cells, with a significant loss of width in some areas. The epidermis cells appeared with a patent loss of the structured disposition described for the previous stages. The same phenomena occurred in the outer, the inner integument, and the transversal fibers (**Figures 4A,B,D**).

The Formation of the Endosperm Throughout Olive Fruit Development

The outermost layer of the endosperm (termed aleurone) was observed to be composed by longitudinal shaped cells that laid over the cells of the endosperm with a high content in lipids. At the green fruit stage (105 DAA), this layer was well developed (**Figures 2A,D**). At the veraison stage (130 DAA), no significant changes were observed in the aleurone layer, with the exception of minor modifications in the shape. The cells set off slight penetrations in the vicinity of the endosperm cells (**Figures 3A,E**). At the mature fruit stage (210 DAA), the aleurone layer seemed with a less-structured disposition compared to the previous stages. The lipid-rich cells forming the upper part of the

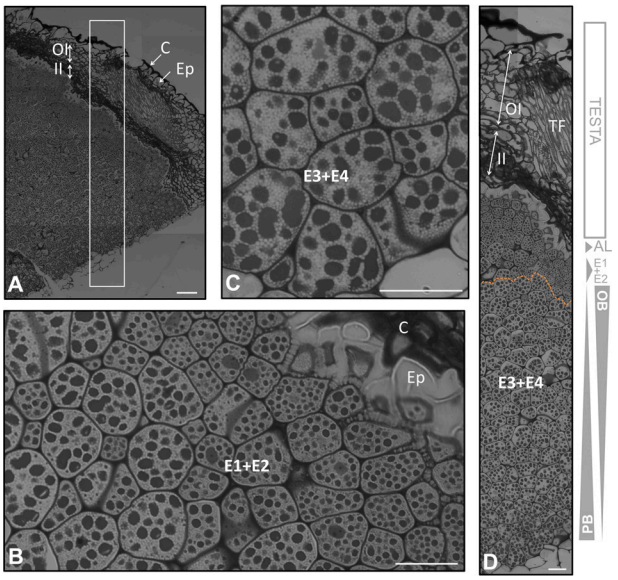

FIGURE 4 | Anatomical structure of the coats and the endosperm of the olive seed 210 DAA, obtained from mature fruits. **(A)** Cross section of the testa and endosperm. **(B,C)** Cell of the endosperm close to the testa, **(C)** cells in the middle part of the endosperm. **(D)** Testa and endosperm cross section. Orange dotted lines demarcate the different layers of the endosperm. OBs, oil bodies; PBs, protein bodies; C, cuticle; Ep, epidermis; Ol, outer integument; II, inner integument; TF, transversal fibers; AL, aleurone layer; E1, endosperm layer 1; E2, endosperm layer 2; E3, endosperm layer 3; E4, endosperm layer 4.

endosperm appeared interweaved with those from the aleurone (**Figures 4A,D**).

In the olive endosperm two main types of reserve material were detected along the seed/fruit development: lipids and proteins. These substances have been already described during the olive seed formation and in the olive seedling, where they have been related in unspecified areas of the endosperm and embryo. The proteins have been reported to accumulate forming PBs, surrounded by lipids that form oil bodies (OBs) (Jiménez-López and Hernández-Soriano, 2013; Jiménez-López et al., 2015).

In the present study we have observed that the endosperm was composed by isodiametric cells with uneven distribution of PBs/OBs (**Figures 1E**, **2D**, **3D**). The gradient of PBs/OBs accumulation followed a similar pattern during the three stages considered (from 105 to 210 DAA). The cells enriched in OBs were present predominantly near the testa, with a gradual decrease of lipids in the area near the embryo. The opposite tendency was observed in the PBs. It was detected the presence of differentially stained PBs within the endosperm cells. Thus, even when considering one single cell, differential types of PB staining was noticed. Cytokinesis phenomena occurred along the tree stages of development as phragmoplasts were detected.

Attending to the disposition and the PBs/OBs content within the endosperm cells, a classification of this tissue into four layers was performed. The first layer (adjacent to the aleurone) was named endosperm 1 (E1). It was detected as a monolayer of isodiametric cells with an arranged disposition. These cells contained small PBs surrounded by small OBs (**Figures 1C,E**). Following the E1, the cells were bigger and with an untidy disposition. This area was named as endosperm 2 (E2) and was the most lipid-enriched layer (**Figures 1C,E**). The area named as endosperm 4 (E4) was highly enriched in PBs, with an increment in their size. The area named as endosperm 3 (E3) was considered as a transition between E2 and E4 as regard to the size and quantity of PBs/OBs.

Noticeable modifications in the pattern of accumulation of reserve substances were observed in the endosperm 130 DAA, corresponding to the veraison stage. The E1 layer was not so clearly differentiated from the E2 as it was in the green fruit stage. The E1 cells lost their arrangement and contained larger PBs (**Figures 3A,E**). The differences between E1, E2, and E3 were not so apparent (**Figure 2D**). However, the transition between the E3 and E4 layers was still perceptible (**Figures 3C,D**).

At the mature stage, the main characteristic of the endosperm was an increment in the homogeny of the cellular size and PB/OBs composition. The aleurone and the E1 layers were interweaved. A conspicuous differentiation could be observed, with the E1+E2 representing a single layer and E3+E4 another one (**Figures 4A,C,D**). The distribution of the storage material was similar to that described by other authors at the same stage of development (Jiménez-López and Hernández-Soriano, 2013).

The Formation of the Cotyledon Throughout Olive Fruit Development

As described for the endosperm, the olive embryo also stocks two main kinds of storage material: lipids and proteins (Jiménez-López and Hernández-Soriano, 2013; Jiménez-López et al., 2015) that build up OBs and PBs, respectively. A deep scrutiny on the embryo histology showed an uneven distribution of this storage material mainly in the cotyledon and the radicle.

Observation of cross sections of the embryo at the cotyledon level (lines named "2" at **Figure 1**) 105 DAA showed the presence of a storing premesophyll tissue that appeared to consist of two zones with cells differing in shape, OBs/PBs distribution and intracellular spaces. Taking into account the orientation of the cotyledon sections, the two areas were identified as the future abaxial/adaxial sites of the leaf. In both zones, the premesophyll was covered by one/two layer of cells corresponding to the upper and lower pro-epidermis, respectively (**Figure 5**).

The pro-epidermal cells of the adaxial side, called upper pro-epidermis were cubic in shape and possessed small and strongly stained PBs surrounded by OBs. On the other hand, the under pro-epidermis was a monolayer of long shaped cells with transversal disposition. Underneath, it was noticed the presence of three layers of isodiametric cells forming the upper premesophyll (UPM). The UPM had densely packed cells with larger PBs compared to the upper pro-epidermal cells. The PBs were also surrounded by OBs. The lower premesophyll (LPM) cells occupied approximately two third parts of the cotyledon section, and their PBs were larger than those from the UPM cells. In the abaxial side it was noticed the presence of intracellular spaces. Interestingly, the PBs showed different stain intensity in both parts of the premesophyll. The lower pro-epidermis was

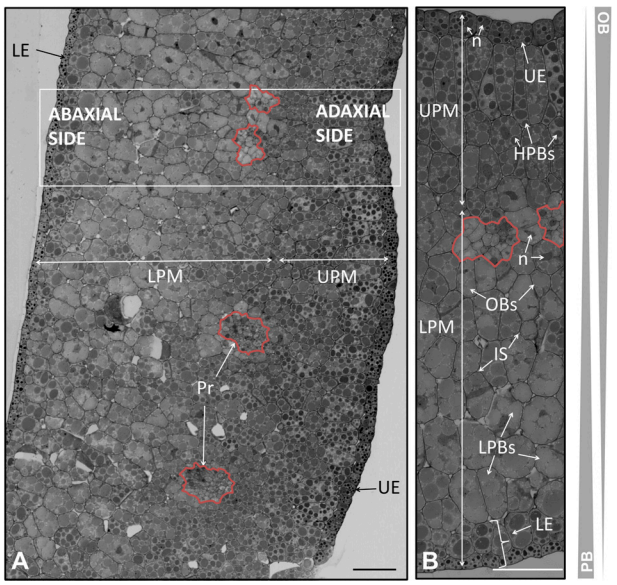

FIGURE 5 | Anatomical structure of the cotyledon of the olive seed 105 DAA. **(A)** Low magnification picture of the cotyledon. **(B)** Large magnification of the cotyledon revealing the precursors of the vascular system (pointed out with a red line). OBs, oil bodies; PBs, protein bodies; UE, upper epidermis; LE, lower epidermis; LPM, lower premesophyll cells; UPM, upper premesophyll cells; Pr, procambium; IS, intracellular spaces; n, nucleus.

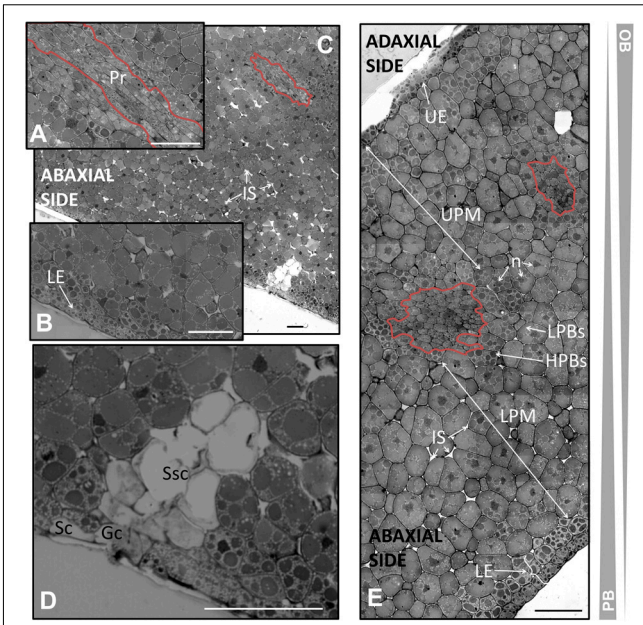

FIGURE 6 | Anatomical structure of the cotyledon of the olive seed 130 DAA. **(A)** Low magnification of the cotyledon. **(B,C)** Large magnification of the cotyledon at the procambium (pointed out with a red line) and lower pro-epidermal cells, respectively. **(D)** Stoma. **(E)** Cotyledon section showing the abaxial and adaxial sites and the cells encompassing them. OBs, oil bodies; PBs, protein bodies; HPBs, highly stained protein bodies; LPBs, low stained protein bodies; UE, upper epidermis; LE, lower epidermis; LPM, lower premesophyll cells; UPM, upper premesophyll cells; Pr, procambium; IS, intracellular space; n, nucleus; Gc, guard cells; Sc, subsidiary cells; Ssc, substomatal cavity.

composed by two layers of cells with different characteristics. The outermost layer possessed cubic cells with small and intensely stained PBs. Next, a layer of cells with half-way characteristics of the outermost epidermal cells and the LPM cells was observed. This layer was considered a transition as regard the cell size, PBs size, PBs stain intensity, and cell shape. The presence of nucleus was detected in all the cells along the cotyledon section (**Figure 5B**).

In between the UPM and the LPM cells, the presence of clusters of cells with irregular shape and size were distinguished, corresponding to the precambium. These cells appeared as densely packed, with nucleus, and without storage material within them (**Figure 5**).

The analysis of the histology of the olive seed cotyledon at the veraison stage of the fruit revealed changes in the premesophyll, precambium, and epidermal cells, which were characterized by changes in the disorganization of the storage material. The nucleus was observed in the cells of all the tissues. At this stage, the presence of structures considered precursors of stoma was detected (**Figure 6**).

The upper pro-epidermis was formed at 130 DAA by a single layer of cubic cells with parallel disposition and with small and intensely stained PBs surrounded by OBs. The layer of cells under the upper pro-epidermis had suffered transversal divisions giving rise to isodiametric cells similar to those of the rest of the UPM below. The cells from the adaxial side appeared densely packed with a tendency toward homogeneity in the cell size, PBs size, and PBs staining intensity. Concerning the LPM cells, the presence of subtle changes in the size and PBs/OBs disposition was detected, leading to a homogenization of the internal organization from the UPM and LPM. The two zones were not so clearly differentiated as in the previous stage with the exception of the presence of the precambium. Moreover, in the UPM cells there was noticed a combination of low- and highly stained PBs within the same cell, being predominant the latest ones. This phenomenon also was evident in the LPM cells, where the low-stained PBs were the most abundant ones in this case. It was observed that the LPM possessed several distinctive attributes: the intracellular spaces, cells slightly bigger than those from the UPM, and PBs occupying most of the volume of the cell.

The cells forming the precambium were detected in the center of the cotyledon section. Noticeable changes in the total area of the UPM and LPM were detected in comparison with the previous stage, with an increment in the UPM and a drop in the LPM area, respectively.

Modifications in the lower pro-epidermis at the veraison stage were detected. Two layers were distinguished, both of them composed by cubic, parallelly arranged cells, and with small PBs intensely stained within them. However, the sizes of the PBs were smaller in the outermost layer than in the internal one. In both cases, PBs size, stain intensity and OBs quantity were clearly different from the cells from LPM (**Figure 6**).

Precursors of stomata were observed at the abaxial side. The lower epidermis was interrupted by the guard and the

subsidiary cells. Below the stoma, a mass of non-stained cells with intracellular spaces was identified. The structure was similar to that described in *Zea mays* (Mauseth, 1988; **Figure 6D**).

The study of the anatomy of the olive cotyledon in the mature seed corresponding to 210 DAA showed a defined structure, with clear precursors of the spongy and palisade mesophyll. The imbalanced distribution of the storage material within the cells of the mesophyll was observed to be the main characteristic of this stage (**Figure 7**).

The upper pro-epidermis contained a monolayer of flattened cells disposed parallel in the plane to the surface. These cells were observed to be highly enriched in OBs and small PBs (**Figure 7C**).

The UPM cells occupied half of the cross section of the cotyledon and they were filled by numerous OBs surrounding the PBs. There was a mixture of highly stained and low stained PBs within the cells, mainly dominated by the highly stained ones. The cells from the LPM had a lower OBs content that surrounds the large PBs. Poles apart, the low stained PBs were dominant over the high stained ones within the cells of the LPM (**Figures 6B,D**).

The precambium appeared among the UPM as a group of long shaped cells without storage material within them (**Figure 7B**). At the mature stage the procambium did not show mature xylem or phloem elements. The lower epidermis was detected to be composed by two layers of cells with a non-arranged disposition, with highly stained PBs and elevated quantities of OBs (**Figure 6D**). The nucleus was observed in all the layers of cells across the cotyledon section.

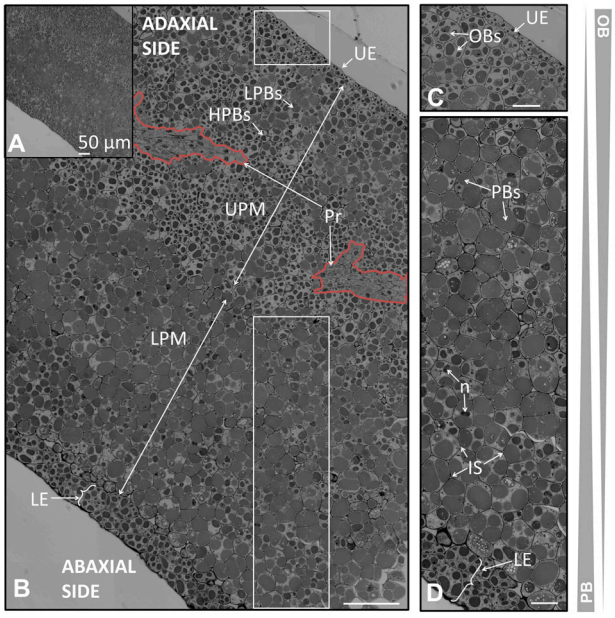

FIGURE 7 | Anatomical structure of the cotyledon of the olive seed 120 DAA. **(A)** Low magnification of the cotyledon. **(B)** Large magnification of the cotyledon. **(C)** Detail of the upper pro-epidermis and UPM cells. **(D)** Detail of the lower pro-epidermis and the LPM cells. OBs, oil bodies; PBs, protein bodies; HPBs, highly stained protein bodies; LPBs, low stained protein bodies; UE, upper epidermis; LE, lower epidermis; LPM, lower premesophyll cells; UPM, upper premesophyll cells; Pr, precambium; IS, intracellular space; n, nucleus.

The Formation of the Radicle Throughout Olive Fruit Development

Sections of the radicle taken form seeds at the green fruit stage showed the presence of three different kind of cells corresponding to the protoderm, ground meristem, and procambium, respectively (**Figure 8A**). The cells from the apex, which form to the ground meristem had a high degree of compaction and possessed large nuclei. The PBs were also large being surrounded by small OBs. The apical ground meristem cells suffered anticlinal divisions (**Figure 8D**). Regarding the protoderm, two layers of long shaped cells were observed, being the PBs small and intensely stained (**Figure 8B**). Underneath, a gradual change in the cell shape and the characteristics of the stored material within the cells was detected, giving rise to isodiametric cells with low-stained and large PBs. These cells were bigger than those forming the protoderm and the presence of intracellular spaces among them was detected. The procambium cells were long-shaped, devoid of storage material, and lacking intracellular spaces among them, which allowed differentiating them from the meristem cells. In between the procambium and the meristem, cells appeared as a transition concerning to the shape and the PBs/OBs content (**Figure 8C**).

At the veraison stage, few changes in the histology of the radicle were observed (**Figures 8E–G**). These changes corresponded mainly to the meristem, which appeared less packed. The cells at the apex of the meristem were an exception, with a high degree of compaction among the cubic-shaped cells.

At the mature fruit stage (210 DAA), we observed the presence of notorious changes in the organization and differentiation of the cells of the radicle (**Figures 8H–K**). The procambium appeared as a central bundle in the midpoint of the radicle. At the distal end of the procambium, the quiescent center was visible, mainly characterized by the disposition of the cells around a central point. In the above part, the cells of the columella and root apex displayed an arranged organization (**Figure 8I**). On the left and right sides of the quiescent center it was noticed that the cells suffered a progressive change in the shape and content of storage material to finally give rise to the meristematic cells. Below the quiescent center, the procambium comprises two areas. The area located in the middle was composed by isodiametric cells containing low-stained PBs. The external area was comprised of long-shaped cells without OBs, nor PBs. In both cases, the presence of the nucleus within the cells was perceptible, as well as the absence of storage material (**Figure 8J**). The protoderm was identified as two layers of long-shaped cells longitudinally arranged. These cells were differentiated from the adjacent meristematic cells since the latter possessed intracellular spaces, large cellular size, large PBs, and non-well-structured cells (**Figure 8K**).

SDS-PAGE protein profiles of whole seeds, isolated endosperm (+testa) and embryo at different DAA were resolved by SDS-PAGE under reducing conditions, as displayed in **Figure 9A**. Conspicuous bands of proteins appear corresponding

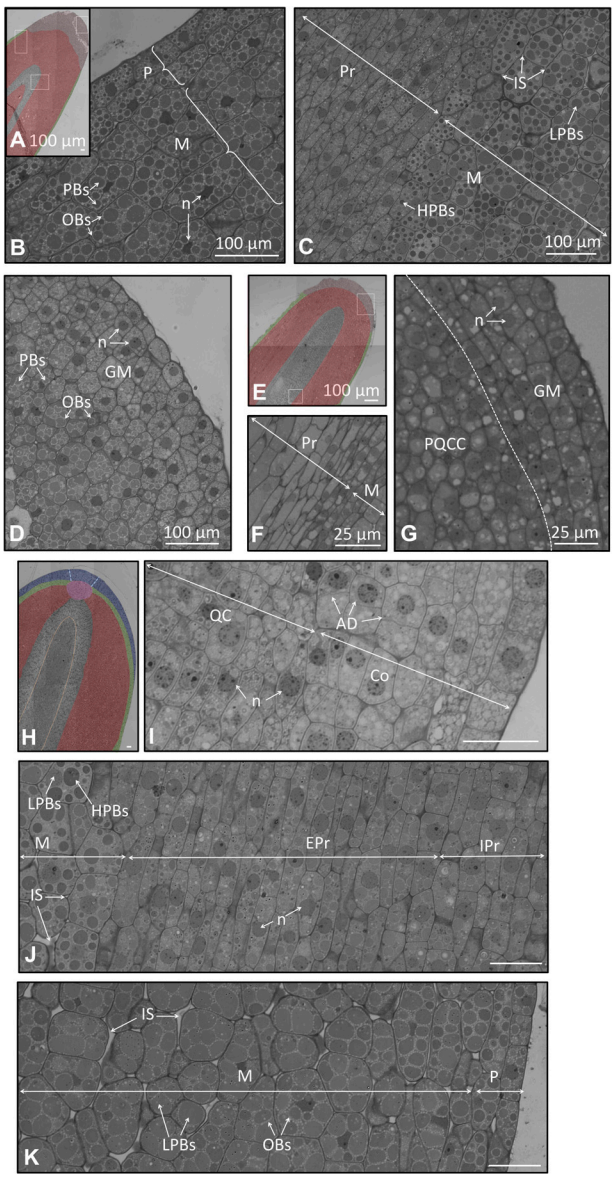

FIGURE 8 | (A–G) Longitudinal section of the embryo at the radicle part in a seed 105 and 130 DAA. **(A)** Low magnification radicle showing the different kind of cells 105 DAA. In light red appears the grown meristem at the apex; in green the protoderm; in gray the procambium. **(B)** Large magnification of the radicle showing a detail of the protoderm and the meristem 105 DAA. **(C)** Large magnification of the cell of the procambium and the meristem 105 DAA. **(D)** Large magnification of the ground meristem at the very apex 105 DAA. **(E)** Low magnification radicle showing the different kind of cells 130 DAA. In light red appear the grown meristem at the apex; in green the protoderm; in gray the procambium. **(F)** Cell of the procambium and the meristem 130 DAA. **(G)** Cell of the procambium and the meristem 130 DAA. **(H–K)** Longitudinal section of the embryo at the radicle in a seed 210 DAA. **(H)** Low magnification radicle section showing the different types of cells. In red appears the meristem; in green the protoderm; in blue the root apex, in gray the procambium. Orange dotted line demarcates two different types of cells within the procambium; blue dotted line demarcates the columella. **(I)** Detail of the radicle at the root apex and quiescent center. **(J)** Large magnification picture of the meristem and the procambium. **(K)** Cells of the protoderm and the meristem. P, protoderm; M, meristem; Pr, procambium; QC, Quiescent Centre; C, columella.

to the peptides p1 to p5 as described by Alché et al. (2006), which represent different peptides integrating the highly abundant 11S SSPs. The Western blotting profile after probing with the anti-β-conglutin primary antibody showed two reactive bands of c.a. 45 and 49 kDa, respectively present in all extracts, although with different relative intensities (**Figure 9B**). Relative quantification of each one of the reactive bands in all samples showed bands of 49 kDa evenly distributed in the endosperm and embryo tissues, with little changes in their intensity through the time developmental course. Contrary, the bands of 45 kDa presented noticeable changes in their intensity, particularly along the developmental stages for a given tissue (endosperm and embryo). The added intensities of both bands for each stage exhibited an increasing trend in the overall amount of β-conglutin along endosperm, embryo, and whole seed development (**Figure 9C**). Relative amount of β-conglutins was higher in the embryo compared to the endosperm.

Immunolocalization studies using the anti-β-conglutin primary antibody yielded an intense labeling by gold particles specifically located in the PBs present in the endosperm and the embryo all-through the seed developmental stages (**Figures 10A–C,A'–C'**). Labeling in the lipid bodies, any other cell structures (cell wall, nucleus, and testa) and in the negative controls processed by either omitting the primary antibody or using the pre-immune serum (not shown) was negligible. A statistically significant and progressive increase of labeling density in the PBs present in both the endosperm and the embryo was observed (**Figure 10D**). The overall density of labeling was significantly higher in the embryo than in the endosperm (**Figure 10D**).

Seed Weight Variability Among Olive Cultivars

The weight of the whole fruit was a variable parameter in the cultivars submitted to the present study (**Supplementary Figures S1A,B**). They ranged from an average of 1.28 g in 'Lechin' to 4.91 g in 'Ombliguillo.' Similarly, the average weights of the mesocarps were comprised between 0.89 g in 'Lechín' to 4.00 g in 'Ombliguillo.' Besides, the data obtained from the average weight of the endocarp oscillated from 0.39 g in 'Lechín' to 0.99 g in 'Gordal de Alhama.' The weights of the whole fruit and that of the pulp (mesocarp + epicarp) showed to have a positive correlation (**Supplementary Figure S1C**). As regard to the number of seeds found within each endocarp, six of the cultivars showed just one seed, whereas in the other six cultivars we managed to observe the presence of two seeds per endocarp in some of the fruits.

Focusing on the seed tissues we identified that the average weights of the complete seeds measured in the 12 cultivars ranged from 0.040 in 'Lechín' to 0.101 g in 'Azul.' The testa ranged from to 0.005 g in the cultivar '3' to 0.030 g in the cultivar 'Hojiblanca.' The endosperm ranged from 0.024 g in 'Picual' to 0.059 g in 'Loaime.' The embryo weights were comprised among 0.011 g in 'Picual' to 0.032 g in 'Nevadillo' (**Supplementary Figure S1D**).

As regard to the olive yield, measured as the ratio complete fruit/pulp weight, the obtained data showed the lowest ratio for 'Llorón' (1.20) while the highest one corresponded

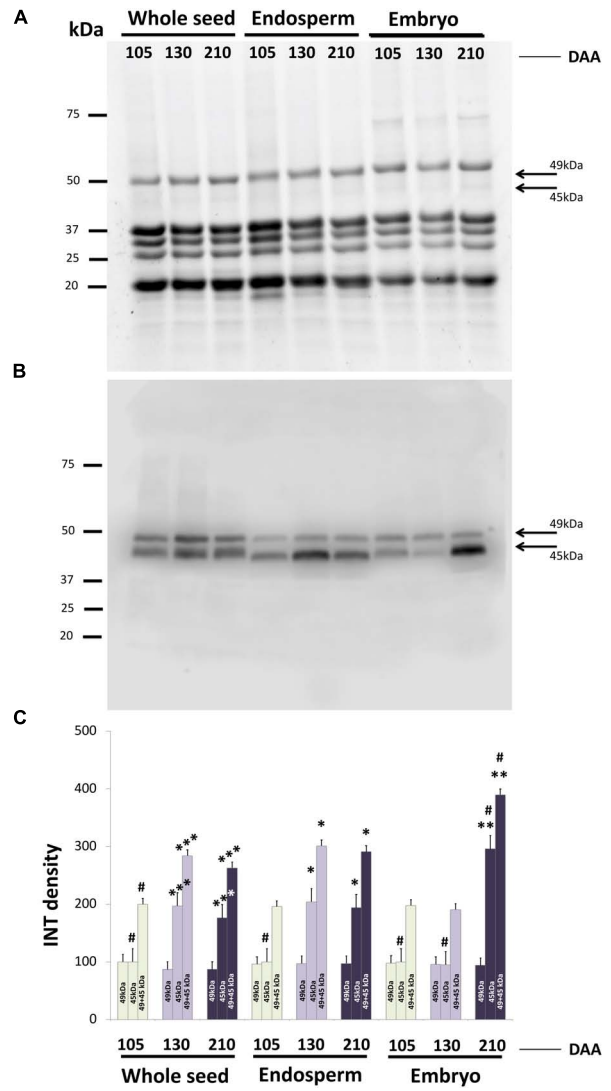

FIGURE 9 | SDS-PAGE profiles of and Western blotting analysis of β-conglutins in samples of whole seeds, isolated endosperm plus testa and embryo from olive fruits at different developmental stages. **(A)** SDS-PAGE profiles showing conspicuous bands corresponding to 11S peptides under denaturing, reducing running conditions. **(B)** Western blotting after using the anti β-conglutin antibody. **(C)** Densitometry of the reactive bands to the antibody. Green bars: green fruit (105 DAA). Light purple bars: veraison fruit (130 DAA). Dark purple bars: mature fruits (120 DAA). $*p < 0.001$ versus the corresponding endosperm sample at 105 DAA; $**p < 0.001$ versus the corresponding embryo sample at 105 DAA; $***p < 0.001$ versus the corresponding whole seed sample at 105 DAA; $^{\#}p < 0.001$ among all samples of the same band category (49, 45, or 49+45 kDa).

to 'Picual' with a ratio of 1.47. On the other hand, the fruit/endocarp ratio oscillated between 3.15 in 'Picual' to 5.77 in 'Llorón' (**Supplementary Figure S1E**). The endocarp/seed ratio showed values among 4.83 in 'Azul' to 14.72 in 'Picual.' The seed/embryo ratios oscillated between 2.15 in 'Nevadillo' to 4.58 in 'Ombliguillo.' In the case of the seeds containing two seeds per endocarp, each seed was weighted as independent sample. Spearman correlation between the weights of endosperm/embryo, seed/testa, seed/endosperm, and seed/cotyledon for different olive cultivars, as well as the registered presence of some fruits of the cultivar containing more than 1 seed is displayed in **Supplementary Figure S1F**.

DISCUSSION

Endocarp morphology is a widely accepted pomological signature for olive tree identification and classification of cultivars based on the presence of morphological differences (Barranco and Rallo, 1984; Rallo et al., 2005), which has been later evidenced to be in accordance with results obtained by molecular methods like simple sequence repeat (SSR) screening (Fendri et al., 2010). Within the endocarp, the olive seed represents a potential source of nutrients and biological elements of high interest, in addition of representing an additional varietal mark as demonstrated in the present work. Such designed potential will allow increasing the added value of this material, which is frequently disposed of concomitantly with olive processing residues. The olive seed can also be used as a source of genetic variability of interest for the development of breeding programs, in combination with the vegetative propagation of the resulting individual of interest (Morales-Sillero et al., 2012).

Histological structure of the olive seed doesn't substantially differ from those of dicots as described here; however, the distribution of the different tissues and their development has to be assessed in order to gain knowledge and establish parameters of maturity, which make easier the analysis of the expression and the presence of the compounds of interest, as is the case of 11S proteins (Alché et al., 2006; Jiménez-López et al., 2015) and the present case of 7S proteins described here. Such studies may help to define further technological developments, i.e., for sub-fractioning olive seed in order to enrich certain components, which could be majority present in a particular fraction. Also, these analyses may help to identify histological parameters relevant for seed and fruit physiology. Thus, the seed coats from different species have been analyzed for a variety of purposes such as the generation of a dichotomous key (Kaplan et al., 2009), or to analyze implications in key physiological roles like viability, dormancy and early control of germination (De Giorgi et al., 2015). The seed coat from the olive tree contains a well-defined cuticle covering the epidermis, which could be involved in key physiological roles. The intense staining might indicate a major presence of proteins, analogously as described in the grape seed coat, which is also rich in polysaccharides (Cadot et al., 2006). Proteome analysis in *Arabidopsis* has revealed the presence of proteins unique to mucilage responsible of alterations of its structure and mechanical alteration of the primary cell wall (Arsovski et al., 2010; Haughn and Western, 2012; Tsai et al., 2016). The key role of the seed cutin has also been associated to soil erosion (Engelbrecht et al., 2014). However, the protein complexity of the seed coat in the olive seed is still unrevealed.

We observed a general laxation and disarrangement of the coats that could be involved in the need to have access to oxygen

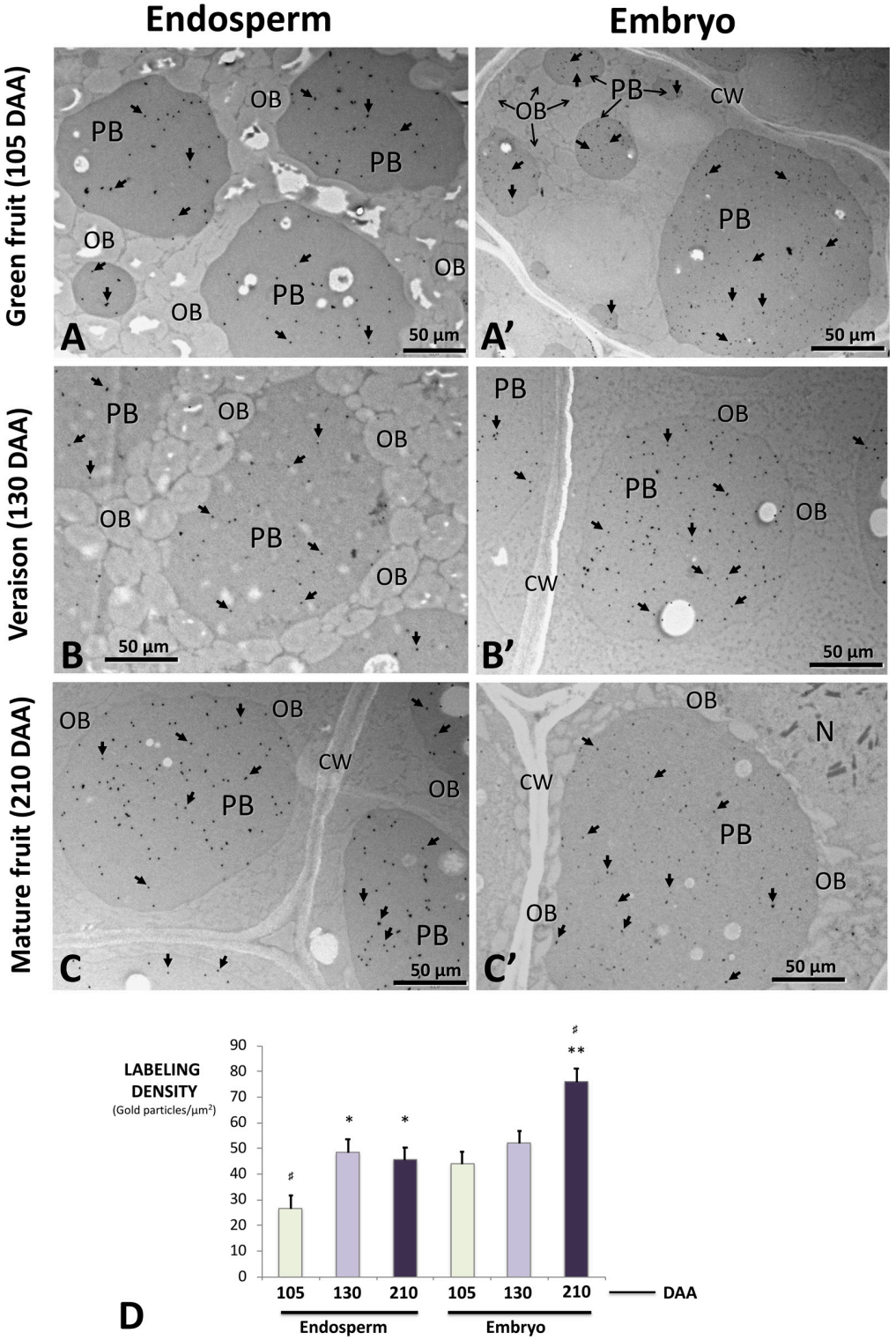

FIGURE 10 | Transmission electron microscope (TEM) immunolocalization of β-conglutins in samples of endosperm plus testa **(A–C)** and embryo **(A′–C′)** from olive fruits at different developmental stages. Gold particles (arrows) are specifically decorating protein bodies of different sizes. Lipid bodies, cell wall, and the nucleus are devoid of gold particles. **(D)** Quantification of labeling density in the protein bodies of both tissues at the different stages analyzed. Green bars: green fruit (105 DAA). Light purple bars: veraison fruit (130 DAA). Dark purple bars: mature fruits (210 DAA). CW, cell wall; OBs, oil bodies; N, nucleus; PBs, protein bodies. Magnification bars: 50 µm. $^{*}p < 0.001$ versus the corresponding endosperm sample at 105 DAA; $^{**}p < 0.001$ versus the corresponding embryo sample at 105 DAA; $^{\#}p < 0.001$ among all samples.

needed in the germination process. The three-dimensional study of the *Arabidopsis* seed revealed a putative network of intercellular air space that allows gas exchange for germination (Cloetens et al., 2006). The X-ray in-line phase tomography performed in maize is as practical tool for the detection of other characteristic non-detectable by conventional microcopy methods, like the metabolic state and the water content (Rousseau et al., 2015). The olive seed presents intracellular spaces and discontinuous in their structures putative involved in the need for the gas exchange, water intake or metabolic activity mainly in the mature stage and prior to the germination process. The presence of a well-defined aleurone layer in the olive seed has been described in the present study. This structure changes form a well-structured disposition in the green stage to disorganization in the mature stage that could be involved in some way in the easy removal of the seed coat at the mature stage.

As regards to the endosperm, Floyd and Friedman (2000) provided the first insight into how different endosperm developmental patterns are evolutionarily and developmentally related. The study of the endosperm possesses an increasing interest further than the long-established role of the endosperm as nourishment and mechanical barrier. The endosperm is capable of sensing environmental signals and interacts with the embryo establishing a bidirectional communication (Yan et al., 2014). The endosperm in the olive tree showed a clear change as regard to the organization and quantity of the OBs/PBs. These data were similar to those of the embryo. Moreover, it was found the presence of differentially stained PBs in both tissues. Thus, the composition of both, the proteins and the lipids could be differentially accumulated. The analysis of the fatty acid composition of the endosperm and embryo was detected to be different in *Arabidopsis* probably due to an hormonal regulation (Penfield et al., 2004) and later confirmed in both, *Arabidopsis* and *B. napus* (Li et al., 2006). These authors also observed that the fatty acid profile was different among embryo tissues. Finally, the apical meristems consist in three types of tissues that correspond to protoderm, ground meristem and procambium. The procambium is differentiated early in the development (60 DAA), however, the proper phloem and xylem did not appear differentiated in the mature seed. These events correlates to those previously described by Zienkiewicz et al. (2011) that pointed out the complete cellular organization of the leaf olive mesophyll is achieved 16 days after germination.

The study of the olive embryo and endosperm reveals the 11S protein as the most abundant one in these tissues (Alché et al., 2006). However, no studies about the presence of other specific proteins are available till the moment, whereas the present study confirms that 7S-type SSPs (β-conglutins) are also relevant constituents of both the endosperm and the embryo. Both proteomic and transcriptomic analysis aimed to agronomical improvements have shown for example the seed coat to function as a specialized secondary cell wall (Haughn and Western, 2012), to be involved in endosperm permeability, seed viability, and seed dormancy which correlates with higher levels of seed lipid oxidative stress (De Giorgi et al., 2015), with implication in specific functions that affects the seed composition, seed permeability, and hormonal regulation (Verdier et al., 2013). The study of both the proteome and the transcriptome of the olive seed (currently being approached) could represent interesting tools for multiple purposes, including the study of specific proteins involved in organoleptic properties of the olive oil. Thus, the presence of seed enzymes involved in the lipoxygenase pathway, enzymatic activities metabolizing 13-hydroperoxides other than hydroperoxide lyase, alcohol dehydrogenase, and alcohol acyltransferase activities among others would provide multiple esters in the olive oil (Luaces et al., 2003, 2007).

Within the increasing demand for plant-derived proteins as components of functional foods in the nutraceutical industry and as an alternative to expensive and less-environmental-friendly production of animal protein, β-conglutins are considered an economical dietary source of good quality protein. Also, they have positive effects on many human health dysfunctions, as many of the seeds containing β-conglutins are protein- and fiber-rich, low in fat and starch, and have a very low glycemic index (Arnoldi, 2008; Duranti et al., 2008). Positive effects have been described for these proteins on blood pressure, risk of cardiovascular disease and the prevention and treatment of type 2 diabetes, by modulating the insulin signaling pathway and diminishing inflammation (Lima-Cabello et al., 2017a). For the olive seed, preliminary work (unpublished) indicates the presence of anti-inflammatory components in the flours derived from this material. However, the direct involvement of β-conglutins in these effects is yet to be analyzed.

Expression of β-conglutins in the olive seed tissues is remarkable as shown here, with at least two forms of the protein reactive to the antibody, which might indicate the presence of a protein maturation process, as it is the case of the olive 11S SSPs (Alché et al., 2006), and has been proposed for β-conglutins (Duranti et al., 2008). The 49 kDa form of the protein shows a constitutive presence in the endosperm, embryo, and whole seed, whereas the 45 kDa form displays developmental changes as well as slight tissue differences. The accumulated presence of both β-conglutin forms indicates that this protein is progressively accumulated in the seed, through the developmental process, and that the relative amount of β-conglutins was higher in the embryo compared to the endosperm. Olive seed development was already characterized as a tissue-dependent process characterized by differential rates of legumin accumulation and PB formation in the main tissues integrating the seed (Jimenez-Lopez et al., 2016) on the basis of the accumulation of 11S legumin proteins. Such developmental pattern is then shared by β-conglutins as well. The relationships between the 45 and 49 kDa forms of the protein must be stablished through future work. Proteomic and transcriptomic work in course will serve the basis for this information, and will help to determine whether these correspond to maturation forms of the protein or the result of the expression of differential genes. These studies will also determine the presence of embryo- and endosperm-specific proteins, as have been recently identified in *Phoenix dactylifera* (Sekhar and DeMason, 2017).

Lupin β-conglutins are located in the endosperm and cotyledonary PBs, as shown by immunocytochemical experiments carried out here, and as it is also the occurrence with olive 11S legumins. As showed by Duranti et al. (2008),

the covalent integrity is not apparently a pre-requisite for β-conglutin to be correctly deposited in these cellular structures, since the mature β-conglutin from lupin dry seeds appeared already proteolytically cleaved in a number of sites, giving rise to complex SDS-PAGE patterns. Immunolocalization of β-conglutins in the olive PBs likely reflects the localization of both the 45 and 49 kDa forms of the protein, as they are both recognized by the antibody. Quantification of the labeling in the PBs is consistent with the quantification of the signal of the 49+45 kDa bands in Western blotting experiments, showing an incremental presence of these proteins through the maturation process, analogous to that of 11S proteins, which is concomitant with the increased presence of PBs in all the tissues analyzed here. Also, the higher presence of ß-conglutins in the embryo compared to the endosperm was verified in the immunocytochemical experiments.

Both the histological features and analytical characteristics and the localization of the olive seed β-conglutins were also determined at longer times after anthesis (240 DAA). Such parameters did not differ substantially from those displayed here for 210 DAA in the cultivar 'Picual' and therefore were not shown in the present work. This may suggest that maturation of the seed ends before the maturation of the pulp in the olive fruit.

The distinctive character of the olive endocarp morphology and size amongst olive cultivars, previously reported by Barranco and Rallo (1984), was also verified in the present work. However, in this case the differences were also assessed as regard to the main parameters of the different constituents of the seed. Although differences among cultivars exist, some general directions can be detected. As an example, the weights of the whole fruit and that of the mesocarp were detected to have a positive correlation for all cultivars, whereas the weight of the whole seed was positively correlated with the weights of the individual components (endosperm and cotyledon) for most cultivars, and on the contrary, no correlation was detected between the weight of the whole seed and the weight of the testa for most cultivars. Such relationships may have particular meaning for future and potential uses of particular cultivars for the extraction of seed derived components, as it is the case of polyphenols (work in progress). In addition, the endocarp is considered to represent an evolutionary strategy for seed protection and dispersal (Dardick et al., 2010). Therefore, their size, and that of the different components of the seed should be further analyzed in relation to their dispersion efficiency, viability, ability of germination and vigor for the different olive cultivars, and particularly for wild olives, which are mainly propagated by seeds. This is one of the objectives of several research projects funding the present work. Also, moderate and severe reductions in water availability proportionately decrease endocarp expansion and prolong the sclerification, delaying the date of physically perceived hardening but not affecting the final degree of endocarp sclerification (Hammami et al., 2013). Therefore, the analysis of the hardening dynamics of the endocarp and the final size of the endocarps might be used as a marker for biological studies and crop management, as well as a marker for cultivar tolerance to water availability.

CONCLUSION

The described anatomy and histological distribution of the olive seed of the 'Picual' cultivar, allows identifying the main features typical of dicots within a developmental time frame. Cell storage structures (PBs and OBs) present a well-defined pattern of accumulation, with complementary distribution in the olive seed tissues.

Seed storage proteins of the 7S-type (β-conglutins) are relevant components of all olive seed tissues, displaying an accumulative pattern concurrent with the development of the seed and fruit. These proteins are present in at least two peptide forms, and are subcellularly associated to PBs in the different tissues analyzed.

Moreover, a panel of other olive cultivars has been used to compare the weight contribution of the different tissues to the seed, seed weight variability, and the number of seeds per fruit.

These hallmarks will help to settle the basis for future studies related to the location of different metabolites along the olive seed and mesocarp development, and therefore helping to assess the appropriate ripening stage for different commercial and industrial purposes.

AUTHOR CONTRIBUTIONS

AZ and JA designed the experimental structure of the work and redacted the manuscript. AZ performed the experiments, observations, image capture, and analysis of the results, whereas MM'-A performed TEM immunocytochemical detection and signal quantitation. JJ-L was particularly involved in the work with the databases and tools on the web servers for prediction of synthetic peptide and the generation of the antibody. EL was responsible for Western blotting experiments and analysis. 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.2018.01481/full#supplementary-material

FIGURE S1 | (A) De-stoned seeds from 18 fruits cv. 'Picual' as an example. The endocarp and seeds are shown. **(B)** Dissection of the tissues from a 'Picual' seed. Up to down: endocarp, testa, endosperm, and embryo. **(C–E)** Representation of the different parameters in a total of 12 olive cultivars, including 'Picual' (arrow). Scale bars: 20 mm. **(F)** Spearman correlation of several weights of the seed tissues form different olive cultivars. (+/−) indicate positive/negative correlation ($p < 0.01$). Y/N (Yes/No) indicates whether some fruits of the cultivar contained more than 1 seed.

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Molecular Control by Non-coding RNAs During Fruit Development: From Gynoecium Patterning to Fruit Ripening

*João Paulo de Oliveira Correa†, Eder M. Silva†‡ and Fabio T. S. Nogueira**

Laboratory of Molecular Genetics of Plant Development, Department of Biological Sciences (LCB), Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ), University of São Paulo, São Paulo, Brazil

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***Correspondence:**
Fabio T. S. Nogueira
ftsnogue@usp.br

†These authors have contributed equally to this work

*‡**Present address:***
Eder M. Silva, Laboratory of Plant Genetic Improvement, Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, Brazil

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Fruits are originated from the transition of a quiescent ovary to a fast-growing young fruit. The evolution of reproductive structures such as ovary and fruit has made seed dispersal easier, which is a key process for reproductive success in flowering plants. The complete fruit development and ripening are characterized by a remarkable phenotypic plasticity which is orchestrated by a myriad of genetic factors. In this context, transcriptional regulation by non-coding small (i.e., microRNAs) and long (lncRNAs) RNAs underlies important mechanisms controlling reproductive organ development. These mechanisms may act together and interact with other pathways (i.e., phytohormones) to regulate cell fate and coordinate reproductive organ development. Functional genomics has shown that non-coding RNAs regulate a diversity of developmental reproductive stages, from carpel formation and ovary development to the softening of the ripe/ripened fruit. This layer of transcriptional control has been associated with ovule, seed, and fruit development as well as fruit ripening, which are crucial developmental processes in breeding programs because of their relevance for crop production. The final ripe fruit is the result of a process under multiple levels of regulation, including mechanisms orchestrated by microRNAs and lncRNAs. Most of the studies we discuss involve work on tomato and *Arabidopsis*. In this review, we summarize non-coding RNA-controlled mechanisms described in the current literature that act coordinating the main steps of gynoecium development/patterning and fruit ripening.

Keywords: tomato, fruit development, microRNAs, lncRNAs, ripening

INTRODUCTION

Fruits are plant organs found solely in angiosperms and are commonly defined as mature ovaries containing seeds. They are also ecologically defined as seed dispersal units, and their diversification and specialization are key events of the adaptive success of angiosperms in a wide range of environments (Seymour et al., 2013). The final characteristics of a mature fruit are determined by events that take place in developmental stages ranging from floral meristem initiation to later stages of fruit ripening. Complex mechanisms of transcriptional regulation of each of these stages ensure proper fruit development. After floral meristem initiation, key events of fruit development include carpel formation, differentiation, patterning and organ boundary formation. Ovule and seed development are also fundamental processes for the completion of fruit maturation. Fruit set

occurs when the signaling triggered by the pollination and fertilization turns a fully developed ovary into a fast-growing fruit that will soon initiate the ripening process.

Although physiological and molecular aspects of fruit development and ripening are well discussed in the available literature (Ferrándiz et al., 2010; Liu et al., 2015), few reviews focused on the role of non-coding RNA-based molecular regulation controlling early and late stages of fruit development. Here, we reviewed the literature focused on the aspects of the regulation by non-coding RNA in different stages of fruit development, including ovule and seed development. Moreover, we discussed aspects of fruit growth and ripening in the light of miRNA and lncRNA-associated mechanisms. One important question that need to be better addressed in future studies is how transcriptional control of fruit development is conserved between dry fruit-bearing and fleshy fruit-bearing species (e.g., *Arabidopsis thaliana* and tomato or *Solanum lycopersicum*, respectively). A better understanding of the non-coding RNA-related transcription hallmarks orchestrating early steps of fruit development and ripening in different species may have the potential to provide novel strategies for crop improvement.

MicroRNA MODULES INVOLVED IN EARLY STEPS OF FRUIT PATTERNING AND GROWTH

The carpel is the female reproductive organ that encloses the ovules in flowering plants. The gynoecium is the innermost floral whorl, formed by the fusion of carpels in the center of the flower. The hypothesis of the origin of the carpels as modified leaves is corroborated by the observation that leaf development-associated factors also have roles in carpel development (Dinneny et al., 2005; Scutt et al., 2006; Alonso-Cantabrana et al., 2007; Ferrándiz et al., 2010; González-Reig et al., 2012; Seymour et al., 2013; Deb et al., 2018). Carpel and fruit development can be broadly divided into two main temporal set of events: an earlier set of events that occur prior to fertilization (differentiation and patterning), and later events, which occur after fertilization (growth, ripening and senescence) (Ripoll et al., 2015; Deb et al., 2018). A fine-tuned molecular regulation of each of these developmental steps is crucial to ensure proper morphological and physiological characteristics of the mature fruit.

MicroRNAs (miRNAs) and their targets (mostly transcription factors; Chen, 2009) are fundamental components of molecular modules (hereafter referred to microRNA modules) belonging to complex circuits that control various aspects of plant development. miRNAs inhibit the activity of their targets by two major mechanisms: *ARGONAUTE1 (AGO1)*-mediated transcriptional cleavage, and translational repression of gene targets (Borges and Martienssen, 2015). At cell and tissue levels, many miRNAs accumulate in a spatiotemporal manner to modulate and/or fine-tune the expression of their targets (Chen, 2009; Rubio-Somoza and Weigel, 2011). For instance, some miRNAs participate in tissue patterning by restricting the expression domain of target genes (Berger et al., 2009; Chen, 2009; Ripoll et al., 2015). On the other hand, miRNAs and targets may be co-expressed in similar domains, where miRNAs ensure proper transcript accumulation by dampening target transcript levels. In this case, miRNAs generally mediate the temporal control of transcript accumulation, in which cells and/or tissues exhibit a gradual decrease or increase in the levels of target transcripts as the organ develops (Aukerman and Sakai, 2003; Wu et al., 2010; Rubio-Somoza et al., 2014; Wang, 2014; Guo et al., 2017; He et al., 2018).

Some miRNA modules had their roles in gynoecium and fruit development described in different model plants, such as *A. thaliana*, which produces dry fruits (silique), and tomato (*S. lycopersicum*), which produces fleshy fruit (berry). Interestingly, alterations in similar miRNA modules produce distinct phenotypic changes in gynoecium and fruits of *Arabidopsis* and tomato (Xing et al., 2013; Silva et al., 2014). Understanding what pathways are directly and/or indirectly regulated by similar miRNA modules in different species, and how they influence distinct fruit morphologies, will shed light on important evolutionary aspects of fruit development. In the next sections, we discussed examples in the literature concerning the roles of miRNA modules in early events of fruit development mostly in tomato and *Arabidopsis*.

The miR164 Module Controls Carpel Development and Leaf Margin Serration Through Similar Mechanisms

MiRNA-associated pathways control many aspects of plant development. Some miRNA-targeted transcriptional regulators that had their roles previously associated with vegetative development, such as leaf development, had similar functions later elucidated in carpel development. For instance, *Arabidopsis* miR164-targeted *CUP-SHAPED COTYLEDON1* and *2* (*CUC1* and *CUC2*) – which belong to the NAC transcription factor family – regulate organ boundary during the separation between organ primordia and meristem, and control leaf margin serration (Laufs et al., 2004; Nikovics et al., 2006; Peaucelle et al., 2007; Hasson et al., 2011; Vialette-Guiraud et al., 2016). Earlier studies showed that *CUC1* and *CUC2* operate during the initial phase of organ initiation inhibiting cell growth in meristem-organ and organ–organ boundaries, facilitating the separation between adjacent vegetative and reproductive organs (Aida et al., 1999; Laufs et al., 2004; Mallory et al., 2004). In this process, miR164 defines boundary domains by restricting the expression of *CUC1* and *CUC2* (the miR164 module), and proper miR164 dosage and/or expression localization is required for organ separation. The miR164 module also operates further in organ development, when organ shape is being determined (Nikovics et al., 2006). In the margins of leaf primordia, *CUC2* and *MIR164A* are spatially and temporally co-expressed, and the balance between their expression controls the degree of *Arabidopsis* leaf margin serration (Nikovics et al., 2006). This module operates similarly in the regulation of leaf complexity in tomato, in which the *CUC2* ortholog miR164-targeted *GOBLET* (*GOB*) plays similar roles during boundary establishment leading to leaflet separation. Interestingly, the regulation of compound leaf development by the miR164 module is conserved in *Aquilegia caerulea*, *Solanum*

tuberosum, *Cardamine hirsuta*, and *Pisum sativum* (Blein et al., 2008).

Like its function in leaf development, the miR164 module is also expressed in the margins of carpel primordium during *Arabidopsis* gynoecium development, and it determines important morphological characteristics of the mature fruit (Ishida et al., 2000; Sieber et al., 2007; Nahar et al., 2012; Kamiuchi et al., 2014; Vialette-Guiraud et al., 2016). *Arabidopsis* gynoecium is formed by two carpels that are already initiated as two fused structures, except by the apical margins, which are fused later to form style and stigma (Sessions and Zambryski, 1995; Nahar et al., 2012). During early gynoecia development, the meristematic tissue called Carpel Margin Meristem (CMM) is originated in the margins of each carpel primordia and is responsible for producing the ovules, the ovary septum, the transmitting tract, and promoting fusion between the apical carpel margins (Alvarez and Smyth, 1999; Nahar et al., 2012; Vialette-Guiraud et al., 2016). Earlier studies showed that *CUC1* and *CUC2* expression is required for the activation of the *KNOX type-I* gene *SHOOT MERISTEMLESS* (*STM*) in different developmental contexts, such as the formation of shoot apical meristem during embryo development and leaf serration in *Arabidopsis* (Takada et al., 2001). In such processes, *STM* expression is required to establish and maintain meristematic tissues. The same mechanism seems to operate in the establishment and maintenance of CMMs during carpel development in *Arabidopsis* (Kamiuchi et al., 2014). Most *cuc1cuc2* double mutants failed to form CMM, producing mature gynoecia with drastically reduced or complete loss of ovules and septum. *Arabidopsis* plants expressing miR164-resistant versions of *CUC1* and *CUC2* showed expanded domain of *STM* expression, resulting in carpel primordia with altered size and number of CMM, of which most initiated in altered positions. These plants produce mature fruits with internal filamentous structures (Kamiuchi et al., 2014). When not regulated by miR164, *CUC1/2* expression is less precise and can expand out of the boundary strips, resulting in incorrect CMM positioning, which leads to carpel and fruit developmental aberrations.

SPATULA (*SPT*) encodes a basic helix-loop-helix (bHLH) transcription factor, and *Arabidopsis* loss-of-function *spt* mutants produce ovaries with split or incomplete fused carpels and defective CMM-derived tissues (Heisler et al., 2001; Nahar et al., 2012). *cuc1;cuc2* mutations partially suppress the split carpel phenotype of *spt* mutant, indicating that congenital carpel fusion depends on *SPT*-based down-regulation of *CUC1* and *CUC2*. Thus, the coordinated interaction among *SPT*, *CUC1*, and *CUC2* regulates *Arabidopsis* ovule and septum development during the progression of fruit growth (Nahar et al., 2012). It was recently shown that SPT enables cytokinin signaling, which provides meristematic properties to CMM. *SPT* seems to play a role in the interaction between auxin and cytokinin pathways, as *SPT* induces *ARABIDOPSIS RESPONSE REGULATOR 1* (*ARR1*) directly. *SPT* and *ARR1* induce the expression of the auxin transporter *PIN-FORMED 3* (*PIN3*) and the auxin biosynthesis gene *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* (TAA1, Reyes-Olalde et al., 2017).

The role of the tomato *CUC2* homolog *GOB* was studied in detail during leaf development and complexity, although little is known about the function of *GOB* in reproductive development. Loss-of-function *GOB* mutant (*gob-3*) produces fruits with fewer locules, whereas gain-of-function *GOB* mutant (which contains a miR164-resistant version of *GOB,* the *Gob-4d*) displays fruits with extra carpels and increased number of locules (Berger et al., 2009). Since leaf complexity was the main objective of this work, no mechanism was proposed of how the miR164 node (miR164-targeted *GOB*) controls locule number in tomato fruits. On the other hand, tomato miR164-targeted *NO APICAL MERISTEM 2* (*SlNAM2*), another member of the NAC transcription factor family, was shown to have an important role in organ boundary maintenance during floral development (Hendelman et al., 2013). Unlike *GOB*, *SlNAM2* is not expressed in boundaries between floral meristem and organ primordia, as *SlNAM2* expression was not detected before carpel fusion in flower buds. Data thus far suggest that *GOB* functions during the formation of the boundaries, being expressed at earlier stages of organ primordia development, whereas *SlNAM2* is expressed at later stages of floral whorl development, being responsible for the maintenance of the boundaries established by *GOB* (Hendelman et al., 2013). Plants overexpressing *mSlNAM2* (a miR164-resistant version of *SlNAM2*) produced gynoecia with shorter stamen and styles and wide pistil, the latter likely due to the extra carpel formation. Although weaker, *mSlNAM2* phenotypes were similar to *Gob-4d* phenotypes, which is consistent with the proposed *SlNAM2* role in boundary maintenance, but not boundary formation (Berger et al., 2009; Hendelman et al., 2013). In summary, the functions of the miR164 module in *Arabidopsis* and tomato gynoecium patterning illustrates the crucial importance of boundary formation and maintenance during fruit development. Proper function of the miR164 module is essential for the establishment and maintenance of gynoecium development, not only in syncarpous species such as *Arabidopsis* and tomato, but also in monocarpous species like *Medicago truncatula* (Berger et al., 2009; Vialette-Guiraud et al., 2016).

The Role of miR156/miR157 in Carpel and Fruit Development

MiR156 targets members of the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SBP/SPL*) transcription factor family. In *Arabidopsis* and tomato, 11 out of 17 *SBP/SPLs* harbor the miR156 recognition site (Salinas et al., 2012; Preston and Hileman, 2013). The miR156 module (miR156-targeted *SBP/SPLs*) defines the evolutionary conserved age-dependent floral pathway in several plants, including tomato (Silva et al., 2018). Interestingly, the miR156 module has been proposed as a main target for crop improvement, aiming to enhance agronomic traits such as the timing of vegetative and reproductive phase change, leaf development, tillering/branching, panicle/tassel architecture, fruit development and fertility (Wang and Wang, 2015).

In terms of gynoecium and fruit development, it was demonstrated that *Arabidopsis SPL8* (which is not targeted by

miR156) acts redundantly with miR156-targeted *SPLs* in the control of carpel development (Xing et al., 2013). Transgenic plants overexpressing miR156 (*p35S::MIR156b*) produce flowers with reduced ovary size but unaffected structure, while ovaries of *spl8-1* mutant show a slight reduction in size and resembles wild-type (WT). Conversely, the double mutant *p35S::MIR156b spl8-1* show extremely modified gynoecia. The gynoecium shape of *p35S::MIR156b spl8-1* is completely altered, displaying an enlarged upper region and a narrower basal region, abnormal septum development, and absence of transmitting tissue to support pollen tube growth into the ovary (Xing et al., 2013). Considering that *SPL8* and the miR156-targeted *SPLs 2, 6, 10, 11, and 13* are expressed in overlapping domains during gynoecium development, this data supports the idea that they have partly redundant roles in the patterning of the gynoecium and fruit development. Furthermore, seed production decreased about 60% in *p35S::MIR156b* plants in comparison with WT and *spl8-1* (which show unaltered seed production), whereas *p35S::MIR156b spl8-1* produces approximately 96% less seeds than WT (Xing et al., 2013). Together, these data indicate that the function of at least one of these *SPLs* is crucial for proper gynoecia development. Another study showed that *Arabidopsis squint* (*sqn*) mutants contain loss-of-function alleles for *Cyclophilin40* (*CyP40*), which increases the activity of miR156 by promoting *AGO1* activity. *sqn* plants showed elevated expression of miR156-targeted *SPLs* and produce siliques with increased carpel number (Smith et al., 2009).

Interestingly, the miR156 module may function by different mechanisms or have different roles in dry fruit and fleshy fruit-bearing species. As mentioned above, ovaries of *Arabidopsis p35S:MIR156b* plants do not present extra carpels or undetermined growth (Xing et al., 2013). On the other hand, the overexpression of miR156 (*p35S:MIR156b*) in tomato plants led to the production of extremely modified ovaries formed by multiple fused extra carpels and undifferentiated tissue inside the post-anthesis ovaries (Silva et al., 2014). After fruit set, the undifferentiated tissue inside the ovaries of *p35S:MIR156b* plants continues to grow, forming fruit-like structures growing from the stylar end of the fruits. Furthermore, mature fruits show increased number of locules due to the presence of extra carpels in the ovary (**Figure 1**; Silva et al., 2014). Floral identity genes like *FUL1/TDR4*, *FALSIFLORA* (*FA*, *Arabidopsis LEAFY* ortholog; Lozano et al., 2009) and *MACROCALLYX* (*MC*, *Arabidopsis APETALA1* ortholog; Lozano et al., 2009) were strongly down-regulated in tomato *p35S:MIR156b* ovaries (Silva et al., 2014). *Arabidopsis FUL*, *AP1* and *LFY* are direct targets of SPL3 (Yamaguchi et al., 2009), although it is still unknown whether their tomato orthologs are direct targets of SlSBP3. Interestingly, the *CUC2* and *STM* orthologs *GOB* and *TKN2,* respectively, are up-regulated in tomato *p35S:MIR156b* ovaries. MiR164-targeted *GOB* and *TKN2* are associated with leaf complexity in tomato but both can also regulate the number of locules per fruit (Parnis et al., 1997; Berger et al., 2009). This finding indicates a link between miR156 and miR164 modules and suggests that tomato miR156 module controls boundary formation and establishment as well as locule number through *GOB* and perhaps other NAC domain-containing genes (such as *SlNAM2*). As expected, tomato plants overexpressing miR164 lead to *GOB* down-regulation and the production of fruits with normal shape but reduced locule number (Silva et al., 2014).

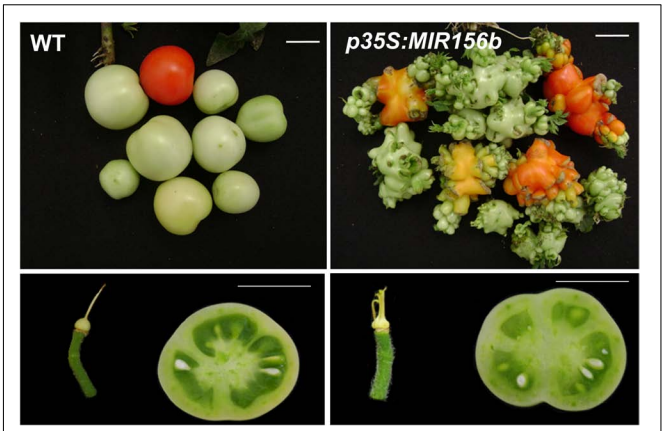

FIGURE 1 | *MIR156* overexpression alters tomato fruit determinacy and locule number. **(Upper)** Wild-type (WT) fruits and undetermined *p35S::MIR156b* fruits. **(Lower)** Three-locular WT fruits and four-locular *p35S::MIR156b* fruits. Bars: 1 cm.

Most plant genomes also contain miR157, a miR156 closely related miRNA which differs from miR156 by three nucleotides (Reinhart et al., 2002). MiR157 overexpression in *Arabidopsis* generates plants phenotypically similar to miR156 overexpressors, but miR157 specific functions are still unknown (He et al., 2018). MiR157 seems to be more abundant but less effective on *SBP*/*SPL* repression, perhaps because it is less efficiently loaded onto *AGO1* (He et al., 2018). Transgenic cotton plants overexpressing miR157 produced smaller gynoecium, with less ovules per ovary and decreased seed production in comparison with WT (Liu et al., 2017). These plants showed reduced expression of two MADS-box transcription factors (orthologs of *AtAGL6* and *SlTDR8*). In addition, auxin response was attenuated in ovaries of miR157-overexpressing cotton plants. It is possible that miR156 and miR157 modules regulate gynoecium development by overlapping but also specific mechanisms, although additional studies are needed to unravel miR157 specific functions in reproductive development.

miR396 Module Regulates CMM Meristematic Competence and Pluripotency During Gynoecium Development

The *GROWTH-REGULATING FACTORs* (*GRFs*) belong to a plant-specific transcription factor family that has nine members in *Arabidopsis*, seven of which (*GRF1*, *GRF2*, *GRF3*, *GRF4*, *GRF7*, *GRF8*, and *GRF9*) are targeted by miR396, representing the miR396 module (Liang et al., 2014; Lee et al., 2014, 2017). MiR396 module regulates several developmental processes, such as leaf development, floral development, and root cell reprograming during nematode infection (Lee et al., 2009; Hewezi et al., 2012).

Arabidopsis plants overexpressing miR396 (*p35S:MIR396a*) show gynoecium developmental defects such as gynoecia formed by only one carpel and siliques (dry fruits) producing a reduced number of seeds (Liang et al., 2014; Lee et al., 2017). *Arabidopsis* GRFs interact physically in the nucleus with the transcriptional co-activators GRF-INTERACTING FACTOR1, 2 and 3 (GIF1, GIF2, and GIF3) (Liang et al., 2014). Because GIF-GRF complexes are crucial for meristematic competency and pluripotency of CMM cells (Lee et al., 2017), high miR396 levels may lead to low *GRF*s available to form these heterodimers, hence CMM loses its meristematic competence and pluripotency over time (Liang et al., 2014; Lee et al., 2017). Single *GRF* loss-of-function mutants produce WT-like siliques, whereas g*if1* single mutant produces normal pistil and siliques but with reduced size. Siliques of the double transgenics *p35S:MIR396a;p35S:mGRF7* and *p35S:MIR396a;p35S:mGRF9* (both expressing miR396-resistant versions of *GRF7* and *9* transcripts, respectively) can recover WT silique phenotypes, indicating that at least miR396-targeted *GRF7* and *9* have roles in fruit development (Liang et al., 2014). The phenotypes of gynoecium and siliques of the triple mutant *gif1 gif2 gif3* phenocopy those of the double mutant *p35S:MIR396a grf5* (*GRF5* is not targeted by miR396), producing extremely short and almost sterile siliques, generally lacking valves, whereas some *GRF* triple mutants (e.g., *grf1/grf2/grf3* and *grf7/grf8/grf9*) present WT-like siliques (Liang et al., 2014). The triple mutant *grf1 grf3 grf*5 show single-valve gynoecia and slight defects on floral organ separation and number, but these defects were strongly enhanced by the addition of *grf2* mutation to this background (generating the quadruple mutant *grf1grf2 grf3 grf5*). Together, these findings indicate that *GRFs* act redundantly to modulate *Arabidopsis* gynoecium patterning and fruit development.

The mechanisms by which GRF-GIF dimers promote CMM meristematic capacity in *Arabidopsis* gynoecium were not well elucidated, but available data suggest that they may be associated with polar auxin transport (PAT) (Lee et al., 2017). *Arabidopsis* PAT mutants (*pin-formed1* and *pid*) and some auxin biosynthesis mutants (*yuc1*, *yuc4* and *wei8 tar2*) produce gynoecia phenotypes identical to *gif p35S:MIR396a* plants and *grf* multiple mutants. The addition of *gif* mutations to a *pid-3* mutant (a *PINOID* mutant with weak developmental defects) or treatment of *gif* mutants with N-1-Naphthylphthalamic Acid (NPA, an auxin polar transport inhibitor) synergistically enhance gynoecium developmental defects of *pid-3* or NPA-treated WT plants (Lee et al., 2017). These findings indicate an interplay between miR396, *GRF-INTERACTING FACTORs* and auxin during gynoecium patterning.

Unlike *Arabidopsis*, the possible role of the miR396 module in tomato fruit development has not been described in detail. The only study in tomato thus far showed that miR396 down-regulation (or *GRF* de-regulation) seems not to affect CMM formation but rather it leads to a significant increase in fruit size (Cao et al., 2016). This is consistent with the main role of GRFs in modulating cell proliferation and cell expansion in several developmental contexts (Lee et al., 2009). Since neither fruit shape nor ripening was altered in the transgenic tomato plants down-regulating miR396 (Cao et al., 2016), the authors proposed that these plants might provide a new way to enhance tomato fruit yield.

MicroRNA160 Module Controls Carpel Development by Modulating Auxin Responses

Some microRNAs, such as miR160, are crucial for auxin signaling during several developmental processes. MiR160, which targets the AUXIN RESPONSE FACTORS *ARF10*, *16,* and *17* (Hendelman et al., 2013; Damodharan et al., 2016), is another example of a miRNA module that apparently has different roles in the regulation of dry and fleshy fruit development.

The *Arabidopsis floral organs in carpels* (*foc*) mutant contains a *Ds* transposon insertion in the 3′ regulatory region of the *MIR160a* gene, which disrupts its native expression pattern, leading to the accumulation of *ARF10*, *16,* and *17* and low auxin responses in various organs (Liu et al., 2010). These regulatory disruptions lead to abnormal embryo, seed, and flower development. *foc* plants show some degree of indeterminacy during gynoecium patterning, which is observed by the production of floral organs inside the siliques and sometimes whole inflorescences emerging from siliques. Furthermore, *foc* mutant produces abnormal seeds and viviparous seedlings. It was also shown that 3′ regulatory region bears three putative auxin-responsive elements (AuxRE) and *MIR160a* expression is positively regulated by auxin. Thus, the disruption of this regulatory region impairs the induction of *MIR160a* expression by auxin, impacting fruit development (Liu et al., 2010).

The miR160 module (miR160 and their targets) seems also to have an important, but different, role in tomato fruit development. Transgenic tomato plants (*STTM160*-expressing plants) with knocked-down miR160 expression generated by the Short Tandem Target Mimic (STTM) approach (Teotia and Tang, 2017) produce ovaries with elongated morphology and thinning of the placenta, which developed into fruits with abnormal pear-shaped fruit morphology. These changes were associated with miR160 depletion and concomitant de-regulation of *SlARF10B* and *SlARF17*, and mostly *SlARF10A* in *STTM160*-expressing plants (Damodharan et al., 2016). Nevertheless, unlike *Arabidopsis foc* mutant, no indeterminacy was observed in gynoecia of *STTM160*-expressing tomato plants. Such discrepancy between phenotypes of tomato and *Arabidopsis* miR160 loss-of-function plants may be due to the fact that *SlARF16* is not de-regulated in *STTM160*-expressing tomato plants, despite the miR160 legitimate site observed in *SlARF16* (Damodharan et al., 2016).

MiR160-guided cleavage of some ARFs is also needed for proper leaf development in tomato and *Arabidopsis*. Interestingly, *STTM160* tomato plants and *5mARF17* (plants expressing a miR160-resistant version of *ARF17*) *Arabidopsis* plants showed similar leaf phenotype, which is reduced leaf blade and strongly lobbed leaflet/leaf margins (Mallory et al., 2005; Damodharan et al., 2016).

miR172 Limits the Growth-Repressing Activity of *APETALA2*-Like Genes During Fruit Expansion

All microRNA modules discussed so far are mostly associated with very early stages of carpel development, such as patterning and differentiation, and the proper control of these stages have great impact on mature fruit morphology and fertility. On the other hand, the miR172 module seems to control not only fruit patterning, but also fruit growth, which comprises a developmental stage after pollination, when the ovary is fully developed. In *Arabidopsis*, the miR172 module comprises the microRNA172 and its targets [*APETALA2-LIKE* (*AP2-like*) transcription factors]: *APETALA2* (*AP2*), *TARGET OF EAT1, 2* and *3* (*TOE1*, *TOE2*, and *TOE3*), *SCHLAFMUTZE* (SMZ), and *SCHNARCHZAPFEN* (*SNZ*) (Wu et al., 2010). Interestingly, pioneer studies showed that miR172 can guide not only *AP2-like* transcript degradation but also its translational repression (Chen, 2004).

Arabidopsis fruit undergoes dramatic increase in fruit size after fertilization, and different tissues grow at different rates (for review please see Ferrándiz et al., 2010). MiR172 module seems to be crucial to specify which regions of the carpel will go through dramatic expansion and which region will arrest fruit growth. *AP2* encodes an AP2/EREBP transcriptional repressor, which was shown to repress valve margin and replum growth post-fertilization by repressing the expression of genes that confer identity to valve margin (*INDHEISCENT* and *SHATTERPROOF*) and replum (*BREVIPEDICELLUS* and *REPLUMLESS*) (Ripoll et al., 2011). In this context, *AP2* prevents replum overgrowth and overproliferation of the layer of lignified cells (LL) (which are associated with fruit dehiscence; Rajani and Sundaresan, 2001; Liljegren et al., 2004) in the valve margin. Consistent with this, *ap2* mutants produce siliques with oversized replum and slightly delayed dehiscence due to increased number and size of LL (Ripoll et al., 2011). Nevertheless, after pollination the valves undergo a conspicuous cell expansion stage, increasing dramatically fruit size. This pollination-dependent valve growth was shown to be blocked in plants with decreased miR172 activity – via target mimicry (*MIM172*) approach (Franco-Zorrilla et al., 2007) – and in plants expressing a miR172-resistant *AP2* version, resulting in smaller fruits (Ripoll et al., 2015). For proper valve expansion, *AP2* and *TOE3* activities must be inhibited by miR172 only in the valves. The MADS-box transcription factor *FRUITFULL* (*FUL*) displays similar expression pattern as miR172, being expressed in the valves, and *ful* mutants resemble *MIM172* plants, presenting arrested growth phenotype in the valves. Furthermore, analysis of different degrees of homo and heterozygosity of *ARF6* and *ARF8* mutant alleles *arf6* and *arf8* in double mutants show that fruit valve expansion decreases with the increasing *ARF* mutant allelic dosage. Valve growth is even more limited when *arf6/8* are introduced in *ful*, and *arf6 arf8 ful* triple mutants produce siliques with extremely impaired growth. *FUL*, *ARF6*, and *ARF8* are expressed only in the valves (except valve margins), where they form protein complexes that bind to the *MIR172C* promoter and activate its expression. *AP2* and *TOE3* are expressed in the whole carpel, but miR172 induction in the valves restricts *AP2* activity to the valve margins and replum, allowing it to repress cell elongation in these locations but not in the valves. Through this mechanism, miR172 fine-tunes fruit patterning and growth by restricting the activity of *AP2-like* genes to certain locations within the fruit (Ripoll et al., 2015). Considering that miR167 negatively regulates *ARF6* and *ARF8* (Wu et al., 2006), it will be interesting to determine whether this miRNA participates in this mechanism by specifying *ARF6/8* expression pattern.

Although high levels of miR172 have a positive effect on *Arabidopsis* fruit growth (Ripoll et al., 2015), this is not always the case for other species. For instance, over-expression of a *MIR172* gene has a negative influence on fruit growth in apple (*Malus domestica*), resulting in a dramatic reduction in fruit size (Yao et al., 2016). Unlike *Arabidopsis* and tomato fruits, which are both derived from ovaries, apple fruits are mostly derived from the hypanthium that is hypothesized to consist of the fused bases of the sepals, petals, and stamens (Pratt, 1988). Interestingly, over-expression of the same *MIR172* gene in tomato results in carpel-only flowers which developed into parthenocarpic fruits (Yao et al., 2016). These examples nicely illustrate that the influence of a particular miRNA module on fruit growth depends on the fruit type and plant species.

MicroRNA-CONTROLLED PATHWAYS MODULATING OVULE AND SEED DEVELOPMENT DURING FRUIT GROWTH

The ovule is the female sexual organ in higher plants and a strict control of ovule development is crucial for plant reproductive success. Ovule is required to enclose the female gametophytes and, more importantly, it is from the fertilized ovules that seeds arise. Ovule structures are conserved in most plants, and comprise the embryo sac, the nucellus, the integument (which originates the seed coat) and the funiculus, which makes the connection between the ovule and placenta. Ovule and seed development are under control of genetic (e.g., transcription factors, non-coding RNAs), physiological (hormones) and epigenetic factors (i.e., chromatin remodeling and DNA methylation) (Skinner et al., 2004; Kelley and Gasser, 2009; Yamaguchi et al., 2013; Cucinotta et al., 2014). In this part of the review, we will discuss the findings of how some small RNAs modules act to modulate ovule and seed development, which are crucial developmental processes that take place during fruit development and ripening.

It was recently shown by our research group that the miR159 module is crucial for ovule and seed development in tomato (da Silva et al., 2017). The miR159 module comprises the microRNA159 and its targets, *SlGAMYB1* and *SlGAMYB2*, which belong to the R2R3 MYB domain transcription factor family. *GAMYB-like* genes are regulated by gibberellin and by the microRNA159 family in different tissues and developmental contexts (Gubler et al., 1995; Tsuji et al., 2006; Alonso-Peral et al., 2010). MiR159 and its targets are expressed early during

tomato placenta and ovule development, which suggest that the miR159 module may be involved in the initial steps of ovule development. Likewise, the overexpression of *SlMIR159* (*p35S::SlMIR159*) disrupts ovule development and induces obligatory parthenocarpy. Such phenotype is more severe than what is shown in *AtMIR159a*-overexpressing Arabidopsis plants, which generates fertile siliques when pollinated with WT pollen (Achard et al., 2004). Tomato, transgenic plants harboring the *p35S::SlMIR159* construct displays defects in the establishment of the embryo sac, which may be due to the observed lower expression of *AINTEGUMENTA*-like genes (da Silva et al., 2017). *AINTEGUMENTA* (*ANT*) gene is an *APETALA2-like* transcription factor required for ovule and integument initiation (Elliott et al., 1996). Although tomato lacks known *ANT* mutants, it was shown in rice that *ANT* was also strongly repressed in *gamyb* mutants displaying ovule developmental defects (Tsuji et al., 2006). MiR159 module interacts with tomato *AINTEGUMENTA-like* genes to drive developmental progression of ovules and, thus, modulates tomato fruit set. Moreover, our work showed that miR159 module interacts with the miR167 module. Down-regulation of miR167 and concomitant *SlARF8* de-regulation in *p35S::MIR159* plants may be also responsible for the arrested ovule development (da Silva et al., 2017), illustrating the link between the miR159 module and auxin during fruit set.

Parthenocarpy, the developmental process in which fruits develop in the absence of fertilization (Varoquaux et al., 2000), can be easily induced in grapevine (*Vitis vinifera*) by exogenous gibberellin (GA) application (Wang C. et al., 2018). These authors show that *VvmiR159c* and its target *VvGAMYB* are dynamically and opposing expressed during flowering and fruit set. GA treatment is capable of inducing *VvmiR159c* and, consequently, down-regulating *VvGAMYB* in reproductive organs. These observations led the authors to suggest that the miR159 module is associated with GA-induced parthenocarpy in grapevine (Wang C. et al., 2018), similarly to what we have discovered in tomato (da Silva et al., 2017).

The use of high-throughput sequencing approaches also provided evidences of the activity of miRNA modules during ovule development. In cotton (*Gossypium hirsutum*), small RNAs profiles of developing ovaries showed distribution of several small RNA signatures, including microRNAs (Abdurakhmonov et al., 2008). Several conserved microRNA families were identified in cotton ovules, including miR156/157, miR159, miR164, miR168, and miR395. These results are important to provide initial information for future functional experiments. In addition, several predicted miRNA targets were validated via degradome sequencing (a modified version of 5′-Rapid Amplification of cDNA Ends that is combined with high-throughput, deep sequencing to detect transcript ends; Ma et al., 2015), reinforcing the idea that conserved miRNA modules may be important in ovule development of cotton (Xie et al., 2015).

MicroRNAs are also required for embryogenesis, which is a key developmental step for plants to establish the seed set. To complete its development, the embryo undergoes specific stages, which in *Arabidopsis* are defined by its morphology as globular, heart, torpedo, and walking stick stages (Jürgens, 2001). Such developmental stages are known to be regulated by transcription factors, small regulatory RNAs, signal transduction orchestrated by kinases, auxin gradients, and epigenetic mechanisms (i.e., DNA methylation, histone acetylation, among others). Thus, these regulatory pathways are key determinants of the fate of primordia cell lineages, and also drive inheritance that is programmed via mitosis at early stages of the embryo development (Willemsen and Scheres, 2004).

DICER-LIKE1 (DCL1) is a key enzyme for the pri-/pre-miRNA processing (Reinhart et al., 2002; Kurihara and Watanabe, 2004; Park et al., 2005). Genome-wide transcriptional profiling of the *Arabidopsis* mutant *dicer1 (dcl1)* shed some light regarding the importance of microRNA modules during early embryo development. At the early globular stage, *dcl1* embryo display about 50 miRNA targets de-repressed due to the lack of miRNA regulation. Some of these targets (usually transcription factors) are required for differentiation at later stages of embryogenesis (Nodine and Bartel, 2010). In addition, in *dcl1* embryos, miR156-targeted *SPL10* and *SPL11* are highly up-regulated, which suggest that the de-regulation of these transcription factors is at least in part responsible for the *dcl1* embryo abnormalities (morphological defects and arresting growth at the globular stage). Thus, one of the first roles of plant microRNAs is to repress its targets at early developmental stages to prevent precocious differentiation during embryogenesis (Nodine and Bartel, 2010). This idea is further supported by the finding that *Arabidopsis* double mutant *ago1*/*ago10* displays embryo lethality, probably due the highly activity of small RNAs targets (Lynn et al., 1999; Mallory et al., 2009). Argonaute (AGO) proteins are part of the RNA-induced silencing complex (RISC), and are required for the repression of microRNA targets (Rhoades et al., 2002; Zilberman et al., 2003).

MiRNA module may also affect seed development. MiR397 negatively regulates members of the Laccase family. MiR397-targeted *Laccase4* is a member of the blue copper oxidase/p-diphenol:dioxygen oxidoreductase family and participates in lignin biosynthesis (Gavnholt and Larsen, 2002; Mayer and Staples, 2002). The miR397/*Laccase4* module has been implicated in the control of the number of seeds and seed size. Overexpression of *MIR397b* in *Arabidopsis* leads to reduce lignin deposition. Interestingly, in terms of fruit development, transgenic plants with less lignin produce bigger siliques with more and enlarged seeds. Similar results are observed in transgenic rice plants overexpressing *MIR397a* and *MIR397b*, which are able to produce enlarged grains (Zhang et al., 2013; Wang C.J. et al., 2014). Such studies highlight that miR397-mediated development via regulating *laccase* genes might be a potential tool not only for engineering plant biomass production with less lignin, but also for manipulating plant seed yield.

NON-CODING RNAs IN THE REGULATION OF FRUIT RIPENING

In the first section of this review, we discussed the main microRNA modules involved in diverse aspects of early fruit development, which is summarized in **Figure 2A**. In this last section, we will discuss a few examples available in the

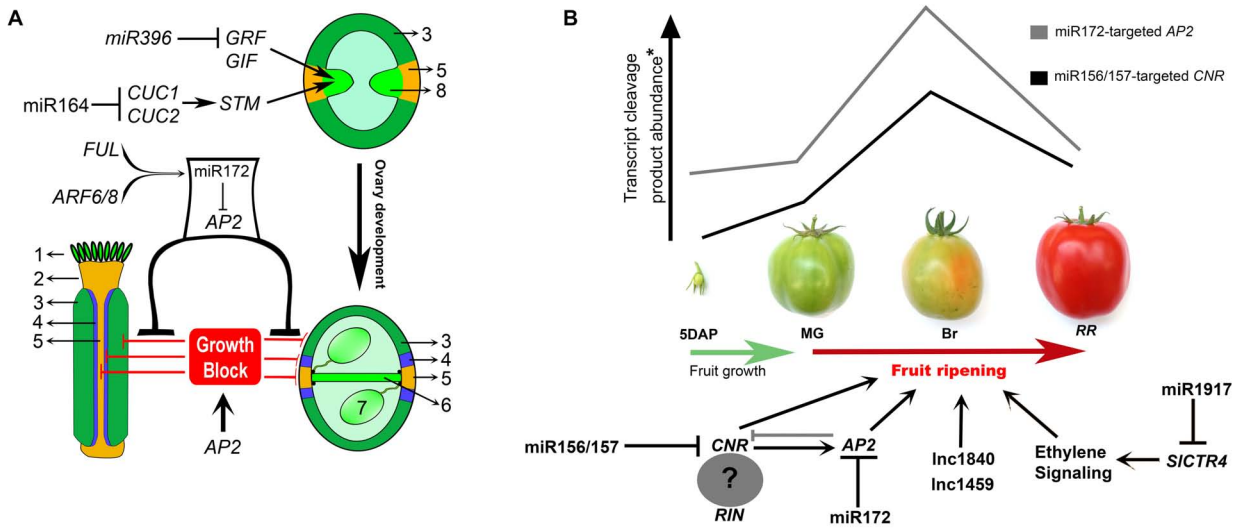

FIGURE 2 | Non-coding RNA networks associated with carpel patterning and fruit ripening. **(A)** Summary of miRNA modules that control early (e.g., CMM establishment and maintenance) and late aspects of *Arabidopsis* fruit development. MiR396 and miR164 modules have important regulatory roles in CMM maintenance. *MIR172C* is induced by AUXIN RESPONSE FATORS ARF6/8 and FRUITFULL (FUL) specifically in the valves, and this specificity is necessary for proper fruit growth after pollination. MiR172-guided *APETALA2* (*AP2*) mRNA cleavage in the valves (but not valve margins) promotes valve growth due to the repression of *AP2* growth-blocking activity. Growth is blocked by *AP2* in valve margins and replum, where miR172 is not expressed. 1 – stigma, 2 – style, 3 – valve, 4 – Valve margin, 5 – replum, 6 – septum, 7 – ovule, 8 – carpel margin meristem (CMM). GRF, GROWTH-REGULATING FACTOR; GIF, GRF-INTERACTING FACTOR; STM, SHOOT MERISTEMLESS; CUC1/2, CUP-SHAPED COTYLEDON1 and 2. **(B)** Graphic shows the accumulation of miRNA-cleaved transcripts of *COLORLESS NON-RIPENING* (*CNR*) and *APETALA2* (*AP2*) through four stages of fruit development/ripening: 5 days after pollination (5 DAP), Mature green (MG), Breaker (Br), and Red ripe (RR) (adapted from Karlova et al., 2013). MRNA cleaved product accumulation occurs in the breaker stage, coinciding with an ethylene peak production. Lnc1840 and lnc1459: long non-coding RNAs. Black lines in the transcriptional networks denote direct regulation, whereas gray lines denote indirect regulation. Question mark denotes that is uncertain if CNR forms a complex with RIN. SlCTR4, tomato *CONSTITUTIVE TRIPLE RESPONSE 4*.

literature that reinforce the fundamental roles of non-coding RNA-mediated regulation also in fruit ripening.

Conserved and Solanaceae-Specific miRNA Modules Control Tomato Fruit Ripening

Tomato plants bearing the dominant mutation *Cnr* (*COLORLESS NON-RIPENING*) produce fruits with characteristics associated with impaired ripening, such as inhibited softening, yellow skin, and pericarp lacking pigments because of the arrested biosynthesis of ripening-related pigments (Thompson et al., 1999). Furthermore, mutant plants produce lower amounts of ethylene and exogenous ethylene application does not recover this phenotype. Positional cloning showed later that a *SPL*/*SBP* gene (called *SlSBP3*/*CNR*) containing a potential miR156/157 binding site resides in the *Cnr* locus. *Cnr* is an epimutation caused by spontaneous heritable hypermethylation of cytosine residues of the *SlSBP3*/*CNR* promoter, leading to *SlSBP3*/*CNR* repression (Manning et al., 2006). Although the mechanism by which *SlSBP3*/*CNR* controls fruit ripening remains unclear, recent data suggest that the MADS-box transcription factor *RIPENING INHIBITOR* (*RIN*) and *CNR* may be part of the same protein complex that induces the expression of ripening-related genes (Martel et al., 2011). *RIN* controls both ethylene-dependent and independent ripening regulatory pathways, interacting directly with the promoter of many known genes associated with key ripening processes, such as ethylene biosynthesis, perception and signal transduction, cell wall metabolism, and carotenoid biosynthesis. Nevertheless, *CNR* is required for RIN promoter binding activity, as RIN does not interact with the promoters of ripening-related genes in the *Cnr* mutant (Martel et al., 2011; Qin et al., 2012; Fujisawa et al., 2013). Although CNR and RIN proteins do not interact, it is possible that these transcription factors are part of the same protein complex that modulates the expression of key ripening genes. Substantiating this hypothesis, *rin* and *Cnr* mutants have similar fruit phenotypes such as blocked ripening and impaired response to exogenous ethylene (Vrebalov et al., 2002; Martel et al., 2011).

Virus-induced gene silencing (VIGS)-based delivery of mature miR157 in tomato fruits reduced *CNR* transcript accumulation and delayed ripening in the injected fruit areas (Chen et al., 2015). Degradome analyses indicate that miR156 cleaves *CNR* in different stages of fruit ripening (Karlova et al., 2013). Surprisingly, VIGS-based delivery of miR156 does not produce any alteration in fruit ripening until the breaker stage, and these fruits show early softening (Chen et al., 2015). These observations suggest that the miR156/miR157 module may be necessary for proper control of fruit ripening and that the closely related miR156 and miR157 play different roles in the temporal control of the ripening-associated processes.

Tomato miR172-targeted *AP2a* appears to have complex functions in the control of diverse ripening-related processes, regulating mostly genes associated with ethylene biosynthesis and

signaling (Karlova et al., 2011). *AP2a* silencing through RNAi leads to the production of fruits that ripe, but never turn from orange to red, showing altered levels of various carotenoids and increased chlorophyll levels, although they produce high levels of ethylene. *AP2a* seems to act downstream to *RIN* and *CNR*, as its expression is negatively regulated in *rin* and *Cnr* mutants and CNR binds to *AP2a* promoter. Thus, *CNR* induces *AP2a* expression directly, although *AP2a* represses *CNR* expression in a negative feedback loop (Karlova et al., 2011). Taken together, the evidences in tomato thus far indicate that both miR156/miR157 and miR172 modules and the interaction between their targets (*CNR* and *AP2a*) are important to proper fruit ripening. In fact, degradome analysis showed that levels of the *CNR* and *AP2a* miRNA-guided cleavage products vary among different ripening stages, showing peak accumulation of cleavage transcripts during breaker stage, which is also the peak of ethylene production (Karlova et al., 2013). It will be interesting to determine whether these miRNAs have specific roles in fine-tuning spatially and/or temporally the expression of their targets during fruit ripening.

Recently, a novel miRNA identified as Solanaceae-specific was implicated in regulating ethylene signaling and hence fruit ripening in tomato (Wang Y. et al., 2018). The microRNA miR1917 targets three splicing variants of the *CONSTITUTIVE TRIPLE RESPONSE 4* (*SlCTR4*, homolog of *Arabidopsis CTR1*), an ethylene signaling repressor that interacts with ethylene receptors (Wang Y. et al., 2018). Tomato plants overexpressing the miR1917 (*p35S::MIR1917*) display higher levels of ethylene signaling, leading to enhanced ethylene production. These plants also have increased ethylene responses in the absence of ethylene, including accelerated pedicel abscission and fruit ripening (Wang Y. et al., 2018). The complementary expression pattern of miR1917 and the splicing variants *SlCTR4*sv3 observed in the pedicel abscission zone by *in situ* hybridization suggests that miR1917 restricts the expression of its targets to the vascular bundle and surrounding cells during pedicel abscission. Thus, miR1917 and its targets represent a novel miRNA module belonging to the intricate ethylene-associated signaling network.

New Evidences of the Role of Long Non-coding RNAs (lncRNAs) in Fruit Ripening

Long non-coding RNAs are broadly present in plant, animal and fungi transcriptomes and emerging evidences show that they play key roles in diverse developmental processes. They are RNAs longer than 200 nt originated from transcription of intergenic regions, introns or antisense coding sequences and do not have any detecting coding potential (Chekanova et al., 2007; Kapranov et al., 2007; Fatica and Bozzoni, 2014; Chekanova, 2015). LncRNAs may modulate gene expression by multiple mechanisms that were extensively reviewed in Chekanova (2015). Although the knowledge of the regulatory roles of lncRNAs in plants is still limited, lncRNAs have been associated with the control of flowering time, male sterility, seedling morphogenesis and, more recently, fruit ripening (Ding et al., 2012; Wang Y. et al., 2014; Berry and Dean, 2015; Li R. et al., 2018).

RNA-seq analyses comparing transcriptomes of tomato cv Ailsa Craig and *rin* fruits identified over 3000 tomato lncRNAs, several of which were differentially expressed in *rin* (Zhu et al., 2015). In the same study, two lncRNAs (lncRNA1459 and lncRNA1840) strongly down-regulated in *rin* were chosen for VIGS-based silencing assays in fruits. Silencing of both lncRNAs produced non-ripening sections in the injected areas of the fruit, similarly to the effect observed in VIGS-based silencing of *RIN*. To better understand the functional role of lncRNA1459, which is a sense intergenic lncRNA, Li R. et al. (2018) generated loss-of-function mutants for lncRNA1459 using clustered regularly interspaced short palindromic repeats (CRISPR)/-associated protein 9 (Cas9)-induced genome editing technology (Feng et al., 2013; Doudna and Charpentier, 2014). Mutant fruits display delayed ripening phenotype associated with repressed ethylene and carotenoid biosynthesis, as well as down-regulation of ripening-associated genes.

In addition to tomato, lncRNAs involved in fruit ripening have been identified and studied in few other species. Sea buckthorn (*Hippophae rhamnoides*) is a plant for land reclamation, and its berry-type fruits have high nutritional value due to the significant amounts of natural anti-oxidants including ascorbic acid, tocopherols, carotenoids, and flavonoids (Zakynthinos et al., 2016). By using high throughput RNA sequencing, Zhang et al. (2018) identified over 9000 lncRNAs expressed in distinct sea buckthorn fruit developmental stages, from mature green to red-ripe. Interesting, the authors identified two lncRNAs (LNC1 and LNC2) that may function as target mimics of miR156 and miR828 during fruit ripening, therefore indirectly affecting the expression of these miRNA targets, *SPL9* and *MYB114*, respectively. By modulating *SPL9* and *MYB114* expression, LNC1 and LNC2 seem to control the biosynthesis of anthocyanin during fruit ripening (Zhang et al., 2018).

Despite the examples given above, the functions of the majority of ripening-associated lncRNAs are still unclear. More functional studies are needed to confirm the function of lncRNAs and their possible target genes. On possibility to be further explored is that lncRNAs can interact with microRNAs to modulate gene expression level (Gorospe et al., 2014), thus combining the "power" of two ncRNAs to modulate fruit ripening (**Figure 2B**).

CONCLUSION

During plant development, multiple microRNA modules are required to control meristem identity, leaf margin serration, polarity, complexity, root development, and flowering time. As summarized here, miRNA modules have key roles in fruit development, ranging from carpel establishment and patterning to fruit ripening. Disruption of miRNA transcription or processing frequently generate pleotropic consequences for the plant. Indeed, their activity are essential for plants to complete their life cycle, since they are active from seed to flower production. Interestingly, evolution of miRNA modules brought about adaptative advantages to plants by using similar pathways to orchestrate different developmental processes. A good example

presented here is the miR164 module, which is required for proper leaf and carpel/fruit development, corroborating the hypothesis of the evolutionary origin of carpel as modified leaves. It is interesting to consider that evolution has also hijacked similar microRNAs modules to control unrelated developmental programs such as the role of the miR156 module in flowering time and fruit development and ripening. In addition, due to their multiple roles in plant development, microRNA modules may also provide promising molecular tools to be explored in an agricultural context. Therefore, the better understanding of the mechanisms that control miRNA and target expression and their spatiotemporal regulatory roles could be an outstanding step toward the application of microRNA-targeted regulation of important fruit traits, including size, shape, seed production, and ripening. For instance, the use of novel CRISPR/Cas9-based technologies (Li C. et al., 2018) might allow subtle changes in miRNA target gene expression which have a potential to quantitative modify fruit traits. Additionally, it would be interesting to investigate whether there are more specific microRNAs modules (e.g., Solanaceae-specific microRNA mentioned in this review) in others crops that might be associated with fruit quality traits. Although there are open questions of how microRNA modules function during fruit development, lncRNA-associated pathways are probably one of the less understood so far, involving multiple and complex origins and modes of action. As mentioned in this review, microRNAs and lncRNAs act during fruit ripening, and they can interact during this process. In addition, since they may have overlapping functions during ripening, it would be interesting to investigate whether these two classes of non-coding RNAs interact in early steps of carpel development and fruit patterning as well. The identification of additional lncRNAs and miRNAs and the understanding of how they interact with each other to control fruit development and ripening would be an important step toward the improvement of fruit production. The use of next generation sequencing technologies combined with functional genomics may help to achieve this goal.

AUTHOR CONTRIBUTIONS

JC, ES, and FN conceived the review. JC and ES wrote the review. FN helped writing and correcting the final version of the review.

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Overexpression of *PpSnRK1α* in Tomato Promotes Fruit Ripening by Enhancing RIPENING INHIBITOR Regulation Pathway

Wen Yu[1], *Futian Peng*[1]*, *Yuansong Xiao*[1]*, *Guifang Wang*[2] *and Jingjing Luo*[1]

[1] State Key Laboratory of Crop Biology, College of Horticulture Science and Engineering, Shandong Agricultural University, Tai'an, China, [2] Shandong Institute of Pomology, Tai'an, China

As a conserved kinase complex, sucrose non-fermenting-1-related protein kinase 1 (SnRK1) is a major regulator of plant growth and development. In our previous study, overexpression of *MhSnRK1* in tomato (*Solanum lycopersicum* L.) modified fruit maturation: the transgenic fruit ripened earlier than the wild type (WT). However, the mechanism by which fruit maturation is regulated by SnRK1 is not clear; therefore, the test materials used were the transgenic tomato lines (OE-1, OE-3, and OE-4) overexpressing the coding gene of peach [*Prunus persica* (L.) Batsch] SNF1-related kinase α subunit (*PpSnRK1α*). The activity of SnRK1 kinase in transgenic tomato lines OE-1, OE-3, and OE-4 was higher than that in the WT at different periods of fruit development; in the pink coloring period the SnRK1 kinase activity increased the most, with 23.5, 28.8, and 21.4% increases, respectively. The content of starch and soluble sugars in red ripe transgenic fruit significantly increased, while the soluble protein and titratable acid content decreased significantly. We also found that the tomatoes overexpressing *PpSnRK1α* matured approximately 10 days earlier than the WT. Moreover, the yeast-two-hybrid assay showed that PpSnRK1α interacted with the MADS-box transcription factor (TF) SIRIN, which acts as an essential regulator of tomato fruit ripening. The BiFC technology further validated the location of the PpSnRK1α interaction sites within the nucleus. The quantitative real-time PCR analysis showed that *RIN* expression was up-regulated by *PpSnRK1α* overexpression; the expression of RIN-targeted TF genes *NOR* and *FUL1* increased during different stages of fruit development. The expression of key genes, *ACS2*, *ACS4*, and *E8*, in ethylene synthesis also changed accordingly, and the ethylene emitted by the red ripe fruit increased by 36.1–43.9% compared with the WT. These results suggest that PpSnRK1α interacts with SIRIN, increasing the expression of *RIN*, thereby regulating the expression of downstream ripening-related genes, finally promoting fruit ripening.

Keywords: SnRK1 protein kinase, RIN, fruit ripening, peach, tomato

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***Correspondence:**
Futian Peng
pft@sdau.edu.cn
Yuansong Xiao
yuansongx2013@163.com

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Abbreviations: ACS2, 1-aminocyclopropane-1-carboxylic acid synthase 2; ACS4, 1-aminocyclopropane-1-carboxylic acid synthase 4; ADPase, ADP-glucose pyrophosphorylase; AP2a, APETALA2a; CNR, colorless non-ripening; ChIP, chromatin immunoprecipitation; DTT, dithiothreitol; E8, ripening-associated ACO homolog; FUL1/TDR4, fruitfull 1; HEPES, 4-(2-Hydroxyethyl)-1-piperazine-ethanesulfonic acid; NOR, non-ripening; RIN, ripening inhibitor; SNF1, sucrose non-fermenting 1; SnRK1, sucrose non-fermenting-1-related protein kinase; SS, sucrose synthase; TF, transcription factor; WT, wild type.

INTRODUCTION

Sucrose non-fermenting 1 kinase (SNF1)-related kinase (SnRK1) in plants belongs to a conserved family that includes SNF1 in yeast and AMP-activated protein kinase in animals (Crozet et al., 2014). SnRK1 is a heterotrimeric protein complex that is an important kinase in the signal transduction of carbon and nitrogen and is one of the regulatory hubs in plant physiological activities (Le Guen et al., 1992; Halford and Hardie, 1998; Polge and Thomas, 2007). Previous studies have shown that SnRK1 may play a key role in the overall regulation of the intracellular sugar signaling pathway and metabolism, and regulates plant carbohydrate metabolism (Ramon et al., 2013; Emanuelle et al., 2016). Recently, many studies have shown that plant SnRK1 is involved in many metabolic pathways including carbohydrate metabolism, stress, organogenesis, and senescence pathways (Purcell et al., 1998; Laurie et al., 2003; Jossier et al., 2009; Broeckx et al., 2016).

Most research on the function of SnRK1 has been conducted in *Arabidopsis thaliana* and crop plants, while research on the function of SnRK1 in fruit trees has rarely been reported. Our previous study showed that overexpression of Pingyitiancha (*Malus hupehensis* Rehd. var. *pingyiensis* Jiang) *MhSnRK1* in tomato can improve the photosynthetic rate, fruit soluble sugar content, starch content and utilization, and also influence the process of growth and development of fruit—for example, transgenic tomato fruit matured 10 days earlier than WT fruit (Li et al., 2010; Wang et al., 2012). This study was also the first to show that SnRK1 affects fruit ripening (Wang et al., 2012).

Tomato is a climacteric fruit, and early studies of the molecular genetic mechanism during the maturation process focused on signal transduction of ethylene biosynthesis and ethylene receptor mediated regulation. With a deep understanding of the ethylene pathway, researchers have gradually realized that if a fruit only has ethylene, it is not mature. Only up to a certain stage of development is a fruit sensitive to ethylene stimulation (Wilkinson et al., 1995). Therefore, the problem of upstream regulation of the ethylene pathway has become a new research focus. As typical of many mutants, the *rin* mutation exists in the upper reaches of the ethylene regulatory pathway, and this is not regulated by ethylene. The MADS-box TF RIN was cloned in the tomato *rin* gene locus using map-based cloning described by Vrebalov et al. (2002), and a homologous gene was also found in strawberry (a model plant for studying the non-respiratory climacteric pathway). Thus, it was inferred that RIN may be a conserved regulatory factor for two types of fruit (Vrebalov et al., 2002). Previous research has shown that RIN is a member of the MADS-box gene family and is a very important factor in the regulation of tomato fruit ripening, affecting almost all relevant metabolic pathways (Vrebalov et al., 2002; Martel et al., 2011). The RIN protein can not only directly regulate fruit ripening-related genes, such as lipid and cell wall metabolism genes, but also can influence ethylene synthesis pathways and other ripening-related TFs (e.g., CNR, NOR, FUL1, and AP2a), indicating that RIN has a very important function in tomato fruit ripening (Fujisawa et al., 2011, 2013).

Our previous study found that the overexpression of *MhSnRK1* in tomato can promote fruit ripening (Wang et al., 2012); however, the exact molecular mechanism by which SnRK1 regulates fruit maturation is not clear. Does SnRK1 interact with the TF RIN, regulating fruit ripening? Using *PpSnRK1*α overexpressing tomato lines (OE-1, OE-3, and OE-4) and WT tomato as test material, we examined the relationship between SnRK1 and RIN and we speculate that SnRK1 regulates fruit maturation by affecting the RIN regulation pathway.

MATERIALS AND METHODS

Plant Material and Treatments

We previously obtained the transgenic lines OE-1, OE-3, and OE-4 overexpressing *PpSnRK1*α. Seeds of transgenic and WT tomatoes (*Solanum lycopersicum* 'Sy12f') were germinated and grown in a plant growth chamber at 30°C for 3 weeks. These transgenic tomatoes (T2) were confirmed using the Plant PCR Kit (Takara, Japan). The primers *PpSnRK1*α-F (5′-GCTCTAGAATGGATGGATCGGTTG-3′) and *PpSnRK1*α-R (5′-GCGTCGACTTAAAGGACCCG-3′) were used to detect *PpSnRK1*α overexpressing tomato plants. The PCR-positive tomato plants were transplanted into pots with soil, and both WT and transgenic tomato plants were grown under natural light. Fifteen tomato plants per genotype were used (one plot per five plants, three plots per line treatment). All tomato fruits of the WT and transgenic plants used for analysis were tagged at the date of anthesis, and the fruit ripening time was observed. For fruit diameter and ripening time analysis, fifteen fruit samples per plot were used and three independent replicates were performed. The WT and transgenic fruits were harvested at different ripening stages, viz., green mature (GM), breaker (BK), pink coloring (PK), and red ripe (RR) stage, to analyze the SnRK1 activity, the gene expression level and ethylene emission as well as the soluble sugar, starch, soluble protein, and titratable acid content. In each case, three biological replicates were performed and each replicate contained at least 10 fruits.

Quantitative Real-Time PCR

Total RNA was extracted from the tomato fruits at different stages of development using the RNA plant Plus Reagent kit (TIANGEN, China). The RNA was then reverse-transcribed to cDNA using a Primescript^{TM} RT reagent kit (Takara, Japan). qRT-PCR was performed using SYBR Premix Ex Taq^{TM} (Takara, Japan). The *r18S* gene was used as loading controls. The calculation method for qRT-PCR is $2^{-\Delta\Delta CT}$. Three independent biological replicates were analyzed per sample. The specific primers used for the PCR analysis are listed in **Table 1**.

SnRK1 Activity Assays

Fruit tissue (1 g) was ground in 1 mL of cold extraction buffer consisting of 100 mmol·l^{-1} HEPES, pH 8, 25 mmol·l^{-1} NaF, 2 mmol·l^{-1} sodium pyrophosphate, 0.5 mmol·l^{-1}ethylene diamine tetra acetic acid, 0.5 mmol·l^{-1} ethylene glycol tetra acetic acid, 1 mmol·l^{-1} anisole, 5 mmol·l^{-1} dithiothreitol, 25 mmol·l^{-1} β-mercaptoethanol, and 1 mol·l^{-1} pepstatin A. The suspension

TABLE 1 | Primers used in this study.

Gene	Acc. no	Primer sequences (5′–3′)
SIRIN	AF448522	F: CATGGCATTGTGGTGAGCAAAGTGT R: AGCATCATGTGTTGATGGTGCTGC
SINOR	AY573802.1	F: AGAGAACGATGCATGGAGGTTTGT R: ACTGGCTCAGGAAATTGGCAATGG
SIFULI	X60757.1	F: ACTGGACTCTCCTCACCTTGGGG R: AGCTGCACCTTGCTGCTGTGA
SIACS2	X59139	F: AAGCGCGATGAGGTTAGGTA R: AAAGTGGACGCAAATCCATC
SIACS4	M88487	F: AAATCTCCACCTTCACTAACGAAC R: CCTAAGTCCTTGGAAAGACTAGACAC
SIE8	DQ317599	F: TGGCTCCGAATCCTCCCAGTCT R: GTCCGCCTCTGCCACTGAGC
SISNF1	AF143743	F: CGCAGATTTTGGTTTGAGCAA R: GTTTGGGCTTCCGCAACTT
Slr18S	X51576	F: GCCCGGGTAATCTTTGAAAT R: AGTAAGCGCGAGTCATCAGC
PpSnRK1α	ppa004347m	F: CTCTTG GTATTGGTTCTT R: TCTCTTCTCACTTTCTCT
PpSnRK1α (JM)	ppa004347m	F: GAATTCATGGATGGATCGGTTGGC R: GTCGACTTAAAGGACCCGAAGTTGT
SIRIN (JM)	AF448522	F: TCCCCCGGGGTACAATATGGGTAGAGGGAAAG R: AAACTGCAGTCAAAGCATCCATCCAGGT

was transferred to two cold microfuge tubes and clarified by centrifugation for 5 min at 12,000 × *g* at 4°C. The supernatant (750 μL) was desalted on a 2.5 mL centrifuge column (Sephadex G-25 medium columns; GE Healthcare, United Kingdom) treated with equilibration solution. Using AMARA polypeptide as the substrate (Zhang et al., 2009), the SnRKl activity was measured using a Universal Kinase Activity Kit (R&D Systems, Minneapolis, MN, United States).

Yeast Two-Hybrid Assay

For the yeast two-hybrid experiments, the plasmids pGAD424 and pGBT9 were used, which contain the GAL4 activation domain and GAL4 DNA-binding domain, respectively. *SIRIN* was amplified and then cloned into the pGBT9 vector. *PpSnRK1α* was amplified and then inserted into pGAD424. The BT-RIN and AD-SnRK1α plasmids were co-transformed into the yeast strain Y2HGold (Clontech, Palo Alto, CA, United States) using the PEG/LiAC method as described in the Clontech Yeast Protocol Handbook. The transformed colonies were selected on synthetic drop-out medium lacking leucine and tryptophan (SD-Leu-Trp). The colonies from the double selection plates were then screened for growth on quadruple selection SD medium lacking adenine, histidine, leucine, and tryptophan (SD-Ade-His-Leu-Trp). To further confirm the positive interactions, X-alpha-Gal was used to assay for beta-galactosidase activity. Primers are listed in **Table 1**.

Bimolecular Fluorescence Complementation (BiFC) Assay

Full-length *PpSnRK1α* and *SIRIN* were transferred from their respective entry clones into the vectors pSPYNE and pSPYCE. Plasmids were co-transfected into Agrobacterium. Following the methods described by Boruc et al. (2010), the instantaneous expression of Agrobacterium-mediated tobacco leaves was used to detect protein interactions, and the fluorescence results were observed with a confocal laser scanning microscope (Zeiss 510 Meta).

Analyses of Soluble Sugar and Starch in Fruit

Soluble sugar was extracted from 1 g of each sample, which was placed in 10 mL of water at 100°C, and then extracted twice at 100°C with the same volume of water (Wang et al., 2012). The total amount of soluble sugar was determined using the anthrone method (van Herwaarden et al., 1998). Starch was determined in the remaining sample after the soluble sugars were extracted. The tissue residue was digested with 0.92 mol·L^{-1} perchloric acid in 20 mL water at 100°C for 30 min to convert starch to glucose. This digestion was repeated twice. The amount of glucose was then determined using the anthrone method.

Measurements of Titratable Acid and Soluble Proteins

Following the methods presented by Wang et al. (2012), titratable acid content of isolated juice sacs was determined by titration. Fresh tissue (50 g) was ground completely using a mortar and pestle and placed in 5 mL of 80% ethanol at 80°C for 1 h. Aliquots of the ethanol extracts were titrated to a neutral endpoint with 0.1 mol·L^{-1} sodium hydroxide, indicated by phenolphthalein. Proteins were extracted from 0.5 g of tissue sample with 5 mL of enzyme assay buffer (5 mmol·L^{-1} cysteine, 5 mmol·L^{-1} EDTA-Na_2, and 25 mmol·L^{-1} potassium phosphate buffer at pH 7.5), ground with a mortar and pestle, and centrifuged

(4,000 r/min) for 15 min. The protein content of the supernatant was measured at 500 nm following the method described by Lowry et al. (1951) using bovine serum albumin as the standard protein.

Determination of Ethylene Emittance

The determination of ethylene emitted by tomato fruit referenced the method described by Jin et al. (2006). The Shimadzu GC-9A gas chromatograph was used with N_2 as the carrier, and the separation column and detector temperatures were 40°C and 120°C, respectively. Samples were taken from three glass containers and the ethylene emitted was calculated according to the peak area method.

Statistical Analysis

Three independent biological replicates were performed for each experiment. The statistical analysis was performed with

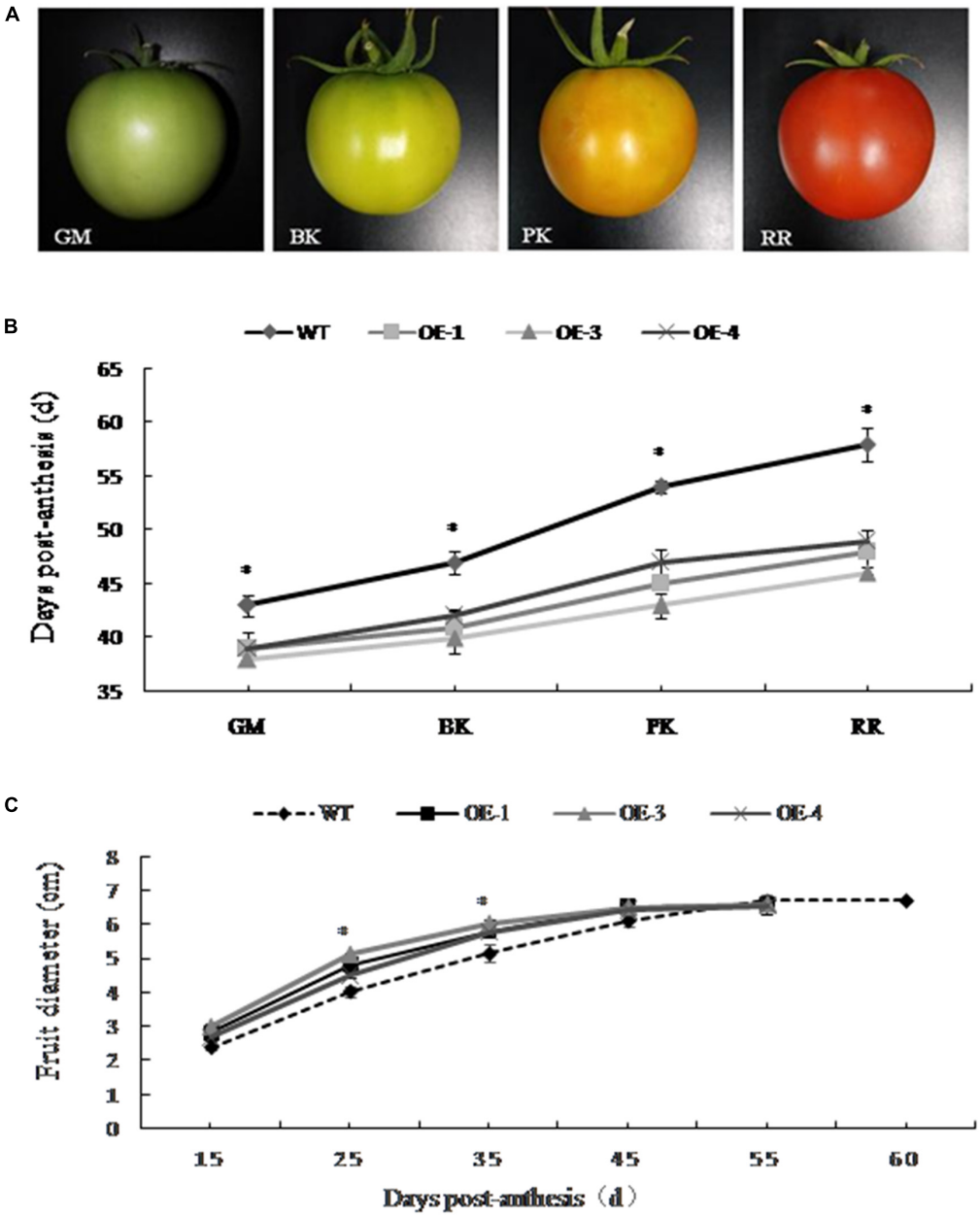

FIGURE 1 | Effect of *PpSnRK1*α overexpression on transgenic tomato fruit development. **(A)** Changes in the appearance of fruit color on which the four developmental stages were divided. Tomato fruit developmental stages: green mature (GM); breaker (BK); pink coloring (PK); red ripe (RR). **(B)** Days required for the different fruit developmental stages; **(C)** Changes in fruit diameter in transgenic and wild type (WT) tomato plants. Error bars represent the SD based on three independent biological replicates. An asterisk (*) on top of the error bar designates a significant difference between transgenic lines and WT at $P < 0.05$.

Microsoft Office Excel 2007 software. Comparison of the means was calculated according to the Duncan multiple range test using the SPSS 20.0 statistical program. Significance was defined as $P < 0.05$.

RESULTS

PpSnRK1α Transgenic Fruit Ripens 10 days Earlier Than WT Fruit

The development of *PpSnRK1α* transgenic tomato plants (OE-1, OE-3, and OE-4) and WT fruit was observed in real time. The changes of fruit diameter and color and the number of days required for fruit development to reach different stages were recorded (**Figures 1A–C**). According to the changes in the appearance of fruit color, four different developmental stages were divided (**Figure 1A**). The development of *PpSnRK1α* overexpressing tomatoes was significantly faster than that of WT tomatoes, and the fruit matured approximately 10 days earlier than WT fruit (**Figure 1B**). In the early stage of fruit development, the diameter of the transgenic tomato fruit was significantly larger than that of the WT, but there was no significant difference in fruit size between the two since the green mature stage (**Figure 1C**).

Higher Starch and Soluble Sugar Content in *PpSnRK1α* Transgenic Tomato Than WT

The soluble sugar content in red ripe fruit from the OE-1, OE-3, and OE-4 tomato lines was significantly higher than that of the WT, increasing by 33.9, 38.4, and 32.5%, respectively (**Table 2**). The starch content in the fruit also significantly increased, and the starch content in the OE-3 fruit was nearly twice that of the WT. The soluble protein and titratable acid content in fruit was lower than that of the WT. The overexpression of *PpSnRK1α* affects the accumulation and distribution of carbohydrates in tomato fruit.

Expression Analysis of SnRK1 Gene and Activities of SnRK1 in Transgenic and WT Tomato

The expression of the tomato SnRK1 encoding gene (*SISNF1*) in transgenic tomatoes OE-1, OE-3, and OE-4 was consistent with the WT fruit; however, the expression of *PpSnRK1α* was higher in transgenic tomato lines, and no expression was detected in the WT (**Figure 2A**), indicating that *PpSnRK1α* was successfully expressed in transgenic tomato lines and did not affect the expression of *SISNF1* in tomato. The SnRK1 activity was significantly higher in OE-1, OE-3, and OE-4 fruit than that of WT tomatoes at different periods, and the SnRK1 activity showed the greatest increase at the pink coloring stage, increasing by 23.5, 28.8, and 21.4%, respectively (**Figure 2B**).

TABLE 2 | The starch, soluble sugar, soluble protein, and titratable acid content in WT and transgenic tomato fruit.

	Starch ($mg \cdot g^{-1}$ FW)	Soluble sugar ($mg \cdot g^{-1}$ FW)	Soluble protein ($mg \cdot g^{-1}$ FW)	Titratable acid (% FW)
WT	4.23 ± 0.28 c	26.62 ± 1.38 b	0.51 ± 0.04 a	0.40 ± 0.03 a
OE-1	7.32 ± 0.36 b	35.64 ± 0.40 a	0.34 ± 0.05 b	0.32 ± 0.01 b
OE-3	8.26 ± 0.45 a	36.83 ± 0.70 a	0.36 ± 0.03 b	0.33 ± 0.04 b
OE-4	7.13 ± 0.77 b	35.27 ± 0.43 a	0.34 ± 0.03 b	0.34 ± 0.02 b

Data in the table are the average of three samples; lowercase letters indicate a significant difference at $P < 0.05$.

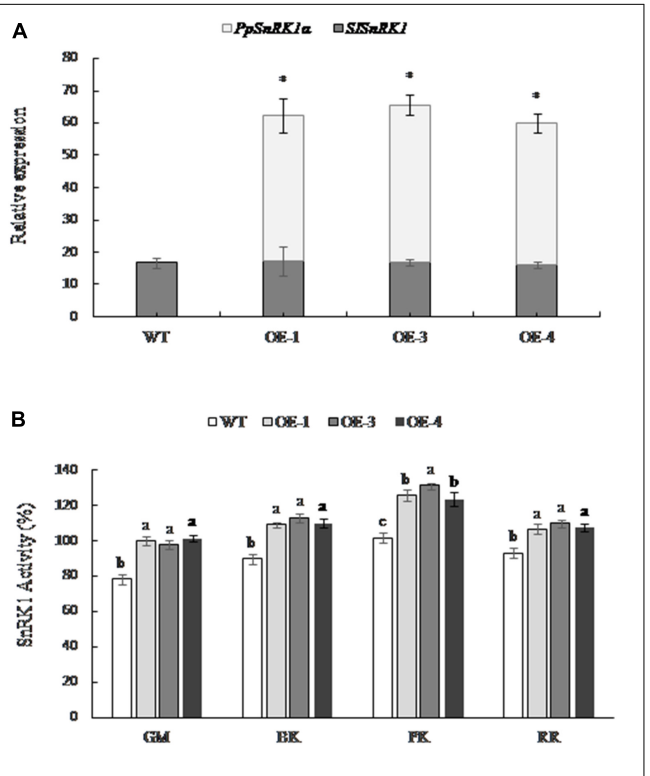

FIGURE 2 | The expression of *SnRK1α* and the activity of SnRK1 in fruit from *PpSnRK1α* overexpressing tomato lines (OE-1, OE-3, and OE-4) and the WT during different developmental stages. **(A)** The expression of the *PpSnRK1α* gene and *SlSNF1* gene in tomato fruit. The error bars represent the SD of three biological replicates. An asterisk (*) on top of the error bar designates a significant difference between transgenic lines and the WT at $P < 0.05$. **(B)** The SnRK1 kinase activity in tomato fruit at different developmental stages. The error bars represent the SD of three biological replicates. Different lowercase letters in the same developmental stage indicate a significant difference at $P < 0.05$.

SnRK1–RIN Interaction Identified by the Yeast Two-Hybrid System

We performed yeast two-hybrid (Y2H) assays to determine whether PpSnRK1α interacts with SlRIN. For Y2H assays, the full-length *SlRIN* was inserted into a pGBT9 vector as bait. The results indicated that RIN protein did not show auto-activation (**Figure 3**). The bait construct carrying the BD-RIN fusion protein was co-transformed with

the prey construct harboring the AD-SnRK1α fusion protein, indicating that PpSnRK1α interacted with SIRIN (**Figure 3**).

SnRK1–RIN Interaction Identified by BiFC

On the basis of the Y2H experiment, this study used a bimolecular fluorescence complementary (BiFC) test to further prove the interaction between PpSnRK1 and the TF SIRIN *in vivo*. As shown in **Figure 4**, tobacco leaves were co-infected with Agrobacterium containing PpSnRK1α-pSPYNE and SIRIN-pSPYCE recombinant plasmids, and yellow fluorescence (YFP) signal was observed in the epidermal nucleus of tobacco leaves. The positions of YFP signal and blue fluorescence signal were exactly the same. The results showed that PpSnRK1α protein could interact with SIRIN protein in plants, and their interaction sites were in the nucleus.

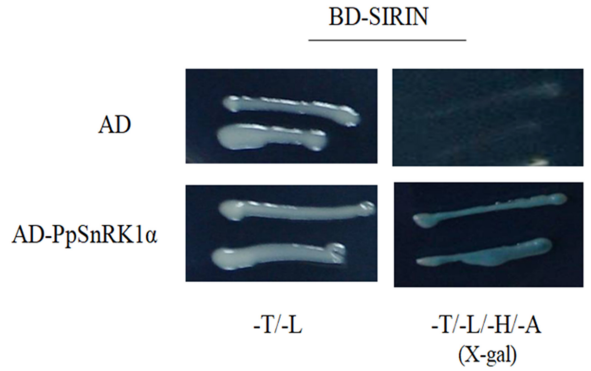

FIGURE 3 | Yeast two-hybrid experiments showing the interaction between PpSnRK1α and SIRIN protein. Results show a representative experiment out of three independent biological replicates.

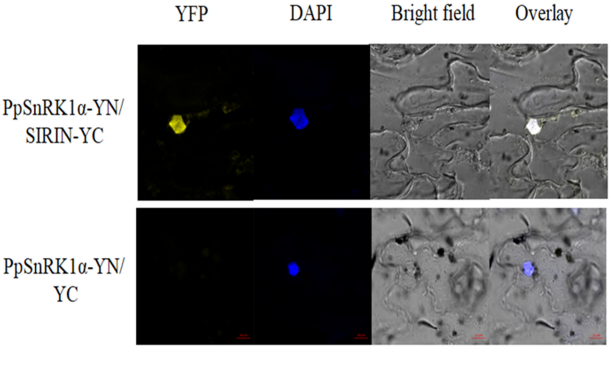

FIGURE 4 | Analysis of interaction between PpSnRK1α and SIRIN by BiFC assay. Visualization of the protein complex using BiFC in tobacco leaf epidermal cells. Results show a representative experiment out of three independent biological replicates.

Expression of RIN at Different Developmental Stages of Tomato Fruit Overexpressing *PpSnRK1α*

The expression level of *RIN* differed significantly among the fruit developmental stages (**Figure 5**). The *RIN* gene was expressed at a low level in the green mature stage; its expression gradually increased with the maturity of the fruit and was the greatest in the red ripe stage. The expression of the *RIN* gene in transgenic fruit was significantly higher than that in WT tomato from the breaker stage. The up-regulation of *RIN* expression was most significant at the pink coloring stage in *OE-1, OE-3,* and *OE-4*, which was up to 1.86, 2.06, and 1.98 times the *RIN* expression in the WT, respectively.

In *PpSnRK1α* Overexpressing Fruit, RIN Regulates Gene Expression in the Maturity Pathway

To explore the effects of changes in *RIN* expression on fruit ripening, the expression levels of RIN-targeted genes associated with maturation were examined. We select RIN-targeted TF genes *NOR* and *FUL1* and key genes for ethylene synthesis, *ACS2*, *ACS4*, and *E8*, which are considered to be directly regulated by RIN (Eriksson et al., 2004; Ito et al., 2008; Fujisawa et al., 2011, 2013). As shown in **Figure 6A**, the expression levels of the TF *NOR* and *FUL1* genes were different during fruit development; the expression of *NOR* was the highest in the breaker period, while the expression of *FUL1* was the highest at the pink coloring stage. At different stages of fruit development, the expression levels of *NOR* and *FUL1* in *PpSnRK1α* overexpression tomato lines (OE-1, OE-3, and OE-4) were significantly higher than that of the WT. The expression was the most significantly up-regulated at the breaker stage in OE-1, OE-3, and OE-4 compared with that of the WT, and *NOR* was up-regulated by 1.15 times, 1.03 times, and 0.98 times, respectively, while

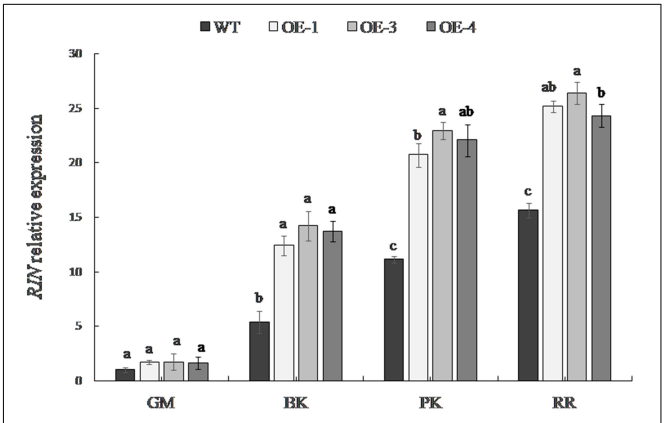

FIGURE 5 | *RIN* expression in WT and the *PpSnRK1α* overexpressing tomato fruit. Averages of three biological replicates ± SD are shown. Different lowercase letters in the same developmental stage indicate a significant difference at $P < 0.05$.

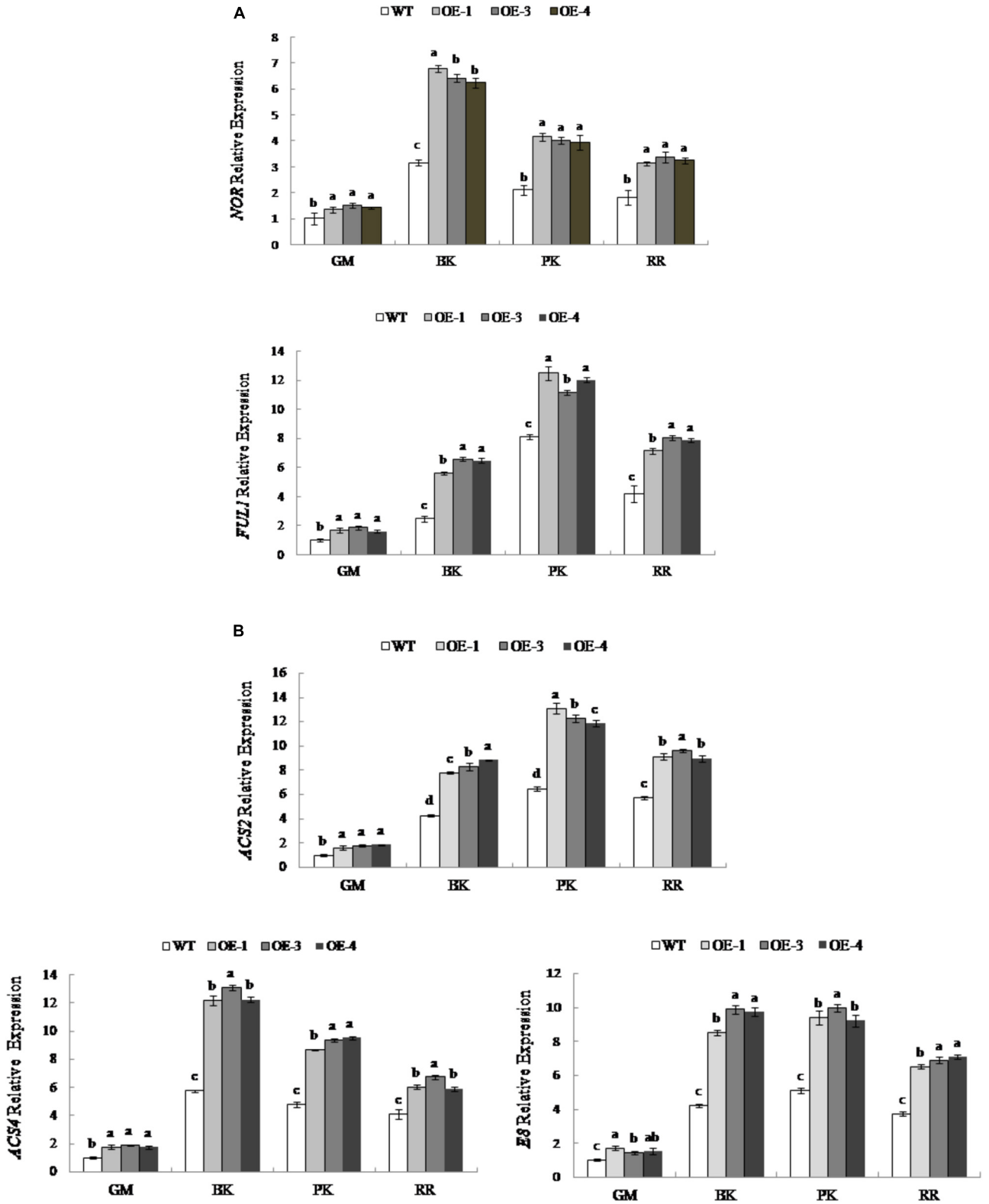

FIGURE 6 | Expression of RIN-targeted genes in WT and *PpSnRK1α* overexpressing tomato fruit during different fruit developmental stages. **(A)** ripening-related transcription factors; **(B)** ethylene synthesis enzymes. Error bars represent the SD based on three independent biological replicates. Different lowercase letters in the same developmental stage indicate a significant difference at $P < 0.05$ level.

FUL1 increased by 1.24 times, 1.62 times, and 1.58 times, respectively.

The expression levels of the ACC synthase encoding genes *ACS2* and *ACS4* are different during fruit development. *ACS2* had the highest expression in the pink coloring stage, while *ACS4* had the highest expression in the breaker stage; the expression of the ACC oxidase encoding gene *E8* was similar to that of *ACS2* (**Figure 6B**). The expression levels of *ACS2*, *ACS4*, and *E8* in transgenic tomatoes OE-1, OE-3, and OE-4 were significantly higher than those in the WT at different stages of fruit development. In OE-1, OE-3, and OE-4, the expression of *ACS2* was the most up-regulated in the pink coloring stage, which, compared with the WT, increased by 2.01 times, 1.89 times, and 1.82 times, respectively. The expression of *ACS4* was up-regulated the most during the breaker stage in OE-1, OE-3, and OE-4, with values 2.12 times, 2.25 times and 2.13 times that of the WT, respectively. Similarly, *E8* was up-regulated the most during the breaker stage, with values in OE-1, OE-3, and OE-4 up to 2.02 times, 2.35 times, and 2.31 times that of the WT, respectively (**Figure 6B**). However, the genes not under the control of RIN were not up regulated (**Supplementary Figure S1**).

Ethylene Emitted From Red Ripe Fruit of Transgenic and WT Tomato

The ethylene emitted from OE-1, OE-3, and OE-4 fruit was significantly higher than that of the WT, increasing by 37.9, 43.9, and 36.1%, respectively (**Figure 7**).

DISCUSSION

As a conserved energy sensor, SnRK1 plays an important role in plant metabolism, stress signal response, and plant growth and development (Hedbacker and Carlson, 2008; Carling et al., 2012). SnRK1 controls the early growth of pea cotyledons by coordinating metabolic, hormonal, and developmental signals

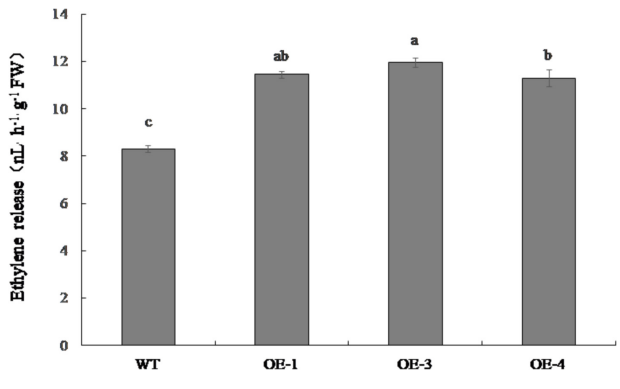

FIGURE 7 | Ethylene release in red ripe fruit of *PpSnRK1α* overexpression tomato lines (OE-1, OE-3, and OE-4) and WT tomatoes. Error bars represent the SD based on three independent biological replicates. Different letters indicate statistically significant differences between the samples ($P < 0.05$).

that influence seed maturation (Radchuk et al., 2010). In our previous study, overexpression of *MhSnRK1* in tomato improved the photosynthetic rate, fruit soluble sugar content, starch content and utilization, and the transgenic tomato fruit matured 10 days earlier than the WT fruit (Li et al., 2010; Wang et al., 2012). In order to further explore the effect of SnRK1 on fruit maturation, transgenic tomatoes over expressing *PpSnRK1α* were studied. The results of the present study showed that overexpression of *PpSnRK1α* significantly increased the content of soluble sugar and starch in the fruit, and the fruit ripening period was 10 days earlier than WT fruit, which confirmed our previous results.

Hou (2009) performed a yeast two-hybrid system sieve library on the tomato TF RIN, finding that one of the proteins interacting with RIN is tomato LeSNF1/AMPK, which has phosphorylation activity. However, whether the combination of the two is just a coincidence of structural matching has not been further verified by the authors. In this study, the interaction between PpSnRK1α protein and tomato SIRIN protein was tested by a yeast two-hybrid experiment and BiFC assay. The results showed a positive interaction between SnRK1α and RIN. We also analyzed the expression of *RIN* at the transcriptional level and found that its expression level was significantly increased in *PpSnRK1α* overexpressing tomato, suggesting that SnRK1 regulated RIN at both the protein and transcriptional level.

RIN belongs to the family of typical MADS-box TFs, which are responsible for a series of physiological and biochemical processes such as respiration, photosynthesis, and nutrient metabolism. RIN are inhibited in *rin* mutants, and some genes related to fruit maturation are aberrantly expressed, indicating that RIN has a very important role in fruit ripening (Ng and Yanofsky, 2001; Vrebalov et al., 2002). Recently, researchers used ChIP, proteomics, gene chips, and other experimental techniques to show that the RIN protein directly targets genes involved in ethylene synthesis and signal transduction pathways, cell wall metabolism, and fruit softening; while in the tomato ripening process, many TF genes, such as *NOR*, *CNR*, *FUL1*, and *HB-1*, are also directly regulated by RIN (Fujisawa et al., 2013). The transcriptional levels of target genes related to maturation of RIN were also investigated in this study. The expression of *NOR* and *FUL1* TF genes in the tomato fruit breaker and pink coloring stages had corresponding increases; the expression levels of *ACS2*, *ACS4*, and *E8* in the ethylene pathway also increased with elevated *RIN* expression. FUL1 is a MADS-box TF with an expression pattern that suggests a possible role during tomato fruit ripening (Busi et al., 2003). NOR is a NAC-domain TF, and when mutated, shows a non-ripening phenotype similar to *rin* (Giovannoni, 2007). *ACS2*, *ACS4*, and *E8* were also directly regulated by RIN. The increase in fruit ethylene production is largely driven by the biosynthetic genes *ACS2*, *ACS4*, *ACO1*, and *E8* (Lincoln et al., 1987; Barry, 2007). This series of expression changes in key maturity genes will ultimately affect the process of fruit ripening.

Overall, our results suggest that PpSnRK1α interacts with SIRIN, increasing the expression of *RIN*, regulating the expression of downstream ripening-related genes and promoting

the fruit ripening. However, the process of fruit ripening is a very complicated regulation network, in which SnRK1 and RIN may play a key role. Phosphorylation of RIN by SnRK1 may achieve a series of downstream regulations of maturation, but it is not known if it is also possible to directly regulate RIN-targeted TFs or activities of the key enzymes in the ethylene pathway. In addition, SnRK1 plays an active role in promoting plant photosynthesis and accumulation of plant sugars and other metabolites, and whether these are also reasons for early fruit maturation still require further verification.

AUTHOR CONTRIBUTIONS

FP and WY conceived and designed the experiments. WY, GW, and JL performed the experiments. GW and YX contributed reagents, materials, and analysis tools. WY and FP wrote the paper.

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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.2018.01856/full#supplementary-material

FIGURE S1 | The expression levels of *CTR2* and *ETR1* genes in the WT and transgenic fruits during different fruit developmental stages.

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MINI REVIEW
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Genome Editing as a Tool for Fruit Ripening Manipulation

*Carmen Martín-Pizarro and David Posé**

Laboratorio de Bioquímica y Biotecnología Vegetal, Facultad de Ciencias, Departamento de Biología Molecular y Bioquímica, Instituto de Hortofruticultura Subtropical y Mediterránea, Universidad de Málaga-Consejo Superior de Investigaciones Científicas, Málaga, Spain

Over the last few years, a series of tools for genome editing have been developed, allowing the introduction of precise changes into plant genomes. These have included Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR/Cas9, which is so far the most successful and commonly used approach for targeted and stable editing of DNA, due to its ease of use and low cost. CRISPR/Cas9 is now being widely used as a new plant breeding technique to improve commercially relevant crop species. Fruit ripening is a complex and genetically controlled developmental process that is essential for acquiring quality attributes of the fruit. Although the number of studies published to date using genome editing tools to molecularly understand or improve fruit ripening is scarce, in this review we discuss these achievements and how genome editing opens tremendous possibilities not only for functional studies of genes involved in fruit ripening, but also to generate non-transgenic plants with an improved fruit quality.

Keywords: fruit ripening, fruit quality, crops, tomato, genome editing, TALENs, CRISPR/Cas9

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****Correspondence:***
David Posé
dpose@uma.es

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INTRODUCTION

Fruit ripening is a complex and irreversible developmental process that involves numerous metabolic, biochemical, physiological and organoleptic alterations. Among these changes, ripening leads to fruit softening, accumulation of sugars, volatile compounds and pigments, reduction of organic acids, etc., making the fruit more attractive for animal consumption, and therefore, facilitating seed dispersal (Gapper et al., 2013).

Fleshy fruits are classified as climacteric or non-climacteric, depending on whether or not they produce autocatalytic ethylene, respectively. Thus, climacteric fruits such as tomato, apple, avocado, and banana are characterized by an increase in the respiration rate and a burst of ethylene at the onset of ripening (Giovannoni, 2004). In contrast, in non-climacteric fruits, which include strawberry, grape, citrus, and pepper among others, ethylene production remains at low levels and there is no dramatic change in respiration (Symons et al., 2012).

The role of phytohormones and the transcriptional regulation of climacteric and non-climacteric fruit ripening have been extensively reviewed in the last few years (Gapper et al., 2013; Cherian et al., 2014; Karlova et al., 2014; Kumar et al., 2014). In particular, ethylene perception and signaling have been very well characterized, especially in tomato (*Solanum lycopersicum*), which is the most studied model system for fruit ripening (Giovannoni, 2001; Barry and Giovannoni, 2007). In contrast, the regulatory network involved in non-climacteric fruit ripening has been much less studied. Nevertheless, it is known that abscisic acid rather than ethylene is essential in the control of ripening in strawberry (Chai et al., 2011; Jia et al., 2011), the established model for non-climacteric fruits.

Fruit ripening is of major economic importance for agriculture. One of the main challenges for producers is to offer a product at the ripening stage with a good flavor and nutritional value, while also having sufficient shelf life to maintain its quality until it is consumed. This is especially relevant for climacteric fruits highly sensitive to ethylene, and for non-climacteric fruits such as strawberry, which become quickly inedible. Thus, improved ripening and shelf life has been a focus of interest for many scientists in recent decades, using conventional breeding and genetic modification. However, the latter relies on the generation of transgenic plants, which have a very limited commercial use due to the current skepticism of consumers and restrictive government policies. Moreover, transgenic strategies have been based on the modulation of gene expression, which may lead to temporary and/or incomplete knockdown effects, unpredictable off-target effects, and too much background noise (Martin and Caplen, 2007). However, the availability of genome editing tools offers new opportunities to overcome these drawbacks.

THE EMERGENCE OF GENOME-EDITING TECHNOLOGY

In the past decade, new and powerful approaches have emerged enabling the precise editing of a gene of interest. These approaches are based on the use of nucleases that are targeted to a specific sequence to generate a double-strand break (DSB). DSBs trigger two different repair mechanisms: (i) error-prone non-homologous-end-joining (NHEJ) and (ii) homology-directed repair (HDR). While NHEJ repair results in InDel mutations of variable lengths, HDR can be used to introduce specific point mutations or a sequence of interest, through recombination supplying an exogenous donor template. To obtain DSBs for genome editing, four major classes of customizable DNA-binding proteins have been engineered so far: meganucleases (Smith et al., 2006), zinc-finger nucleases (ZFNs) (Urnov et al., 2005), transcription activator-like effector nucleases (TALENs) (Christian et al., 2010), and RNA-guided DNA endonuclease Cas9 (Jinek et al., 2012). Meganucleases, ZFN, and TALEN rely on the binding and recognition of the nucleases to specific sequences of DNA. Therefore, these approaches require complex engineering processes to produce custom nucleases that target the sequence of interest. However, methods based on the bacterial CRISPR (clustered regularly interspaced short palindromic repeats)/Cas system have opened up tremendous possibilities, since the specificity does not lie in the endonuclease, but on a simple and cheap design of a single guide RNA (sgRNA) that is complementary to the target sequence.

Despite the enormous number of reported studies using genome editing technology for gene functional studies in plants and crop improvement (Malzahn et al., 2017), only a small handful of studies, summarized in this review (**Table 1**), have focused on improving or identifying key regulators of fruit ripening as an essential developmental process and an economically relevant trait.

FIRST GENOME EDITING APPROACHES IN TOMATO

In the case of dicot crops, tomato became the ideal candidate for gene editing because of its several advantages, i.e., (i) diploid and high-quality sequenced genome, (ii) ease of transformation, (Van Eck et al., 2006), and (iii) economic importance, being the fourth most important commercial crop in the world. The first reports on genome editing in tomato appeared in 2014 when CRISPR/Cas9 was applied to effectively perform gene functional analysis by stable root transformation, using *Agrobacterium rhizogenes* (Ron et al., 2014). This study was followed by two others, where CRISPR/Cas9 and TALENs were applied to generate mutations in complete plants for the first time, in particular for the *ARGONAUTE7* (*SlAGO7*) and *PROCERA* (*PRO*) genes, respectively (Brooks et al., 2014; Lor et al., 2014). As in most of the pioneer studies of genome editing in any species, both genes had been functionally characterized already, allowing the functional validation of these new approaches. Particularly, *PRO* encodes for a DELLA protein that acts as a negative regulator of gibberellin (GA) signaling (Bassel et al., 2008; Jasinski et al., 2008). Lor et al. (2014) characterized the vegetative stage of the TALEN-induced *pro* mutants, which showed a phenotype consistent with an increased GA response, such as tall and slender plants. Besides their role in plant growth and development, the role of GAs in fruit set (Kumar et al., 2014) and fruit ripening (Dostal and Leopold, 1967) have been widely studied. In fact, a previous report where the spontaneous *pro* mutant was phenotypically characterized, showed that fruit ripening was significantly delayed and that the Brix index value was higher in the mutant (Carrera et al., 2012), consistent with a higher GA response in these plants. Therefore, it would be expected that fruit ripening was also altered in the TALEN-induced *pro* mutants, though Lor et al. (2014) did not characterize the fruit phenotype.

Two years later, the ZFNs gene editing tool was applied for the first time in tomato to mutagenize the *LEAFY COTYLENDON1-LIKE4* (*LIL4*) gene, which encodes for a subunit of a heterotrimeric transcription factor (Hilioti et al., 2014). Mutation in *LIL4* resulted in a pleiotropic phenotype, including fruits with different sizes and shapes, a reduced number of locules, and absence of placenta. Furthermore, fruits with a paler color and slower ripening were obtained (Hilioti et al., 2016), although how *LIL4* regulates this processes is still unknown.

RIN – HOW CRISPR/CAS9 CONVERTED A LOSS-OF-FUNCTION INTO A GAIN-OF-FUNCTION MUTATION

A large number of studies have been focussed in the role of different transcription factors (TFs) involved in the ripening process. One of the most investigated TFs for ripening is *RIPENING-INHIBITOR* gene (*RIN*), a member of the *SEPALATA* (*SEP*) class of the MADS-box gene family, first discovered half a century ago when a mutation in this locus (*rin*) was found to cause a failure to ripen in tomato (Robinson and Tomes, 1968;

TABLE 1 | List of applications of genome editing technologies to study tomato fruit ripening, and CRISPR/Cas9 in various fruit crop species.

Technology	Gene edited in tomato	Character	Reference
TALEN	*PROCERA* (*PRO*)	GA metabolism	Lor et al., 2014
TALEN	*LEAFY COTYLEDON1-LIKE4* (*LIL4*)	Pleiotropic effects	Hilioti et al., 2016
CRISPR/Cas9	*RIPENING-INHIBITOR* (*RIN*)	Fruit ripening	Ito et al., 2015, 2017
CRISPR/Cas9	*PECTATE LYASE* (*PL*)	Fruit firmness	Uluisik et al., 2016
CRISPR/Cas9	*SELF-PRUNING 5G* (*SP5G*) and *SELF-PRUNING* (*SP*)	Photoperiodic response	Soyk et al., 2016
CRISPR/Cas9	LONG-NON CODING RNA (*lncRNA1459*)	Fruit ripening	Li R. et al., 2018
CRISPR/Cas9	*ORGANELLE RECOGNITION MOTIF* (*SlORRM4*)	Mitochondrial function	Yang et al., 2017
Species	**Gene edited by CRISPR/Cas9**	**Character**	**References**
Grapevine (*Vitis vinifera*)	*MLO-7*	Pathogen resistant	Malnoy et al., 2016
	IdnDH	Tartaric acid biosynthesis	Ren et al., 2016
	VvPDS	Carotenoid biosynthesis	Nakajima et al., 2017
Watermelon (*Citrullus lanatus*)	*ClPDS*	Carotenoid biosynthesis	Tian et al., 2016
Cucumber (*Cucumis sativus*)	*eIF4E*	Virus resistance	Chandrasekaran et al., 2016
Banana (*Musa* × *paradisiaca)*	*PDS*	Carotenoid biosynthesis	Kaur et al., 2018
Kiwifruit (*Actinidia Lindl*)	*AcPDS*	Carotenoid biosynthesis	Wang et al., 2018
Sweet orange (*Citrus sinensis*)	*CsPDS*	Carotenoid biosynthesis	Jia and Wang, 2014
	CsLOB1	Pathogen resistant	Peng et al., 2017
Duncan grapefruit (*Citrus* × *paradisi*)	*CsLOB1*	Pathogen resistant	Jia et al., 2016
	CsLOB1	Pathogen resistant	Jia et al., 2017
Apple (genus *Malus*)	*PDS*	Carotenoid biosynthesis	Nishitani et al., 2016
	DIPM1, *2* and *4*	Pathogen resistant	Malnoy et al., 2016
Woodland strawberry (*Fragaria vesca*)	*TAA1* and *ARF8*	Auxin biosynthesis and signaling	Zhou et al., 2018
Cultivated strawberry (*Fragaria* × *ananassa*)	*FaTM6*	Flower development	Martín-Pizarro et al., 2018

Vrebalov et al., 2002). *RIN* is induced early during ripening, and regulates ethylene-dependent and ethylene-independent pathways that promote ripening (Karlova et al., 2014). The effect on ripening of the *rin* mutation has been commercially exploited as hybrid cultivars (*RIN*/*rin*) with an extended shelf life (Kitagawa et al., 2005). Due to the importance and clear phenotype of the *rin* mutation, the *RIN* locus has been recently targeted using CRISPR/Cas9 to validate the functionality and inheritance of mutations mediated by this approach in tomato. Ito et al. (2015) designed three CRISPR/Cas9 constructs to mutagenize three different regions within the *RIN* locus. As expected, CRISPR/Cas9-mediated novel mutations at the *RIN* locus resulted in an inhibition of fruit ripening at the T0 generation (Ito et al., 2015). However, and contrary to the *rin* mutant, these CRISPR mutants partially initiated the ripening process, and this was interpreted as the result of the presence of wild-type RIN in the T0 generation. Previously, Vrebalov et al. (2002) generated knockdown *RIN* plants using RNA interference, resulting in a fruit ripening that was only partially suppressed, in contrast to the green *rin* mutant phenotype, and interpreted as due to residual expression of *RIN*. Thus, RIN has been considered so far to function as an essential regulator of ripening, and the models have always been based on the idea that *rin* was a loss-of-function mutation. However, a recent paper has overturned this model (Ito et al., 2017). Firstly, unlike the *rin* mutant, homozygous CRISPR/Cas9-mediated knockout *rin* mutants (*RIN*-KO) did not fail to ripen, reaching a pale red color. Moreover, a molecular and physiological characterization of these lines showed that most ripening-related parameters were less affected than in the *rin* mutant. These results suggested that, contrary to what has been considered so far, ripening can be initiated independently of RIN. Furthermore, they also suggested that the *rin* mutant protein may have gained a new function, as a partial dominant negative mutation that blocks the initiation of ripening. This hypothesis was supported by the fact that *rin* mutant allele encodes for a in-frame fusion of *RIN* and *Macrocalyx* coding sequences (Vrebalov et al., 2002), the latter containing a putative repression motif that might convert *rin* into a transcriptional repressor. This repressor activity was actually confirmed *in vitro* (Ito et al., 2017). Consistent with this

hypothesis, use of CRISPR/Cas9 to generate additional mutations in the *rin* mutant allele (*rin*Δ*N*) resulted in fruits that recovered the initiation of ripening, showing a similar phenotype to that of *RIN*-KO lines. Furthermore, a molecular and physiological characterization of *rin*Δ*N* lines showed a partial recovery of most of the ripening markers. Thus, this study proposes that the *rin* mutant protein would impair the DNA-binding and activation of ripening-related genes by other master regulators such as NONRIPENING (NOR), COLORLESS NONRIPENING (CNR) (Giovannoni, 2004; Manning et al., 2006), or other SEP homologs.

In conclusion, the use of a gene editing approach such as CRISPR/Cas9 has allowed generating alternative knockout alleles, which have changed our current model of fruit ripening, with RIN being necessary to initiate this process, and *rin* being a loss-of-function mutation. This implies that many results obtained in the past should be reconsidered, and that further experiments should be carried out now that we are closer to defining the actual mechanism.

TARGETING FRUIT TEXTURE

While *RIN*/*rin* hybrid plants are widely used by tomato breeders, the incomplete ripening of these hybrid fruits leads frequently to a poor flavor and a reduced nutritional value. Hence, to modify texture characteristics for an improved shelf life, without reducing tomato organoleptic and nutritional quality, has been a challenge for researchers and breeders for many years. Fruit softening depends on cell-wall modifying proteins such as polygalacturonase, pectin methylesterase, endo-β-(1,4)-glucanase, β-galactosidase, and expansin. A number of studies have characterized the effect of silencing the expression of genes encoding these proteins in strawberry (Posé and García-Gago, 2011), and tomato (Sheehy et al., 1988; Smith et al., 1990, 1988; Tieman et al., 1992; Hall et al., 1993; Tieman and Handa, 1994; Brummell et al., 1999; Smith, 2002). Silencing of the polygalacturonase gene had no apparent effect on tomato fruit softening (Sheehy et al., 1988; Smith et al., 1990, 1988), but it did affect the firmness of strawberry fruits, which even showed a slightly higher °Brix (Quesada et al., 2009). For the rest of the genes, silencing of their expression has had only very small or no detectable effects on both tomato or strawberry fruit ripening (Tieman et al., 1992; Hall et al., 1993; Tieman and Handa, 1994; Brummell et al., 1999; Smith, 2002). However, silencing another cell-wall related protein, the pectate lyase (PL), has been successfully applied for the modulation of fruit firmness in both species. *PL* silencing increased fruit firmness without changes in color, size, total soluble solids, or metabolites influencing taste and aroma in both strawberry (Jiménez-Bermúdez et al., 2002), and tomato (Uluisik et al., 2016). Particularly in tomato, preliminary analysis of CRISPR/Cas9-induced *pl* mutants has shown an effect on fruit firmness without altering color and soluble solids content (Uluisik et al., 2016). A further characterization would be necessary to confirm that these CRISPR/Cas9 mutant lines maintain other important agronomical characteristics such as aroma, flavor, yield, color, and resistance to pathogens, all required traits for a successful introduction to the market.

TARGETING PHOTOPERIODIC RESPONSE

An appropriate timing of flowering is not only essential for plant reproductive success but also to optimize yield in agriculture. In a search for the genetic component controlling the different day-length sensitivities regulating flowering in tomato, Soyk and colleagues identified *SELF PRUNING 5G* (*SP5G*) as the responsible gene (Soyk et al., 2016). *SP5G* is a *FLOWERING LOCUS T*-like gene that works as a floral repressor controlling flowering under long-day conditions (Cao et al., 2015). In this study, the authors generated CRISPR/Cas9-mediated mutations for this gene, obtaining plants that flowered earlier under long-day conditions. Another gene, *SELF-PRUNING* (*SP*), is an ortholog of Arabidopsis *TERMINAL FLOWER 1* (*TFL1*) and encodes for another flowering repressor in tomato (Pnueli et al., 1998). The *sp* mutation revolutionized tomato cultivation since it leads to determinate plants with a synchronized burst of flowering and fruit ripening. In order to obtain faster-flowering and determinate growth plants, Soyk and collaborators used CRISPR/Cas9 to generate double *sp5g sp* mutants, which showed an earlier flowering burst and an earlier fruit ripening than that of the *sp* single mutant and the wild-type (Soyk et al., 2016). However, the earlier tomato ripening in the double *sp5g sp* mutant might be caused simply by the earlier flowering time phenotype of these plants. Therefore, further studies on fruit ripening dynamics need to be performed to clarify whether SP5G actually modulates actively this process.

TARGETING POST-TRANSCRIPTIONAL REGULATION

There are several previous studies demonstrating the importance of post-transcriptional regulation by non-coding RNAs in the control of fruit ripening (Moxon et al., 2008; Zhu et al., 2015; Wang et al., 2016). To investigate further, CRISPR/Cas9 has been employed in two studies to identify and characterize post-transcriptional regulators of tomato fruit ripening.

In plants, long non-coding RNAs (lncRNAs) are important regulators of gene expression, as they interact with DNA, RNA and proteins (Zhu and Wang, 2012). Interestingly, two lncRNAs, lncRNA1459 and lncRNA1840, have been recently associated with tomato fruit ripening (Zhu et al., 2015). To investigate further the role of lncRNA1459 in fruit ripening, this gene was stably knocked-out by Li X. et al. (2018) using CRISPR/Cas9, obtaining CR-*lncRNA1459* mutant lines with a delayed fruit ripening. A molecular characterization of this mutant showed that key ripening-related genes involved in lycopene and ethylene biosynthesis, and in signal transduction were down-regulated. Consistently, CR-*lncRNA1459* mutant fruits showed a reduction in lycopene accumulation and an inhibition of ethylene production (Li R. et al., 2018). However, the mechanism and

target genes of lncRNA1459 in its regulation of fruit ripening still need clarification.

Another post-transcriptional regulation involved in ripening that has been recently explored is RNA editing. In flowering plants, RNA editing by cytidine-to-uridine (C-to-U) conversion is a widespread process that occurs only in plastids and mitochondrial transcripts and plays an important role in developmental processes such as organelle biogenesis, adaptation to environmental changes and signal transduction (Ichinose and Sugita, 2017). In a recent report, Yang et al. (2017) aimed to identify RNA editing factors that might play an essential role in the regulation of tomato fruit ripening. A virus-induced gene silencing (VIGS) assay was performed, targeting 11 RNA editing factor genes putatively related to ripening, positively identifying *SlORRM4*, which is located in mitochondria (Yang et al., 2017). Consistently, CRISPR/Cas9-mediated stable *slorrm4* mutants resulted in a delay of ripening, and in a diminution of the respiratory rates and ethylene production compared with the wild-type. Further molecular characterization showed that *slorrm4* mutation results in a down-regulation of genes associated with the Krebs cycle and mitochondrial function, and a decrease in the level of proteins essential for the mitochondrial respiratory chain, supporting the essential role of mitochondria in the regulation of ripening. However, the specific mechanisms linking RNA-editing in mitochondria with ripening requires further investigation.

FUTURE PERSPECTIVES

Besides ripening, other interesting agronomic traits have been modulated recently in tomato using CRISPR/Cas9, such as parthenocarpy (Klap et al., 2017; Ueta et al., 2017), lycopene content (Li X. et al., 2018), and fruit size, inflorescence branching and plant architecture (Rodríguez-Leal et al., 2017). Especially relevant is the work of Rodríguez-Leal et al. (2017), in which, instead of editing CDS loci, they targeted *cis*-regulatory elements (CREs) in promoters, obtaining quantitatively different phenotypes (Rodríguez-Leal et al., 2017). CRE mutations are widespread in nature and have notably contributed to crop domestication through the alteration of gene expression levels (Meyer and Purugganan, 2013). Thus, targeting CREs with genome-editing technologies offers the possibility to fine-tune gene expression without the common pleiotropic effects observed in complete loss-of-function mutants, opening the door to enhance variability for important agronomic and quality traits. However, a lack of precise knowledge about functional motifs in CREs hampers the current application of this approach.

In addition to tomato, the CRISPR/Cas9 gene-editing strategy has been successfully applied in several fruit crop species to date, including examples of climacteric ripening species, such as apple (Malnoy et al., 2016; Nishitani et al., 2016), banana (Kaur et al., 2018) and kiwifruit (Wang et al., 2018), and non-climacteric ripening species, such as sweet orange (Jia and Wang, 2014; Peng et al., 2017), Duncan grapefruit (Jia et al., 2016, 2017), grapevine (Malnoy et al., 2016; Ren et al., 2016; Nakajima et al., 2017), watermelon (Tian et al., 2016), cucumber (Chandrasekaran et al., 2016), and the woodland (Zhou et al., 2018) and cultivated strawberries (Martín-Pizarro et al., 2018; **Table 1**). Most of these studies have targeted either the *Phytoene Desaturase* gene (*PDS*), or genes to improve pathogen resistance. However, they have opened up the possibility of using CRISPR/Cas9 technology to study or improve fruit ripening in these crops. Among them, strawberry is a species of particular interest because the fast softening of the berries is the main cause of its short shelf life and the source of commercial losses (Perkins-Veazie, 1995). Hence, the successful application of gene editing using the CRISPR/Cas9 approach may provide effective solutions for these postharvest issues.

It is important to highlight that all these studies are based on the generation of new random mutations mediated by the NHEJ mechanism. However, homologous recombination-based gene targeting (GT) allows a more precise genome editing. GT has already been successfully achieved in several crops, including tomato (Čermák et al., 2015; Filler Hayut et al., 2017). In a recent study, GT has been used to extend tomato shelf life by the incorporation of *alcobaca* (*alc*) (Yu et al., 2017), an allelic mutation of *NOR* with a lower impact on fruit quality than *nor* and *rin* mutations (Casals et al., 2011), into a wild-type genotype. Despite these successful studies, GT is still a very challenging approach due to its low efficiency. Thus, the optimization of GT in higher plants in general, and in crop species in particular, would provide of a much wider spectrum of possibilities for breeding, allowing the introgression of genes of interest into elite breeding germplasm.

In conclusion, genome editing strategies, especially CRISPR/Cas9 are becoming rapidly more efficient and precise. Their application to the coding sequences of TFs, hormones or

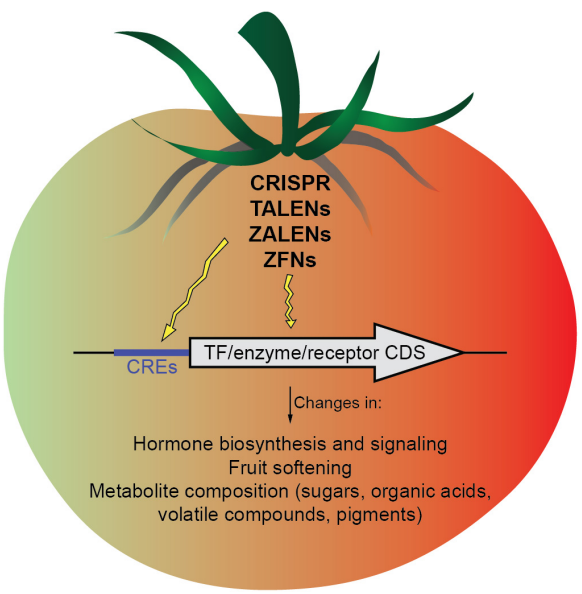

FIGURE 1 | Scheme of the possible targets (*cis*-regulatory elements and gene coding sequences) that might be modified by genome-editing approaches to modulate different aspects of fruit ripening.

metabolites biosynthetic enzymes, and hormone receptors, or, alternatively to CREs of these genes may allow a more precise fruit ripening modulation (**Figure 1**). Importantly, genome editing tools have the possibility of removing transgenes through self- or backcrossing, an important advantage compared to traditional approaches for genetic modification. Moreover, preassembled Cas9 protein-gRNA ribonucleoproteins (RNPs) remove the likelihood of inserting recombinant DNA into the host genome. This particular approach has been demonstrated in the protoplasts of several plant species (Woo et al., 2015; Malnoy et al., 2016; Svitashev et al., 2016; Liang et al., 2017) as a strategy that could be potentially operated outside existing GMO regulatory criteria and gain acceptance from consumers. However, the recent decision of the Court of Justice of the European Union is a major setback to innovation in EU agriculture, considering the "process" instead of the "product" and putting crops created using gene-editing techniques under GMO regulations (Directive 2001/18/EC). Hopefully, these regulatory decisions will be reconsidered in the future, as there are unquestionable advantages of gene editing to address the challenge of ensuring sufficient food supply for an increasing global population in the current changing climatic conditions.

AUTHOR CONTRIBUTIONS

All authors conceived and wrote the manuscript.

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