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Research Article

Hormone balance in a climacteric plum fruit and its non-climacteric bud mutant during ripening

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ARTICLE INFO

Keywords:

ABA
Auxin
Climacteric
Ethylene
Fruit ripening
Hormone balance
Non-climacteric
Plum

ABSTRACT

Hormone balance plays a crucial role in the control of fruit ripening. We characterized and compared hormone balance in two Japanese plum cultivars (*Prunus salicina* Lindl.), namely Santa Rosa, a climacteric type, and Sweet Miriam, its non-climacteric bud-sport mutant. We assessed hormonal changes in gene expression associated with hormone biosynthesis, perception and signaling during ripening on-the tree and throughout postharvest storage and in response to ethylene treatments. Non-climacteric fruit displayed lower ethylene levels than climacteric fruit at all stages and lower auxin levels during the initiation of ripening on-the-tree and during most of post-harvest storage. Moreover, 1-MCP-induced ethylene decrease also resulted in low auxin contents in Santa Rosa, supporting the role of auxin in climacteric fruit ripening. The differences in auxin contents between Santa Rosa and Sweet Miriam fruit could be the consequence of different routed auxin biosynthesis pathways as indicated by the significant negative correlations between clusters of auxin metabolism-associated genes. Ethylene induced increased ABA levels throughout postharvest storage in both ripening types. Overall, ripening of Santa Rosa and Sweet Miriam fruit are characterized by distinct hormone accumulation pathways and interactions.

1. Introduction

Fleshy fruit ripening involves a series of irreversible physiological and biochemical modifications that determine the overall fruit quality, making the fruit palatable for consumption [1–3]. These modifications include changes in color, taste, texture and aroma [4–7]. Based on differences on their respiratory patterns and ethylene production rates, fleshy fruit ripening behavior has traditionally been categorized as either climacteric or non-climacteric. Climacteric fruit display a peak in respiration and a burst of autocatalytic ethylene production, while fruit not showing these changes are defined as non-climacteric [6,8–10]. Nevertheless, this classification might be too general since a conserved

program controlling both ripening types might exist [11–14]. This notion is supported by the small ethylene changes occurring during ripening of non-climacteric fruit such as grapes and strawberries [15–17] and the capacity of externally applied ethylene to stimulate color break in citrus [18]. Climacteric and non-climacteric ripening types can be found in the same species, as for example in melons [13] as well as in Japanese plums [19,20] and a ubiquitous role for ethylene in both ripening types was hypothesized, suggesting that the sensitivity towards ethylene could be altered based on the interaction of ethylene with other plant hormones and the environment [17,21–24]. Also, other hormones, together with transcriptional and epigenetic regulators, have shown to be key players in the control of fruit ripening [25,26], such

Abbreviations: SR, Santa Rosa; SM, Sweet Miriam; DAFB, days after full bloom; 1-MCP, 1-methylcyclopropene; SAMS, S-adenosyl-L-methionine synthase; ACS, 1-aminocyclopropane-1-carboxylate synthase; ACO, ACC oxidase; ERS, ethylene-response sensor; ETR, ethylene receptor-type; EIN, ethylene-insensitive; ERF, ethylene response factor; IPyA, indole-3-pyruvic acid pathway; NCED, 9-cis-epoxycarotenoid dioxygenase; GA2OX, gibberellin 2-oxidase; IPT, isopentenyl transferase; CRE, cytokinin response element; ICS, isochorismate synthase

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<https://doi.org/10.1016/j.plantsci.2018.11.001>

Received 4 October 2018; Received in revised form 4 November 2018; Accepted 7 November 2018

Available online 14 November 2018

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that changes in the overall fruit hormone balance had direct impacts on the ripening process. For instance, abscisic acid (ABA) demonstrated to have a positive effect in ripening of both fruit types [27,28], while auxins (IAA) have been reported to inhibit or delay non-climacteric ripening [29–33] but enhance climacteric ripening in different species of the Rosacea family [31,34,35]. Consequently, synergistic interactions between ABA and ethylene biosynthesis, as well as synergistic and antagonistic interactions between IAA and ethylene biosynthesis during ripening of both climacteric [36–39] and non-climacteric [17,30,38,40] fruit have been proposed.

The Japanese plum Sweet Miriam (SM), is a non-climacteric bud mutant of the climacteric cultivar Santa-Rosa (SR). Previous analyses showed that SM fruit ethylene contents on the tree [20] and during post-harvest storage [41], are considerably lower than those of SR. Comparative analyses of these two cultivars offer a unique opportunity to study the involvement of hormones in the fruit ripening process. Here, we characterized and compared the hormone balance controlling climacteric and non-climacteric fruit ripening on-the-tree as well as throughout postharvest storage, and the fruit response to ethylene and 1-MCP (1-methylcyclopropene; SmartFresh™) an inhibitor of ethylene binding to its receptors [42,43] treatments. We assessed hormonal changes and integrated these with changes in the expression of hormone metabolism-associated genes in each cultivar during ripening on-the-tree as well as throughout postharvest storage.

2. Materials and methods

2.1. On-the-tree ripening fruit material

Fruit from Japanese plum [*Prunus salicina* Lindl.] SR and SM cultivars were harvested throughout two growth seasons from a commercial orchard located in the California Central Valley production area (Parlier, CA, USA) as previously defined [19]. Fruit growth and development patterns were monitored weekly as described before [19,20]. Six biological replications, each consisting of 20 fruit taken from random positions in five independent trees randomly located throughout the orchard, were collected at four ripening-related stages on-the-tree: S3/S4 (mature), S4-I (commercial harvest), S4-II (fully-ripe) and S4-III (overripe) (Table 1; [19]) and immediately transported to the laboratory. For each biological replication, six fruit were used for the evaluation of physicochemical parameters and ripening patterns, while the rest of the fruit were washed, peeled, cut into small pieces, pooled, frozen in liquid nitrogen and stored at -80°C for further analyses.

Table 1
Ripening-related stages collected in this study in fruit of Santa Rosa (SR) and Sweet Miriam (SM) Japanese plum cultivars.

Ripening-related stage	Description/Name	Fruit Flesh Firmness (N)	Harvest date (days)	
			DAFB ^a (Santa Rosa)	DAFB ^a (Sweet Miriam)
S3/S4	Mature	40.6 ± 1.7	~110	~152
“Well-mature”	“Well-mature”	37.2 ± 0.6	~112	~170
S4-I	Commercial harvest	30.8 ± 1.8	~116	~199
S4-II	Fully ripe	19.6 ± 1.2	~123	~223
S4-III	Overripe	SR:7.5 ± 1.5; SM:16.6 ± 1.4	~126	~243

^a DAFB: Days after full bloom.

2.2. Postharvest storage ripening fruit material and treatments

Fruit were harvested at the ‘well-mature’ stage [44] with a flesh fruit firmness of $\sim 37\text{N}$ which was reached $\sim 112\text{d}$ after full bloom (DAFB) for SR and $\sim 170\text{ DAFB}$ for SM and corresponded with the preclimacteric stage of SR (Table 1) [41]. Fruit with uniform size, absence of visual blemishes, bruises and/or diseases were chosen and quickly transported to the laboratory. Fruit within each cultivar were collected from random positions in thirty SR and SM trees randomly distributed throughout the orchard, and randomized and divided into 3 groups of 420 fruit each and commercially packed in cardboard boxes [41]. Fruit from the first group were treated with $0.5\ \mu\text{L}^{-1}$ 1-MCP (1-methylcyclopropene; SmartFresh™) an inhibitor of ethylene binding to its receptors [42,43] at 20°C for 24 h. Following treatment, fruit were left to ripen under humidified, ethylene-free air at a flow rate of $2\ \text{Lmin}^{-1}$ in 330-L aluminum tanks completely sealed and connected to a flow-through system. Fruit from the second group were left to ripen under humidified, ethylene-free air containing 500 ppm of propylene (ethylene analogue, Praxair Inc., Danbury, CT) and fruit from the third group (control), were left to ripen under humidified, ethylene-free air [41]. Fruit from all groups were stored at 20°C and 90% relative humidity throughout a total of 14 d. Evaluations were carried out at harvest (0) and after 1, 3, 5, 7, 10, and 14 d of storage. For each evaluation period, 6 biological replications from each group were assessed. For each biological replication, 6 fruit were used for the analysis of physicochemical parameters and ripening patterns, while 4 fruit were washed, peeled, cut into small pieces, pooled together, frozen in liquid nitrogen and stored at -80°C for further analyses.

2.3. Hormone extraction and analysis

Six biological replications of SR and SM fruit, throughout each ripening-related stage on-the-tree and postharvest storage period for each treatment, were used to quantify hormone levels. Abscisic acid (ABA), indole-3-acetic acid (IAA), cytokinins (*trans*-zeatin) (CK), salicylic acid (SA), and gibberellins (GA_1 , GA_3 and GA_4), were analysed according to [45] with some modifications. Briefly, freeze-lyophilized plant material (50 mg) was homogenized into 1 ml of cold (-20°C) extraction mixture of methanol/water (80/20, v/v). After 10 min of vortex and 10 min of sonication, solids were separated by centrifugation (20 000 g, 15 min) and re-extracted for 30 min at 4°C in additional 1 ml of the same extraction solution. Pooled supernatants were passed through a Sep-Pak Plus C_{18} cartridge (SepPak Plus, Waters, USA) to remove interfering lipids and part of plant pigments and evaporated at 40°C under vacuum to near dryness. The residue was dissolved in 1 ml methanol/water (20/80, v/v) solution using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter Millex filters with $0.22\ \mu\text{m}$ pore size nylon membrane (Millipore, Bedford, MA, USA). Ten μl of filtrated extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, MA, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. Mass spectra were obtained using the Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA). For quantification of the plant hormones, calibration curves were constructed for each analysed component (1, 10, 50, and $100\ \mu\text{g l}^{-1}$) and corrected for $10\ \mu\text{g l}^{-1}$ deuterated internal standards. Recovery percentages ranged between 92 and 95%.

2.4. Measurement of IAA-Glu and IAA-Asp

Conjugated IAAs were quantified by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS; Agilent 6410 TripleQuad LC/MS system). Approximate 100–200 mg frozen samples were extracted with 80% (v/v) acetone containing [d_5]-indole-3-acetyl-L-[^{15}N] aspartic acid (DN-IAA-Asp), and [d_5]-indole-3-acetyl-L-[^{15}N] glutamic acid (DN-IAA-Glu) as internal standards (Olchemim, <http://www.olchemim.cz>), followed by solid phase extraction using Oasis cartridge columns, HLB and MCX (Waters, <http://www.waters.com>), and subjected to LC-ESI-MS/MS analysis. An LC (Agilent 1200 series) equipped with a 2.1- x 50-mm, 1.8- μ m Zorbax Eclipse XDB-C18 column was used for separation. A liner gradient with increasing solvent B (acetonitrile/0.05% acetate) against solvent A (water/0.1% acetate) from 15% to 50% for 6 min at a flow rate of 0.2 ml min⁻¹ was used to separate metabolites. The retention time of IAA-Glu and IAA-Asp are 2.65 min and 2.27 min, respectively. The calibration curves were constructed by using stable isotope labeled compounds (i.e. DN-IAA-Asp and DN-IAA-Glu) against corresponding non-isotope labeled authentic compounds (Olchemim). MS/MS settings were described in [46].

2.5. Real-time quantitative RT-PCR Analysis

RNA was isolated from each of the 6 biological replicates of SR and SM plum fruit from each of the 4 ripening-related stages on-the-tree as well as throughout postharvest storage for each treatment, using the CTAB/NaCl method [47] with some modifications [48]. The integrity of RNA samples was assessed on 1.0% agarose gel electrophoresis (Supplemental Fig. S1) and RNA concentration and purity were determined using a NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). First-strand complementary DNA synthesis, primer design, and quantitative PCR were performed as described before [48]. The sets of primers used for the amplification of the different target genes are listed in Supplemental Table S1. The target genes analyzed in this study were selected based on two conditions: (i) been differentially expressed between fruit of SR and SM cultivars and throughout on-the-tree developmental stages, using the RNASeq dataset and methodology for identification of differentially expressed genes, as described in our previous work [19], and (ii) have been previously reported in literature as key genes related to pathways of hormone biosynthesis, perception or signaling. Gene nomenclature is based on the closest well-annotated peach genome (*Prunus persica* version 2.1) provided on Phytozome12.1 (<http://www.phytozome.net/peach.php>), Genome Database for Rosaceae (GDR) (<http://www.rosaceae.org/>), [49]. Analysis of the relative gene expression was performed according to the Comparative Cycle Threshold Method as described by [50]. The expression of the SAND protein-related trafficking protein (*MON*) was used as a reference as it was previously validated as a reference gene for precise transcript normalization across different Japanese plum tissue samples and developmental stages [48]. The primer sequences used for the amplification of *MON* reference gene can be found in our previous publication [48].

2.6. RNA-Seq reads mapping and gene annotation

RNA-Seq analysis was previously performed for the following on-the-tree stages: SR-S2, SR-S4-II, SM-S2, and SM-4II [19], based on the same material as in the current study. In this work, we used the raw data set of the previous analysis. In addition, RNA-Seq analysis was performed for the following postharvest stages/treatments: SR harvest

control, SR 1 d control, SR 3 d control, SR 3 d with 1-MCP, SM harvest control, SM 3 d control, SM 3 d with propylene, and SM 10 d control. Illumina RNA-Seq reads from each replicate (3 per each ripening stage on-the-tree and postharvest storage) were submitted to adaptor trimming and contaminant sequence removal using fastqc (www.bioinformatics.babraham.ac.uk/projects/fastqc/) and fastx-toolkits (hannonlab.cshl.edu/fastx_toolkit/). The closest well-annotated peach genome (*Prunus persica* version 2.1) provided on Phytozome12.1 was used as a primary reference and the transcriptome fasta was obtained from the genome and annotation (gff3 file). Genome assembly and transcriptome mapping was achieved by using software STAR aligner version 2.5.1 [51]. Gene counts were calculated using HTSeq version 0.6.1 [52]. Differential expression analyses were carried out applying a statistical test evaluating the negative binomial distribution provided in the R package DESeq [53]. Genes associated with auxin biosynthesis and auxin inactivation were identified based on gene ontology and queried for in the Arabidopsis database TAIR (<https://www.arabidopsis.org/>) and for peach using PpersicaCyc of PlantCyc (<http://www.plantcyc.org/>).

2.7. Correlation analysis of genes associated with auxin synthesis, conjugation and oxidation

Prunus persica genes corresponding to auxin biosynthesis and inactivation via conjugation and oxidation, were identified within the processed RNASeq data for all on-the-tree and postharvest storage ripening/treatments. Their normalized counts as computed by DESeq were extracted from the dataset and used for further analysis. The normalized counts were averaged for the different ripening stages followed by log₂ transformation. In addition, free-auxin and Asp- and Glu-auxin conjugates levels at the corresponding ripening stages on-the-tree and postharvest were loaded. Since, no hormone levels were measured for stage S2, data imputation was performed. All data was log₂ transformed. The log₂ transformed data was then used to estimate correlation coefficients and their corresponding p-values applying the Pearson product moment correlation. Genes were clustered together based on the Euclidean distance to each other. For the purpose of visualizing differential gene expression for the candidate genes, also the log₂ transformed fold-changes values and their corresponding adjusted p-values as computed by DESeq were extracted as well.

2.8. Statistical analysis

The software package JMP® (ver.10.0, SAS Institute) was used for the statistical analyses. For fruit ripening on-the-tree, a post-hoc Tukey's test was used to compare between cultivars (Santa Rosa and Sweet Miriam) and ripening-related stages (S3/S4, S4-I, S4-II, S4-III) using means of the 6 biological replications in all cases at an adjusted significance level for multiple hypotheses testing at $p \leq 0.05$. Additionally, for fruit ripening throughout postharvest storage, a post-hoc Tukey's test was used to compare between SR and SM cultivars, treatments (1-MCP, propylene and control) and time in postharvest storage using means of the 6 biological replications in all cases at an adjusted significance level for multiple hypotheses testing at $p \leq 0.05$.

3. Results

3.1. Ethylene metabolism and the response of SR and SM fruit to ethylene

Fruit from SR and SM cultivars were harvested at the S3/S4, S4-I, S4-II and S4-III stages of development (defined in Table 1; [19]). Fruit

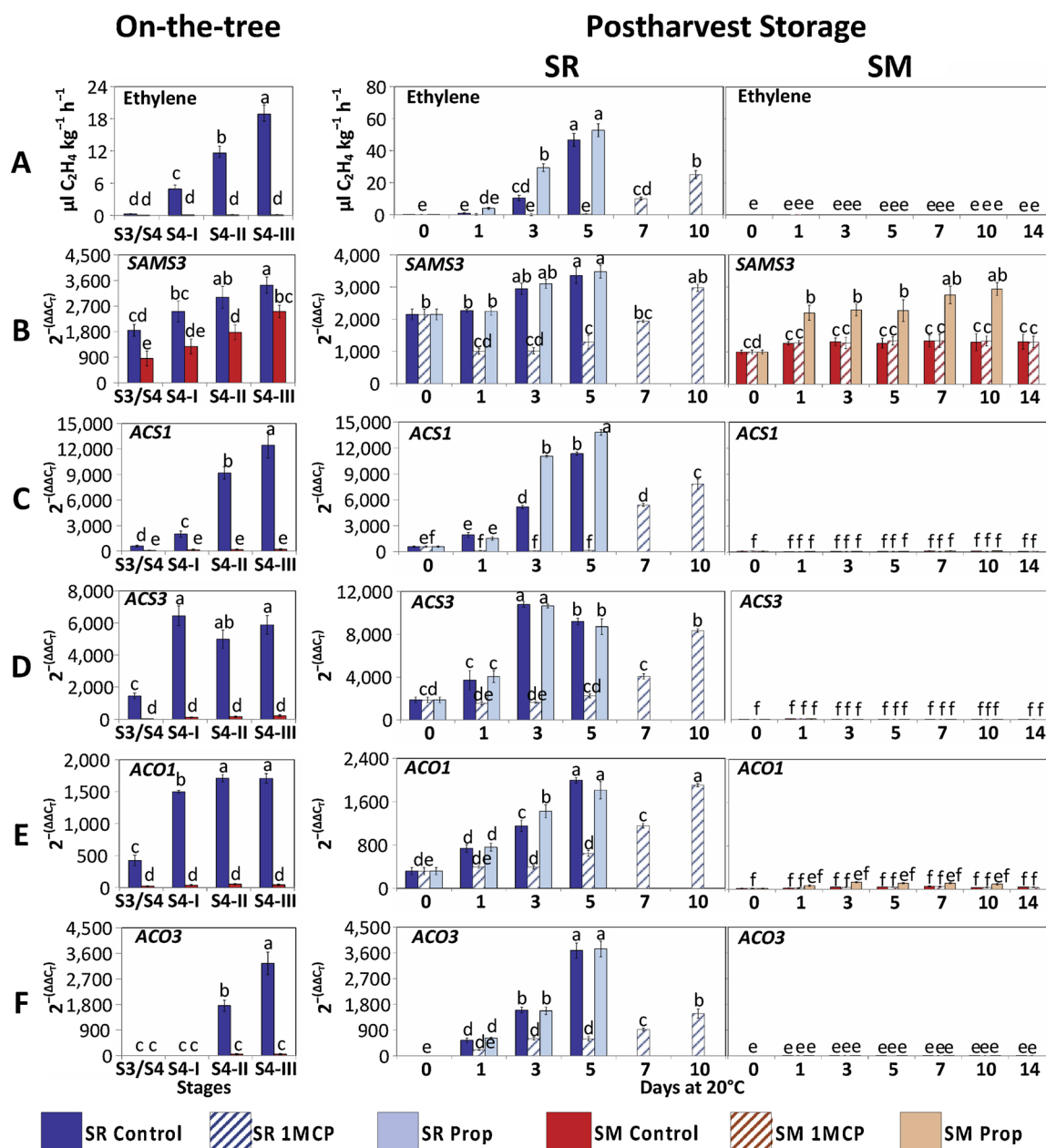


Fig. 1. Ethylene production levels and relative gene expression levels of ethylene biosynthesis-related genes in Santa Rosa (SR) and Sweet Miriam (SM) Japanese plum cultivars during ripening on-the-tree and throughout postharvest storage at 20 °C. Measurements were made throughout S3/S4 (mature), S4-I (commercial harvest), S4-II (fully-ripe), S4-III (overripe) stages on-the-tree; SR (blue bars) and SM (red bars). Throughout postharvest storage (right panel) ethylene production and relative gene expression levels were determined in SR fruit (left graph) and SM fruit (right graph) submitted to: No treatment (SR control, blue bars; SM control, red bars), 1-MCP treatment (SR 1-MCP, dashed light blue bars; SM 1-MCP, dashed light red bars) and propylene treatment (SR, light blue bars; SM, light red bars) after 0,1,3,5,7,10, and 14 d at 20 °C. (A) Ethylene production; (b) *SAMS3* (S-adenosyl-L-methionine synthase), (C) *ACS1* (1-aminocyclopropane-1-carboxylic acid synthase), (D) *ACS3* (1-aminocyclopropane-1-carboxylic acid synthase), (E) *ACO1* (1-aminocyclopropane-1-carboxylic acid oxidase) and (F) *ACO3* (1-aminocyclopropane-1-carboxylic acid oxidase). Values are the Mean \pm SE (n = 6). Different letters indicate significant differences ($p \leq 0.05$) according to posthoc Tukey's test (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

firmness, the main maturity index [19,20], decreased from ~ 40 N to ~ 7.5 in SR and ~ 16.6 in SM (Table 1). SM fruit took ~ 42 , ~ 83 , ~ 100 and ~ 117 more days to reach S3/S4, S4-I, S4-II and S4-III stages than SR fruit, respectively (Table 1). Fruit from both cultivars were additionally harvested at the “well-mature” stage [44], with a flesh firmness of ~ 37 N, that was reached ~ 112 and ~ 170 d after full

bloom (DAFB) in SR and SM, respectively (Table 1). These fruit were treated immediately after harvest with propylene and 1-MCP and kept in postharvest storage at 20 °C for a maximum of 14 d, as described before [41]. Climacteric SR fruit ripening on-the-tree, as well as control fruit ripening in postharvest storage, maintained higher respiration rates than SM fruit, displaying the typical respiratory burst [41]. SR

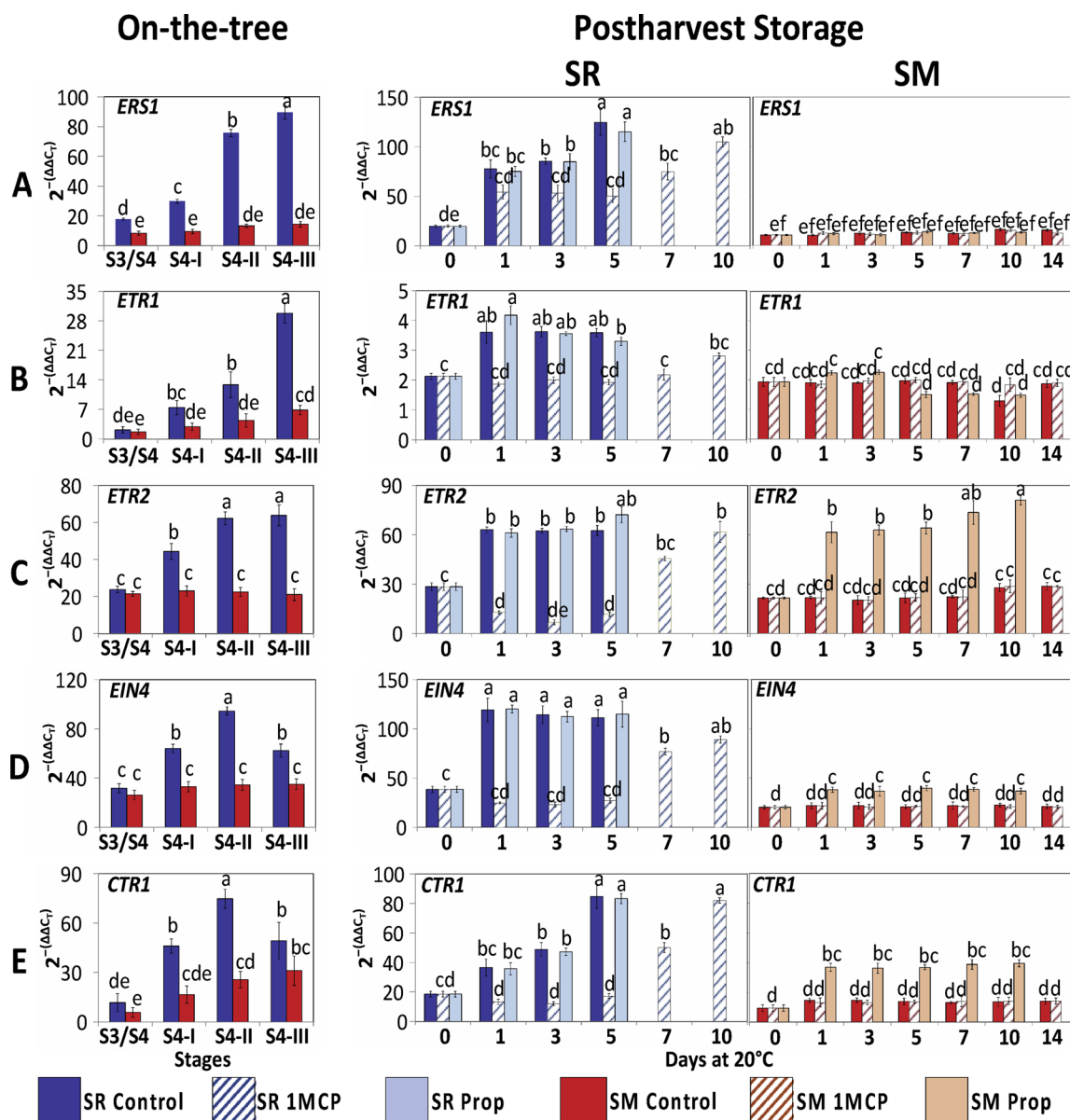


Fig. 2. Relative gene expression levels of ethylene perception-associated genes. SR (blue bars) and SM (red bars). Throughout postharvest storage (right panel) relative gene expression were determined in SR (left graph) and SM (right graph) cultivars submitted to: No treatment (SR control, blue bars; SM control, red bars), 1-MCP treatment (SR 1-MCP, dashed light blue bars; SM 1-MCP, dashed light red bars) and propylene treatment (SR, light blue bars; SM propylene, light red bars) after 0, 1, 3, 5, 7, 10, and 14 d at 20 °C. (A) *ERS1* (Ethylene response sensor 1), (B) *ETR1* (Ethylene receptor 1), (C) *ETR2* (Ethylene receptor 2), (D) *EIN4* (Ethylene insensitive 4) and (E) *CTR1* (Constitutive triple response 1). Values are the Mean \pm SE (n = 6). Different letters indicate significant differences (p \leq 0.05) according to posthoc Tukey's test (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

fruit presented increased ethylene production rates during ripening while non-climacteric SM fruit displayed constant and lower ethylene production rates throughout development on-the-tree and during postharvest storage (Fig. 1A).

3.1.1. Ethylene biosynthesis-associated genes

S-adenosyl-L-methionine (SAM), the precursor of ethylene, is synthesized from methionine and ATP in a reaction catalyzed by S-adenosyl-L-methionine synthase (SAMS) [54]. Although *SAMS3* expression increased in both SR and SM fruit during ripening on-the-tree,

transcript levels were consistently lower in SM than in SR. An increase in *SAMS3* expression was also detected in the SR fruit control and propylene treated fruit up to 5 d into postharvest storage (Fig. 1B). *SAMS3* expression in SM fruit increased upon treatment with propylene (Fig. 1B) and decreased initially in SR fruit upon treatment with 1-MCP up to 5 d into postharvest storage, followed by a significant increase thereafter (Fig. 1B). The biosynthesis of ethylene results from the conversion of SAM to 1-aminocyclopropane-1-carboxylate (ACC) through the action of ACC synthase (ACS) followed by the oxidation of ACC to ethylene, catalyzed by ACC oxidase (ACO) [11,55]. The

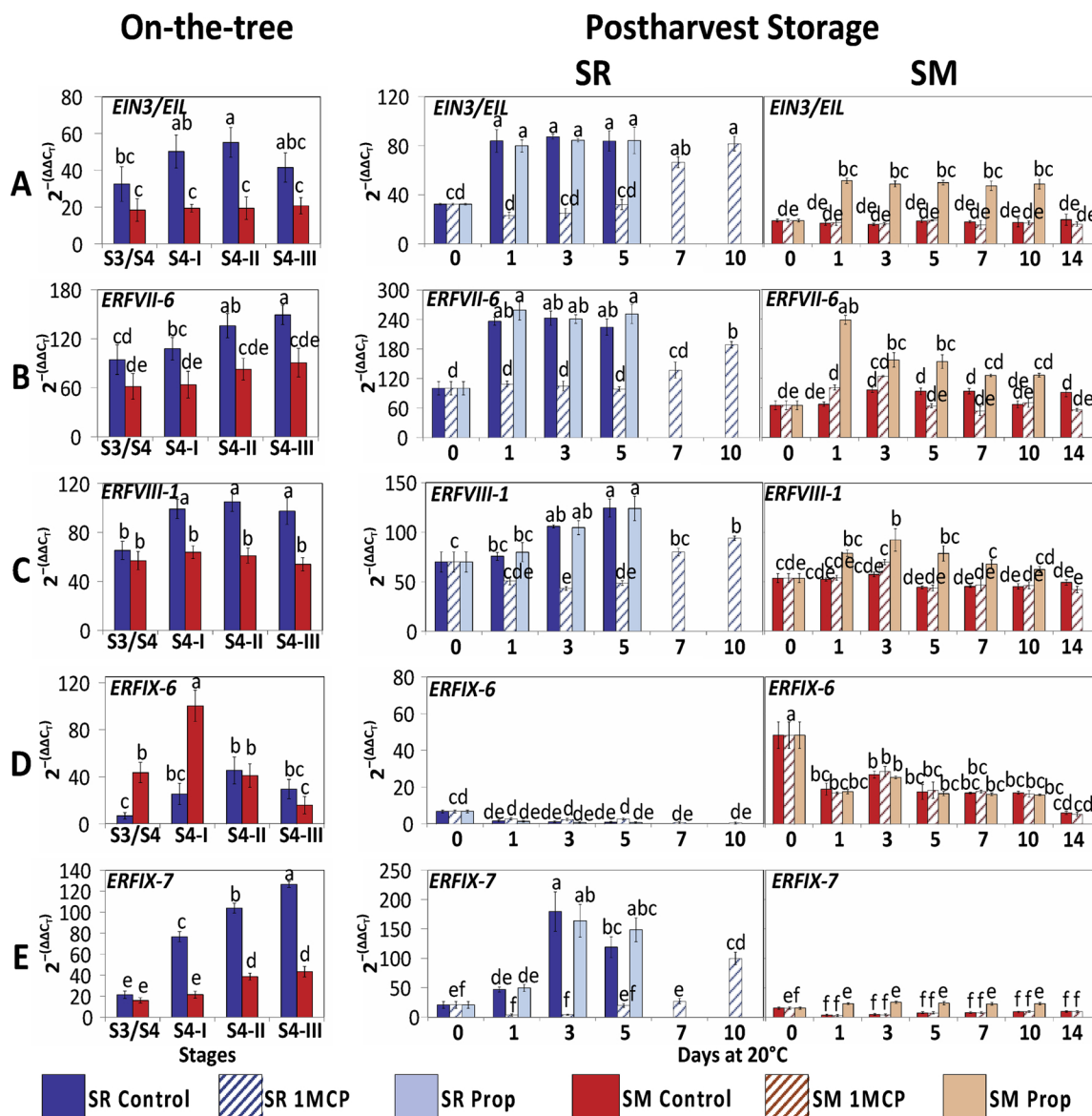


Fig. 3. Relative gene expression levels of ethylene signaling-related genes. SR (blue bars) and SM (red bars). Throughout postharvest storage (right panel) relative gene expression were determined in SR (left graph) and SM (right graph) cultivars submitted to: No treatment (SR control, blue bars; SM control, red bars), 1-MCP treatment (SR 1-MCP, dashed light blue bars; SM 1-MCP, dashed light red bars) and propylene treatment (SR, light blue bars; SM propylene, light red bars) after 0, 1, 3, 5, 7, 10, and 14 d at 20 °C. (A) *EIN3/EIL* (Ethylene insensitive-like gene), (B) *ERFVII-6* (Ethylene response factor), (C) *ERFVIII-1* (Ethylene response factor), (D) *ERFIX-6* (Ethylene response factor), and (E) *ERFIX-7* (Ethylene response factor). Values are the Mean \pm SE (n = 6). Different letters indicate significant differences ($p \leq 0.05$) according to posthoc Tukey's test (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

expression of *ACS1*, *ACS3*, *ACO1* and *ACO3* increased in SR fruit on-the-tree and throughout postharvest storage (Fig. 1C–F), following a similar pattern as observed for ethylene production (Fig. 1A). On the other hand, both ethylene and the expression of the above-mentioned genes, were barely detectable in SM fruit (Fig. 1A, C–F). Treatments with propylene did not affect these results, while the exposure to 1-MCP induced a significant decrease in the expression of these genes in SR fruit (Fig. 1C–F).

3.1.2. Ethylene perception and signaling-associated genes

Once ethylene is synthesized, it is perceived by a family of copper-

binding membrane-associated receptors with ethylene-binding capacity [12]. We assessed the expression levels of four ethylene receptors, including *ERS1* (ethylene-response sensor), *ETR1*, *ETR2* (ethylene receptor-types), *EIN4* (ethylene-insensitive) and *CTR1* - a Raf-like serine/threonine-protein kinase – that interacts with ethylene receptors [56]. In SR fruit, a general increase of expression of *ERS1*, *ETR1*, *ETR2*, *EIN4* and *CTR1* during ripening on-the-tree and postharvest storage was detected (Fig. 2A–E). In SM fruit, with exception of *ETR1* and *CTR1* (Fig. 2B, E), the expression levels of the ethylene receptors remained unchanged (Fig. 2A, C, D). Propylene treatments did not affect significantly the expression of the ethylene receptors in SR (Fig. 2A–E), but

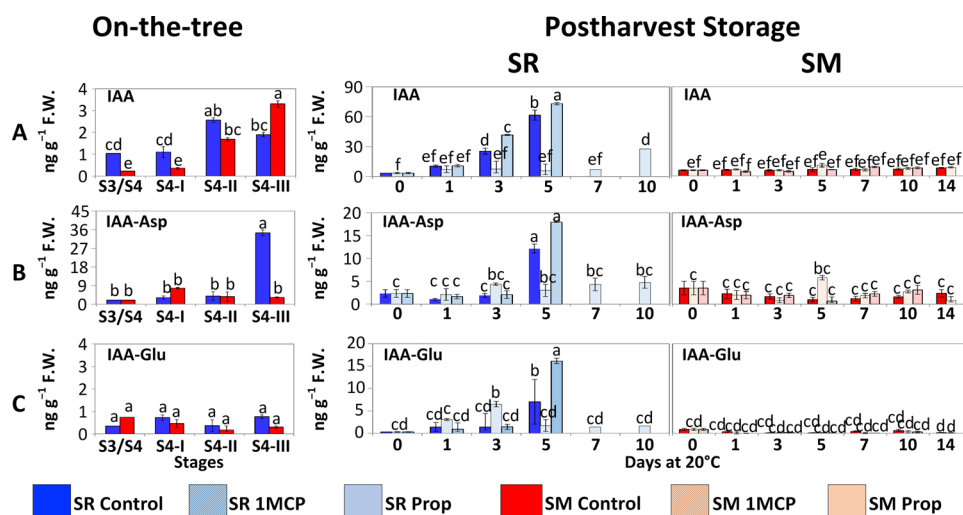


Fig. 4. Levels of free-IAA (A), IAA-Asp (B), IAA-Glu (C) in SR and SM Japanese plum cultivars during ripening on the tree and throughout postharvest storage at 20°C. SR (blue bars) and SM (red bars) cultivars on-the-tree. Throughout postharvest storage (right panel) IAA, IAA-Asp and IAA-Glu levels were determined in fruits from SR (left graph) and SM (right graph) cultivars submitted to: No treatment (SR control, blue bars; SM control, red bars), 1-MCP treatment (SR 1-MCP, dashed light blue bars; SM 1-MCP, dashed light red bars) and propylene (SR propylene, light blue bars; SM propylene, light red bars) after 0, 1, 3, 5, 7, 10, and 14 days at 20°C. Values are the Mean \pm SE (n = 6). Different letters indicate significant differences ($p \leq 0.05$) according to posthoc Tukey's test (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

significantly increased the expression of *ETR2*, *EIN4* and *CTR1* in SM fruit (Fig. 2C–E). Following 1-MCP treatments, the expression of the 5 tested genes decreased until 5 d of storage in SR fruit and increased afterwards, as the inhibitor action was released (Fig. 2), while in SM no significant changes were observed.

We evaluated ethylene signal transduction by assessing the expression of *EIN3/EILs* (ethylene-insensitive-like genes), transcription factors acting downstream of *CTR1* [2] and of ethylene response factors (ERFs) [12,57,58]. Expression levels of *EIN3/EILs* were higher in SR fruit than in SM fruit during most developmental stages on-the-tree and during post-harvest treatments (Fig. 3A), supporting the role of *EIN3/EILs* as positive regulators of ethylene responses in fruit [58,59]. *EIN3/EILs* mRNA level was induced in SM by propylene, while it decreased in SR by 1-MCP treatment (until 5 d) (Fig. 3A). In terms of ERFs, whose numbering system is based on [49], transcript levels of *ERFVII-6* and *ERFVIII-1* (Fig. 3B, C) and *ERFVIII-2* and *ERFVIII-4* (not shown) increased in SR and remained constant in SM fruit throughout ripening on-the-tree and postharvest storage with higher levels in SR than in SM fruit on-the-tree. *ERFIX-7* expression increased in both cultivars throughout ripening on-the-tree, with constant higher expression in SR than in SM (Fig. 3E). An increase in *ERFIX-7* expression was also detected in SR during postharvest, while 1-MCP treatment caused a significant reduction. In SM fruit, *ERFIX-7* transcript levels were low but appeared induced by propylene treatments (Fig. 3E). Noteworthy, the expression of *ERFIX-6* in SM was higher than in SR during the two earliest ripening stages assayed on-the-tree, with a significant peak in expression at stage S4-I (Fig. 3D). A very low and constant *ERFIX-6* expression was observed in SR during postharvest, while SM transcript levels tended to decrease throughout postharvest, lacked induction after propylene treatment, but presented higher expression levels than SR in all postharvest periods (Fig. 3D). In general, our results confirmed the notion of ERFs acting as positive regulators of ethylene-induced responses [60].

3.2. Auxin metabolism is affected by ripening behavior and ethylene treatments

Levels of IAA in SR fruit peaked towards S4-II and increased by about 20-fold during postharvest storage (Fig. 4A). In SM fruits, IAA

contents increased in stage S4-II on-the-tree and at stage S4-III they were higher than SR. During postharvest storage IAA levels were constant in SM fruit and significantly lower than in SR fruit after three days into postharvest storage (Fig. 4A). 1-MCP treated SR fruits did not increase their IAA levels at all stages tested, while the application of propylene had no significant effects, as expected by its climacteric nature (Fig. 4A). Auxin perception, measured through the expression of *TIR1* encoding an auxin receptor [61], paralleled IAA contents in SR fruit ripening-on-the tree and in both cultivars during postharvest storage (Fig. 4A, Fig. 8A). SM fruit ripening on-the-tree displayed opposing trends between IAA contents and *TIR1* expression levels (Fig. 4A, Fig. 8A). Auxin inactivation is regulated via oxidation (oxIAA) or via conjugation with amino acids, such as glutamate (IAA-Glu) or aspartate (IAA-Asp) [62,63]. In the current study, two auxin conjugates were detected; IAA-Asp and IAA-Glu (Fig. 4B, C). During fruit development on-the-tree, the contents of IAA-Glu and IAA-Asp remained constant in both cultivars, with exception of SR fruit at stage S4-III (override), where a large increase in IAA-Asp was observed. During post-harvest storage, IAA-Asp and IAA-Glu contents increased in SR fruit (Fig. 4B, C). 1-MCP treatments induced the increase of IAA-Glu conjugates after 3 d of storage in SR fruit, while propylene treatments increased IAA conjugates contents after 5 d of storage only in SR fruit (Fig. 4B, C).

We assessed the relationship between fruit free- and conjugated-auxin and the expression of genes associated with IAA metabolism (synthesis and inactivation) in SR and SM transcriptomes that were analyzed during stage S2 and stage S4-II on-the-tree [19], during postharvest storage and during propylene and 1-MCP treatments. IAA may be synthesized via four different tryptophan-dependent pathways: i) the Tryptamine pathway (TAM), ii) the indole-3-acetaldoxime pathway (IAOx), iii) the indole-3-acetamide pathway (IAM) and iv) the indole-3-pyruvic acid pathway (IPyA) [64]. A total of 24 genes, putatively associated with IAA biosynthesis and its inactivation, were identified (Table S2), of which 13 genes (shaded cells within Table S2) showed differential expression levels in SR and SM fruit during on-the-tree development and postharvest storage. To identify patterns of coordinated behavior, a pairwise-correlation analysis between the 13 identified genes, the fruit contents of free-IAA and the detected IAA-conjugates, IAA-Glu and IAA-Asp, was carried out across all available RNA-Seq data stages (Fig. 5, Fig. S3). The resulting correlation

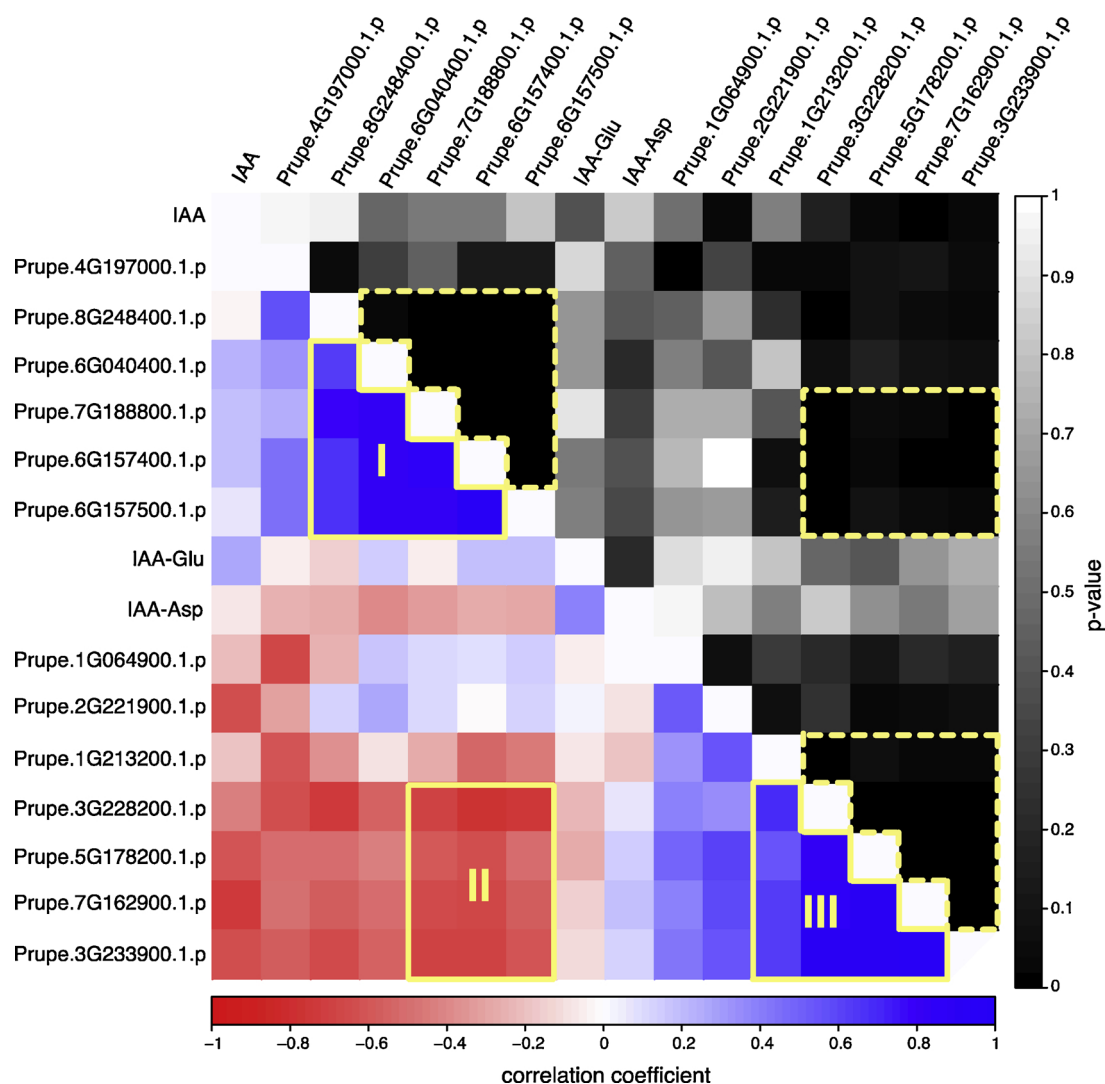


Fig. 5. Correlation analysis of genes putatively involved in auxin synthesis and conjugation/oxidation. Heatmap representation of the correlation analysis of 13 genes associated with auxin synthesis and oxidation/conjugation and IAA, IAA-Asp, and IAA-Glu levels. The lower triangle illustrates the correlation coefficients, where a red rectangle represents a negative correlation and a blue rectangle a positive correlation. The upper triangle (shaded area) represents the corresponding p-values. Variables on the x and y axes are ordered as determined by hierarchical clustering. Highly correlated variables (clusters) are highlighted within. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

coefficients were ordered according to hierarchical clustering. The analysis revealed two clusters of genes of strong positive correlations to each other (marked as I and III, Fig. 5), while the expression of genes of cluster I were negatively correlated to the expression of genes of cluster III (cluster II, Fig. 5). A positive trend (positive correlations) of free IAA was detected with genes of cluster I, while a negative trend was detected with genes of cluster III. Similar trends, although less significant according to corresponding p-values, were recorded for IAA-Glu. A negative trend (albeit less significant) of IAA-Asp content was detected with genes of cluster I, while a positive trend was detected with cluster III genes.

A schematic overview of the genes in clusters I and III and their involvement in IAA synthesis is presented in Fig. 6, emphasizing a putative ‘either/or’ mechanism in plum fruits of the IAOx versus the IPyA IAA synthesizing pathways. Two of the five genes of cluster I are

directly associated with the IPyA Trp-dependent IAA synthesis pathway (red arrows); *Prupe.6G157400.1.p* and *Prupe.6G157500.1.p*, encoding YUC/YUCCA, that regulate the conversion of IPyA to IAA (Fig. 6); while two genes correlating strongly to the YUC/YUCCA genes are involved in auxin regulation [64], namely *Prupe.7G188800.1*, encoding DAO1 that mediates IAA oxidation and *Prupe.8G248400.1.p*, encoding GH3.17 that regulate IAA conjugation. The fifth gene within the cluster, *Prupe.6G040400.1.p*, encodes a trans-cinnamate 4-monooxygenase, whose function has not been yet determined. The four genes of cluster III are associated with the IAOx-routed Trp-dependent pathways (blue arrows in Fig. 6); *Prupe.3G228200.1.p* encoding a Trp decarboxylase that mediates the conversion of L-Trp to Tryptamine (TAM) [64]; *Prupe.7G162900.1.p*, coding for CYP79B2/CYP79B3, regulating the conversion of L-Trp to IAOx; *Prupe.5G178200.1.p*, encoding CYP71A13 that mediates the conversion of IAOx to IAN; and *Prupe.3G233900.1.p*,

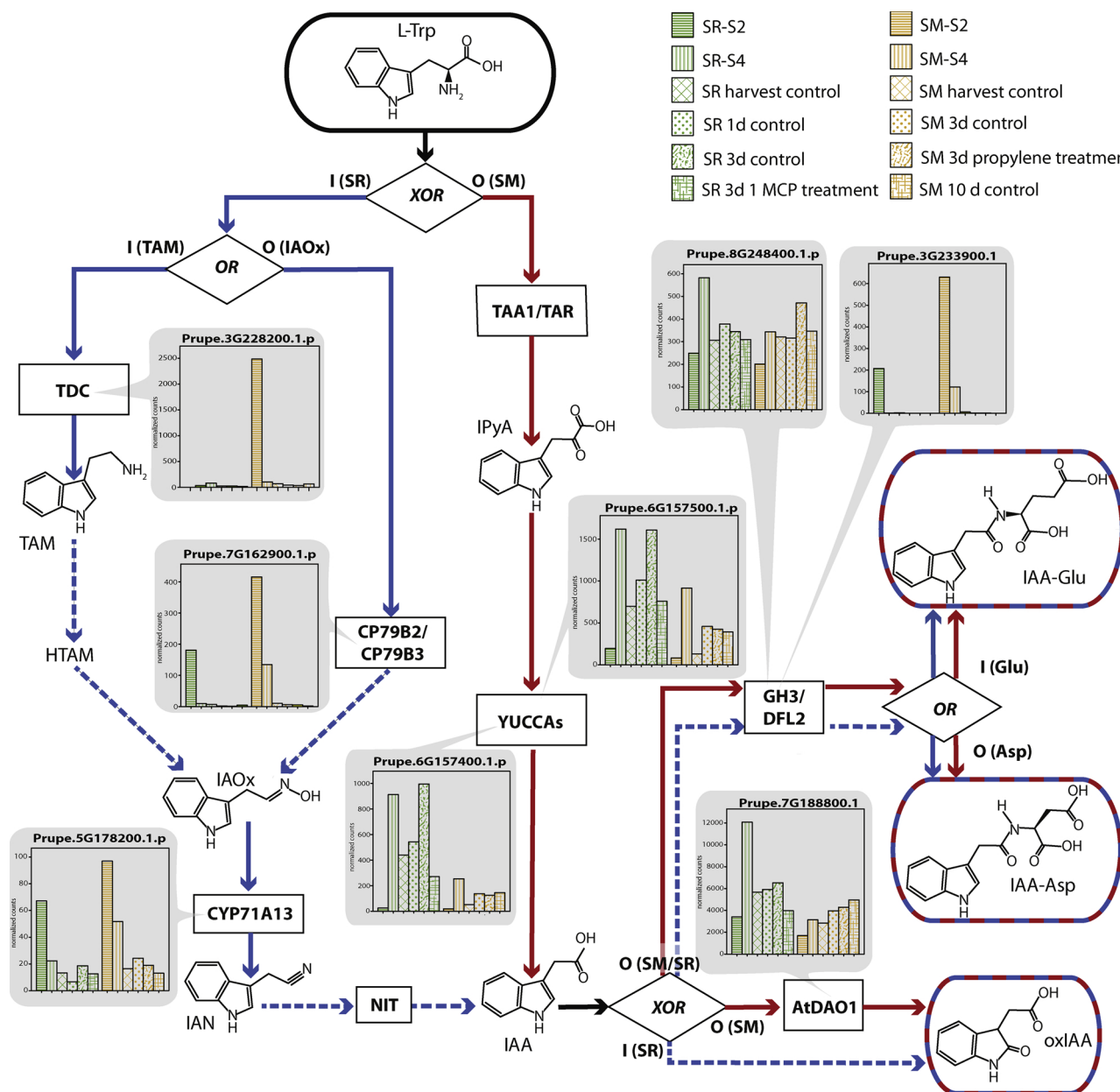


Fig. 6. Schematic overview of the detected genes in plum that are involved in the Trp-dependent auxin biosynthesis. Eight of the 13 genes associated with IAA synthesis and its oxidation/conjugation could be directly linked to Trp-dependent pathways. The scheme highlights the IAOx (blue arrows) and IPyA-routed pathways (red arrows) and displays the normalized counts of genes associated with them. The x-axes represent the different on-the-tree and postharvest storage stages, where green bars correspond to SR and yellow bars to SM, in the following order: SR-S2, SR-S4, SR harvest control, SR 1 d control, SR 3 d control, SR 3 d 1-MCP treatment, SM-S2, SM-S4, SM harvest control, SM 3 d control, SM 3 d propylene treatment, SM 10 d control; the y-axes represent the normalized gene counts. A flowchart-type representation was chosen to emphasize the anti-correlative behavior of genes of the IAOx-routed pathway to the genes of the IPyA-routed pathway, revealing that ‘either/or’ but not both can be chosen (exclusive or [XOR]). Rounded rectangles represent endpoints of the IPyA- and IAOx-routed pathways (red and blue dashed lines) on the oxidation/conjugation of IAA. Solid and dashed arrows indicate processes for which the genes have or have not been identified or for which gene function has yet to be determined, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

encoding DFL2, associated with IAA conjugation [65].

3.3. Response of other hormones to ethylene treatments

In contrast to ethylene and IAA, ABA levels in SR fruit remained constant during ripening on-the-tree (Fig. 7A), while during postharvest

storage ABA peaked at 3 d of storage and decreased afterwards (Fig. 7A). SM fruit displayed an increase in ABA levels during ripening on-the-tree and throughout postharvest (Fig. 7A). Upon propylene treatments, SM fruit exhibited considerable increase in ABA contents reaching 2 to 3-fold higher levels than SR and SM control fruit (Fig. 7A). The application of 1-MCP delayed the appearance of the ABA peak from

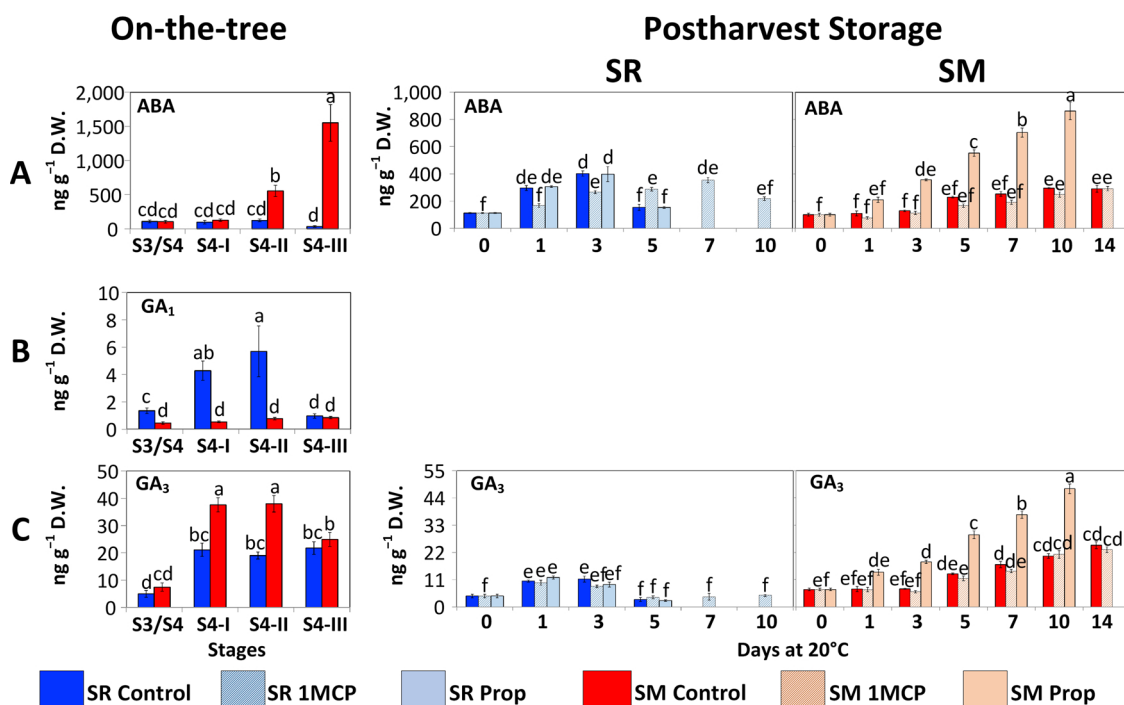


Fig. 7. Hormone levels of SR and SM cultivars during ripening on-the-tree and throughout postharvest storage at 20 °C. During ripening on the tree (left panel) hormone levels were determined in SR (blue bars) and SM (red bars) fruit throughout S3/S4 (mature), S4-I (commercial harvest), S4-II (fully-ripe), S4-III (overripe) stages on-the-tree. Throughout postharvest storage (right panel) hormone levels were determined in SR (left graph) and SM (right graph) cultivars submitted to: No treatment (SR control, blue bars; SM control, red bars), 1-MCP treatment (SR, dashed light blue bars; SM, dashed light red bars) and propylene treatment (SR, light blue bars; SM, light red bars) after 0, 1, 3, 5, 7, 10, and 14 days at 20 °C. (A) Abscisic acid (ABA); (B) Gibberellins (GA₁) and (C) Gibberellins (GA₃). Values are the Mean \pm SE (n = 6). Different letters indicate significant differences ($p \leq 0.05$) according to posthoc Tukey's test (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3 to 7 d in SR fruit and decreased ABA contents until 3 d of storage (Fig. 7A). The expression of *NCEDs* (*NCED2*, *NCED3*), coding for an enzyme mediating the rate-limiting step in ABA biosynthesis [40] was paralleled with ABA levels of each cultivar (Fig. 8B, C) on-the-tree and during post-harvest treatments.

Three active forms of gibberellins (GA) have been identified in *Rosacea*, GA₁, GA₃ and GA₄ [66,67]. GA₁ and GA₃ were identified in SR and SM fruit on-the-tree while GA₃ was also detected throughout postharvest storage (Fig. 7B, C); GA₄ was not detected, neither in SR nor SM fruit at any stage. GA₁ levels increased towards S4-II in SR fruit while GA₁ levels were low in SM fruit on-the-tree (Fig. 7B). The decrease in GA₁ levels at S4-III in SR fruit was paralleled by the high expression of *GA2ox* (Fig. 8D), encoding an enzyme converting active GA forms to inactive forms through hydroxylation [68]. GA₁ was not detected throughout the postharvest storage of SR and SM fruit, suggesting that its role was associated to fruit on-the-tree. GA₃ was the most abundant GA form in both SR and SM fruit. GA₃ contents were higher in SM than in SR fruit, increased during postharvest storage of SM fruit and upon propylene treatment. These statistically significant trends were paralleled by a decreased *GA2ox* expression.

The levels of *trans*-zeatin, an active form of cytokinin [69], and salicylic acid (SA) remained constant throughout postharvest for all treatments in both cultivars and only significantly increased throughout ripening on-the-tree in the case of SA (Fig. S2A, B). Cytokinin contents were higher in SM fruit than in SR fruit during ripening on-the-tree and postharvest storage (Fig. S2A) and these results were well paralleled

with the expression of *IPT5* and *CRE1* (Fig. S2C, D), encoding the rate-limiting enzyme in cytokinin biosynthesis and a cytokinin receptor, respectively [70,71]. The levels of SA were higher in SM than in SR fruit only throughout postharvest storage corresponding with the expression of *ICS* encoding isochorismate synthase (ICS) which mediates the synthesis of salicylic acid from chorismate [72] (Fig. S2E).

4. Discussion

In this study, we used 'Santa Rosa', a climacteric Japanese plum cultivar and 'Sweet Miriam', its non-climacteric bud-sport mutant [19,20,73] to characterize and compare the fruit hormone balance and the expression profiles of key hormone metabolism-associated genes as well as their response to ethylene and an inhibitor of ethylene perception. SM and SR fruit, sharing a common genetic background but displaying different ripening behavior, provided a unique tool to evaluate and compare climacteric and non-climacteric ripening behavior patterns and hormone interactions during fruit ripening on-the-tree and throughout postharvest storage.

4.1. Ethylene and non-climacteric fruit ripening

ACC synthase catalyzes the rate-limiting reaction in the ethylene biosynthetic pathway [74,75]. The considerably lower ethylene contents in the non-climacteric SM fruit during ripening on-the-tree and throughout postharvest storage were probably a consequence of the

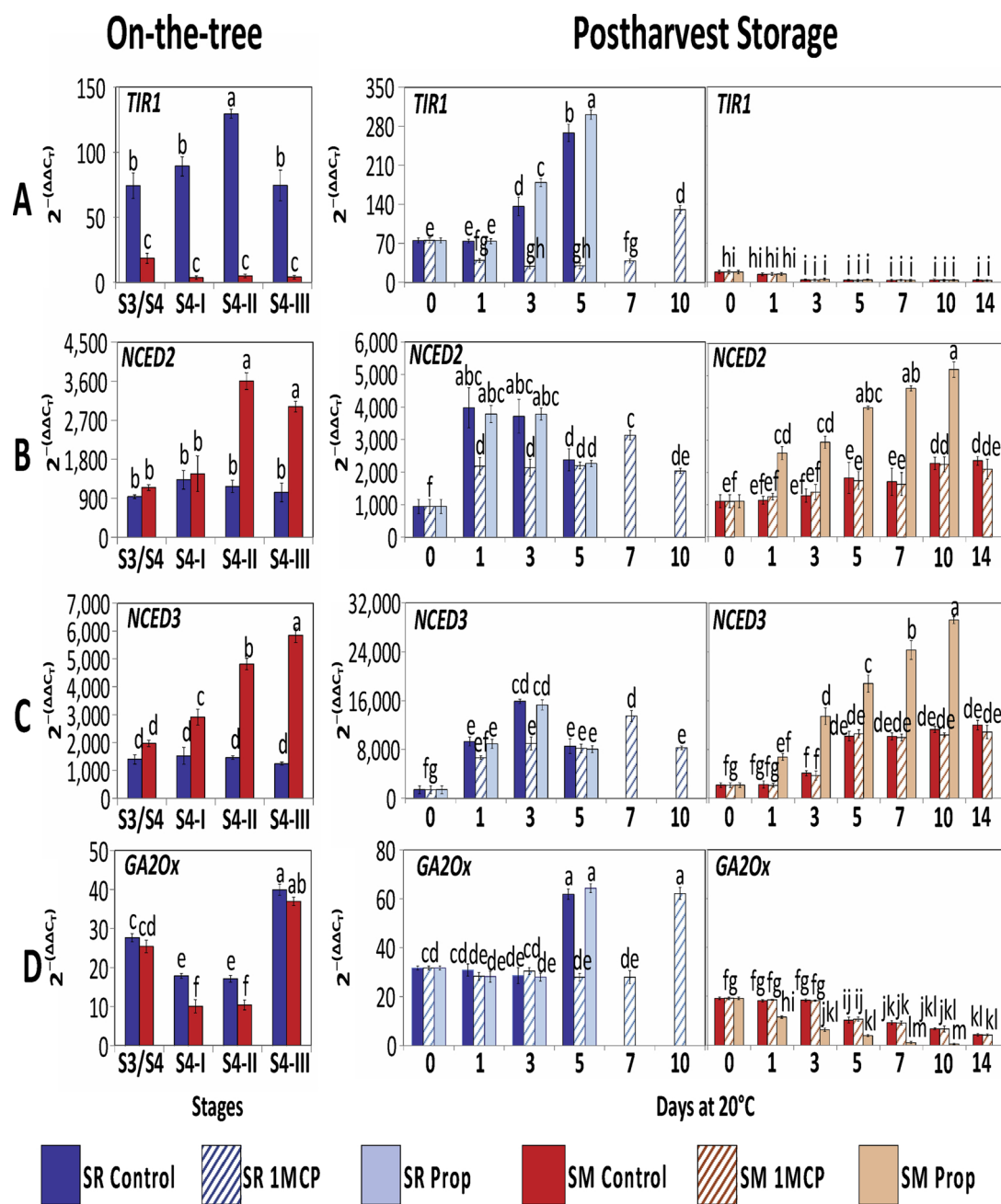


Fig. 8. Relative gene expression levels of genes associated with auxin perception and abscisic acid and gibberellins metabolism in SR and Sweet Miriam SM fruit during ripening on-the-tree and throughout postharvest storage at 20 °C. During ripening on-the-tree (left panel) relative gene expression levels were determined in SR (blue bars) and SM (red bars) fruit throughout S3/S4 (mature), S4-I (commercial harvest), S4-II (fully-ripe) and S4-III (overripe) stages. Throughout postharvest storage (right panel) relative gene expression levels were determined in fruit submitted to: No treatment (SR control, blue bars; SM control, red bars), 1-MCP treatment (SR, dashed light blue bars; SM, dashed light red bars) and propylene treatment (SR, light blue bars; SM, light red bars) after 0, 1, 3, 5, 7, 10, and 14 days at 20 °C. (A) *TIR1*; (B) *NCED2*; (C) *NCED3*; (D) *GA20x*. Values are the Mean ± SE (n = 6). Different letters indicate significant differences (p ≤ 0.05) according to posthoc Tukey’s test (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

decreased expression levels of *ACS* and *ACO* (Fig. 1), as both showed tight coordinated behaviors. In other non-climacteric fruit, such as peppers [76] no accumulation of *ACS* and *ACO* transcripts was observed during ripening. It was shown recently, using copy number variation analysis (CNV) [77] that the copy number of two *ACS* genes

was higher in SR than in SM, which may result in higher expression of these genes in the climacteric cultivar. SM also differed from suppressed-climacteric plum cultivars, where ethylene treatments restored their climacteric ripening [75,78,79]. Based on the above and on the System-1 and System-2 ethylene biosynthesis model [80], it suggested

that during ripening of climacteric SR fruit *ACS1*, *ACS3*, *ACO1* and *ACO3* involved in the maintenance of System-2 autocatalytic ethylene production stage due to their high transcript accumulation and responsiveness to ethylene treatments; with *ACO3* transitioning from System-1 to System-2 after S4-I in SR fruit. In SM fruits these genes are locked in System-1, as suggested to occur in non-climacteric fruit [12] due to their low expression levels and lack of response to ethylene treatments. Comparative analysis between the *SAMS3* expression patterns in SR control fruits and SM fruit treated with propylene (Fig. 1B) suggested that methionine synthesis (precursor of ethylene) was not affected in the SM fruit and that ethylene synthesis was the affected pathway, since the propylene treatments induced the increased expression of *SAMS3* in SM fruits, but did not affect the expression of genes encoding *ACS1*, *ACS3*, *ACO1* or *ACO3*.

Transcript levels of genes encoding for ethylene receptors increased during ripening of SR fruit, both on-the-tree and during postharvest storage (Fig. 2), similar to other climacteric fruit [81,82]. Based on the negative regulation model, increased presence of receptors reduce ethylene sensitivity [3,11]. The binding of ethylene to receptors triggers the degradation of the receptor protein, thus levels of receptor proteins drop and the suppression of the ethylene signaling pathway is removed allowing downstream responses to occur [11,82]. Our results support the notion that in climacteric SR fruit the increased expression of ethylene receptor genes is a homeostatic response to the high ethylene production rates in this fruit, thus modulating the ripening process. The above was also supported by the higher *CTR1* expression levels in SR (Fig. 2E). *CTR1* interacts downstream with ethylene receptors, acting as a negative regulator of ethylene responses. The increased *CTR1* transcript accumulation during ripening and its response to ethylene treatments has been also reported in other species [83–85]. *ETR2* and *EIN4*, encoding type-II ethylene receptors with a weaker affinity to *CTR1* [86,87], were the most abundantly expressed in SM during ripening on-the-tree and throughout postharvest (Fig. 2C, D). Moreover, similarly to what was observed in non-climacteric strawberries [17], *ETR2* and *EIN4* were the only receptors, together with *CTR1*, displaying increased transcription after propylene treatments in SM fruit. These results, together with the overall reduced expression of genes encoding receptors of SM as compared to SR, suggest a higher sensitivity to ethylene of the non-climacteric SM fruit, where the low amounts of ethylene produced could be enough to trigger the release of *CTR1* and induce ethylene-related physiological responses.

Although *EIN3/EILs* and *ERFs* expression levels were lower in SM than in SR fruit, they were expressed during ripening on-the-tree and throughout postharvest (Fig. 3), indicating that the ethylene-mediated signaling pathways are well conserved in the non-climacteric SM fruit. Nevertheless, the differential *ERF6* and *ERF7* expression in SR and SM plum fruit would indicate that ethylene is not the only regulator of *ERFs* expression (Fig. 3), in agreement with what was observed during the ripening of a climacteric plum [88].

4.2. Climacteric fruit ripening is associated with auxin synthesis

High auxin levels have been shown to be associated with climacteric fruit ripening and the external application of auxin enhanced ripening in peach and plum [31,34,37]. Non-climacteric fruit like strawberry [32] and grape [30] displayed low auxin levels during ripening, and the application of IAA during the pre-ripening stage delayed fruit ripening [89]. Consistent with these observations, SR fruit displayed higher IAA contents than SM fruits during the mature (S3/S4) and commercial harvest (S4-I) stages of ripening on-the-tree and remarkably higher

levels during postharvest storage. In agreement with previous work [90], the high auxin contents of the climacteric fruit were correlated with increased *TIR1* expression. The interactions between auxin and ethylene during fruit ripening appear to depend on the fruit species under study. In some climacteric fruit such as tomato [91] and apple [92], inverse ethylene and auxin levels were reported during fruit ripening. On the other hand, a positive correlation between ethylene and auxin production was reported in stone fruit such as peach [31,35,37] and climacteric plum [34]. Treatments with 1-MCP blocked the increase in IAA content of SR fruit supporting these observations. The increase in IAA-Asp conjugate contents during the S4-III stage in SR fruit during ripening on-the-tree, was not correlated with an increase in IAA contents (Fig. 4B), suggesting an altered IAA metabolism due to the overripe stage of the fruit.

A correlation analysis of the expression of genes associated with IAA metabolism revealed the existence of two highly correlated clusters. Cluster I comprised two genes directly involved in the IPyA Trp-dependent IAA biosynthesis pathway and two genes regulating the conjugation and oxidation of IAA. The relatively higher expression of genes associated with the IPyA-mediated Trp-dependent IAA synthesis pathway, at all fruit development stages on-the-tree and ripening in postharvest storage, hinted at its predominance in SM fruit (Fig. S3). In addition, these genes negatively correlated to IAA-Asp fruit contents. Consequently, during postharvest storage, the relatively low IAA synthesis together with the continuous IAA-Asp formation resulted in an overall low free IAA content in SM fruit. Cluster III, on the other hand, comprises four genes associated with the IAOx-mediated Trp-dependent IAA synthesis pathway [93] and IAA oxidation (IAOx – Fig. 6). Elevated expression levels of these genes were particularly associated across all postharvest stages of SR fruit (Fig. S3). The positive trends of genes in cluster III with IAA-Asp contents suggested an opposite mode of action in SR than in SM fruit, i.e. high IAA synthesis and IAA-Asp conjugation, resulting in an overall high free IAA content in SR fruit. The strong negative correlations between the expression of genes of Cluster I versus genes of Cluster III (Fig. 5) could indicate a limitation on their simultaneous activation; an ‘either/or’ regulation of the IAOx- and IPyA-Trp-dependent IAA synthesis pathways.

4.3. Hormone balance in non-climacteric fruit

ABA has been reported to positively regulate ripening in both climacteric and non-climacteric fruit [38,40]. This notion was supported by the ABA increase in SR fruit during postharvest storage and in SM fruit at the S4-III stage and following propylene treatments - increases that were paralleled by *NCED2* and *NCED3* expression. The increase in ABA contents during ripening was also observed in other non-climacteric fruit [27,29,94].

GA₁ and *GA₃* were the main GA forms detected in both SR and SM fruit. *GA₁* contents were high in maturing SR fruit consistent with *GA₁* role in promoting ripening [67]. Interestingly, *GA₁* was not detected in SR nor SM fruit during postharvest storage. On the other hand, *GA₃* contents were higher in SM than in SR fruit during ripening on-the-tree and during postharvest storage. The effects of propylene treatment on *GA₃* contents in SM fruit, together with the marked decrease in expression of *GA2ox* expression suggested a positive effect of ethylene on *GA₃* formation in the non-climacteric fruit. The increase in *GA₃* together with the higher contents of *transZeatin* and SA in SM fruit correspond with the role(s) of these hormones in delaying ripening in non-climacteric fruit [95–98].

In summary, we assessed hormonal contents and the expression of

key genes, associated with hormone metabolism, in climacteric SR and non-climacteric SM fruits during ripening on-the-tree and throughout postharvest storage as well as in response to ethylene treatments. The low ethylene production in SM fruit was the result of almost undetectable expression of genes associated with ethylene biosynthesis, since perception and ethylene signaling-related genes were actively expressed and responded to ethylene treatments. Auxin contents enhanced fruit ripening, with SM fruit showing significant lower IAA contents than SR at the initiation of ripening on-the-tree and after most of post-harvest storage. Our data showed a positive relationship between auxin and ethylene as supported by the low IAA contents when ethylene perception was inhibited in the climacteric SR fruit throughout postharvest storage. The differences in auxin contents between SR and SM fruit could be the consequence of different routed auxin biosynthesis pathways. Ethylene induced increased ABA levels throughout postharvest storage in both ripening types. Overall, ripening of SR and SM fruit are characterized by distinct hormone accumulation pathways and interactions.

Author contributions

MF and EB designed the experiments; MF, NS, RMR, EN and AS conducted the experiments; AF, DT and MF analyzed the expression data; DT, MF, EN, AS and EB wrote the article.

Funding information

This work was supported by the Will W. Lester Endowment from the University of California.

Declaration of interests

The authors declare no conflict of interests.

Acknowledgments

We thank Ayako Nambara (University of Toronto) for her technical support in the measurements of IAA-conjugates and Dr. Swen Koenig for technical assistance. M.F. was a fellowship recipient from Programa Formacion de Capital Humano Avanzado, CONICYT, Chile. This work was supported by the Will W. Lester Endowment from the University of California.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.plantsci.2018.11.001>.

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