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Non-climacteric ripening and sorbitol homeostasis in plum fruits

Ho-Youn Kim[a], Macarena Farcuh[a], Yuval Cohen[b], Carlos Crisosto[a], Avi Sadka[b], Eduardo Blumwald[a,*]

[a] Department of Plant Sciences, University of California, Davis, CA 95616, USA
[b] Department of Fruit Tree Sciences, Institute of Plant Sciences, A.R.O. Volcani Center, PO Box 6, Bet Dagan 50250, Israel

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ABSTRACT

During ripening fruits undergo several physiological and biochemical modifications that influence quality-related properties, such as texture, color, aroma and taste. We studied the differences in ethylene and sugar metabolism between two genetically related Japanese plum cultivars with contrasting ripening behaviors. 'Santa Rosa' (SR) behaved as a typical climacteric fruit, while the bud sport mutant 'Sweet Miriam' (SM) displayed a non-climacteric ripening pattern.

SM fruit displayed a delayed ripening that lasted 120 days longer than that of the climacteric fruit. At the full-ripe stage, both cultivars reached similar final size and weight but the non-climacteric fruits were firmer than the climacteric fruits. Fully ripe non-climacteric plum fruits, showed an accumulation of sorbitol that was 2.5 times higher than that of climacteric fruits, and the increase in sorbitol were also paralleled to an increase in sucrose catabolism. These changes were highly correlated with decreased activity and expression of NAD^+-dependent sorbitol dehydrogenase and sorbitol oxidase and increased sorbitol-6-phosphate dehydrogenase activity, suggesting an enhanced sorbitol synthesis in non-climacteric fruits.

1. Introduction

Japanese plums [*Prunus salicina* Lindl.] belong to the genus *Prunus* of the family *Rosaceae* and include most commercial fresh-market plums worldwide [1]. In addition to their wide environmental adaptation, there is also a high variability of Japanese plum cultivars with respect to their fruit developmental and ripening patterns [2].

Traditionally, fruit ripening has been defined as either climacteric or non-climacteric. Climacteric fruits are characterized by increased levels of autocatalytic ethylene production and respiration rates during ripening. Once ripening is initiated, fruits continue to ripen past harvest. Non-climacteric fruits show no increase or autocatalytic ethylene production or respiration rates during ripening [3–5]. Although Japanese plums have been classified as climacteric fruits, there are differences in ripening patterns among cultivars [2,6]. While some cultivars, such as 'Santa Rosa' are climacteric [7]; other cultivars, such as 'Shiro' or 'RubyRed' have suppressed-climacteric ripening [8]. Lower ethylene production during ripening and a reduced respiratory peak characterize the latter. Nevertheless, when these cultivars are exposed to exogenous ethylene, typical climacteric ripening is restored [9]. These variations in ethylene production rates among plum cultivars result from differences in their capacity to synthetize ethylene as well as to respond to ethylene, due to differences in ethylene perception and signal transduction pathways [6].

Ethylene biosynthesis requires the activities of two key enzymes: 1-aminocyclopropane-1-carboxylic acid synthase (EC 4.4.1.14, ACS), which mediates the synthesis of 1-amino-cyclopropane-1-carboxylic acid (ACC), and 1-amino-cyclopropane-1-carboxylic acid oxidase (EC 4.4.17.4, ACO), which produces ethylene from ACC [10,11]. Once synthesized, ethylene interacts with a family of membrane-bound receptors (ETR and ERS) [12] that in the absence of the hormone, actively suppress ethylene responses [13]. Upon ethylene binding, the response's suppression is removed, and the signal is transmitted into the nucleus and consequently amplified by a transcription factor cascade, which

Abbreviations: ACO, 1-amino-cyclopropane-1-carboxylic acid oxidase; CA, control air; CP, control propylene; CWI, CI, VI, cell wall,, cytosolic and vacuolar invertase, respectively; DAFB, day after full bloom; EOL, ethylene-overproducer-like; ERF, ethylene response factor; ETR, ethylene receptor; MA, 1-MCP air; MP, 1-MCP propylene; NAD^+-SDH, NAD^+-dependent sorbitol dehydrogenase; SM, Sweet Miriam; SOT, sorbitol transporter; SOX, sorbitol oxidase; S6PDH, sorbitol-6-phosphate dehydrogenase; SPS, sucrose phosphate synthase; SR, Santa Rosa; Susy, sucrose synthase.

* Corresponding author at: Department of Plant Sciences – Mail Stop 5, One Shields Avene, Davis, CA 95616, USA. Tel.: +1 530 752 4640; fax: +1 530 752 2278.
E-mail addresses: jsakim@ucdavis.edu (H.-Y. Kim), mfarcuh@ucdavis.edu (M. Farcuh), vhyuvalc@volcani.agri.gov.il (Y. Cohen), chcrisosto@ucdavis.edu (C. Crisosto), vhasadka@volcani.agri.gov.il (A. Sadka), eblumwald@ucdavis.edu (E. Blumwald).

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includes Ethylene Insensitive (EIN) and EIN-like-proteins (EILs) [6,10,14]. Finally, members of the AP2/ERF transcription factor family, which include ERFs (Ethylene Response Factors), are involved in a feedback loop that stimulates autocatalytic ethylene synthesis [13] and bind *cis*-elements found in the promoters of target genes, modulating their transcription [15] and thereby inducing downstream ethylene responses that lead to fruit ripening [10].

Japanese plum ripening, like that of other fleshy fruits, is a complex and highly coordinated developmental process [13]. During ripening, fruits undergo several physiological and biochemical modifications that influence properties associated with fruit quality, such as texture (fruit softening), color (chlorophyll degradation and accumulation of non-photosynthetic pigments), aroma (production of volatile compounds) and taste (increase in sugars and decline in organic acids) [5,16,17]. Within taste, sugars are important determinants of sweetness and thus of fruit quality due to their direct association with palatability. In Japanese plums, as in other *Rosaceae* family members, the sugar-alcohol sorbitol is translocated to the fruit along with sucrose. Differences in sugar metabolism with concomitant variations in sucrose, sorbitol, glucose and fructose have been reported [18]. Sucrose synthesis can occur through the enzymatic activity of sucrose phosphate synthase (SPS) or sucrose synthase (SuSy). The first enzyme uses UDP–glucose and fructose-6-phosphate as substrates [19,20], while the second uses UDP–glucose and fructose [21]. Sucrose degradation results from the reversible activity of SuSy and from the activity of cell wall, vacuolar and cytosolic invertases (CWI, VI and CI, respectively), which break down sucrose into glucose and fructose [22]. The sugar-alcohol sorbitol is synthesized by the enzyme sorbitol-6-phosphate dehydrogenase (S6PDH), which reduces glucose-6-phosphate to sorbitol-6-phosphate [23]. Sorbitol is taken up into parenchyma cells via sorbitol transporter (SOT), and once in the cytosol, sorbitol catabolism results from the activity of NAD^+-dependent sorbitol dehydrogenase (NAD^+-SDH) and sorbitol oxidase (SOX), which convert sorbitol to fructose and glucose, respectively [24].

Here, we study the differences in ethylene and sugar metabolism between two Japanese plum cultivars with contrasting ripening behaviors, but sharing the same genetic background (Farcuh et al., unpublished). 'Santa Rosa' (SR) is a typical climacteric fruit, while 'Sweet Miriam' (SM) has a non-climacteric ripening pattern [25]. The existence of these cultivars provides a very attractive system to study fruit ripening and the interactions between ethylene biosynthesis, signaling and sugar metabolism during ripening. Gene expression, enzymatic activity and metabolite concentrations of the cultivars were compared at an early (end of pit hardening) and late (fully ripe fruit) stage of development. Our aim is to study the relationship between ethylene production and fruit sugar homeostasis during fruit development. The comparison between climacteric and non-climacteric behavior in two genetically related plum varieties offers an ideal model system for the study of the relation between ethylene and fruit ripening and senescence, and could provide valuable insight that would aid breeders in the improvement of fruit quality.

2. Materials and methods

2.1. Plant material

Japanese plum fruits (*Prunus salicina* cv. Lindl.) were collected during two seasons from a commercial orchard located in the California Central Valley production area (Parlier, CA, USA). Two different cultivars were used: 'Santa Rosa' (SR) and 'Sweet Miriam' (SM). Twenty fruits per each of six biological replications were harvested and immediately transported to the laboratory. Six fruits were used for the evaluation of fruit quality and ripening patterns for each replication and the remaining fruits were peeled, cut into small pieces, frozen in liquid nitrogen and stored at −80 °C until further use.

2.2. Fruit growth and development

Fruit sampling started immediately after the natural fruit drop occurring approximately 80–85 days after full bloom (DAFB). Fruit growth patterns were monitored weekly by measuring fruit diameter (size), skin color and firmness. To assess diameter, two dots were initially labeled on a total of 20 fruits and the distance between these was measured weekly. Based on this data, we collected samples of SR and SM plums at specific developmental stages (Table 1). Stages S2, S3, and Stage S4-1 were defined based on fruit size and skin color changes [6,26]. In SR fruits, stage S4-2 was defined based on the high production of ethylene and high respiration rates according to El-Sharkawy et al. [6,26]. Since SM fruits did not display a repiratory burst or a burst in ethylene synthesis, stage S4-2 in these fruits was determined based on the fruit firmness, since firmness is also the parameter of ripening index [27].

For fruit quality and ripening pattern evaluations, fruits from all the stages were harvested. For sugar analysis, relative gene expression and enzymatic assays fruits from S2 and S4-2 were used (Table 1 and Fig. 1A).

2.3. Fruit quality evaluations

For each cultivar and sampling date, fruit weight, diameter, flesh firmness, skin and flesh color, soluble solids content (SSC), titratable acidity (TA), and pH were measured on six fruits from each biological replication. Fruit weight and diameter were quantified using an electronic balance (Sartorius, AG Gottingen, Germany) and a digital caliper (Manostat Co., NY, USA). Skin was removed on two opposite sides of each fruit along the equatorial axes and then the chroma (c^*), lightness (L) and a^* and b^* values were measured using a colorimeter (Konica Minolta CR400 Chroma Meter, Konica Minolta Sensing, Inc., Osaka, Japan). The hue angle (H°) that represented changes in primary colors was calculated [28]. Flesh firmness was measured using a Güss FTA Penetrometer with an 8 mm tip (Güss, Strand, Western Cape, South Africa). A wedge from each fruit was removed and pooled to create a composite sample of each replication. Juice was extracted from these composite samples with a hand press, filtered through cheesecloth, and the soluble solids content (SSC), pH and titratable acidity (TA) were determined. SSC was measured using a digital refractometer (AR6 Series Reichert Technologies, Reichert, Inc., NY, USA) and expressed as %, while pH and TA were computed by automatic titration (TIM 850 TitraLab, Radiometer Analytical SAS, Lyon, France) with 0.1 N sodium hydroxide solution to an end point of pH 8.2, and was expressed as % malic acid.

2.4. Ripening patterns

At each collection date, fruit ethylene (C_2H_4) and carbon dioxide (CO_2) evolution were measured using a static system. Each fruit was sealed in a 1 l airtight container and at 20 °C, and ethylene production was calculated by measuring ethylene concentration in the gas phase of the containers, determined by withdrawing a 10 ml headspace gas sample from each container and injecting into a 2 ml fixed sample volume valve of a gas chromatograph (model Carle AGC-211, EG&G Chandler Engineering, Tulsa, OK, USA) equipped with two stainless steel columns (1.22 m and 0.305 m) packed with 8% NaCl on Alumina F-1 80/100 DV (EG&G Chandler Engineering, Tulsa, OK, USA) and a flame ionization detector. Nitrogen (N_2) was used as the carrier gas at a flow rate 30 ml min^{-1} while O_2 and H_2 were used to create the flame of the detector at flow

Table 1
Developmental stages of SR and SM fruits.

Developmental stage	Description	DAFB[a] in SR	DAFB in SM	Length (days) of each period (SR/SM)
S2	Pit hardening	90–96	109–118	6/9
S3	2nd exponential growth phase	96–114	118–194	18/76
S4-1	Full red color stage	114–118	194–207	4/13
S4-2	Fully ripe stage	118–125	207–236	7/29

[a] DAFB = days after full bloom.

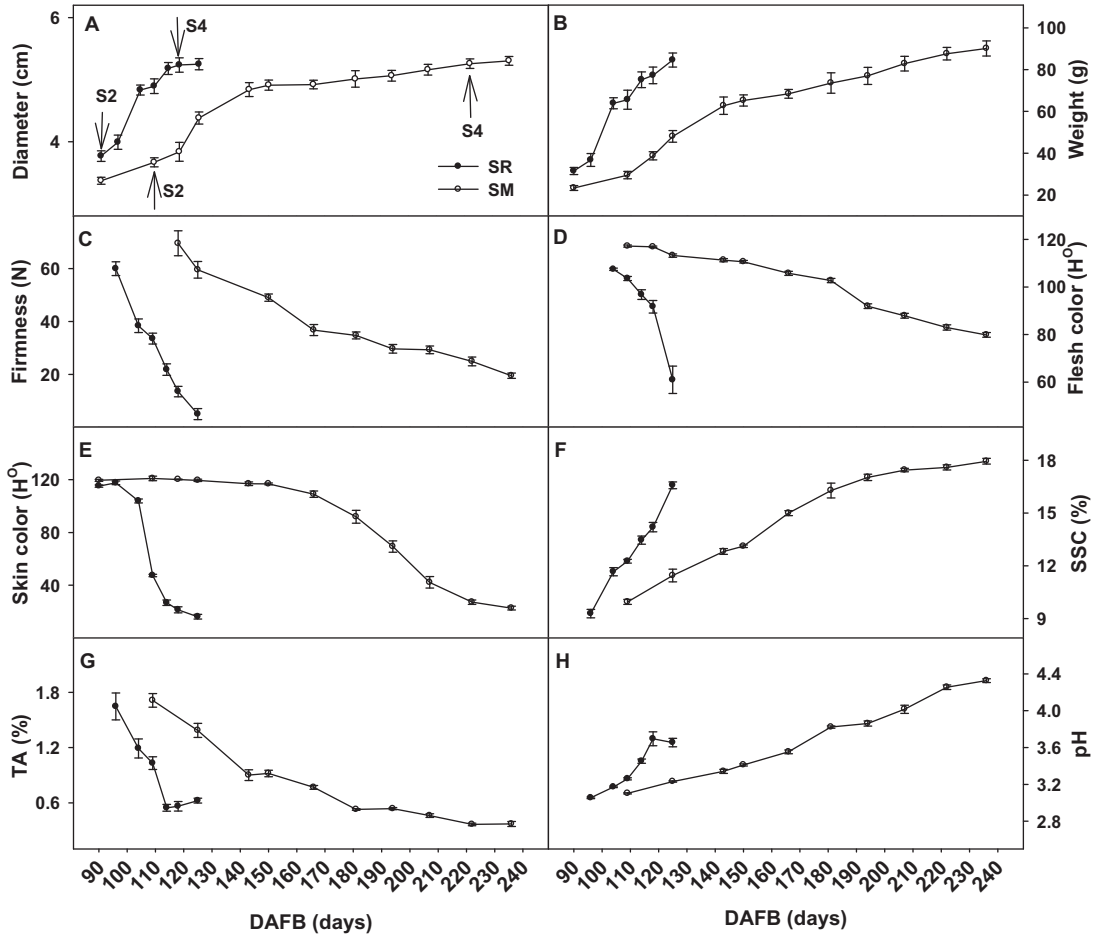

Fig. 1. Fruit quality traits. (A) Fruit diameter; (B) fruit weight; (C) fruit firmness; (D) fruit flesh color; (E) fruit skin color; (F) fruit soluble solid contents; (G) fruit titratable acidity and (H) fruit pH. SR, Santa Rosa (closed circles); SM, Sweet Miriam (open circles). Values are the mean ± SE ($n = 6$). Arrows indicate stages S2 and S4 in each cultivar.

rate 300 and 30 ml min^{-1}, respectively. Injector, oven and detector temperatures were at 80 °C. Respiration rate was calculated by carbon dioxide concentration in the gas phase of the containers, determined by using an infrared gas analyzer (Horiba PIR-2000R, Horiba Instruments Inc., Irvine, CA, USA) similar to the ethylene collection method. Nitrogen was used as the carrier gas at a flow rate 20 ml min^{-1}. Ethylene production and respiration rates were expressed as C_2H_4 μl kg^{-1} h^{-1} and CO_2 ml kg^{-1} h^{-1}, respectively.

2.5. Analyses of sugars and organic acids

Sugars and organic acids in fruit tissues (pit hardening, stage S2 and fully riped stage S4-2) were quantified as described elsewhere [29] with some modifications. Lyophilized fruit tissue (~0.1 g) was homogenized in 1 ml of distilled water that was passed through a Milli-Q water purification system (Biocel A10 Millipore, MA, USA). The homogenates were centrifuged at 15,000 × *g* for 10 min and the supernatant was filtered through a 0.45 μm syringe filter (Millex-HP filter, Millipore Co., MA, USA). One ml of each supernatant was passed through a pre-conditioned C_{18} Sep-Pak Cartridge (Waters, Milford, MA, USA) with methanol and water to clean the extracts before analysis. Finally, the extracted sample was filtered through a 0.2 μm syringe filter (Millex-HP filter, Millipore Co., MA, USA), collected in 1 ml glass vial and used for high-performance liquid chromatography (HPLC) analysis.

Reverse phase-liquid chromatography (Agilent HPLC 1100 series, USA) was performed to determine individual sugars and organic acids. Separation of sugars was performed with water as a mobile phase flowing at 0.6 ml min^{-1} using an Aminex HPX-87C column (300 mm × 7.8 mm; Bio Rad Laboratories, Hercules, CA, USA) which was preceded by a micro-guard cartridge (Carbo-C, pH range 5–9, 30 mm × 4.6 mm; Bio Rad Laboratories, Hercules, CA, USA) and maintained at 80 °C. Ten microliter extract was injected by an auto-sampler and sugars were detected using a refractive index detector (Agilent G1362A) [30]. Organic acids were eluted through an Aminex HPX-87-H column (300 × 7.8 mm; Bio Rad Laboratories, Hercules, CA, USA) at 35 °C with micro-guard cartridge (Carbo-H, pH range 1–3; Bio Rad Laboratories, Hercules, CA, USA) and were

detected by diode array and multiple wavelength detectors (DAD, Agilent G1315B, USA) at 210 nm. Mobile phase was 0.1% phosphoric acid at a flow rate of 0.6 ml min^{-1}. The concentrations of sugars (fructose, glucose, sorbitol, sucrose and glucose-6-phosphate), and organic acids (malate, citrate, oxalate and succinate) were expressed as mg/g of dry weight.

2.6. RNA isolation

Total RNA was isolated as described before Chang et al. [31] with some modifications. One gram of tissue was homogenized in 4 ml of extraction buffer containing 2% CTAB, 2% PVP, 25 mM EDTA, 2.0 M NaCl, 0.5 g/L spermidine, 2% β-mercaptoethanol and 100 mM Tris–HCl, pH 8.0. Samples were extracted twice with equal volumes of chloroform:isoamylalcohol (24:1, v/v) at 10,000 × *g* at 4 °C and RNA was selectively precipitated with 1/4 volume of 10 M LiCl overnight at 4 °C. RNA was harvested by centrifugation at 10,000 × *g* for 20 min, and the pellet was dissolved in a solution containing 500 μl SSTE buffer: 1.0 M NaCl, 0.5% SDS, 1 mM Tris–EDTA, pH 8.0 and 10 mM Tris–HCl, pH8.0. RNA was extracted with an equal volume of chloroform:isoamylalcohol (24:1) and precipitated with two volumes of ethanol at −20 °C for 2 h. Following centrifugation, the RNA was resuspended in RNAse-free water. Genomic DNA was eliminated by treating each sample with RNase-free DNase I (TaKaRa, Japan), according to the manufacturer's instructions. The concentration of isolated total RNA was calculated from absorbance at 260 nm with a Nano-dropper (ND-1000, Thermo scientific, DE, USA). RNA purity was verified by optical density and RNA integrity was evaluated by electrophoresis on 1.0% agarose gels. Intact rRNA subunits of 18S and 28S were observed on the gel and the absence of smears indicated minimal RNA degradation.

2.7. Isolation and in silico analysis of plum cDNA sequences

To isolate plum homologues of ACO1 and SOT, cDNA was synthesized using 1 μg of total DNase-treated RNA. An aliquot of 2 μl of cDNA was used in a PCR reaction with the appropriate degenerate primers. Primers of ACO1 were designed from the conserved regions of *Prunus persica* [32], *Prunus armeniaca* [33] and *Prunus mume* [34]. In the case of SOT, primers were designed from *P. persica*, *Prunus cerasus* [35] and *Malus domestica* [36]. The primers for the rest of the genes were designed from *Prunus salicina* [6,11,15]. Gene alignments for design of primers were performed with ClustalW [37]. The Neighbor-Joining tree was built with MAFFT [38].

2.8. Real-time quantitative RT-PCR

Complementary DNA (cDNA) was synthesized from 1 μg total RNA with the QuantiTect Reverse Transcription kit (Qiagen). Quantitative PCR was performed on the StepOnePlus™ (Applied Biosystems, Foster City, CA), using SYBR® GREEN. Primer sets were designed using Primer Express software (Applied Biosystem, Foster City, CA) and these are specified in Table S1. A total reaction volume of 15 μl was used. This included 3 μl template, 0.2 μl forward primer, 0.2 μl reverse primer, 7.5 μl SYBR Green Master Mix and 4.1 μl RNA-free water. qPCR assays were performed using the following conditions: 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. The $2^{-\Delta\Delta CT}$ method [39] was used to normalize and calibrate transcript values relative to endogenous actin, whose expression did not change across different cultivars and developmental stages. The expression of the endogenous actin gene was used as a reference because its expression did not change across different cultivars and developmental stages [6,26].

2.9. Enzyme assays

2.9.1. Sucrose phosphate synthase (SPS), sucrose synthase (SuSy) and invertases

Tissue for SPS analysis was extracted and assayed as described [21] with some modifications. Fruit flesh tissue (~1 g) was homogenized in 4 ml ice-cold extraction buffer containing 5 mM EDTA, 5 mM $MgCl_2$, 0.05% Triton X-100, PVPP, 5 mM β-mercaptoethanol and 50 mM HEPES, pH 7.5. The homogenate was filtered through four layers of cheesecloth and centrifuged at 20,000 × *g* for 20 min. The supernatant was applied to a PD10 column (GE Healthcare, Buckinghamshire, UK) that was pre-equilibrated with 100 mM Tris–HCl (pH 8.0). Forty microliter extract was incubated for 1 h at 37 °C with 30 μl reaction buffer containing 15 mM $MgCl_2$, 25 mM fructose-6-phosphate, 25 mM glucose-6-phosphate, 25 mM UDP–glucose and 50 mM HEPES, pH 7.5. The reaction was stopped by boiling the samples for 10 min. Sucrose was quantified using Anthrone [40]. Absorbance was measured using a microplate reader (SynergyMx Monochromator-Based Multi-Mode Microplate Reader, BIOTek®, Winooski, VT, USA) at 620 nm.

Both SuSy-mediated sucrose synthesis and degradation were assayed as described [21,41] with some modification. Fruit flesh tissue was homogenized in an extraction buffer as described above and desalted with a PD10 column. Thirty microliter reaction buffer containing 50 mM HEPES (pH 7.5), 15 mM $MgCl_2$, 25 mM fructose and 25 mM UDP–glucose and 40 μl of desalted extract were incubated for 1 h at 37 °C. Sucrose concentrations were determined as described above. Sucrose cleavage was assayed for 30 min at 37 °C in 100 μl extract and 200 μl reaction buffer, containing 100 mM sucrose and 4 mM UDP and 50 mM HEPES, pH 7.5. Reducing sugars were determined as described [42]. Absorbance was measured using a microplate reader (SynergyMx Monochromator-Based Multi-Mode Microplate Reader, BIOTek®, Winooski, VT, USA) at 540 nm. A standard curve was prepared using fructose as a standard.

Cell wall invertase (CWI), cytosolic invertase (CI) and vacuolar invertase (VI) activities were assayed as described before [43,44]. Protein content was determined according to Bradford [45].

2.9.2. NAD^+-dependent sorbitol dehydrogenase, sorbitol-6-phosphate dehydrogenase and sorbitol oxidase

The activities of NAD^+-dependent sorbitol dehydrogenase (NAD^+-SDH), sorbitol-6-phosphate dehydrogenase (S6PDH) and sorbitol oxidase (SOX) were measured according to previously published methods [23,44] with some modifications. For NAD^+-SDH measurements, fruit flesh tissue (~10 g) was homogenized in 40 ml extraction buffer containing 1 mM EDTA, 10 mM sodium ascorbate, 1 mM DTT, 0.1% Tween 20, 5% PVPP, 2 mM PMSF and 200 mM potassium phosphate buffer, pH 7.8). The homogenate was filtered through four layers of cheesecloth and centrifuged at 17,000 × *g* for 30 min. The supernatant was adjusted to 70% ammonium sulfate and centrifuged at 17,000 × *g* for 30 min. The precipitated proteins were resuspended in extraction buffer and desalted with a PD10 column (GE Healthcare, Buckinghamshire, UK) pre-equilibrated with 100 mM Tris–HCl, pH 8.0. The eluate was assayed for SDH activity. The reaction mixture (1 ml) contained: 10 μl 1 mM NAD^+, 150 μl 300 mM sorbitol, 100 μl 100 mM Tris–HCl buffer, pH 9.5, 100 μl enzyme solution and 640 μl water. Enzyme activity was measured as change in absorbance at 340 nm at 37 °C and expressed as nmol NADH per mg of protein per min.

For S6PDH activity assays, fruit flesh tissue (~10 g) was homogenized in 40 ml extraction buffer containing 10 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, 1% Tween 20, 5% (w/v) glycerol and 50 mM Hepes–NaOH, pH 7.5. The slurry was filtered through four layers of cheesecloth. After centrifugation at 4000 × *g* for 10 min, the supernatant was used for enzyme assays. S6PDH activity was

determined as described previously [23,46]. Net rates were calculated by subtracting the rate for a control (containing water instead of G6P). Sorbitol was detected by HPLC and the unit was expressed as 1 μmol sorbitol per mg of protein per min.

For SOX activity assays, fruit flesh tissue (~10 g) was homogenized in 40 ml extraction buffer containing 10 mM dithiothreitol (DTT), 3 mM Mg acetate, 8% (v/v) glycerol, 0.1% (v/v) Tween 20, 1% (w/v) PVPP and 0.2 M HEPES buffer, pH 7.5. The homogenates were centrifuged at 3000 × *g* for 15 min and the supernatants were desalted with a PD10 column (GE Healthcare, Buckinghamshire, UK) pre-equilibrated with 100 mM Tris–HCl (pH 8.0). SOX activity was assayed by determining the amount of sorbitol degraded. The SOX reaction mixture (2.0 mL) contained 235 mM sorbitol, 59 mM acetate buffer, pH 4.0 and 1 ml enzyme extract. After 30 min of incubation at 37 °C, the reaction was stopped by boiling for 10 min. The sorbitol degraded was measured using a HPLC system (Agilent HPLC 1100 series) and the unit was expressed as 1 mmol sorbitol per mg of protein per min.

2.10. Statistical analysis

Statistical analysis was carried out using the software package JMP® (ver.10.0, SAS Institute) by two-way analysis of variance using Tukey's test to compare between cultivars and developmental stages (S2 and S4-2) at a probability level of 5%.

3. Results

3.1. Fruit growth and development

The growth patterns of Japanese plum fruit from two commercial cultivars, SR and SM were characterized using fruit size, color and firmness to assess their developmental stages (Fig. 1). Growth of both cultivars followed a double sigmoidal growth pattern [26,47] that is typical of stone fruits. Fruit growth can be divided into four stages: pit-hardening, second exponential growth phase, full red color and fully ripe stage, which were defined as stages 2 (S2), 3 (S3), 4–1 (S4-1) and 4–2 (S4-2), respectively [6]. The lengths of the stages were determined based on fruit diameter and expressed as a function of days after full bloom (DAFB) (Table 1). Although fruit weight and size were similar in both cultivars at the end of the season, SR fruits reached their fully ripe stage (S4-2) after 125 DAFB while SM fruits needed approximately 240 DAFB. SM fruits had longer S3 and S4 periods than SR. While the second exponential growth phase (S3) lasted ~76 DAFB in SM, it only required 18 DAFB for SR fruits (Table 1).

Fruit firmness was also measured during fruit development, although the analysis excluded the early stage S2 (90 and 109 DAFB in SR and SM, respectively) due to the high mechanical rigidity imposed by the flesh at this stage. In both cultivars, fruit firmness declined during fruit development while the fruit hang on the tree (Fig. 1C). During the phase of fruit development (S4), from the full red color stage (S4-1) to the fully ripe stage (S4-2) SR fruits softened very quickly: over ~11 days firmness decreased from 33.5 N to 5.1 N. On the other hand, SM fruits softened slowly: over ~40 days the firmness dropped from 36.7 N at stage 4–1 to 19.5 N at stage S4-2 stage.

Fruit color changes were measured using a colorimeter and expressed as hue (*H*°) angles. *H*° represents primary color changes ranging from red (0°) through yellow (90°), green (180°), and blue (270°), ending back in red (360°). Skin and flesh remained green until the end of the expansion period. Red skin and yellow flesh appeared at stage S4-1 (Fig. 1D and E). Fruit skin and flesh color changes correlated well with firmness and development in both cultivars (Fig. 1) [27].

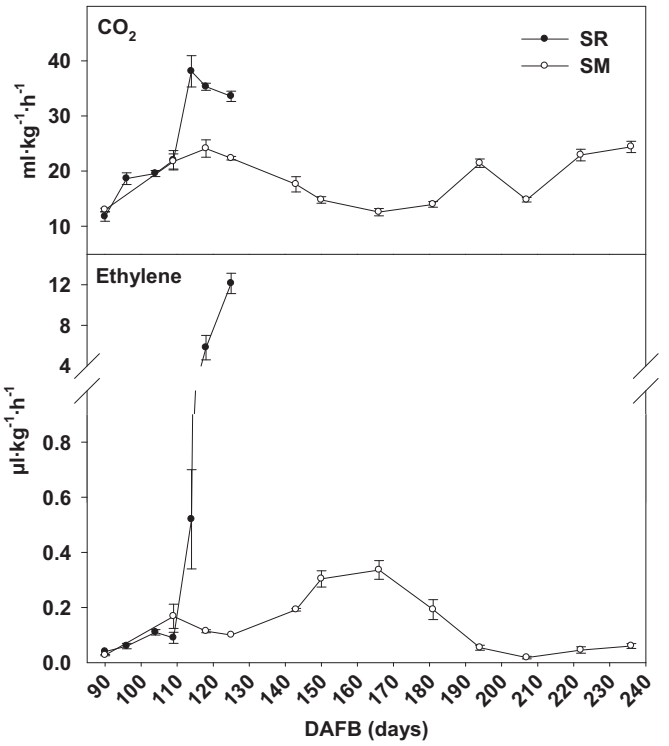

Fig. 2. Ethylene and CO_2 production rates during maturation and ripening on the tree. SR, Santa Rosa (closed circles); SM, Sweet Miriam (open circles). Values are the mean ± SE (n = 6).

In ripe fruits, SSC and TA are key factors determining fruit quality, because they directly affect fruit flavor and consumer acceptance [48,49]. SSC, TA and pH changed similarly in both cultivars during fruit development. SSC increased up to 16.6% and 18% in SR and SM, respectively. TA decreased from 1.8% to 0.6% and 0.4% with a concomitant increase in pH from 3.0 to 3.7 and 4.3 in SR and SM, respectively. (Fig. 1F, G, and H)

3.2. Ripening patterns

Most Japanese plums are climacteric fruits because they display autocatalytic ethylene production and a respiratory burst during ripening [8]. SR showed a typical climacteric pattern characterized by a burst of ethylene and carbon dioxide at 114 DAFB. However, SM fruits had minimal ethylene evolution and no burst of respiration was detected during ripening (Fig. 2). The low ethylene production rates (0.16–0.31 $\mu l\,kg^{-1}\,h^{-1}$) in SM fruits were similar to ethylene emission in non-climacteric fruits [50,51]. Moreover, it has been shown that during postharvest ripening of the SM cultivar, the ethylene production rates remained low under normal air conditions as well as when fruits were exposed to a continuous exogenous source of ethylene (propylene) [25]. In addition, the respiration rates of SM fruits exposed to propylene were not different that the rates recorded at harvest [25].

3.3. Sugar concentrations and metabolism

Differences in sugar accumulation between cultivars at two developmental stages were detected by HPLC analysis (Fig. 3). In both Japanese plums cultivars, five major sugar peaks were detected: glucose-6-phosphate (G6P), sucrose (Suc), glucose (Glu), fructose (Fru) and sorbitol (Sor). The abundance of these sugars in plum cultivars has been reported previously [29]. At S2, G6P and Sor concentrations were lower in SR than in SM fruits, while Suc, Glu,

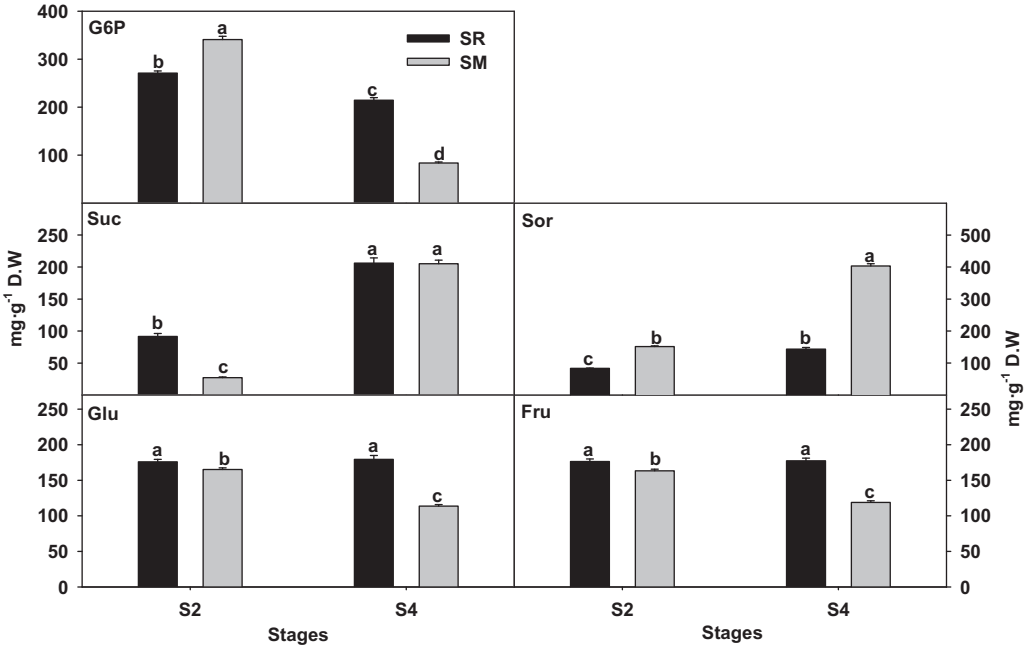

Fig. 3. Fruit sugar composition during pit hardening (S2) and fully ripe stage (S4) on the tree. SR, Santa Rosa (black bars); SM, Sweet Miriam (gray bars). Values are the mean ± SE ($n = 6$). Different letters indicate significant differences ($p < 0.05$).

and Fru concentrations were higher. During S4, there were not differences in Suc concentration between the two cultivars, but G6P, Glu, and Fru concentrations remained lower in SM than in SR. Sor was approximately 2.5 times higher in SM than in SR (Fig. 3).

Fruit sucrose concentration is mainly regulated by translocation form source leaves to fruits [29], and once unloaded from the phloem into the fruit, sucrose degradation is mediated by invertases (cell wall, cytosol, vacuolar) (EC 3.2.1.26) and sucrose synthase (SuSy, degradation) (E.C. 2.4.1.13), while sucrose synthesis is mediated by the concerted action of sucrose phosphate synthase (SPS) (EC 2.4.1.14) and sucrose synthase (SuSy, synthesis) (EC 2.4.1.13). Sucrose synthesis via SPS was high in SR fruits at S2 and S4, but low in SM fruits (Fig. 4). In both SR and SM cultivars, synthesis via SuSy was low at S2 and increased during S4. Sucrose degradation via SuSy was high in SR fruits at S2 and decreased at S4, while SM fruits showed the opposite trend. Sucrose degradation via cell wall-bound and cytosolic invertases was low in SR fruits at both stages, but high in SM at S2. The apparent discrepancy between a relative low SPS activity in SM fruits at stage S4 and the amounts of Suc, comparable to those of SR fruits, could be a consequence of the longer time that SM fruits in the tree, and the strong dependence of fruit Suc content on sink strength. On the other hand, vacuolar invertase activities were high in SR fruits at S2 but low at S4, and SM fruits displayed low vacuolar invertase activities during all stages of development (Fig. 4). Although these results would indicate differences in sucrose degradation mediated by SuSy activity, the results should be taken with caution since the direction of reversible enzyme activity in vitro would depend on the kinetic control effected by substrate availability in the assay reaction.

Two sorbitol catabolic pathways operate in fruits, NAD^+-dependent sorbitol dehydrogenase (NAD^+-SDH) (EC 1.1.1.14) that converts sorbitol to fructose, and sorbitol oxidase (SOX) that converts sorbitol to glucose [44]. NAD^+-SDH activity at S2 was similar in both cultivars, but significantly decreased in the SM fruits during S4 (Fig. 5A). *NAD^+-SDH* gene expression followed similar patterns (Fig. 5B). SOX activity was reduced in SM fruits S2 and remained at the same level in both fruits at S4 (Fig. 5C). Sorbitol synthesis is mediated by the activity of sorbitol-6-phosphate dehydrogenase (S6PDH) (EC 1.1.1.200) [52]. Although many studies on S6PDH activity and *S6PDH* gene expression have examined leaves and source tissues [23,53,54], S6PDH activity was also reported in flesh tissues from loquat fruits [55]. Both SR and SM fruits displayed S6PDH activity, which was higher in SM fruits at both stages (Fig. 5E). Expression of *S6PDH* was higher in SR fruits at S2, but higher in SM fruits at S4-2 (Fig. 5F). S6PDH activity was higher in SM fruits in both early and late stages of development, suggesting higher Sor synthesis in SM fruits (Fig. 5E). The expression of *SOT* in SR fruits did not change during S2 and S4. In contrast, the expression of *SOT* in SM was significantly higher than SR at S2 and decreased dramatically at S4 (Fig. 5D).

3.4. Organic acid concentrations

Malic, citric, oxalic and succinic acids were the main organic acids detected in plum fruits (Fig. S1). At the S2 stage of development, malic was the major acid and similar amounts (205.7 and 219.4 mg/g DW in SR and SM, respectively) was found in both cultivars. Malic acid decreased during fruit development, and the decrease was higher in SM than in SR (115.7 and 55.4 mg/g DW, respectively). A similar pattern was observed with succinic acid, although the relative amounts were lower in both fruits. Oxalic acid contents were relatively higher in SR than in SM fruits at S2, but were not detected at S4. Citric acid contents were similar in both SR and SM fruits at stage S2, remained constant at S4 in SR fruits but decreased significantly in SM fruits (Fig. S1).

3.5. Ethylene biosynthesis, perception and signal transduction pathways

The expression of genes associated with ethylene biosynthesis and perception in SR and SM cultivars was assessed by qPCR (Fig. 6). Ethylene synthesis is mediated by two enzymes ACC synthase (ACS) that catalyzes the conversion of SAM (S-adenosyl-L-methionine) to ACC (1-aminocyclopropane-1-carboxylic acid), and ACC oxidase (ACO), which converts ACC to ethylene [56]. SR and SM plum fruits displayed very low expression of *ACO1* at S2 (Fig. 6A). At S4-2, SR fruits has a high *ACO1* expression with respect to S2 (800-fold

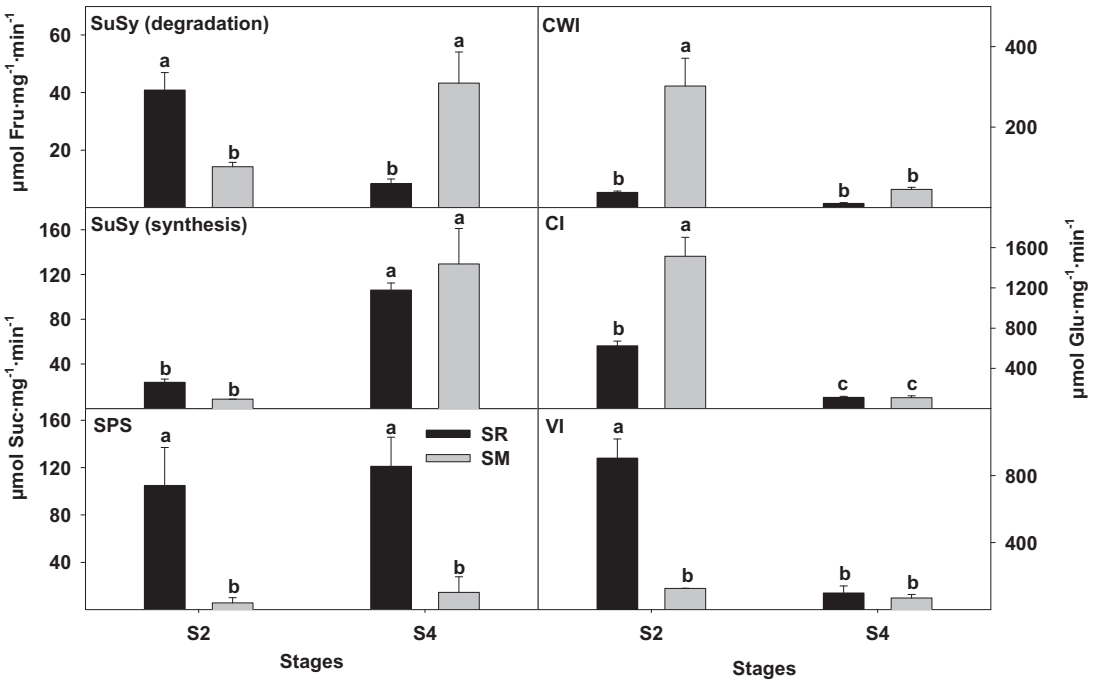

Fig. 4. Activities of enzymes involved in sucrose metabolism. Sucrose synthase (SuSy), synthesis and degradation and sucrose phosphate synthase (SPS); and cell wall (CWI), cytosolic (CI) and vacuolar (VI) invertases. SR, Santa Rosa (black bars); SM, Sweet Miriam (gray bars). Values are the mean ± SE ($n = 3$). Different letters indicate significant differences ($p < 0.05$).

change), that correlated well with the increased ethylene production (Fig. 2), while the expression of *ACO1* in SM fruits was lower and only slightly increased from S2 (6-fold change) in comparison to SR, as expected (Fig. 2).

The expression of ACS1 and was not detected either in SR or SM fruits. Nevertheless, the expression of ACS3 was detected in both fruits and followed a similar trend to that of ACO1, i.e. low levels of expression at stage S2 in both SR and SM fruits and highly expressed at stage S4-2 in SR fruits only (results not shown). The lack of expression of ACS1 is intriguing and requires further investigation. Although the ACS1 expression was reported in Japanese plums [10,11], these primers did not amplify any transcripts either in SR or in SM fruits (results not shown). Furthermore, RNA sequencing, using the peach genome as a reference, of SM and SR fruits failed to detect ACS1 transcripts (Kim et al., unpublished).

It has been shown that ACS proteins are post-translationally regulated through the phosphorylation of their C-terminus [57]. Ethylene-overproducer 1 (ETO1) protein is a negative regulator of ethylene production that interacts with a region of the ACS C-terminus (called TOE, target of ETO1). This interaction contributes to degradation of the protein via the ubiquitin-26S proteasome pathway [58]. We assessed the expression of *EOL1* (*ETO1*-like) in both SR and SM (Fig. 6B). Both cultivars had similar EOL1 expression levels during S4-1 but expression increased significantly in SM during S4-2. The expression of two ethylene receptors (*ERS1* and *ETR1*) was assessed (Fig. 6C and D). Expression of both receptors increased during S4-2 in SR fruits, but remained low in SM. Downstream of the ethylene receptors, the signals are transduced by ethylene-responsive factors (ERFs). ERF1 is a transcriptional activator for GCC box-dependent transcription in *Arabidopsis* that responds to both

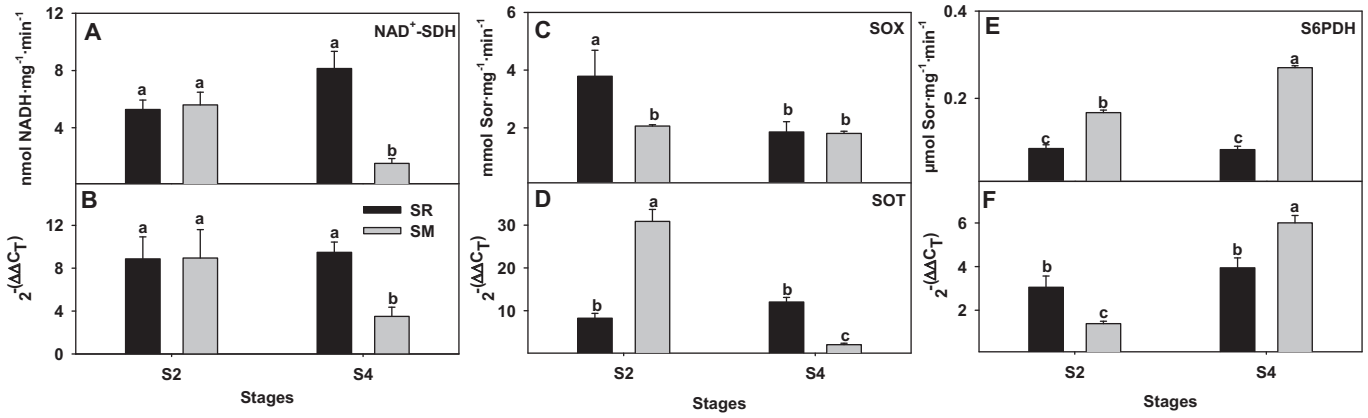

Fig. 5. Enzyme activity and gene expression involved in sorbitol metabolism and transport. (A) Enzyme activity and (B) gene expression of NAD$^+$-dependent sorbitol dehydrogenase (NAD$^+$-SDH); (C) the activity of sorbitol oxidase (SOX); (D) gene expression of a sorbitol transporter (SOT); (E) enzyme activity and (F) gene expression of sorbitol-6-phosphate dehydrogenase (S6PDH). SR, Santa Rosa (black bars); SM, Sweet Miriam (gray bars). Values are the mean ± SE ($n = 3$–6). Different letters indicate significant differences ($p < 0.05$).

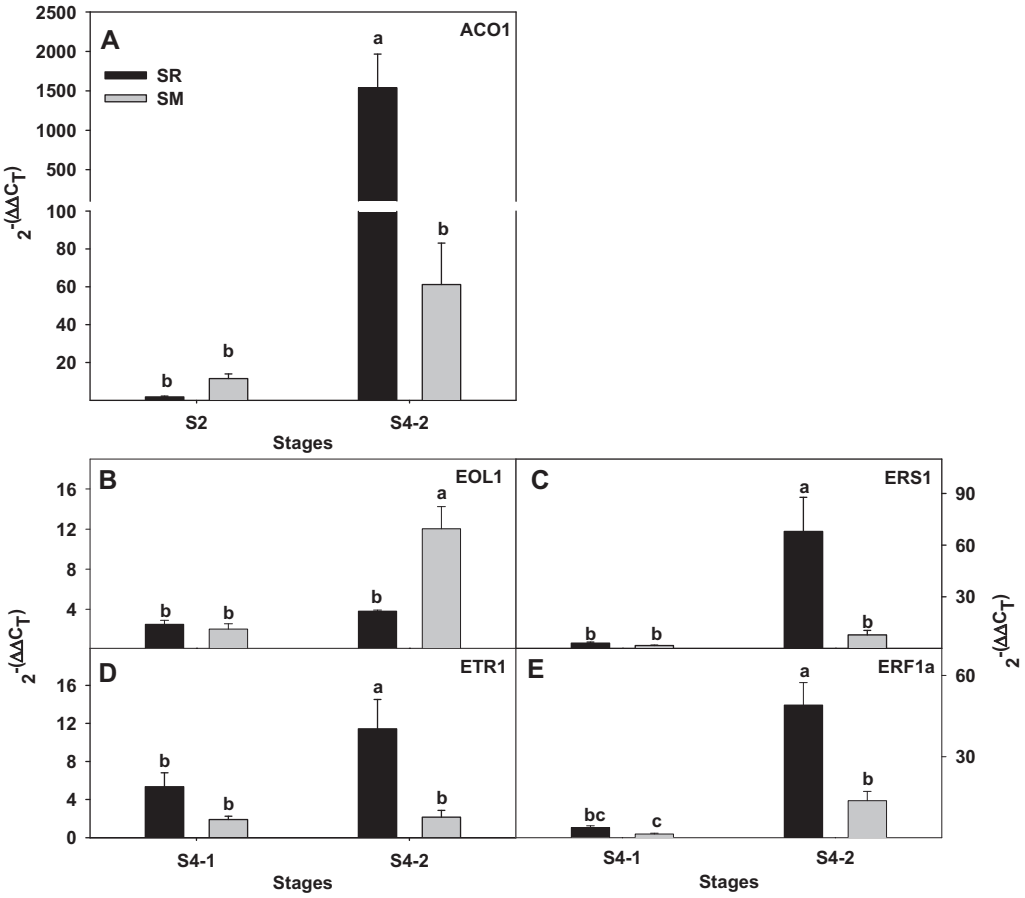

Fig. 6. Expression of genes associated with ethylene synthesis, sensing and signaling. (A) 1-Aminocyclopropane-1-carboxylic acid oxidase (ACO1); (B) ethylene overexpression-like 1 (EOL1); (C) ethylene receptor (ERS1); (D) ethylene receptor (ETR1); and (E) ethylene-responsive transcriptional factor 1a (ERF1a). SR, Santa Rosa (black bars); SM, Sweet Miriam (gray bars). Values are the mean ± SE (n = 3–6). Different letters indicate significant differences ($p < 0.05$).

ethylene and abiotic stresses [59]. ERFs are also involved in the feedback loop that stimulates autocatalytic ethylene synthesis [13] and bind to *cis*-elements found in the promoters of target genes, modulating their transcription [15], and thus inducing downstream ethylene responses that lead to fruit ripening [10]. *ERF1a* expression increased greatly in SR fruits during S4-2 (Fig. 6E) but only moderately in SM fruits.

4. Discussion

We characterized and compared physiological, biochemical and molecular attributes of two Japanese plum cultivars with substantial differences in their ripening patterns. 'Santa Rosa' is a typical climacteric cultivar and one of the oldest plum varieties [1] while 'Sweet Miriam' has the same genetic background as 'Santa Rosa' (Farcuh et al., unpublished) but displays non-climacteric ripening. The cultivars showed differences in growth patterns and physiological attributes (color, pH, sugar content, softening, etc.) constituting an appropriate system for the study of ethylene-induced ripening processes.

Other Japanese plum cultivars with a suppressed climacteric behavior, unable to induce autocatalytic ethylene production, have been reported [2,6,8]. These cultivars did not produce enough ethylene to control fruit ripening, but responded to exogenous ethylene (or propylene) treatments with resumed ripening [8]. In these suppressed climacteric plum cultivars, changes in CO_2 production were smaller than in climacteric cultivars and were not associated to changes in ethylene biosynthesis. When attached to the tree, the fruits were ripe with similar properties to climacteric fruits [8]. The SM cultivar described here showed marked differences with respect to both, climacteric and suppressed climacteric fruits described by Abdi et al. [8]. Although fully ripe fruits from SR and SM cultivars reached a similar final size and weight, SM fruits took ~120 days longer to ripen than climacteric fruits (SR). SM fruits showed no climacteric ethylene production and a small increase of respiration rate during ripening on the tree (Fig. 2) as well as during postharvest ripening [8,25].

The critical difference between climacteric and non-climacteric fruits rests in their relative abilities to produce ethylene in response to low concentrations of ethylene or propylene [60]. Since the treatment of SM fruits with propylene did not induce autocatalytic ethylene production or an increase in respiration rate [25], these results indicated that SM fruits behave non-climacteric, a behavior not observed before in Japanese plum fruits.

Although SM fruits were firmer than SR fruits (Fig. 1C), they were able to soften under continuous supply of propylene [25]. Fruit softening during ripening is a complex process that depends on cell wall disassembly [61]. It typically involves extensive depolymerization and solubilization of the polysaccharides comprising the primary cell wall [62,63] and the middle lamella, leading to a decrease in cell-to-cell adhesion, increase in cell separation and an overall loss of biomechanical strength [64]. Given the occurrence of fruit softening when fruits from SM were exposed to exogenous propylene, our results would suggest that the expression of genes encoding cell wall modifying enzymes could be, in part, regulated by ethylene.

The higher soluble solid content (SSC) at the end of fruit development in SM fruits in comparison to SR fruits could be a result of the longer time that the SM fruit remained attached to the tree.

Guis et al. [65] found that transgenic melons with decreased ethylene production were unable to form abscission zones, remaining attached to the vine for a longer time and therefore accumulated more total sugars. The low titratable acidity of the SM fruits correlated well with the decreased organic acid concentrations at S4 (Fig. S1). These results support the notion that organic acid metabolism in fruits is ethylene-independent and associated with fruit respiratory metabolism [65].

Our results also suggest that the SM cultivar has mutation(s) in the autocatalytic ethylene biosynthesis pathway. This notion is supported by the increased expression of *EOL1*, the decreased expression of *ACO1*, and the reduced expression of ethylene receptors (*ESR1* and *ETR1*) and the ethylene-responsive factor *ERF1* in the SM cultivar with respect to the SR.

Several reports have indicated that Suc, Fru, Glu and Sor are the predominant soluble sugars (or sugar-alcohol in the case of Sor) present in plum cultivars [2,66]. Interestingly, Suc contents at stage S4 were similar in both SR and SM, although SPS activities were relatively lower in SM fruits. These results highlight the role of Suc transfer from leaf sources to the fruits. Since SM fruits remained in the tree for an extended period of 120 days with the concomitant relative high levels of Suc in the fruits. Moreover, sugar relative concentrations can vary due to differences in the activities of sugar metabolism enzymes regulated by hormonal action [18]. In some cases, the effect of ethylene on sugar concentrations has been demonstrated. For example, ethylene can influence the concentrations of Suc and Fru in apples [67]. Experiments using transgenic apples suppressed for ethylene biosynthesis and applications of 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception, suggested the existence of a complex interaction between ethylene and sugar metabolism in fruits, highlighting that sugar accumulation was under ethylene regulation during ripening (Defilippi et al. [67]. Working with grape berries, a non-climacteric fruit, Chervin et al. [68] found that blocking ethylene perception during ripening decreased sucrose accumulation. Our results showed that in fully ripe SM fruits, Sor accumulation correlated with inhibition of ethylene production. Decreased Glu and Fru contents paralleled the increased Sor content in SM fruits. These changes were well correlated with the decreased activity of NAD^+-SDH and SOX, enzymes associated with Sor catabolism. Moreover, the high Sor concentration, the sharp decrease in Glu-6-P and the increased S6PDH activity strongly suggest the occurrence of enhanced Sor synthesis in SM fruits. The lack of correlation between S6PDH activity and *S6PDH* expression at stage S2 would suggest possible posttranscriptional regulation of the transcripts and/or posttranslational modifications of the enzyme activity. The presence of S6PDH in mature fruits was demonstrated by suppression subtractive hybridization of shaded apple fruits [69] and by proteomics of apple fruits [70]. Although our results would suggest a link between the non-climacteric response of SM fruits and Sor production, more research is needed to establish a relationship between ethylene biosynthesis/signaling and Sor homeostasis. Interestingly, recent analysis of apple fruits treated with ethylene showed decreased S6PDH, further suggesting regulation of Sor synthesis/catabolism by ethylene [70].

In conclusion, we characterized two genetically related Japanese plum cultivars, SR and SM, that show contrasting ripening behavior. The non-climacteric variety displayed an altered sugar metabolism with the enhanced production of sorbitol. Our data shows a link between ethylene and fruit sugar homeostasis, and would suggest the possible regulation of sorbitol synthesis/catabolism by ethylene. The contrasting properties of these two genetically related plum cultivars offer an ideal model system to study the role(s) of ethylene in fruit ripening and fruit senescence. In addition, the high production of Sor in the fruits and the relatively lower Glu and Fru fruit contents suggests the suitability of SM plums as a component of low glycemic index diets.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2014.11.002.

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