

RESEARCH PAPER

Differential regulation of four members of the ACC synthase gene family in plum

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Abstract

The regulation of ACC synthase (ACS) genes was studied in early ('Early Golden') and late ('Shiro') Japanese plum cultivars (*Prunus salicina* L.) in order to determine the role of this gene family in fruit ripening. Of the four *Ps-ACS* cDNAs isolated, two (*Ps-ACS1* and -3) showed differential expression between the two cultivars. *Ps-ACS1* accumulated during fruit ripening of 'Early Golden' ('EG') and 'Shiro' ('SH') in ethylene-dependent and -independent manners, respectively. *Ps-ACS3a* transcripts accumulated throughout fruit development and during 'EG' fruit ripening. *Ps-ACS3b* was detected only during ripening of 'SH' fruit. Furthermore, *Ps-ACS3a* transcript accumulation was negatively regulated by ethylene, whereas *Ps-ACS3b* was positively induced by the hormone. In both cultivars, the expression of *Ps-ACS4* and -5 is under positive and negative feedback control by ethylene, respectively. Genetic analyses of 'EG' and 'SH' cultivars demonstrated that 'EG' is homozygous for *Ps-ACS3a* whereas 'SH' is heterozygous for *Ps-ACS3* (*a/b*). The role of ethylene-overproducer 1-like in delaying fruit ripening by interacting with *Ps-ACS* proteins was also studied. The effect of the plant hormones, auxin, gibberellin, and cytokinin, in regulating ethylene production by promoting the induction of the different *Ps-ACS* mRNAs in plum was investigated. A model is presented in which differences in *Ps-ACS*

alleles and gene expression between early and late plums are critical in determining the ripening behaviour of the cultivars.

Key words: 1-Aminocyclopropane-1-carboxylic acid synthase (ACS), ACS alleles, auxin, cytokinin, double sigmoid curve growth pattern, ethylene-overproducer 1 (ETO1), gibberellin, plum fruit development and ripening, system 1 and system 2 ethylene production.

Introduction

Ethylene, a gaseous phytohormone, regulates diverse developmental and physiological processes throughout the entire life cycle of plants, including seed germination, root initiation, flower and leaf senescence, abscission, wounding response, and disease resistance (Abeles *et al.*, 1992). One of the most studied examples of ethylene regulation is the ripening of climacteric fruit. Climacteric fruit development typically includes a transition phase during which ethylene responsiveness and production levels are dramatically altered. While the outcome of these changes, the ripening process, has been extensively characterized in many plant species (Abeles *et al.*, 1992; Fluhr and Mattoo, 1996), the developmental processes that lead up to and accompany the changes are less well understood (Giovannoni, 2001). Although tomato has generally been the model of choice in the study of

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, 1-aminocyclopropane-1-carboxylic acid oxidase; ACS, 1-aminocyclopropane-1-carboxylic acid synthase; DAB, days after bloom; 'EG', 'Early Golden'; ETO1, ethylene-overproducer 1; EOL1, ETO1-like; GA, gibberellin; PGR, plant growth regulator; 1-MCP, 1-methylcyclopropene; SAM, S-adenosyl-L-methionine; 'SH', 'Shiro'; TOE, target of ETO1.

climacteric fruit ripening (Alexander and Grierson, 2002), species such as plum have distinct features that are economically important and provide opportunities to dissect specific mechanisms.

Fruits can be classified as climacteric or non-climacteric depending on the presence or absence of post-harvest, ripening-associated rise in ethylene evolution and respiration (Biale and Young, 1981). Plum (*Prunus salicina*), like peach (*P. persica*) and apricot (*P. armeniaca*), is a climacteric fruit characterized by a large diversity for the date and rate of ripening. Some late plum cultivars such as 'Shiro' and 'Golden Japan' show a suppressed-climacteric pattern, while 'Early Golden' and 'Santa Rosa' behave as typical climacteric fruit (Abdi *et al.*, 1997; Zuzunaga *et al.*, 2001). However, treatment of such late cultivars with propylene showed that fruit displaying the suppressed-climacteric phenotype ripen as a typical climacteric fruit, so should still be placed in the climacteric class (Abdi *et al.*, 1997).

The cumulative growth pattern of stone fruits (*Prunus* spp.), including plums, is portrayed by a typical double sigmoid curve during fruit development and ripening (Tonutti *et al.*, 1997). Within this developmental period, four distinct stages (S1–S4) are clearly recognized. In the first stage (S1), of intense cell division and differentiation, growth is rapid. A long period of stasis follows (S2) during which the endocarp hardens to form a solid stone coincident with hardly any increase in fruit size. The third stage (S3) is accompanied by rapid cell division resulting in a significant increase in fruit size. Finally the fourth stage (S4), is when the fruit arrive at their fullsize and start to enhance ripening in an ethylene-dependent manner accompanied by changes in colour, texture, and aroma enrichment.

Ethylene is synthesized from *S*-adenosyl-L-methionine (SAM) via 1-aminocyclopropane-1-carboxylic acid (ACC). The conversion of SAM to ACC is catalysed by ACC synthase (ACS), and the subsequent oxidation of ACC to ethylene is catalysed by ACC oxidase (ACO) (Yang and Hoffman, 1984). Although ACS and ACO are the two key enzymes in ethylene biosynthesis, ACS is generally the rate-limiting enzyme in the biosynthetic pathway. In *Arabidopsis*, tomato, and other species, ACS and ACO proteins are encoded by small gene families whose members are differentially regulated at the transcriptional level by various developmental, environmental, and hormonal signals (Fluhr and Mattoo, 1996; Wang *et al.*, 2002). Previous studies demonstrated that treatment of *Arabidopsis* seedlings with the phytohormones, auxin, gibberellin, or cytokinin, increase ethylene biosynthesis by activating ACS transcription only in the case of auxin and gibberellin (Abel *et al.*, 1995; Kaneta *et al.*, 1997), whereas cytokinin increases ethylene biosynthesis via a post-transcriptional mechanism (Vogel *et al.*, 1998; Woeste *et al.*, 1999).

Recent findings suggest that post-translational regulation is an important aspect of the control of ACS expression (Chae *et al.*, 2003). *Arabidopsis* and tomato ACS proteins have been shown to be post-translationally phosphorylated by a wounding-inducible kinase activity within the Val and Ser residues found in the conserved C-terminal region (Tatsuki and Mori, 2001; Chae *et al.*, 2003). Ethylene-overproducer 1 (ETO1) protein, a negative regulator of ethylene evolution, interacts strictly with this conserved C-terminal region, target of ETO1 (TOE) sequence, found uniquely in Type 2 ACS proteins, and degrades them via the ubiquitin–26S proteasome pathway (Chae *et al.*, 2003; Chae and Kieber, 2005; Yoshida *et al.*, 2005). Moreover, phosphorylation of the C-terminus of Type 1 ACS proteins by MPK6 suggests the involvement of the ACS C-terminal region in post-transcriptional regulation (Liu and Zhang, 2004; Chae and Kieber, 2005).

It has been proposed that specific members of ACS and ACO gene families control two systems of ethylene production in plants (Lelièvre *et al.*, 1997). System 1 is the basal low rate of ethylene production that is detected in all tissues including non-climacteric fruits. System 2, or autocatalytic ethylene, operates during climacteric fruit ripening. In tomato fruit, ethylene biosynthesis is primarily regulated by three different *Sl-ACS* mRNAs, *Sl-ACS2*, *-4*, and *-6* (Nakatsuka *et al.*, 1998). *Sl-ACS2* and *-4* are responsible for system 2 autocatalytic ethylene production and *Sl-ACS6* is responsible for the auto-inhibitory, low levels of system 1 ethylene (Barry *et al.*, 2000).

The objective of this study is to understand the role of ACS gene family members in the capacity of the fruit to produce autocatalytic ethylene and ripen. The ethylene evolution and the accumulation pattern of four *Ps-ACS* mRNAs were studied during plum fruit development and ripening in early 'Early Golden' ('EG') and late 'Shiro' ('SH') cultivars. The effects of the phytohormones, auxin, gibberellin, and cytokinin, on the regulation of the different *Ps-ACS* mRNAs were investigated. The role of ETO1-like (EOL1) in controlling the date and rate of fruit ripening by suppressing ethylene production in plum was also studied. The results are discussed in terms of system 1 and system 2 ethylene synthesis, and a model of *Ps-ACS* gene regulation throughout the double sigmoid growth pattern is proposed.

Materials and methods

Plant material and post-harvest treatments

Fruits were harvested from two Japanese plum (*Prunus salicina* L.) cultivars 'Early Golden' ('EG') and 'Shiro' ('SH') grown at the experimental farm of the University of Guelph (Vineland Station, ON, Canada). These two varieties were chosen according to their maturity times, early and late, respectively. Plum fruit were treated as described previously (El-Sharkawy *et al.*, 2007). Fruit tissues from five fruits exhibiting similar ethylene production were used for mRNA extraction and analysis. Since the seed is inseparable in S1

and S2, the whole fruit tissue was used for RNA extraction, while in S3 and S4 stages both fruit tissues (fruit pulp and seed) were carefully separated for RNA analysis. Other tissues such as shoot apex, axillary bud, young leaves, and flowers were collected from the 'EG' cultivar. All plant material was frozen in liquid nitrogen and stored at -80°C .

Plant growth regulator (PGR) treatments and growth conditions

To minimize the effect of possible regulation of *Ps-ACS* mRNAs caused by the presence of endogenous hormones (ethylene, auxin, and/or gibberellin), leaves from the 'EG' cultivar were used for studying the effect of various PGRs. Leaves were thoroughly rinsed in tap water, surface sterilized for 10 min in 20% (v/v) commercial bleach, washed 3 \times with sterile distilled water, and the excess water was gently dried under aseptic conditions. The leaves were cultured in full-strength woody plant basal medium (Lloyd and McCown, 1981) (PhytoTechnology Laboratories, KS, USA) supplemented with 2% (w/v) sucrose, 0.7% (w/v) agar, and the respective PGR treatment as detailed below. The cultures were incubated in a controlled environmental condition (relative humidity 80%, 22 $^{\circ}\text{C}$ and 16 h light/8 h dark cycles) for 4 weeks. After that, the leaves were transferred to suspension cultures in 250 ml flasks containing 100 ml of liquid medium with appropriate PGRs as treated earlier. This ensures sufficient imbibition of PGRs by the leaves. The suspension cultures were incubated on a rotary shaker (100 rpm) and the leaves continued growing for 30 min at room temperature.

The PGR treatments include auxins, 2,4-D and picloram (0.5, 1, and 2 μM), IAA, IBA, and 1-NAA (0.4, 4, and 40 μM), gibberellins, GA₃ and GA₄₊₇ (0.4, 4, and 40 μM), and cytokinin-benzyladenine (0.4, 4, 20, and 40 μM). Leaves cultured in basal medium without any PGR were used as control. At the end of the suspension culture all the plant tissues were removed, briefly blotted dry, immediately frozen in liquid nitrogen, and stored at -80°C for RNA analysis.

RNA isolation

Total RNA from fruit samples was extracted using the methods described by Boss *et al.* (1996). For vegetative tissues and flowers, total RNA was extracted using the PureLink Plant RNA reagent (Invitrogen, Burlington, ON, Canada) as per the manufacturer's instructions. All RNA extracts were treated with DNase I (Promega, Madison, WI, USA), then cleaned up with an RNeasy mini kit (Qiagen, Mississauga, ON, Canada).

Isolation and in silico analysis of plum cDNA sequences

For the isolation of plum homologues of ACC synthase (ACS), first-strand cDNA synthesis was carried out using 20 μg of total DNase-treated RNA in a 50 μl aliquot. One microlitre of cDNA was used in a PCR with the appropriate degenerate primers. Several sets of primers (Jones and Woodson, 1999) were used to isolate plum *Ps-ACS* sequences that shared the structural characteristics associated with functional ACS proteins (Zarembinski and Theologis, 1994; Capitani *et al.*, 1999). To isolate *Ps-EOL1* cDNA, a PCR primer set was synthesized based on the conserved region of the nucleotide sequences of ETO1 (Wang *et al.*, 2004; Yoshida *et al.*, 2006) [Eol1-F 5'-ACCCAACACTCACATWCCCATAC-3'; Eol1-R 5'-CAA-GATTGTTGAGGGCCTGACC-3']. The isolated fragments were cloned in pGEM-T easy vector (Promega, Madison, WI, USA), sequenced and compared with database sequences using the BLAST program (Altschul *et al.*, 1997). Extension of the partial cDNA clones was carried out using the 3'- and 5'-RACE kit (Invitrogen, Burlington, ON, Canada). Full-length amplification of cDNA sequences, designated *Ps-ACS1*, *Ps-ACS3*, *Ps-ACS4*, and *Ps-ACS5*, was carried out using Platinum *Taq* DNA Polymerase High Fidelity

following the instructions provided by the manufacturer (Invitrogen). Alignments of the predicted protein sequences were performed with ClustalX (Jeanmougin *et al.*, 1998) and GeneDoc (Nicholas and Nicholas, 1997). The Neighbor-Joining tree was constructed with PAUP* 4.0b3. Bootstrap values from 1000 replicates were obtained. The tree was visualized with the TreeView program (Page, 1996).

Real time quantitative RT-PCR

DNase-treated RNA (5 μg) was reverse transcribed in a total volume of 50 μl using SuperScript III Reverse Transcriptase (Invitrogen). Real-time quantitative PCR was performed using SYBR GREEN PCR MasterMix (Qiagen) on a Mx4000[®] multiplex quantitative PCR system (Stratagene, La Jolla, CA, USA). Mx4000[®] v. 4.20 software (Stratagene) was used to design gene-specific primers (Table 1). For all the sequences studied here, optimal primer concentration was 200 nM. RT-PCR conditions were as described previously (El-Sharkawy *et al.*, 2007). The PCR products were analysed further by a dissociation curve program at 95–60 $^{\circ}\text{C}$ (16 s). All RT-PCR experiments were run in triplicate with different cDNAs synthesized from three biological replicates. Each sample was run in three technical replicates on 96-well plates. For each sample, a Ct (threshold constant) value was calculated from the amplification curves by selecting the optimal ΔRn (emission of reporter dye over starting background fluorescence) in the exponential portion of the amplification plot. Relative fold differences were calculated based on the comparative Ct method using the β -actin as an internal standard (El-Sharkawy *et al.*, 2007). To demonstrate that the efficiencies of the different gene primers were approximately equal, the absolute value of the slope of log input amount versus ΔCt was calculated for *Ps-ACS1*, *Ps-ACS3*, *Ps-ACS4*, *Ps-ACS5*, *Ps-EOL1*, and β -actin sequences, and was determined to be <0.1 . To determine relative fold differences for each sample in each experiment, the Ct value for the five mRNAs studied was normalized to the Ct value for β -actin and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta\text{Ct}}$. The calibrator is the sample that exhibited the minimum level of transcripts in the whole experiment (fruits 47 DAB for *Ps-ACS3*, fruits 52 DAB for *Ps-ACS1*, -4, and -5, and 'EG' fruits 82 DAB for *Ps-EOL1*; Figs 3–7) and control tissues (Figs 8–10).

Promoter isolation

Genomic DNA was extracted from immature leaves according to the DNeasy Plant Maxi Kit (Qiagen). Promoters of *Ps-ACS3* [*Ps-DACS3a* (EU034651), *Ps-DACS3b* (EU034652)] were isolated from 'EG' and 'SH' genotypes using the Universal Genome Walker Kit (Clontech, Palo Alto, CA, USA). In order to identify the presence of the different

Table 1. Real-time quantitative PCR primers

Name	Sequence
Ps-ACS1-F	5'-ACCGCGTTCACGTTGTTT-3'
Ps-ACS1-R	5'-TGCCACAACCATGTCGTC-3'
Ps-ACS3-F	5'-GTGCAACTGCAGCCAATG-3'
Ps-ACS3-R	5'-GGTTGGCACAAGCAAAGC-3'
Ps-ACS4-F	5'-GCGTCGATGCTCTTGGAT-3'
Ps-ACS4-R	5'-AAAACCCCGTGCCTCTTC-3'
Ps-ACS5-F	5'-GATCTTGGATGGCGAACG-3'
Ps-ACS5-R	5'-AGCCTCCAAGGCTGCTCT-3'
Ps-EOL1-F	5'-TGATGCAGCAAAGGTGTTTC-3'
Ps-EOL1-R	5'-ACTTTTCATGCTCGCTGGAT-3'
Ps-actin-F	5'-CTGGACCTTGCTGGTTCGT-3'
Ps-actin-R	5'-ATTTCCCGCTCAGCAGTG-3'

Ps-ACS3 promoters in ‘EG’ and ‘SH’ cultivars, four specific primers for *Ps-DACS3a* and *b*, ACS3a-F [-1078] (5’-GCGTAATTTATGAA-TACAATATCCCTAC-3’), ACS3a-R (5’- CCCAGTTGGAAGCT-TCTGAGTGCTTCC-3’) [331], ACS3b-F [-1456] (5’-ATC-TACTGATTCCAAGTCGTTAATAC-3’), and ACS3b-R [-810] (5’-GAGAATTTACGTATATAAGGTAATAGAG-3’), were designed from each promoter sequence. PCR contained ~150 ng of template genomic DNA and were carried out using an Advantage Genomic PCR Kit (Clontech).

Results

Protein structure and organization

ACS cDNAs and proteins have been isolated from a wide variety of plant species. Although sequence identity can be as low as 45% in *ACS* proteins, there are highly conserved signature elements. Four novel *Ps-ACS* sequences were isolated from two plum cultivars using an RT-PCR approach in order to investigate the involvement of *ACS* proteins in fruit ripening (Fig. 1). All *Ps-ACS* isozymes contain the seven conserved boxes found in *ACS* from *Arabidopsis*, tomato, and other plant species. Eleven out of 12 amino acid residues conserved in aminotransferase and *ACS* proteins, as well as the glutamate (E) residue in box 1 that is responsible for substrate specificity (McCarthy *et al.*, 2001), are present in all of the plum sequences (Mehta *et al.*, 1989;

Yamagami *et al.*, 2003). All *Ps-ACS* predicted proteins, except *Ps-ACS3*, have the Ser residue that is phosphorylated by a calcium-dependent protein kinase, CDPK (Tatsuki and Mori, 2001), whereas only *Ps-ACS4* and -5 predicted proteins have the three Ser residues that are targets of the protein kinase MPK6 phosphorylation (Liu and Zhang, 2004) (Fig. 1). Interestingly, *Ps-ACS1* predicted protein has only one out of the three Ser residues (S465) important for phosphorylation by MPK6 (Fig. 1).

The relationships between the predicted amino acid sequences, as indicated by percentage similarity over the whole sequence, are presented in Table 2. Although there was considerable divergence among the *Ps-ACS* mRNAs, there were also highly homologous sequences putatively coding for closely related isozymes. Sequences related to the isolated plum cDNAs have been identified previously in pear (El-Sharkawy *et al.*, 2004). Strong sequence similarity between specific plum and pear sequences (89–93%; Table 2) indicates that these sequences are likely to be orthologous.

Arabidopsis and tomato *ACS* proteins have been shown to be post-transcriptionally and/or post-translationally regulated by the phosphorylation of target residues found in the conserved C-terminal region (Tatsuki and Mori, 2001; Chae *et al.*, 2003; Liu and Zhang, 2004; Yoshida *et al.*, 2005). A classification of ACC synthase based on

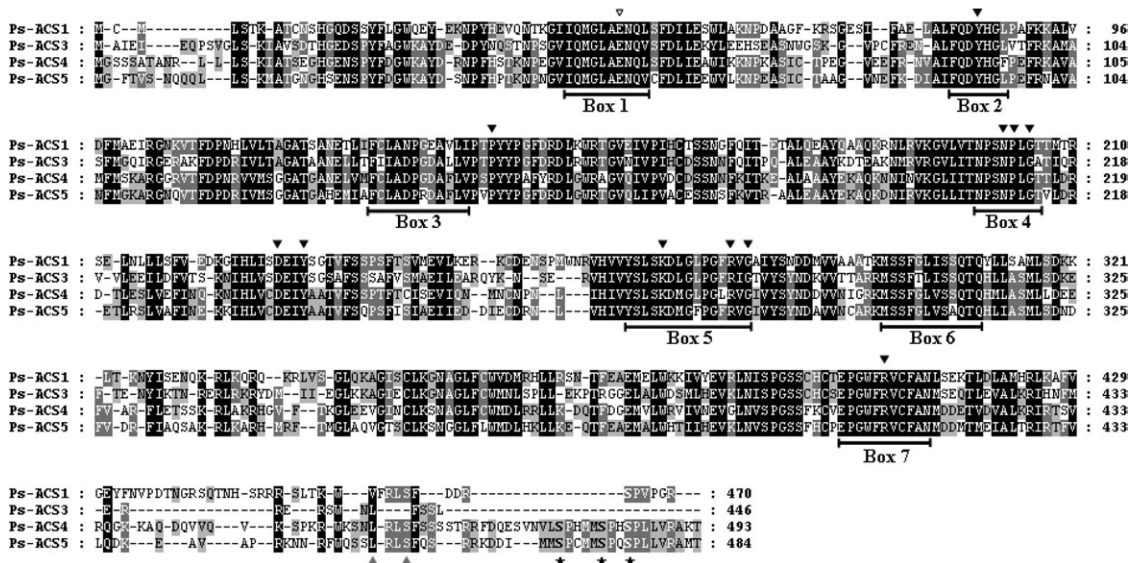


Fig. 1. Amino acid sequence alignment of *Ps-ACS1* (EU034649), *Ps-ACS3* (EU034650), *Ps-ACS4* (EU034653), and *Ps-ACS5* (EU034654) using the ClustalX program. Conserved residues are shaded in black. Dark grey shading indicates similar residues in three out of four of the sequences and light grey shading indicates similar residues in two out of four of the sequences. The 11 black arrows designate the residues that represent the conserved amino acids in aminotransferases. The conserved glutamate residue (E) marked with an open arrow is involved in substrate specificity (McCarthy *et al.*, 2001). The two grey arrows indicated the Val and Ser residues important for interaction with ETO1 and CDPK phosphorylation (Tatsuki and Mori, 2001; Yoshida *et al.*, 2005). The Ser residues that are targets of the protein kinase MPK6 phosphorylation (Liu and Zhang, 2004) are marked with asterisks.

Table 2. Amino acid sequence comparison between the predicted full-length plum and pear ACC synthase cDNAs

	Protein size (no. of aa)	Amino acid similarity percentage			
		Ps-ACS1	Ps-ACS3	Ps-ACS4	Ps-ACS5
<i>Prunus salicina</i>					
Ps-ACS1	470	100			
Ps-ACS3	446	68	100		
Ps-ACS4	493	64	65	100	
Ps-ACS5	484	65	65	73	100
<i>Pyrus communis</i>					
Pc-ACS1	473	93	68	62	65
Pc-ACS2	446	67	89	63	63
Pc-ACS3	446	68	91	64	64
Pc-ACS4	495	64	65	91	72
Pc-ACS5	487	64	64	72	93

the similarity of DNA sequences and the C-terminal consensus motif has been reported (Yoshida *et al.*, 2005). Predicted proteins of both Ps-ACS4 and -5 are members of Type 1 ACC synthases that have the Ser residue in the 'RLSF' motif, necessary for CDPK phosphorylation (Tatsuki and Mori, 2001), followed by a long C-terminal tail (27–33 aa) containing the three conserved Ser residues proved to be the targets of MPK6 phosphorylation (Liu and Zhang, 2004) but lack the Val in the 'WVF' motif (Fig. S1 in Supplementary data available at *JXB* online). In both of these predicted proteins the 'WVF' motif is degenerated to 'WKSNL' and 'WQSSL', respectively (Fig. 1). Ps-ACS1 predicted protein belongs to Type 2 ACS isozymes that possess the conserved TOE sequence (Yoshida *et al.*, 2006), which includes the 'WVF' sequence just before the 'RLSF' motif, followed by a short tail (nine amino acids) rich in basic and acidic amino acids (Supplementary Fig. S1). The predicted Ps-ACS3 protein is a member of Type 3 isozymes, where the C-terminus is truncated by 24–47 amino acids and consequently lacks all the residues important for phosphorylation and the 'WVF' motif degenerates to 'WNLF' (Fig. 1).

The phylogenetic tree presented in Fig. 2 was obtained by comparing the C-terminal amino acid sequences of 23 ACS proteins from four species—plum, pear, *Arabidopsis*, and tomato. It indicates that ACS proteins can be divided into three main subfamilies (1, 2, and 3). Sequences corresponding to the first subfamily, Type 1, comprising all the At-ACS proteins, have been shown to be post-transcriptionally regulated by MPK6 phosphorylation (Liu and Zhang, 2004). The second subfamily, Type 2, isozymes containing all the *Arabidopsis* and tomato ACS proteins, have been shown to confer a strong ability to bind ETO1 and to be post-translationally regulated by CDPK phosphorylation (Vogel *et al.*, 1998; Chae *et al.*, 2003; Yoshida *et al.*, 2005). The third subfamily, Type 3, contains the ACS proteins that have a long C-terminal (Sl-ACS4) or a short

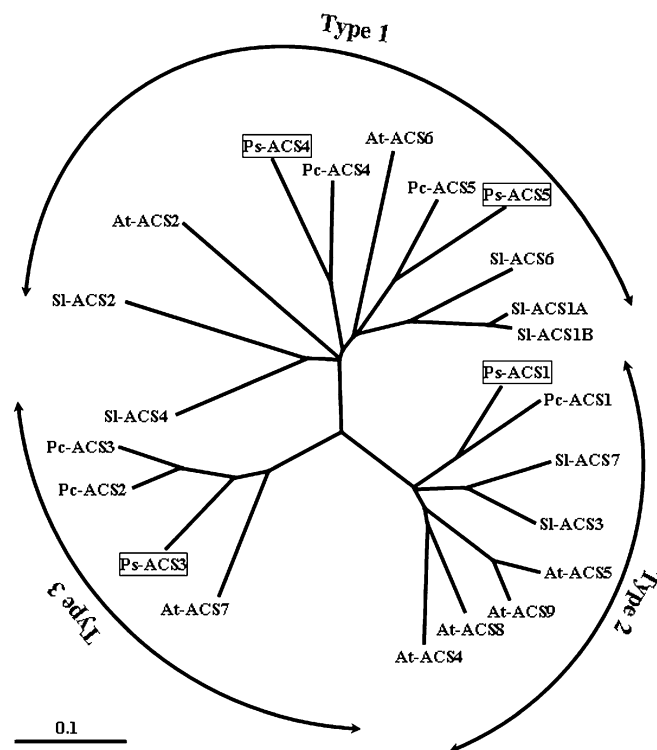


Fig. 2. Phylogenetic relationships between *Prunus salicina* [*Ps-ACS1*, *Ps-ACS3*, *Ps-ACS4*, *Ps-ACS5*], *Arabidopsis thaliana* [*At-ACS2* (Q06402), *At-ACS4* (NP179866), *At-ACS5* (AAG50098), *At-ACS6* (T13019), *At-ACS7* (AAG48754), *At-ACS8* (AAG50090), *At-ACS9* (AAG48755)], *Solanum lycopersicon* [*Sl-ACS1A* (AAF97614), *Sl-ACS1B* (AAF97615), *Sl-ACS2* (CAA41855), *Sl-ACS3* (AAB48945), *Sl-ACS4* (AAA03164), *Sl-ACS6* (BAA34923), *Sl-ACS7* (AAC32317)], and *Pyrus communis* [*Pc-ACS1* (X87112), *Pc-ACS2* (AF386519), *Pc-ACS3* (AY388988), *Pc-ACS4* (AF386518), *Pc-ACS5* (AF386523)] based on amino acid sequences. Type 1, Type 2, and Type 3 show the different ACS protein subfamilies.

C-terminal tail (Ps-ACS3, Pc-ACS2, Pc-ACS3, and At-ACS7) but in both cases lack all the residues important for post-transcriptional and/or post-translational regulation.

A plum homologue of the *Arabidopsis* ethylene-overproducer 1-like (*At-ETO1*) has also been isolated. The partial *Ps-EOL1* sequence encoded a putative protein of 245 amino acid residues. The predicted protein displayed strong homology (92% identity, 97% similarity) to the tomato *Sl-EOL1* sequence (Yoshida *et al.*, 2006). Analysis of the partial *Ps-EOL1* amino acid sequence revealed the presence of four out of six TPR (tetratricopeptide repeat) motifs which are involved in diverse protein–protein interactions and can serve as a scaffold for assembly of multi-protein complexes (D'Andrea and Regan, 2003).

Ethylene production during fruit development and ripening

Low levels of ethylene production, ranging from 0.8 to 2 $\text{nl g}^{-1} \text{h}^{-1} \pm 0.2$, have been detected during S1 of fruit development (22–37 DAB). In both studied cultivars,

'EG' and 'SH', the whole fruit produced ethylene throughout S4 fruit ripening (Fig. 5A, B). No ethylene emission was detected from the separated S3 and S4 seeds (data not shown). 'EG' fruit displayed an early, rapid ripening, and a short and rapid (maximal 5 d) ethylene production profile (Fig. 5A). 'SH' fruit exhibited a suppressed climacteric pattern. The fruit ripened more slowly and later than 'EG', with ethylene production in 'SH' fruit reaching a maximum at ~11 d after the onset of ethylene emission (Fig. 5B). 1-Methylcyclopropene (1-MCP) treatment immediately at harvest before the onset of endogenous ethylene production completely abolished the ethylene burst and ripening in the pre-treated fruits (data not shown).

The expression of the Ps-ACS cDNAs during fruit development and ripening

To investigate the possible role of ACC synthase in controlling ethylene production and subsequently in the capacity of plum fruit to ripen, four *Ps-ACS* mRNAs were identified. Real-time PCR analysis of steady-state mRNA levels was carried out to determine their mRNA accumulation patterns throughout fruit development and ripening.

Transcripts of *Ps-ACS1*, -3, and -5 accumulated at constant levels in vegetative tissues, while those of *Ps-ACS4* mRNA remained low or undetectable in such tissues (data not shown). Figures 3 and 4 show the expression pattern of the four cDNAs during flower and early fruit development (0–15 DAB), and throughout fruit development (22–77 DAB), respectively. *Ps-ACS4* transcripts were expressed at a basal low level or were almost undetectable during these periods of fruit development at 0–77 DAB (Figs 3, 4). *Ps-ACS1*, -3, and -5 mRNAs accumulated in the same pattern during the periods mentioned (Figs 3, 4). The three *Ps-ACS* transcripts peaked in opened flowers (~4 DAB), followed by strong inhibition in their transcript levels after fertilization, ~7 DAB (Fig. 3). During early fruit development (7–15 DAB), transcript levels for *Ps-ACS1*, -3, and -5 increased slightly (Fig. 3).

Throughout S1 of fruit development (~27 DAB), another peak of the three transcripts was detected (Fig. 4), declining slightly thereafter (32–37 DAB) to reach their basal levels, and becoming almost undetectable during S2 of fruit development at 42–52 DAB (data not shown). During S3 (57–77 DAB) of fruit development and when the pulp was separated from the seed (endocarp+embryo), high transcript levels of *Ps-ACS1*, -3, and -5 were only detected in the fruit between 62 and 72 DAB (Fig. 4). Markedly higher accumulation levels of the three mRNAs were detected in the seed than in the pulp. The expression levels of *Ps-ACS1*, -3, and -5 were ~4, 150, and 110 times higher, respectively, in the seed than in the pulp (Fig. 4). Only *Ps-ACS1* and -3 transcript levels showed

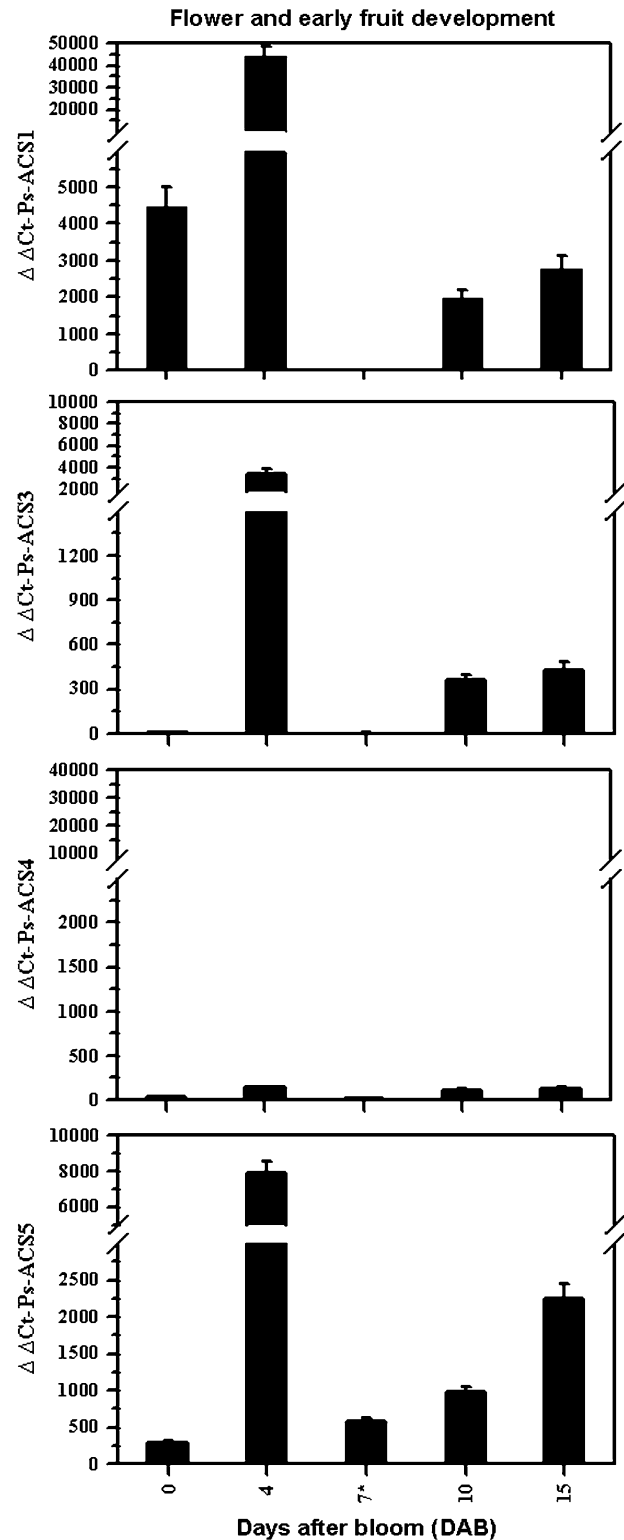


Fig. 3. Steady-state transcript levels of *Ps-ACS1*, *Ps-ACS3*, *Ps-ACS4*, and *Ps-ACS5* assessed by real-time quantitative PCR during flower and early fruit development of the 'EG' cultivar. The fertilized flower stage is marked with an asterisk. The experiments were carried out in triplicate. The x-axis represents the developmental stages indicated by number of days after bloom (DAB). $\Delta\Delta C_t$ on the y-axis of each figure refers to the fold difference in gene expression relative to fruits 47 DAB for *Ps-ACS3* and fruits 52 DAB for *Ps-ACS1*, -4, and -5.

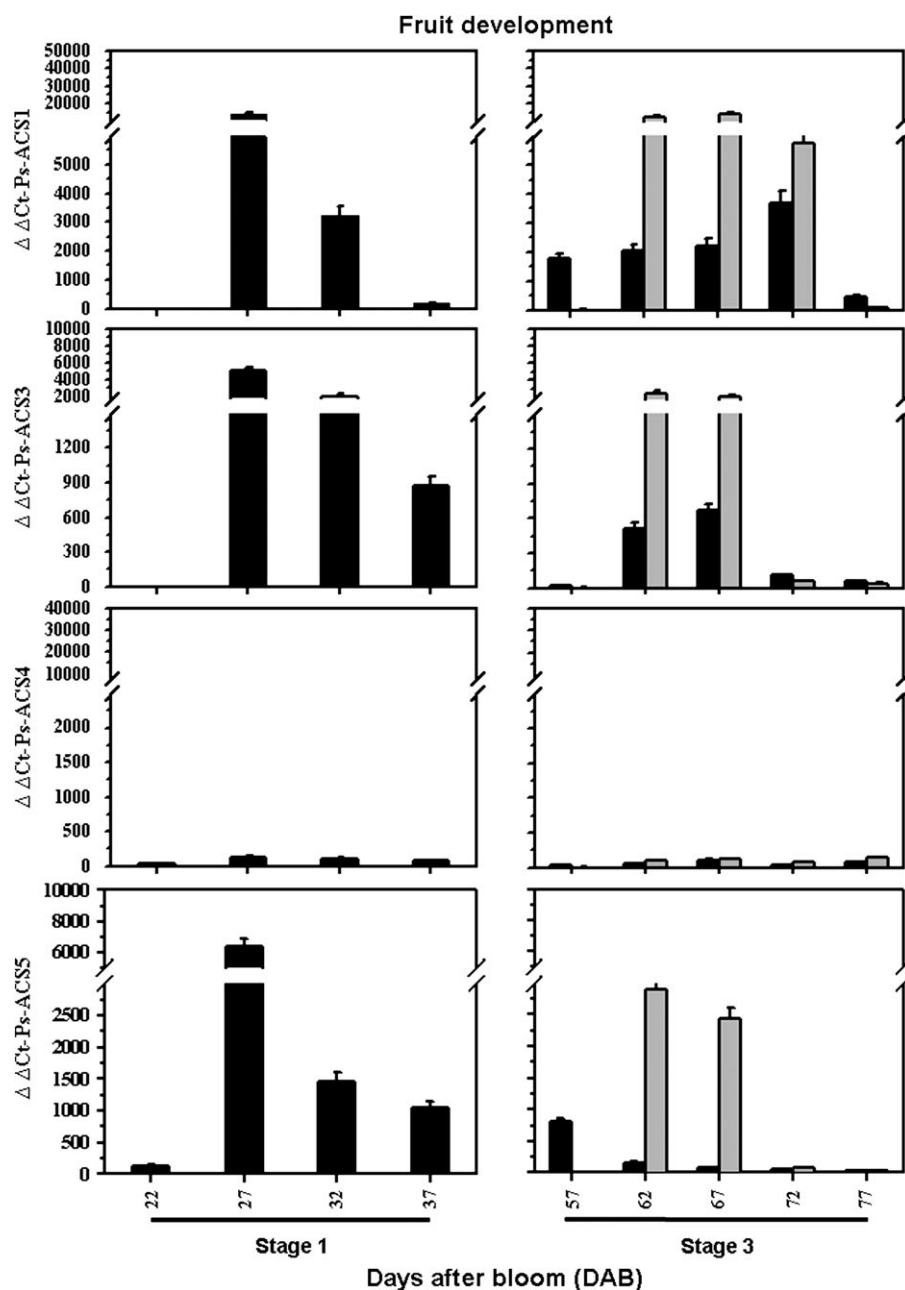


Fig. 4. Steady-state transcript levels of *Ps-ACS1*, -3, -4, and -5 assessed by real-time quantitative PCR during S1 of fruit development using the 'EG' whole fruit. During S3 of fruit development the expression was studied in pulp (black-filled columns) and in seeds (grey-filled columns). The experiments were carried out in triplicate. The x-axis represents the developmental stage indicated by number of days after bloom (DAB) and by the name of the stage. $\Delta\Delta Ct$ on the y-axis of each figure refers to the fold difference in gene expression. Other details are as described in Fig. 3.

a steady increase in the pulp tissue, whereas those of *Ps-ACS5* decreased in abundance. The three transcripts reached a basal level in the whole fruit at the end of S3 at ~77 DAB.

'EG' fruit showed a dramatic increase in the transcript levels of *Ps-ACS1*, -3, and -4 mRNAs throughout S4 at fruit ripening (78–83 DAB). Transcripts of the three cDNAs were low or almost undetectable in the non-climacteric stage of 'EG' fruit at ~78 DAB (Fig. 5A, C,

E, G). As ripening progressed and higher levels of autocatalytic ethylene were produced (Fig. 5A), the expression levels of *Ps-ACS1*, -3, and -4 mRNAs increased in abundance. *Ps-ACS1* transcripts markedly increased in whole fruit but late, during the climacteric stage (~82 DAB), and strongly declined thereafter but only in the seed (Fig. 5C). *Ps-ACS3* transcript levels increased to a peak ~80 DAB and decreased slightly thereafter (Fig. 5E), whereas those for the *Ps-ACS4*

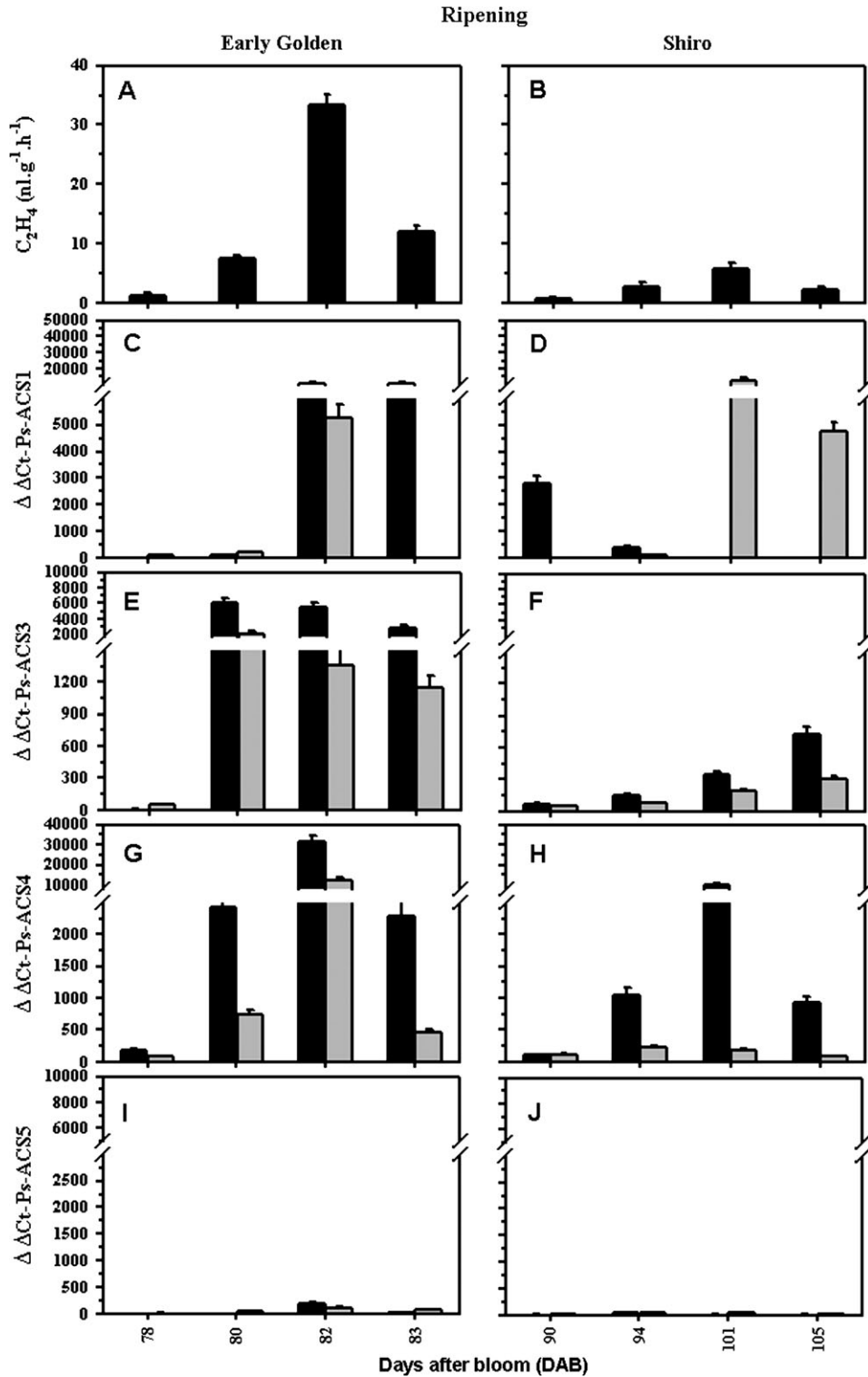


Fig. 5. Ethylene evolution and steady-state transcript levels assessed by real-time quantitative PCR throughout fruit ripening: (A, B) ethylene production; (C, D) *Ps-ACS1*; (E, F) *Ps-ACS3*; (G, H) *Ps-ACS4*; and (I, J) *Ps-ACS5* transcript levels during ripening of early 'EG' (left panel) and late 'SH' (right panel) plum fruits, respectively. The expression was studied in pulp (black-filled columns) and in seeds (grey-filled columns). The experiments were carried out in triplicate. The x-axis represents the developmental stage indicated by number of days after bloom (DAB). $\Delta\Delta Ct$ on the y-axis of each figure refers to the fold difference in gene expression. Other details are as described in Fig. 3.

mRNA accumulation peak correlated well with the increase in ethylene production at ~82 DAB, (Fig. 5A, G).

Throughout ripening of late 'SH' fruit (90–105 DAB), *Ps-ACS1* decreased in abundance in the pulp but steadily increased in the seeds (Fig. 5D). *Ps-ACS3* transcripts slightly accumulated in both fruit tissues to reach their maximum in the post-climacteric stage at ~105 DAB (Fig. 5F). The expression of *Ps-ACS4* in 'SH' fruit has the same pattern as in 'EG' fruit, except that its accumulation in the seed remained constant and low throughout 'SH' fruit ripening (Fig. 5H). In both plum cultivars, *Ps-ACS5* transcripts were absent or expressed in very low levels in the whole fruit throughout ripening (Fig. 5I, J).

Effect of 1-MCP on the expression of the four Ps-ACS genes during fruit ripening

To determine the role of ethylene in regulating *Ps-ACS* gene expression throughout early and late fruit ripening, the expression of the four cDNAs was studied in 1-MCP-pre-treated fruit during ripening.

The increases in *Ps-ACS3* transcripts during early 'EG' fruit ripening were not found to be ethylene dependent, but seemed to be ripening related, since no significant changes in its accumulation level or pattern were observed in the MCP-treated fruits (Fig. 6C). 1-MCP completely inhibited the ethylene-associated transcription of *Ps-ACS1* and -4 in the whole fruit (Fig. 6A, E). The effect of 1-MCP in the inhibition of *Ps-ACS4* mRNA accumulation was stronger in the pulp than that in the seed (Fig. 6E).

In late 'SH' fruit, the *Ps-ACS1* accumulation level or pattern was not significantly affected by MCP treatment (Fig. 6B). 1-MCP treatment strongly reduced the ripening-related increase in *Ps-ACS4* expression and, in contrast to the situation in 'EG', also for *Ps-ACS3* (Fig. 6D, F). Accumulation of both transcripts did not significantly respond to 1-MCP treatment in 'SH' seeds (Fig. 6D, F).

Contrary to the situation during fruit ripening, 1-MCP treatment enhanced the accumulation of *Ps-ACS5* transcripts in both cultivars (Fig. 6G, H). The level of *Ps-ACS5* mRNA was higher in the seed than in the pulp of 'EG' MCP-treated fruit (Fig. 6G); whereas 1-MCP treatment did not affect the transcript level of *Ps-ACS5* in 'SH' MCP-treated seed (Fig. 6H).

The expression of the Ps-EOL1 during fruit ripening

To investigate if the differences between the two plum cultivars in the date and rate of ripening are related to the differences in *Ps-EOL1* mRNA accumulation pattern and/or level, the expression of *Ps-EOL1* mRNA during ripening of early and late plum cultivars was studied.

Throughout ripening of early 'EG' fruit, high levels of *Ps-EOL1* transcripts accumulated in the non-climacteric stage at ~78 DAB (Fig. 7A). As ripening progressed and higher levels of ethylene were produced, the expression

levels of *Ps-EOL1* mRNAs decreased in abundance to reach their basal level at the climacteric stage (~82 DAB). In the post-climacteric stage (~83 DAB), and when climacteric ethylene evolution starts to decline, *Ps-EOL1* transcripts start to increase again (Fig. 7A). Unlike 'EG' fruit, the expression levels of *Ps-EOL1* mRNA increased in abundance during late 'SH' fruit ripening to reach a maximum at the post-climacteric stage (Fig. 7B). In both cultivars, 1-MCP treatment enhanced the accumulation of *Ps-EOL1* transcripts in the whole fruit (Fig. 7C, D).

Effect of cytokinin on the expression of the four Ps-ACS cDNAs

Cytokinin increases ethylene biosynthesis by increasing uniquely Type 2 ACS isozyme activity via a post-transcriptional mechanism (Vogel *et al.*, 1998; Woeste *et al.*, 1999). To explore this effect, the expression level of the four *Ps-ACS* transcripts was studied in leaves treated with different concentrations of cytokinin. From the four *Ps-ACS* mRNAs isolated, only *Ps-ACS1* mRNA showed a steady increase in response to cytokinin treatment (Fig. 8). *Ps-ACS1* mRNA levels did not respond significantly to low cytokinin concentration (0.4 μM ; Fig. 8). A dramatic increase in *Ps-ACS1* transcript levels was detected with increasing cytokinin concentrations (4–40 μM ; Fig. 8).

Effect of auxins and gibberellins (GAs) on the expression of the four Ps-ACS mRNAs

High levels of different *Ps-ACS* transcripts accumulated in the seed during fruit development and ripening. To investigate whether any of the *Ps-ACS* mRNAs under study were induced by auxin and/or gibberellin that strongly occurred during seed development, the expression of the four cDNAs was studied in leaves treated with different types and concentrations of synthetic and natural auxins and gibberellins.

None of the *Ps-ACS* transcripts significantly responded to 2,4-D treatment, at least under the conditions used in this study (data not shown). The transcript levels of the four cDNAs increased along with increasing picloram concentrations (Fig. 9A–D).

Plant tissues treated with IAA, IBA, and NAA exhibited dramatic increases in *Ps-ACS1*, -3, and -5 mRNA levels. IAA- and NAA-treated leaves strongly accumulated *Ps-ACS1* and -3 mRNAs but only with low concentrations of hormones (0.4 μM and/or 4 μM) and declined thereafter with higher concentrations (Fig. 9E, F, M, N). While the *Ps-ACS5* accumulation pattern was similar to those of *Ps-ACS1* and -3 in response to IAA treatment (Fig. 9H), in NAA-treated tissues *Ps-ACS5* mRNA expression levels significantly increased only at high NAA concentrations (Fig. 9P).

Ps-ACS1 and -3 mRNAs were mostly detected only in leaves treated with elevated IBA concentrations (Fig. 9I,

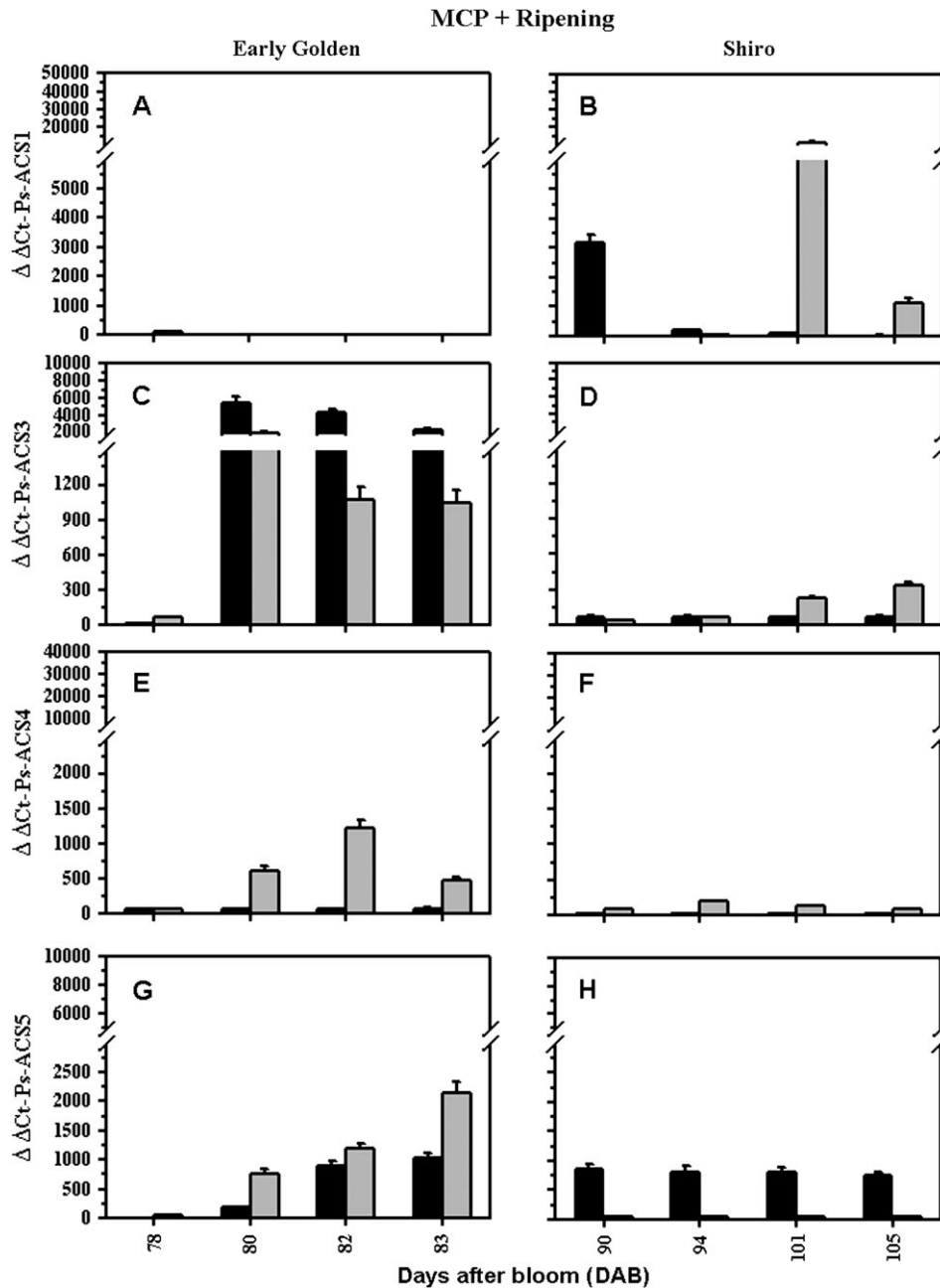


Fig. 6. Ethylene evolution and steady-state transcript levels assessed by real-time quantitative PCR during ripening of 1-MCP-pre-treated fruit: (A, B) *Ps-ACS1*; (C, D) *Ps-ACS3*; (E, F) *Ps-ACS4*; and (G, H) *Ps-ACS5* transcript levels during ripening of early ‘EG’ (left panel) and late ‘SH’ (right panel) plum fruits, respectively. The expression was studied in pulp (black-filled columns) and in seeds (grey-filled columns). Fruits were exposed overnight immediately at harvest (76 DAB and 88 DAB for ‘EG’ and ‘SH’, respectively) to 1-MCP ($1 \mu\text{l l}^{-1}$) before the onset of endogenous ethylene. The experiments were carried out in triplicate. The x-axis represents the developmental stage indicated by number of days after bloom (DAB). $\Delta\Delta\text{Ct}$ on the y-axis of each figure refers to the fold difference in gene expression. Other details are as described in Fig. 3.

J), whereas *Ps-ACS5* expression levels did not significantly respond to IBA treatment (Fig. 9L). The expression of *Ps-ACS4* was not affected by IAA and IBA treatments, but was markedly induced by NAA at high concentrations ($40 \mu\text{M}$; Fig. 9G, K, O).

Ps-ACS4 mRNA levels did not significantly respond to any of the gibberellin treatments used in this study

(Fig. 10C, G). *Ps-ACS1*, -3, and -5 expression levels were slightly induced by low GA_3 concentration ($0.4 \mu\text{M}$) and declined thereafter to reach their basal level with higher concentrations (Fig. 10A, B, D). The effect of GA_3 treatment on the induction of the different *Ps-ACS* mRNAs was significantly higher for *Ps-ACS5* transcripts than those of *Ps-ACS1* and -3.

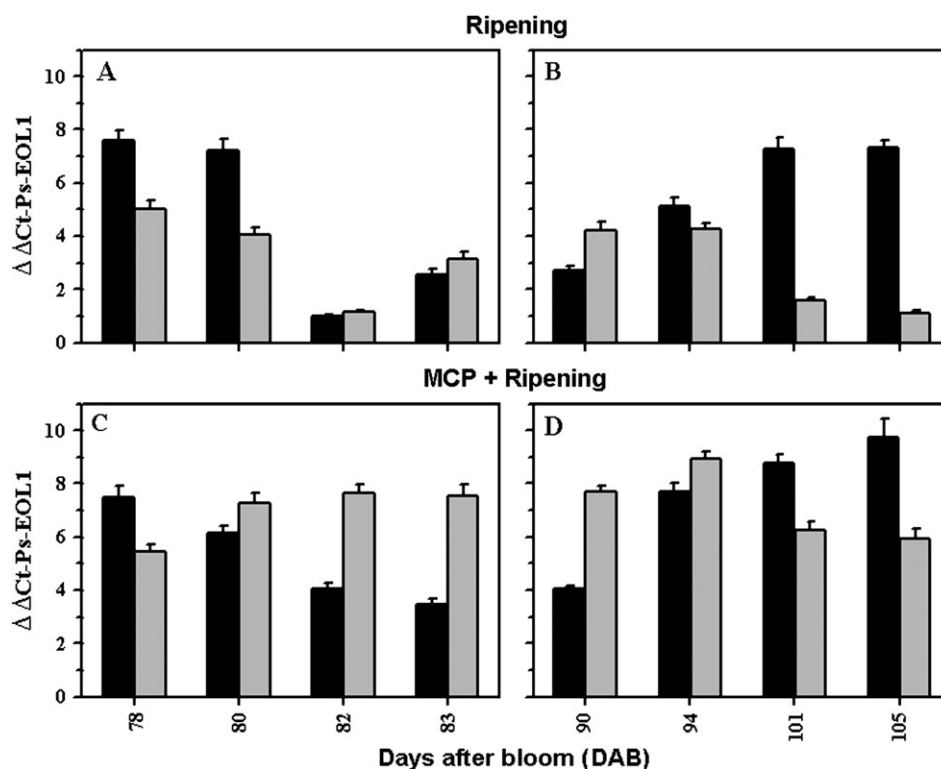


Fig. 7. *Ps-EOLI* (EU176813) gene expression in early 'EG' (left panel) and late 'SH' (right panel) plum fruits. The steady-state transcript levels were assessed by real-time quantitative PCR during ripening of 'EG' (A) and 'SH' (B) fruits, and in MCP-treated 'EG' (C) and 'SH' (D) fruits. The experiments were carried out in triplicate. The *x*-axis represents the developmental stage indicated by number of days after bloom (DAB). $\Delta\Delta\text{Ct}$ on the *y*-axis of each figure refers to the fold difference in *Ps-EOLI* gene expression relative to fruits 82 DAB.

Transcript levels of the three *Ps-ACS* mRNAs increased after treatment with GA_{4+7} (Fig. 10E, F, H). The patterns of transcript accumulation for the three cDNAs were, however, quite different. The levels of *Ps-ACS1* mRNA increased to reach their maximum with 4 μM GA_{4+7} concentrations and declined thereafter at the highest concentration (40 μM) (Fig. 10E). *Ps-ACS3* transcript levels increased dramatically along with increasing GA_{4+7} concentrations (Fig. 10F). *Ps-ACS5* mRNA accumulated in GA_{4+7} -treated leaves in the same manner as *Ps-ACS3* but at much lower levels (Fig. 10H).

Alleles identified for the *Ps-ACS3* promoter region

Since *Ps-ACS3* mRNA showed differential expression between early and late plum cultivars, further genomic characterization was undertaken in order to determine possible genetic linkages with the level of autocatalytic ethylene and the capacity of fruit to ripen.

Two alleles of the 5'-non-coding regions of the *Ps-ACS3* [*Ps-DACS3a* (-1349 bp) and *Ps-DACS3b* (-1496 bp)] gene were identified from the 'EG' and 'SH' cultivars. The percentage similarity between the isolated sections of the 5'-non-coding regions was 57% (Fig. S2 in Supplementary data available at *JXB* online). Interestingly, the analysis of both promoter sequences revealed

the presence of a sequence motif box only in the *Ps-DACS3b* promoter sequence (Supplementary Fig. S2) which shows 87.5% identity with an ERE (ethylene responsive element) regulatory element found in the promoter of tomato *E4* gene (Montgomery *et al.*, 1993).

PCR was performed to determine the presence of the *Ps-DACS3a/b* alleles in the 'EG' and 'SH' genomes. While the early 'EG' genotype was homozygous for *Ps-DACS3a*, the late 'SH' genotype was found to be *Ps-DACS3a/b* heterozygous (Fig. 11).

Discussion

In general Japanese plum fruits are climacteric, characterized by producing a burst of autocatalytic ethylene that triggers and maintains many aspects of the ripening process (Abdi *et al.*, 1997). However, within these plums there are marked differences that distinguish them from the other climacteric fruits. *Prunus* spp., including plum, exhibit a typical double sigmoid growth pattern during fruit development and ripening (Tonutti *et al.*, 1997). Within this developmental period, four distinct stages are clearly recognized.

There are big differences in ripening behaviour and in ethylene production and responses in the plum cultivars

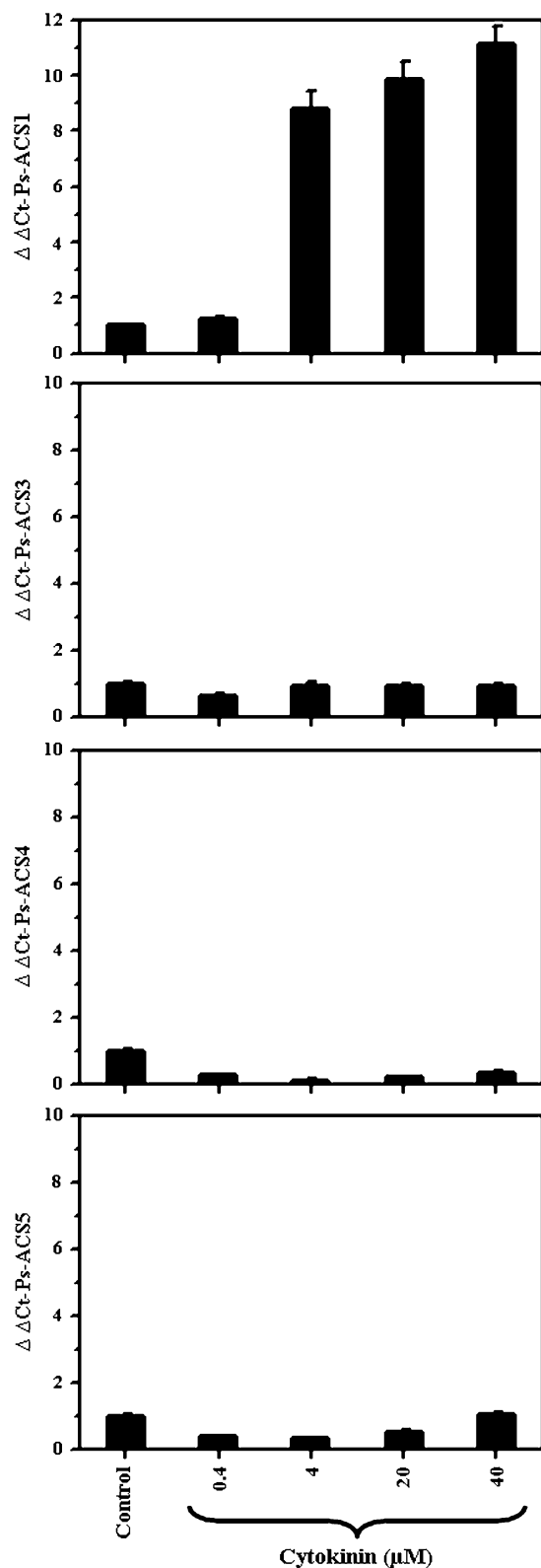


Fig. 8. Steady-state transcript levels of *Ps-ACS1*, -3, -4, and -5 assessed by real-time quantitative PCR in control 'EG' leaves and 'EG' leaves treated with cytokinin using the indicated concentrations. The experiments were carried out in triplicate. The *x*-axis represents the type and concentration (μM) of cytokinin used in this study. $\Delta\Delta\text{Ct}$ on the *y*-axis of each figure refers to the fold difference in gene expression relative to the control.

used in this study. 'EG' fruits showed ripening patterns typical of climacteric fruits, while the late cultivar ('SH') exhibited a suppressed-climacteric pattern with only a slight increase in autocatalytic ethylene production related to ripening.

Some ethylene responses, including *ACO1* gene expression and enzyme activity, can be induced in immature green plum fruits and seeds, which remain unable to produce autocatalytic ethylene and to ripen (Fernández-Otero *et al.*, 2006). This indicates that other factors control the ethylene response and ripening, and consequently control the ethylene levels during later stages of fruit ripening. In tomato, ACS has been shown to control the onset and maintenance of the ripening-related autocatalytic ethylene (Nakatsuka *et al.*, 1998).

Four ethylene biosynthetic mRNAs, putatively encoding ACS isozymes, were isolated and characterized from two plum cultivars in order to determine their role in fruit ripening in terms of the double sigmoid growth pattern. The predicted amino acid sequences had the traits generally associated with ACS post-transcriptional and/or post-translational regulation and related aminotransferase activity (Yamagami *et al.*, 2003; Yoshida *et al.*, 2005).

In the dendrogram, a number of well-defined branches have both *Arabidopsis* and tomato sequences but lack plum sequences, suggesting that there are likely to be as yet unidentified ACS genes within the plum genome. In species where multiple ACS genes have been characterized, each mRNA appears to have a unique mode of transcriptional and/or post-translational regulation (Wang *et al.*, 2002).

Post-translational regulation has been identified in a number of *Arabidopsis* and tomato ACS proteins (Chae *et al.*, 2003; Yoshida *et al.*, 2005). *Ps-ACS4* and -5 predicted proteins are closely related to *At-ACS2* and -6 (Type 1), respectively, that were characterized as lacking the Val residue in the conserved C-terminal 'WVF' motif but have the Ser target of CDPK phosphorylation in the 'RLSF' motif (Tatsuki and Mori, 2001) followed by an extended C-terminal region containing the three conserved Ser residues that are the target for MPK6 phosphorylation (Liu and Zhang, 2004). Replacement of the conserved Val residue with a Leu in all ACS proteins belonging to the Type 1 subfamily (Fig. S1 in Supplementary data available at *JXB* online) suggests that these proteins have special post-translational behaviour. The ethylene-overproducing phenotype of the *eto3* mutation is a result of replacing the Val residue in the 'WVF' motif, which is essential for the regulation of *At-ACS9* activity, with aspartic acid (Chae *et al.*, 2003), suggesting an important role for this small motif in protein stability. ACS isozymes from Type 2 have been shown to possess the TOE sequence that includes the 'WVF', 'RLSF', and R/D/E-rich motifs (Yoshida *et al.*, 2006). Additionally, all ACS isoforms that have been shown to be post-translationally

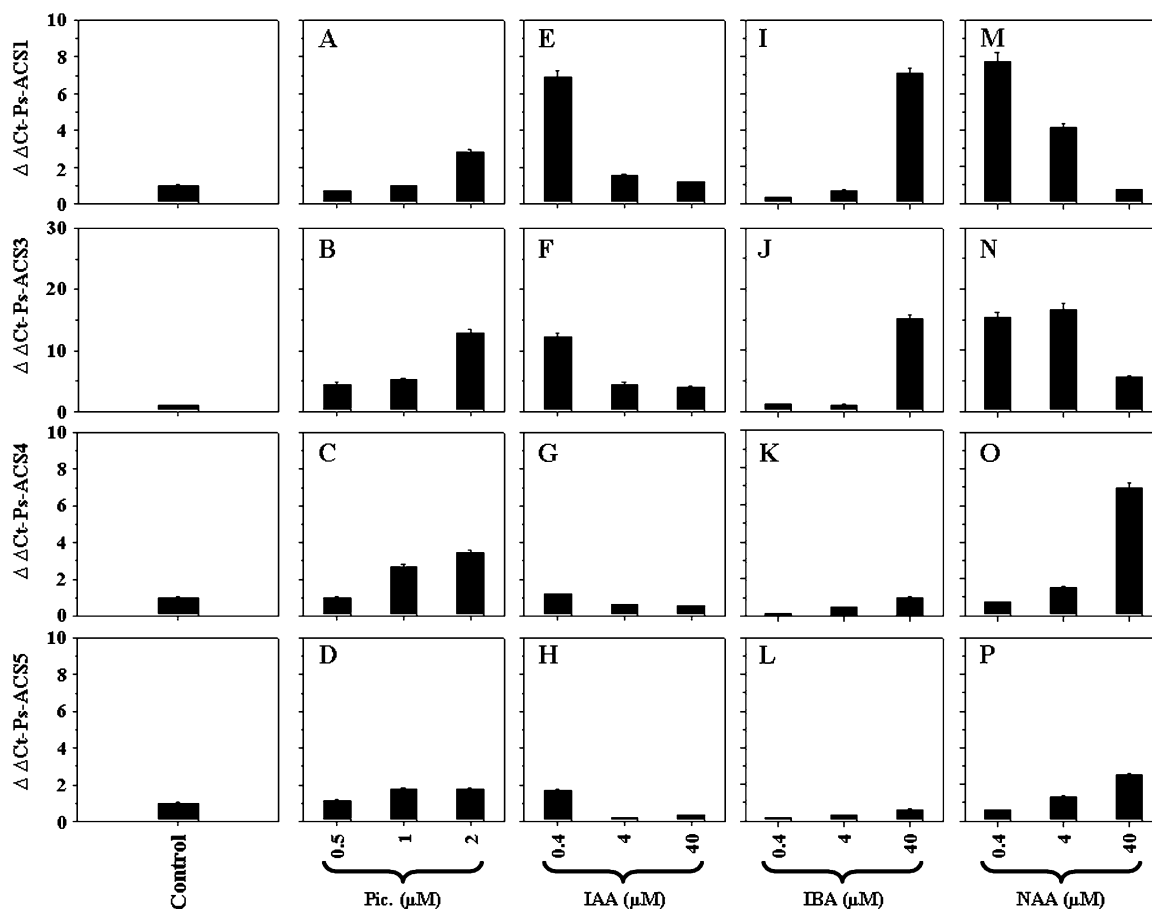


Fig. 9. Steady-state transcript levels of *Ps-ACS1*, -3, -4, and -5 assessed by real-time quantitative PCR in control ‘EG’ leaves and ‘EG’ leaves treated with different types of auxins (picloram, IAA, IBA, and NAA) using the indicated concentrations. The *x*-axis represents the type and concentration (μM) of auxin used in this study. $\Delta\Delta\text{Ct}$ on the *y*-axis of each figure refers to the fold difference in gene expression relative to the control.

regulated by CDPK phosphorylation fall into this group (Chae *et al.*, 2003; Sebastià *et al.*, 2004). *Ps-ACS1* as *At-ACS4*, -5, -8, and -9 and *Sl-ACS3* has the corresponding Val and Ser residues necessary for ETO1 interaction and CDPK phosphorylation (Tatsuki and Mori, 2001; Yoshida *et al.*, 2005). None of the ACS isoforms from Type 2 has the three conserved Ser residues found in Type 1 ACC synthase, and MPK6 does not phosphorylate *At-ACS5* *in vitro* (Liu and Zhang, 2004), indicating a restricted specific regulation of Type 1 ACS isoforms by MPK6. Interestingly, the *Ps-ACS1* predicted protein has one putative phosphorylation site (S465) where the Ser residue was followed by Pro (Fig. S1 in Supplementary data available at *JXB* online), which provides the ability of *Ps-ACS1* protein to serve as an MPK6 substrate. Liu and Zhang (2004) suggested that the phosphorylation of the three Ser residues by MPK6 is independent of each other, but the level of phosphorylation is related to the number of phosphorylation sites. Thus, at least two parallel signalling pathways, containing either a CDPK, for Type 2 ACS isoforms, and/or MPK6 for Type 1 ACS

isozymes, might be involved in modulating ACS function and activity. *Ps-ACS3* (Type 3) has a short C-terminal region compared with the other *Ps-ACS* sequences and consequently lacks all the residues important for protein phosphorylation. It seems that *Ps-ACS3* protein may play an important role in stabilizing ACC levels in plums. Yoshida *et al.* (2005) showed that ETO1 specifically interacts with Type 2 of ACC synthase (*At-ACS5* and *Sl-ACS3*), but not with Type 1 (*Sl-ACS2*) or Type 3 (*Sl-ACS4*) in the yeast two-hybrid system. Mutation of the *Sl-ACS2* C-terminus, by adding the ‘WVF’ motif just before the ‘RLSF’ motif to mimic *Sl-ACS3* protein, was sufficient to recover the strong interaction with ETO1 to a level comparable with *Sl-ACS3* (Yoshida *et al.*, 2005).

Ps-ACS1, -3, and -5 are expressed in very high levels in opened flowers and rapidly decline after fertilization. The complete inhibition of the different *Ps-ACS* transcripts, post-fertilization, correlated well with a large accumulation of free polyamines that occur strongly at fertilization (Pérez-Amador *et al.*, 1995; Antognoni *et al.*, 2002; Aziz, 2003; DeDios *et al.*, 2006). Ethylene and polyamines

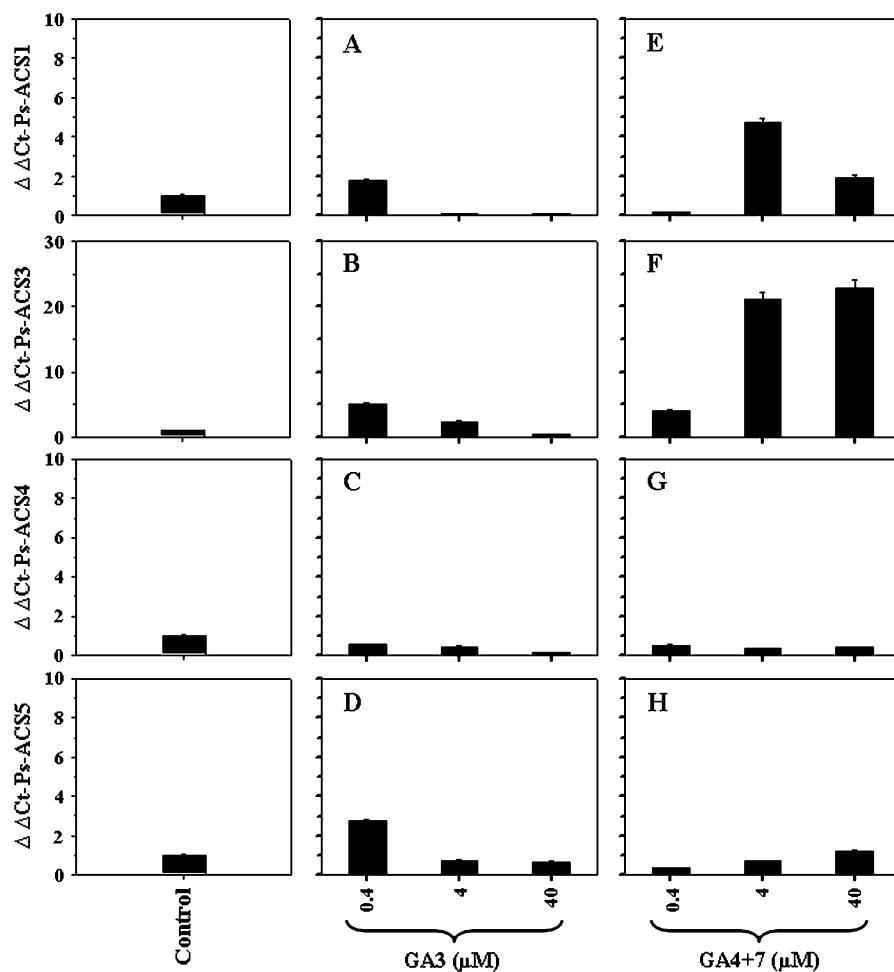


Fig. 10. Steady-state transcript levels of *Ps-ACSI*, -3, -4, and -5 assessed by real-time quantitative PCR in control 'EG' leaves and 'EG' leaves treated with different types of gibberellins (GA_3 and GA_{4+7}) using the indicated concentrations. The experiments were carried out in triplicate. The x-axis represents the type and concentration (μM) of gibberellin used in this study. $\Delta\Delta Ct$ on the y-axis of each figure refers to the fold difference in gene expression relative to the control.

share SAM as a common intermediate. It was shown that ethylene biosynthesis may compete with the biosynthesis of polyamines (Kushad and Dumbroff, 1991; Escribano and Merodio, 1994), and it cannot be ruled out that SAM may be alternatively channelled towards ethylene or the polyamine pathway, constituting a control mechanism for certain physiological processes in higher plants (Walden *et al.*, 1997). The gradual increase in abundance of the three transcripts throughout early fruit development may reflect the important roles of ethylene in petal wilt, flower abscission, and embryo development.

Ps-ACSI, -3, and -5 increased, again, to a peak in the young fruit at ~ 27 DAB. The increase of the three transcripts corresponds to low levels of ethylene production, and high levels of IAA and cytokinin contents, important for cell division during embryogenesis, which occurred throughout S1 of fruit development (Miller *et al.*, 1987; Hartmann *et al.*, 2002; this study). Ethylene production in the S1 stage is perhaps needed for

regulating the abundant cell division and embryo development characterizing this stage of fruit development. It was found that the low ethylene emission throughout this stage of fruit development coincided with high ACC and *N*-malonyl-ACC (MACC) contents detected in the non-lignified endocarp (DeDios *et al.*, 2006). The absence of all the studied *Ps-ACS* transcripts throughout S2 of fruit development coincided with strong down-regulation of four different ethylene perception and signal transduction component elements (El-Sharkawy *et al.*, 2007), suggesting only a minor role for ethylene during this stage.

Although there was hardly any ethylene detected throughout S3 of fruit development, high transcript levels of *Ps-ACSI*, -3, and -5 were detected in the seed from this stage. During S3, the endocarp is almost fully developed to form the highly lignified seed, which could not be involved in any synthesis of ethylene for many reasons: (i) any accumulation of MACC and ACC coincided with very high levels of free polyamines in the lignified seed;

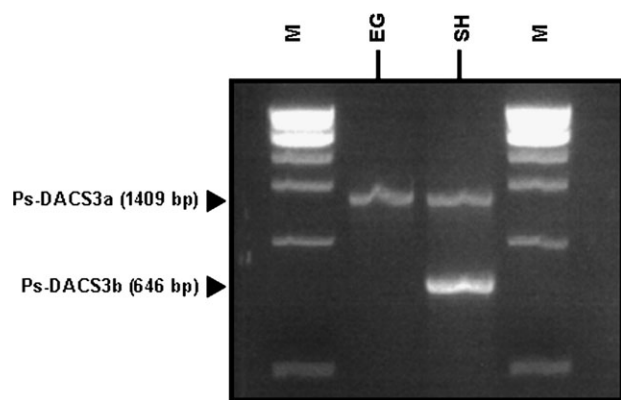


Fig. 11. Diagnosis of the two *Ps-DACS3a/b* alleles in two plum cultivars, 'EG' and 'SH'. DNA from both cultivars was extracted from leaves. Specific primers were used to amplify each 5'-flanking region and the PCR products were viewed on a 1.2% agarose gel. The sizes of the fragments are given on the left side. Bands of 1409 bp and 646 bp correspond to *Ps-DACS3a* (EU034651) and *Ps-DACS3b* (EU034652), respectively.

(ii) the seed, which develops much later than the fruit, does not have *in vivo* ACO activity (DeDios *et al.*, 2006). The present results showed that the strong accumulation of *Ps-ACS1*, -3, and -5 in the seed coincides with the high auxin and gibberellin content detected in the seed during S3 (Jackson, 1968; Miller *et al.*, 1987). The role of seeds in stone fruit development was determined by Jackson (1968) and Miller *et al.* (1987) who provided evidence that seeds stimulate fruit growth and ripening by providing auxins and gibberellins. The accumulation of *Ps-ACS1* and -3 transcripts in the fruit pulp was ethylene independent but seemed to be auxin and/or gibberellin dependent. Early induction of both transcripts suggests their importance for the fruit to ripen.

Differences in the ripening behaviour between the cultivars reflect an altered capacity to produce and respond to ethylene. Mature 'EG' fruit are capable of producing autocatalytic ethylene, and ripen, ~82 DAB, as a typical climacteric fruit. Late 'SH' fruit exhibited a very low ethylene emission and ripened more slowly and later than 'EG' fruit. However, fruits pre-treated with propylene could recover the climacteric pattern and ripen as rapidly as early cultivars (Abdi *et al.*, 1997).

From the different ACS isozymes studied, *Ps-ACS1* and -3, and partially *Ps-ACS4* cDNAs showed a cultivar-specific expression pattern and/or level throughout fruit ripening. In both cultivars studied, *Ps-ACS1*, -3, and -4 expression levels dramatically increased in abundance during the ripening process (S4). Transcripts of the three mRNAs in 'SH' fruits were at least four times lower than in 'EG' fruits.

During 'EG' and 'SH' fruit ripening, *Ps-ACS1* mRNA accumulated in ethylene-dependent and -independent manners, respectively. Isolation of the *Ps-ACS1* promoter sequence (~1.8 kb) from both cultivars showed no significant differences in their homology (data not shown).

In 'EG' fruits, *Ps-ACS3* transcript levels increased early during the onset of ripening in an ethylene-independent manner. By contrast, the induction of *Ps-ACS3* transcripts, throughout ripening of 'SH' fruit, was dependent on ethylene action. Both plum cultivars share a common allele of the *Ps-DACS3* promoter associated with *Ps-DACS3a*, whereas the second allele, associated with *Ps-DACS3b*, was found only in the late 'SH' genotype. It is clear that the *Ps-ACS3* isoform that functions during fruit development and throughout 'EG' fruit ripening is *Ps-ACS3a*, while the *Ps-ACS3b* isoform is only functional during 'SH' fruit ripening. Interestingly, the promoter of *Ps-DACS3b* has two boxes of motif sequences that share high sequence identity with a regulatory element found in the promoter of *Arabidopsis* ERF1 (Solano *et al.*, 1998). This sequence has also been found in the promoter regions of many genes which are up-regulated by ethylene (Itzhaki and Woodson, 1993; Montgomery *et al.*, 1993). The presence of the two boxes in the promoter is necessary for full promoter activity (Tapia *et al.*, 2005). In 'SH' fruit, the low transcript levels of *Ps-ACS3* (*Ps-ACS3b*) expressed during ripening may be related to the low quantities of autocatalytic ethylene produced.

In both cultivars, *Ps-ACS4* mRNA levels increased linearly with the ethylene levels. 1-MCP treatment completely repressed *Ps-ACS4* expression. The transcript levels of *Ps-ACS1*, -3, and -4 appear to play an important role in determining the level of autocatalytic ethylene production and the capacity of the fruit to ripen. *Ps-ACS5* mRNA was negatively regulated by ethylene during ripening of both cultivars studied, since its transcripts only accumulated in non-autocatalytic ethylene-produced tissues such as vegetative tissues, flowers, immature fruit, and MCP-treated fruits.

The high transcript levels of *Ps-ACS1*, -3, and -4 detected in the seeds during ripening have no role in the synthesis of autocatalytic ethylene produced throughout this stage of fruit development, since the endocarp is already lignified at this stage and, as in other seeds (Rodríguez-Gacio and Matilla, 2001), the activity of the last step of ethylene synthesis is inhibited. By contrast, the greatest amount of ethylene produced and ACC and MACC content have been observed in the ripening mesocarp and epicarp (DeDios *et al.*, 2006).

In both plum cultivars, the fruit development process seems to progress in parallel. Once the ripening stage (S4) has started, the ripening process in the late 'SH' cultivar seems to progress much more slowly than in the early 'EG' cultivar. As mentioned previously, there are large differences in ripening behaviour and in ethylene production and responses in the plum cultivars used in this study. ETO1-like protein (EOL1) is a negative regulator of ethylene evolution and interacts with Type 2 ACS proteins to degrade them via the ubiquitin-26-S proteasome pathway (Chae *et al.*, 2003; Chae and Kieber, 2005; Yoshida *et al.*, 2005).

The expression of *Ps-EOL1* throughout 'EG' and 'SH' fruit ripening was studied to explore the possible role of EOL1 protein in suppressing ethylene production and delaying ripening in plum fruit. *Ps-EOL1* transcript levels decreased in abundance along with increasing ethylene evolution. Contrarily, 'SH' fruits significantly accumulated *Ps-EOL1* mRNA during ripening. Interestingly, *Ps-EOL1* transcripts were negatively regulated by ethylene in both the cultivars studied, since 1-MCP treatment increased its transcript accumulation. It seems that late 'SH' fruit produces insufficient amounts of ethylene to inhibit *Ps-EOL1* mRNA induction. However, treatment of 'SH' fruit with propylene restored the climacteric pattern and the fruit ripens as a typical climacteric fruit (Abdi *et al.*, 1997). Recent studies suggest that cytokinin increased ethylene biosynthesis by inhibiting the interaction between Type 2 ACS isozymes and ETO1 protein, which resulted in reducing ACS protein degradation by a proteasome-dependent pathway (Wang *et al.*, 2004; Chae and Kieber, 2005). Cytokinin activity tends to be high in developing fruits but decreases thereafter and becomes difficult to detect in mature fruits (Hartmann *et al.*, 2002). This indicates that cytokinin does not have any role in enhancing ethylene production in plum fruit through the inhibition of EOL1 and Type 2 ACC synthesis interaction (Chae and Kieber, 2005; this study). Taken together, the data suggest that there is a minimum requirement for ethylene quantity necessary to inhibit the negative ethylene regulator, *Ps-EOL1*, transcript levels, but once this minimum requirement is met and *Ps-EOL1* transcripts are down-regulated, the developmental changes required for ripening will be accelerated.

As mentioned before, cytokinin increases ethylene biosynthesis via a post-transcriptional mechanism (Vogel *et al.*, 1998; Woeste *et al.*, 1999) by blocking the interaction of the Type 2 ACS isozymes with ETO1 protein that leads to an increase in ACS protein stability (Chae and Kieber, 2005), whereas the application of other plant hormones such as auxin and gibberellin can affect ethylene levels through increased ACS transcription levels (Abel *et al.*, 1995; Kaneta *et al.*, 1997). Ethylene can also affect its own biosynthesis, either increasing (autocatalysis) or decreasing (autoinhibition) its rate of production (Nakatsuka *et al.*, 1998). All these plant hormones play an important role in the differential accumulation of the various *Ps-ACS* mRNAs in flowers, and throughout fruit development and ripening. Among the four *Ps-ACS* mRNAs characterized, only *Ps-ACS1* is up-regulated by cytokinin. *Ps-ACS1* and -3 transcripts were found to be strongly up-regulated by ethylene, auxin, and gibberellin. Despite the strong accumulation of *Ps-ACS4* mRNA by the synthetic auxins, picloram and NAA, none of the natural auxins or gibberellins induced its expression. It seems that *Ps-ACS4* transcript abundance strongly increased in response to ethylene evolution. Unlike *Ps-*

ACS4, *Ps-ACS5* transcripts were completely inhibited by ethylene; however, auxin and gibberellin significantly induced its mRNA accumulation. Thus the various *Ps-ACS* isozymes seem to regulate distinct developmental processes and environmental responses differentially.

In tomato, it has been proposed that there are two systems of ethylene production. System 1 ethylene is autoinhibitory and operates in immature fruit and vegetative tissues. System 2, or autocatalytic ethylene, is produced during climacteric fruit ripening (Lelièvre *et al.*, 1997; Barry *et al.*, 2000). In tomato and other climacteric fruits, *ACS* genes play a critical role in the transition from system 1 to system 2.

Throughout plum fruit development, *Ps-ACS1*, -3, and -5 transcripts are present well above their basal levels. In both the cultivars studied, autocatalytic ethylene resulted in complete inhibition of *Ps-ACS5* transcripts. *Ps-ACS1* and -3 transcripts were induced in ethylene-independent and/or -dependent manners during ripening of both cultivars. Together, these results suggest that *Ps-ACS5* and, to some extent *Ps-ACS1* and -3, are important for system 1 ethylene production and offer further evidence for the existence of a dual system of ethylene production in plum.

The model presented in Fig. 12 illustrates our current understanding of the control of ethylene production in plum fruit within the two-systems approach. In immature plum fruit, system 1 ethylene relies on the expression of *Ps-ACS5* and partially on *Ps-ACS1* and -3*a*. System 1 ethylene synthesis continues throughout fruit development (0–62 DAB) until the fruit attains a state of physiological maturity, at which point a transition occurs (62–77 DAB). In mature plum fruit, the transition from system 1 to system 2 is induced throughout the induction and/or repression of unknown ethylene-independent factors so that the fruit gains the competence to ripen. These factors could be the increase of plant hormones, auxin, gibberellin, and, in slightly later stages, ethylene, which coincided with the repression of *Ps-EOL1* transcripts. *Ps-ACS1* and -3*a* mRNAs are expressed during this transition period in an ethylene-independent manner but in auxin- and/or gibberellin-dependent manner. This resulted in ethylene production that activates the expression of *Ps-ACS1*, -3*a*, and -4 in 'EG' fruit, and *Ps-ACS1*, -3*b*, and -4 in 'SH' fruit. The elevated levels of ethylene produced in system 2 resulted in negative feedback of the system 1 developmental pathway and consequently reduced *Ps-ACS5* expression. In tomato, transition from system 1 to system 2 is associated with the expression of *Sl-ACS4* which triggers the expression of *Sl-ACS2* and inhibits the expression of *Sl-ACS6* (Alexander and Grierson, 2002).

The results presented here suggest that differences in *Ps-ACS* gene expression between early and late plums are important determinators in the ripening behaviour of the cultivars. However, in addition to *Ps-ACS* expression, it is likely that *Ps-EOL1* is also involved in determining the

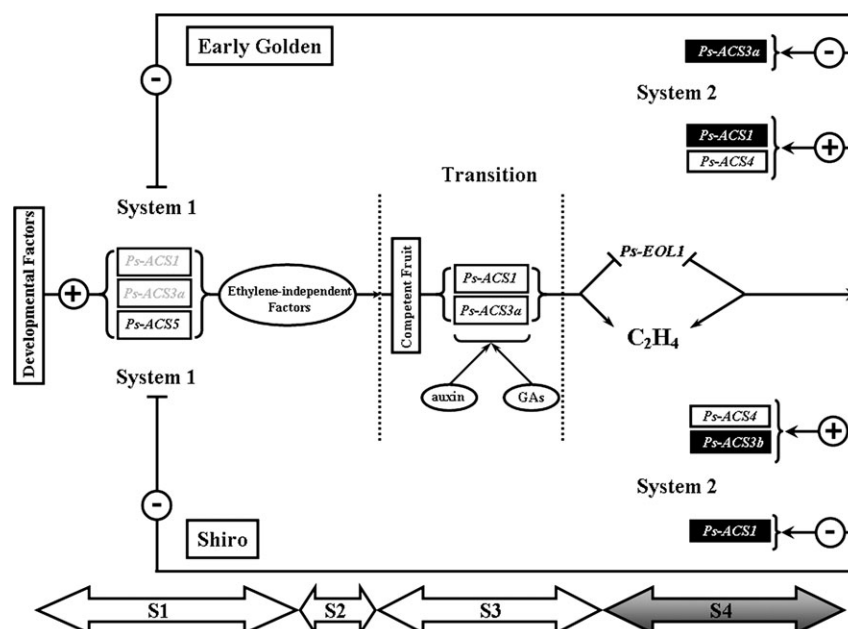


Fig. 12. Model for the regulation of *Ps-ACS* gene expression during fruit development and throughout ripening of early (Early Golden) and late (Shiro) plum cultivars. The plus (+) and minus (-) symbols refer to the positive and negative actions of ethylene, respectively, during system 1 and system 2 ethylene synthesis that leads to the activation (+) or repression (-) of *Ps-ACS* gene expression. Filled black rectangles correspond to mRNAs that are differentially regulated in early and late plum cultivars. Throughout the transition stage both hormones, auxin and gibberellin, from the seed play a major role in initiating the ripening process through increasing *Ps-ACS1* and *-3a* transcript levels, which result in increasing endogenous ethylene levels. High levels of ethylene repress *Ps-EOL1* transcripts and accelerate the autocatalytic ethylene production in the fruit (system 2). The four stages of plum fruit development throughout the double sigmoid growth pattern are indicated by S1 to S4.

suppressed-climacteric pattern in late plum cultivars. In tomato, the *rin* mutant fails to produce autocatalytic ethylene and to ripen (Giovannoni, 2001). This non-ripening phenotype is caused by mutations in transcription factors that control the expression of ripening-related genes. *Sl-ACS4* and *Sl-ACS2* transcript accumulation, critical for the onset of system 2 ethylene, does not occur in *rin* mutant fruit. Partially in 'SH' fruit, an ethylene-independent developmental process which operates to reduce *Ps-ACS* mRNA expression resulted in this late phenotype, 'SH', which appears to result from an inability of the fruit to produce sufficient quantities of ethylene to coordinate the transition from system 1 to system 2 ethylene production and ripening.

Supplementary data

Figure S1: Classification based on C-termini of ACC synthases of plum, tomato and *Arabidopsis*.

Figure S2: Nucleotide sequence alignment of the promoter regions of *Ps-ACS3a* and *Ps-ACS3b* genes using ClustalX program.

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