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Research Note

Ethylene biosynthesis in apricot: Identification of a ripening-related 1-aminocyclopropane-1-carboxylic acid synthase (*ACS*) gene

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ABSTRACT

Apricots are climacteric fruits with a high susceptibility to flesh softening and loss of flavor during postharvest storage, and most of the ripening processes are regulated by ethylene, which also has an effect on its own biosynthesis. To understand this process in apricot, inhibition of ethylene biosynthesis and perception was performed for studying key genes involved in the ethylene biosynthetic pathway. Apricots, cv. "Patterson", were harvested with yellow-green ground color and immediately treated with either the ethylene perception inhibitor 1-methyl cyclopropene (1-MCP) at 10 $\mu L\,L^{-1}$ or the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG) at 1 $g\,L^{-1}$. After treatment, quality and physiological attributes such as firmness, color, total soluble solids, acidity, fruit weight, ethylene production and respiration rates were evaluated every 2 d until they ripened at 20 °C. Gene expression analysis was performed by quantitative polymerase chain reaction (qPCR). Both ethylene inhibitors were effective in reducing ethylene production, respiration rate and fruit softening. Three 1-aminocyclopropane-1-carboxylic-acid synthase (*ACS*) genes were characterized, but only the expression of *ACS2* was highly reduced by ethylene inhibition, suggesting a key role in ethylene synthesis at ripening. Contrarily, *ACS1* and *ACS3* showed a higher expression under ethylene inhibition suggesting that the corresponding genes are individually regulated in a specific mode as observed in other climacteric fruits. Finally, changes in 1-aminocyclopropane-1-carboxylic-acid oxidase genes did not show a consistent pattern of ethylene modulation.

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1. Introduction

Apricot (*Prunus armeniaca* L.) is a climacteric fruit characterized by a peak in ethylene production near the ripe stage. In higher plants, ethylene is synthesized from the amino acid methionine by the conversion to S-adenosyl-L-methionine (SAM). SAM is transformed to 1-aminocyclopropane-1-carboxylic-acid (ACC) by the enzyme ACC synthase (ACS), and in the last step of the pathway, ACC is oxidized to ethylene by the action of the enzyme ACC oxidase (ACO) (Lin et al., 2009). During the ripening of climacteric fruit, ethylene can stimulate its own production, inducing autocatalysis through ACS and ACO activity (Lelièvre et al., 1997). In plants, ACS and ACO enzymes are encoded by a multigene family (El-Sharkawy et al., 2008; Pech et al., 2008), and their expression is regulated by many different signals, such as environment, fruit development and plant hormone levels (El-Sharkawy et al., 2004, 2008; Lin et al., 2009). The role of ethylene in the modulation of gene expression related to ethylene biosynthesis, perception and signal transduction have been studied in several species, but the mechanism is unknown in apricot.

Different strategies have been used to study the effects of ethylene on fruit development and ripening, such as biotechnological approaches using sense/antisense plants for *ACO* and *ACS* genes (Ayub et al., 1996; Dandekar et al., 2004) and the use of ethylene inhibitors, such as aminoethoxyvinylglycine (AVG) and 1-methylcyclopropene (1-MCP) (Defilippi et al., 2005; El-Sharkawy et al., 2008). AVG inhibits the synthesis of ethylene at the level of the ACS and is considered a key enzyme in the biosynthesis of ethylene. The use of AVG has been focused mainly on aspects of pre-harvest fruit management, such as reducing pre-harvest fruit drop and slowing the development of maturity attributes in several fruits, including apricot (Palou and Crisosto, 2003; Valdés et al., 2009). The synthetic cyclopropene 1-MCP blocks ethylene receptors

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for extended periods of time and impedes the physiological effects induced by ethylene. It has been demonstrated that 1-MCP inhibits the maturation of fruit and improves the post-storage quality of climacteric fruit (Watkins, 2006; Valdés et al., 2009). The main objective of this research was to determine the role of ethylene in the modulation of its own synthesis with two different ethylene inhibitors that work at different levels of ethylene biosynthesis and perception. This study analyzed transcript accumulation levels involved in both processes during apricot ripening.

2. Materials and methods

"Patterson" apricots were harvested from a commercial orchard located in the Chilean Central Valley, near Santiago. Fruit were harvested with yellow-green ground color corresponding to a pre-climacteric stage (Valdés et al., 2009). The applications of 1-MCP (SmartFresh™, AgroFresh, Philadelphia, USA) and AVG (ReTain®, Valent BioSciences, Libertyville, USA) were performed according to Valdés et al. (2009). Briefly, 1-MCP was applied at 10 μL L^{-1} for 24 h at 0 °C, and AVG at 1 g L^{-1} of active ingredient was applied by immersion for 10 min at room temperature (Palou and Crisosto, 2003; Valdés et al., 2009). Following the application, fruit were maintained at 20 °C until they ripened. Ripeness was defined for each treatment by a change in the ground color from yellow-green to yellow-orange. Flesh firmness, total soluble solids, titratable acidity and skin color were measured during each evaluation period as described by Defilippi et al. (2009). Ethylene production and respiration rate were determined for intact fruit using a static system as described by Defilippi et al. (2009).

Total RNA was isolated from frozen fruit using the hot borate method (Wan and Wilkins, 1994). First, strands of cDNA were obtained using a reverse transcriptase (Promega, Madison, USA) and oligo dT primers according to standard procedures.

To obtain the full-length cDNA of the three isoforms of *ACS* (*PaACS1*, *2* and *3*), RACE-PCR assays were performed using the procedures from the GeneRacer kit (Invitrogen, Breda, Netherlands). Primers for 3′ RACE and 5′ RACE amplifications were derived from partial sequences of apricot: *PaACS1* (GenBank accession no. AF184076), *PaACS2* (AF184077), and *PaACS3* (AF184078). The primers designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, USA) were: *PaACS*1(f) 5′-ATTCAACCAGGCAAAGAAACGC-3′, *PaACS*1(r) 5′-GATGGAGTGGAAATGGACGAGA-3′; *PaACS*2(f) 5′-AGCACTTGAAGCAGCCTATGAA-3′, *PaACS*2(r) 5′-CAGGAACCTTGCCACAAACTCT-3′; and *PaACS*3(f) 5′-AGTTGGCACCGTGTACTCATAC-3′, *PaACS*3(r) 5′-CACCTCCTCTTGTTGGTTTCTC-3′. The fragments obtained were cloned through TA cloning (using the pGEM-T vector, Promega), purified, sequenced (Macrogen, Seoul, Korea) and compared to sequences deposited at the National Center for Biotechnology Information (NCBI) using BLAST alignment programs. Additionally, the complete sequence annotated (Mbéguié-A-Mbéguié et al., 1999) for *PaACO* (AF026793) was used, and specific primers were designed for posterior analyses: *PaACO1*(f) 5′-GCAACTACCCTCCTTGTCCCA-3′, *PaACO*1(r) 5′-ATCACTCTGTGCTCCACGCTC-3′.

Transcript abundance of the genes in the study was analyzed by real-time PCR with the LightCycler Real-Time PCR System (Roche Diagnostics, Mannheim, Germany), using SYBR Green as a fluorescent dye to measure the amplified DNA products derived from the RNA. Conditions, procedures and analyses for qPCR were performed as described by González-Agüero et al. (2008) and the expression values obtained were normalized against 26S ribosomal RNA gene (*Pa26sRIB*, GenBank accession no. AF003997) abundance. Specific primers from *Pa26sRIB* were designed for posterior analyses: *Pa26sRIB*(f) 5′-AACGCAGGTGTCCTAAGATGAG-3′, and *Pa26sRIB*(r) 5′-GCTGCCACAAGCCAGTTATCC-3′. To test whether *26sRIB* behaves as a housekeeping gene, cDNA samples from the entire set of samples analyzed by qPCR were synthesized with *DAP* (dehydrodipicolinate reductase, obtained from the ATCC; no. 87486) spike mRNA added as internal control (0.01%) prior to the first strand synthesis, according to González-Agüero et al. (2008).

3. Results and discussion

3.1. Respiration rate and ethylene production

Respiration rates in all of the fruit were similar until ripeness, when fruit treated with both ethylene inhibitors showed a lower respiration rate compared to control fruit, especially in the AVG treated apricots (Fig. 1A). Moreover, ethylene production before

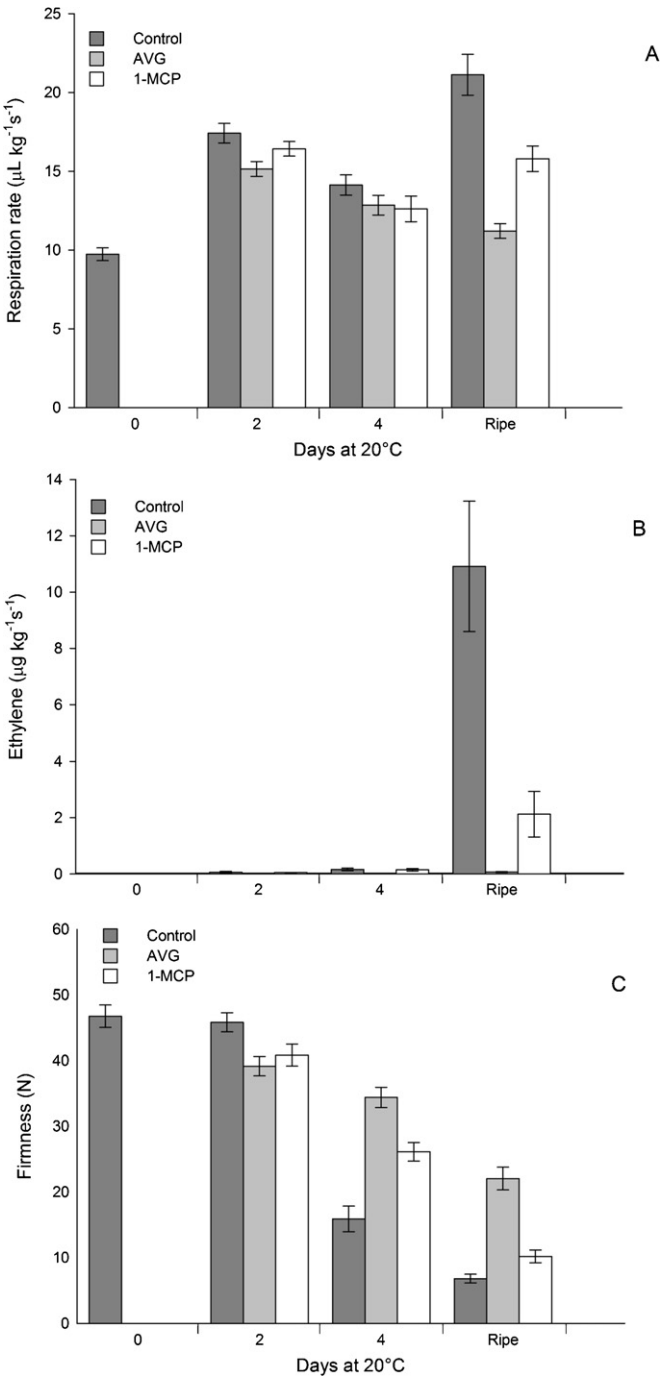

Fig. 1. Ripening and quality parameters during Patterson apricot ripening. Ethylene production (A), respiration rate (B) and firmness (C). Data are means ± SD.

ripening was very low in all the treatments, and no major differences were observed among treatments. However, at the ripe stage, fruit applied with both ethylene inhibitors showed a remarkably lower ethylene production than the control fruit, although the fruit treated with AVG showed the lowest ethylene production (Fig. 1B). Similar results were observed in the peach cultivar 'Akatsuki', where AVG significantly reduced ethylene production throughout ripening at 25 °C (Hayama et al., 2008). These results also indicate that the efficacy of both inhibitors on ethylene production is not affected by the storage temperature, because previous works were performed at 0 °C (Valdés et al., 2009).

3.2. Ripening and quality parameters

As observed in our previous work, there was a delay in the ripening process with the application of both ethylene inhibitors, and the flesh softening progress clearly showed this effect (Valdés et al., 2009). During the ripening period at 20 °C, a slow rate of softening was observed in all the fruit in the first 2 d, but after 4 d at 20 °C, the control fruit reached the maximum softening rate of 0.174 mN s^{-1} between days 2 and 4, followed by the 1-MCP and AVG treated apricots with a rate of 0.085 and 0.028 mN s^{-1}, respectively (Fig. 1C). 'Akatsuki' peaches show a similar decrease in firmness, observed at 2 d after harvest (Hayama et al., 2008). In this work, the authors reported a softening at 25 °C of 71% for the control, 60% for AVG and 25% for 1-MCP-treated fruit. As ripening progressed until apricots reached ripeness, the softening rate increased in fruit treated with ethylene inhibitors, with a 63% and 35% increase for 1-MCP- and AVG-treated fruit, respectively. This delay in ripening affects the time required to attain the ripe stage, which was 8, 9 and 10 d for the control, 1-MCP and AVG treatments, respectively. In fact, the AVG-treated fruit was evaluated before reaching an appropriate firmness due to excessive water loss symptoms in the fruit (Valdés et al., 2009). Therefore, at the ripe stage, only fruit treated with AVG showed a higher firmness compared to control fruit and the 1-MCP-treated fruit. Considering these results, treatments with 1-MCP or AVG were effective for reducing the softening rate during ripening in Patterson apricots. For total soluble solids, titratable acidity and color development, no statistical differences were observed among treatments (data not shown).

3.3. Expression of ethylene biosynthesis genes during apricot ripening

Before analyzing the transcript accumulation levels, for each cDNA the transcript abundances of *Pa26sRIB* and *DAP* were analyzed by qPCR and the ratios of control transcript to the endogenous transcript *Pa26sRIB* were calculated. The results indicated that the abundance of *Pa26sRIB* mRNA remains stable between samples (data not shown). Later we gathered more information about the coding sequences of some of the genes by RACE-PCR. Using the partial information of *ACS*, we have increased the *PaACS1* sequence and obtained the full-length cDNA for *PaACS2* and *PaACS3*. The sequences obtained for *PaACS1* (GenBank accession no. HQ333207), *PaACS2* (HQ333208) and *PaACS3* (HQ333209) are 1008, 1602 and 1747 bp, respectively; increasing sequence information for the last two by 45% and 58%. Several orthologous sequences were found in different plant species, such as *Populus trichocarpa*, *Arabidopsis thaliana*, *Zea mays*, among others, suggesting that these genes are highly conserved within the plant kingdom. As in other plant species, the individual ACS proteins have sequence variations in the C-terminal regions that influence the stability of the corresponding protein through post-translational modification (Lin et al., 2009).

At the amino acid level, at least two *PaACS* isozymes contained the seven conserved boxes found in *ACS* from other plant species

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                          1         10        20        30        40        50        60
                          |         |         |         |         |         |         |
Prunus_armeniaca_ACS2     YFDGWKAYDRNPFHPTKNPEGVIQMGLAENQLSFDLIEERIK--KNPKASICTPEGVEEF  : 58
Prunus_armeniaca_ACS3     ----------------------QMGLAENQVSFDLLEKYLELEKHSKSSNLGSKGVSGF  : 37
Prunus_armeniaca_ACS1     ------------------------------------------------------------  : 00

Prunus_armeniaca_ACS2     KNVAIFQDYHGFPEFRKAVAMFMSKARGGRVTFDPNRVVMSGGATGANELVMFCLADPGD  : 118
Prunus_armeniaca_ACS3     KKNALFQNYHGLLSFKKAMAIFMDQIKGGRAKFDPDRVFLTAGATAANELLTFIIADPGD  : 97
Prunus_armeniaca_ACS1     ------------------------------------------------------------D  : 01

Prunus_armeniaca_ACS2     AFLVPSPYYPAFFRDLGWRTGVQIVPVDCHSSNNFKITKEALEAAYEKAQKNNIKVKGLI  : 178
Prunus_armeniaca_ACS3     ALLVPTPYYPGFDKNLRWRTGVNIVPIHTDSSNNFQVTPQALEAAYKEAEAKNMKVRGVL  : 157
Prunus_armeniaca_ACS1     AFLVPTPYYPGLDKDIKWRTGVEIITVPCRSADKFNLSVTALDRAFNQAKKRGLKVRGII  : 61

Prunus_armeniaca_ACS2     ITNPSNPLGTTLDRDTLESLVEFINQKNIHLVCDEIYAATVFSSPTFTCISEVIQNMNC-  : 237
Prunus_armeniaca_ACS3     ITNPSNPLGATIQRQVLERILGFVTQKNIHLVSDEIYSGSAFSSSEFISVAEILESQQYK  : 217
Prunus_armeniaca_ACS1     ISNPSNPVGTLFSRESLYNLLDFAREKNIHIISNELFAGSTHGSEEFVSMADIVELEDL-  : 120

Prunus_armeniaca_ACS2     NPNLIHIVYSLSKDMGLPGLRVGIVYSYNDDVVNIGRKMSSFGLVSSQTQHMLASMLLDE  : 297
Prunus_armeniaca_ACS3     NSERVHIVYSLSKDLGLPGFRVGTVYSYNDKVVTTARRMSSFTLISSQTQHLLASMLSDK  : 277
Prunus_armeniaca_ACS1     DQNRVHIVYGLSKDLSLPGFRAGVIYSFNKNVLAAAKRLTRFSSISTPSQQLLSSMLSDT  : 180

Prunus_armeniaca_ACS2     EFVARFLETSSKRLANRHGVFTKGLEEVGINCLKSNAGLFCWMDLRRLLKDQTFDGEMVL  : 357
Prunus_armeniaca_ACS3     EFTENYIKTNRERLRKRYDMIIEGLKKAGIECLRGNAGLFCWMNLSPLLEKPTRGGELAL  : 337
Prunus_armeniaca_ACS1     KFVQKFIDANRERLRGMHLKFLTGLKQLGIECTKSNGGFSCWADMSGLIRSYNEKGELEL  : 240

Prunus_armeniaca_ACS2     WRVIVNEVGLNVSPGSSFKCVEPGWFRVCFA  :368
Prunus_armeniaca_ACS3     WDSMLHEVKLNISPGSSCHCSEPGWFRVCFA  :348
Prunus_armeniaca_ACS1     WDRLLNVAKVNVTPGSSCHCIEPGWFRVW--  :269
```

Fig. 2. Amino acid sequence alignment. The sequences were identified using the tools "template identification" and "domain annotation" from the program Swiss-model Version 8.05 released by Expasy (Gasteiger et al., 2003). The box indicates the binding site for pyridoxal phosphate for the three ACS sequences detected in *Prunus armeniaca*.

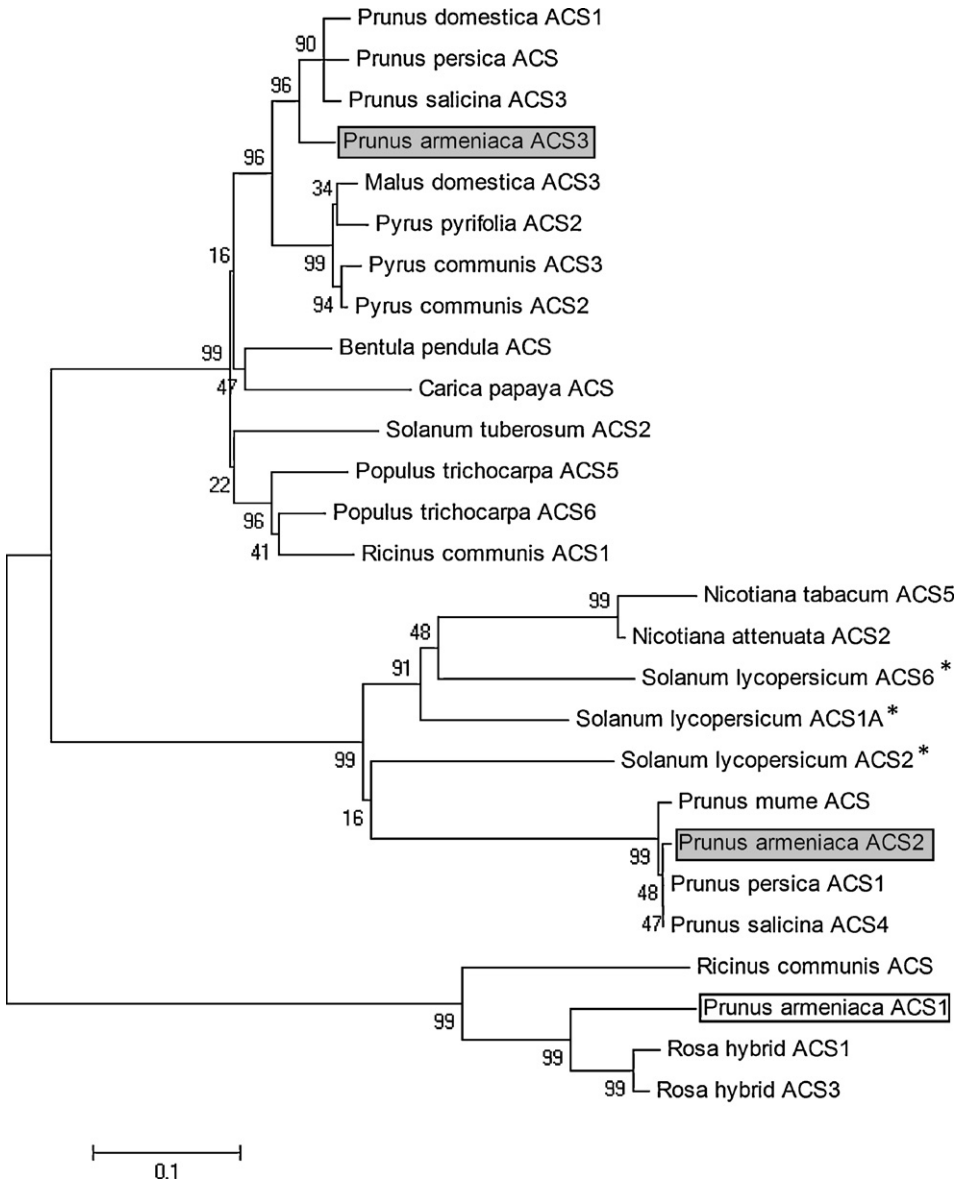

Fig. 3. Phylogenetic analysis of three *PaACS* genes with 24 other sequences. The tree was generated using the following sequences with their respective databank numbers: *Bentula pendula*: ACS (AAM80890); *Carica papaya*: ACS (CAB86187); *Nicotiana attenuata*: ACS2 (AAR99391); *Nicotiana tabacum*: ACS5 (ABW97851); *Malus domestica*: ACS3 (AAB67989); *P. trichocarpa*: ACS5 (XP_002330516), ACS6 (XP_002308289); *Prunus domestica*: ACS1 (CAI64501); *Prunus mume*: ACS (BAH66892); *Prunus persica*: ACS (AAF61233), ACS1 (AAX99362); *Prunus salicina*: ACS4 (ABW03085), ACS3 (ABW03082); *Pyrus communis*: ACS2 (AAR38503), ACS3 (AAR12136); *Pyrus pyrifolia*: ACS2 (BAA76388); *Ricinus communis*: ACS (XP_002509645), ACS1 (XP_002514161); *Rosa hibrid*: ACS (ABY55589), ACS3 (AAQ88100); *Solanum Lycopersicum:* ACS1a (AAB17278), ACS2 (CAA41856), ACS6 (AAK72433); *Solanum tuberosum*: ACS2 (CAA81749). Alignments were made using CLUSTAL W multisequence software. The phylogenetic tree was constructed with the MEGA 3.1 program using the Neighbor-Joining method.

(El-Sharkawy et al., 2008). ACS is a pyridoxal phosphate (PLP)-dependent enzyme, and it requires PLP as cofactor (Lin et al., 2009). Fig. 2 shows the alignment of the three *PaACS* where there is high conservation of amino acid residues and emphasizes the PLP binding site domain. Most PLP-dependent enzymes have a lysine residue in their active site. The Lys276 present in apricot ACS corresponding to the Lys278 is conserved in the nine isoforms in tomato, and this residue serves as the PLP-binding site (Lin et al., 2009). The ACS isoforms are biochemically distinct and function in unique cellular environments for the biosynthesis of ethylene (Yamagami et al., 2003). A phylogenetic tree was elaborated by comparing the C-terminal amino acid sequences of the three apricot ACS proteins with other ACS proteins from nearly ten plant species (Fig. 3). As in plums, this analysis indicates that ACS proteins can be divided into three main subfamilies. According to the classification by El-Sharkawy et al. (2008), the corresponding ACS subfamilies are: *PaACS2*, type 1; *PaACS1*, type 2; and *PaACS3*, type 3.

Among the three *PaACS* genes cloned in apricot, we observed different patterns of expression levels (Fig. 4). In general, the three isoforms in the non-treated fruit showed an increase in expression levels throughout the ripening process, reaching a maximum level between day 4 and the ripe stage. *PaACS1* and *PaACS3* levels did not show a consistent response in the fruit applied with both ethylene inhibitors but in general showed similar or higher levels of expression than the control fruit in the early stages of ripening. Contrarily, *PaACS2* transcript levels were concomitant with ethylene levels in all of the treatments and during the entire evaluation period at 20 °C (Figs. 1B and 4B). It is interesting that at the ripe stage, *PaACS2* expression levels in the AVG- and 1-MCP-treated apricots had the same inhibitory levels as the ethylene production rate values for

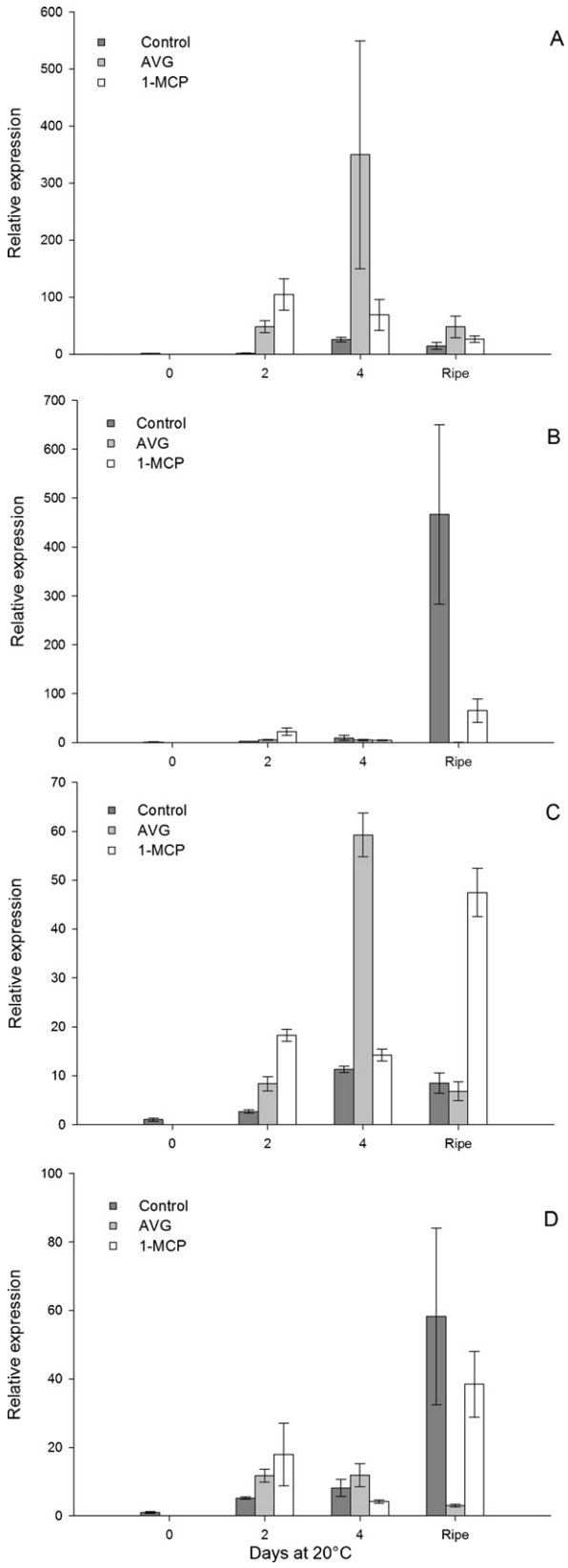

Fig. 4. Gene expression analysis for ethylene-biosynthesis-related genes during apricot ripening. (A) ACC synthase 1 (*PaACS1*); (B) ACC synthase 2 (*PaACS2*); (C) ACC synthase 3 (*PaACS3*); and (D) ACC oxidase (*PaACO*) were assayed by qPCR using cDNAs from four ripening stages. The relative abundance of each mRNA was normalized to the *Pa26sRIB* gene in the corresponding samples. The results are presented as relative expression against the transcript amount of the corresponding gene at 0 d after harvest with a nominal value of 1. Data are means ± SE.

both treatments, which were 99% and 80% for AVG and 1-MCP, respectively. Levels of gene expression inhibition higher than 80% in *ACS* genes have been associated with a lower accumulation of ACC, the direct precursor of ethylene (Defilippi et al., 2005). This result suggests that *PaACS2* would be primarily involved in the synthesis of the ACS enzyme at the ripe stage.

In plum, *PsACS4* mRNA levels increased linearly with the ethylene levels, and their expression is completely repressed with 1-MCP treatment (El-Sharkawy et al., 2008). As in tomato, where *SlACS2* and *SlACS4* are responsible for system 2 autocatalytic ethylene production (Barry et al., 2000), the *PsACS4* form appears to be highly regulated during climacteric fruit ripening. The high sequence identity and phylogenetic proximity of *PaACS2* with *PsACS4*, *SlACS2* and *SlACS4* (Fig. 3) suggests that the *PaACS2* form plays an important role in determining the level of autocatalytic ethylene production and the capacity of the fruit to ripen. On the other hand, the higher level of expression of *ACS1* and *ACS3* under ethylene inhibition would indicate that both isoforms could be responsible for producing the basal levels of ethylene (system 1), which has been described in other species (Barry et al., 2000).

In the case of *PaACO*, the expression increased substantially during apricot ripening, similar to what was observed by Mbéguié-A-Mbéguié et al. (1999); however, this change was not concomitant with ethylene production, so *PaACO* would not be transcriptionally regulated during apricot ripening, as seen in the work of Mbéguié-A-Mbéguié et al. (1999). This discrepancy could be due to the different variety used (Bergeron) and the distinct maturity stages at harvest between the two studies. In addition, *ACO* transcript levels showed a dissimilar response to ethylene inhibition, which were higher than the control fruit in early stages of ripening and similar to or lower than the control at the ripe stage (Fig. 4D). Different than what was observed for *ACS2* during ripening, *ACO* transcript levels were similar between the ethylene inhibition treatments and were not concomitant with ethylene production. Moreover, we also cloned and characterized the expression levels of several ethylene receptors, including putative ethylene receptor 1 (*PaETR1*, non-annotated), putative ethylene-responsive sensor 1 (*PaERS1*, non-annotated), ethylene transcriptional factor 1 (*PaEIL1*, non-annotated), and putative ethylene-responsive element binding factor 1 (*PaERF1*, non-annotated); and no clear differences were observed in response to ethylene inhibition or ripening stage (data not shown).

4. Conclusions

It is clear that ethylene plays a role in apricot ripening, especially close to phase “ready to eat”. Interestingly, the differential behavior of the *ACS* gene expression levels under ethylene inhibition observed in this work reassembles features of ethylene regulation described in other climacteric fruit, such as tomato. From these, it seems *PaACS2* is a key player in determining the capacity of ethylene production in apricot at the ripe stage, and further work is required to study the regulatory aspects of *ACS* gene family in apricot. On the other hand, our work suggests that there are other factors modulating ripening in apricot that should be further considered.

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