Study on differential expression of 1-aminocyclopropane-1-carboxylic acid oxidase genes in table grape cv. Thompson Seedless

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Keywords:
ACO
Ethylene
Non-climacteric
Veraison
Table grape

A B S T R A C T

As a consequence of the non-climacteric status of grapes (*Vitis vinifera*), ethylene biosynthesis and signal transduction have scarcely been studied in this fruit. In spite this drawback, the available information suggests a role for ethylene in ripening grape berries. In this work, we report the identification of three homologous genes that encode 1-aminocyclopropane-1-carboxylic acid oxidase (ACO), a key component of ethylene biosynthesis. A comparison of protein sequences revealed that all three *VvACOs* harbor a 2OG-Fe(II) oxygenase domain, which is typical of the *ACO* gene family; however, *VvACO1* showed a higher amino acid sequence homology with *VvACO2* than with *VvACO3*. The expression pattern of *VvACOs* and the effect of exogenous ethylene on their transcript accumulation were evaluated during table grape berry development in the "Thompson Seedless" cultivar. A peak in *VvACO1* transcript accumulation levels was registered around veraison that was 4-fold higher than at harvest, and this peak was confirmed during a second season in grapes that were harvested from three different vineyards. An enhancement in ethylene production and *VvACO* genes transcript levels was observed in grapes sprayed with ethephon during berry development. However, *VvACO1* transcripts reached the highest accumulation earlier than *VvACO2* and *VvACO3*. Altogether, these data confirmed that ethylene may have a role in some aspects of the grape ripening process, and they also highlighted the potential use of some *VvACO* genes as molecular markers for identifying grape veraison stages in grapes.

1. Introduction

During fruit development, there are many flavor changes caused by the synthesis, transport or degradation of metabolites. The inception of grape berry ripening, which is known as veraison, has been identified as a critical developmental stage. Some of the chemical changes that determine fruit quality are triggered during this stage, including sugar accumulation, an increase in anthocyanin synthesis and a reduction in titratable acidity (Ollat et al., 2002). To identify the veraison stage, several strategies have been used, including berry color change, berry softening, changes in soluble solids and xylem conductance functionality (Bondada et al., 2005). However, some of them, such as berry color change, are suitable only for some varieties while indicators for the others are still under discussion (Chatelet et al., 2008).

Ethylene plays an important role as a ripening modulator in climacteric fruit and is involved either directly or indirectly in the regulation of metabolites that determine quality (Pech et al., 2008). In spite of the identification of grape as a non-climacteric fruit (Kader, 2002; Chervin et al., 2004), ethylene could be involved in the expression of its quality traits, but only in the earlier stages of berry development, i.e., the veraison stage (Tesnière et al., 2004; Chervin et al., 2008). Interestingly, an ethylene increase was detected at veraison that was higher than the physiological threshold required to trigger a metabolic change (Abeles et al., 1992). Research on ethylene biosynthesis in grapes has mainly focused on the final step, i.e., the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene, which is catalyzed by 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) (Chervin et al., 2004; Sun et al., 2010). The ACO enzymes are encoded by a multigene family in all plant species that have been studied for this trait (Binnie et al., 2007; Binnie and McManus, 2009), and the expression of these genes is differentially regulated by developmental and hormonal signals (Choudhury et al., 2008; Lin et al., 2009). In climacteric fruit, such as tomato (*Lycopersicum esculentum*), five members of

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http://dx.doi.org/10.1016/j.postharvbio.2012.10.006

the ACO multigene family have been described (Barry et al., 1996). While the tomato genes *LeACO1* and *LeACO4* were up-regulated at the onset of ripening and continuously expressed throughout ripening, *LeACO3* showed a transient activation only at the breaker stage of fruit ripening. These differences may be explained by several factors involved in the regulation of the *ACO* gene family, as observed in other species (Binnie and McManus, 2009; Lin et al., 2009). In non-climacteric fruit such as the strawberry (*Fragaria ananassa*), Trainotti et al. (2005) reported the isolation of two *ACO* genes (*FaACO1* and *FaACO2*), but only *FaACO1* expression increased during ripening, and it was highly active in parallel with an increase in ethylene production that preceded the visible start of strawberry ripening. An increase in transcript levels of the *ACO* gene, which was identified as the ethylene biosynthesis gene in grapes, was concomitant with an ethylene peak just before veraison in Cabernet Sauvignon (Chervin et al., 2008) and Muscat Hamburg grapes (Sun et al., 2010). These authors also showed that genes responsible for encoding ethylene receptors were up-regulated at veraison, demonstrating that the ethylene signaling pathway was active at this stage. However, little is known about *ACO* regulation in table grapes; therefore, the purpose of the current study was to investigate the expression pattern of ethylene-related genes during berry development in a commercial variety of table grapes through the cloning and expression of *ACO* genes.

2. Materials and methods

2.1. Plant material

During the first year, table grapes of the "Thompson Seedless" variety were obtained from a commercial orchard located in Los Andes (Aconcagua Valley, 32°52′27″ S and 70°38′26″ W), Chile. Grapes were sampled weekly, starting 7 weeks after full bloom (WAFB) and lasting until the time of commercial harvest. Three clusters were obtained from three homogeneous vines at each sampling time. The vines were similar in vigor, age, rootstock and handling. In terms of cultural management, only gibberellic acid was used in early stages of fruit development. Immediately after sampling, grape berries were transported to the laboratory for physiological characterization. For the molecular assays, the whole berries (peel + pulp) were frozen in liquid nitrogen and stored at −80 °C until analysis.

On a second year, three "Thompson Seedless" vineyards under different environmental and cultural management conditions were selected for studying *ACO* gene expression levels. The vineyards were located at Los Andes, Santiago (Central Valley, 33°34′20″ S and 70°37′31″ W) and Rosario (Cachapoal Valley, 34°19′52″ S and 70°55′37″ W). To evaluate the response of *ACO* genes to exogenous ethylene treatment, a trial was performed at the experimental orchard located in Santiago, where no plant growth regulators other than ethephon were applied during fruit growth and development. A concentration of 1040 mg L^{-1} of 2-chloroethyl phosphonic acid (ethephon) (Ethrel 48SL, Bayer CropScience, Santiago, Chile) was applied with a hand-held sprayer at 1000 L of spray solution per hectare to grape clusters and foliage between 7 and 9 WAFB. Sampling for grape berry physiological characterizations and molecular assays was performed in the same way as the first year.

2.2. Maturity parameters

The total soluble solids content (TSS) was measured with a manual temperature-compensated refractometer (ATC-1E, Atago, Tokyo, Japan) and the results were expressed as a percentage (%). Titratable acidity (TA) was obtained by titrating 10 mL of juice from a representative sample of fruit with 0.1 N NaOH until neutralization of organic acids at a pH of 8.2. In this case, results were expressed as a percentage of tartaric acid equivalents. Additionally, berry firmness was assessed by a Firmtech 2 texture analyzer (Bioworks, KS, USA) and results were expressed in N m^{-1}. For TSS and berry firmness, a total of 25 berries per replicate were considered, and for TA a composite sample per replicate was used.

2.3. Ethylene production rate

Ethylene production was determined for intact berry using a static system. At each sampling date, five berries per bunch were detached, weighed and placed in 0.5 L jars. Due to the low level of ethylene produced by grapes (Chervin et al., 2004), the jars were sealed and kept at 20 °C for 10 h prior to ethylene measurements. To avoid CO_2 accumulation, calcium carbonate was present in the jars, which could affect ethylene biosynthesis. The level of ethylene in the jar headspace was then determined using a gas chromatograph (Shimadzu 8A, Tokyo, Japan) equipped with a flame ionization detector (Egea et al., 2007).

2.4. RNA isolation and cDNA synthesis

Total RNA from grape samples was isolated using the hot borate method (Gudenschwager et al., 2012). The first strand of cDNA was obtained by carrying out reverse transcription reactions with 2 μg of total RNA as a template, using MMLV-RT reverse transcriptase (Promega, Madison, WI, USA) and oligo dT primers (Invitrogen, Breda, The Netherlands).

2.5. Isolation and in silico analysis of ACO cDNA sequences

The *ACO* cDNA fragments were amplified using specific primers designed against *VvACO1* (AY211549) (Chervin et al., 2004) and two other putative forms, namely *VvACO2* and *VvACO3*, which were designed from partial sequences annotated with GenBank IDs XM_002275284 and XM_002279710, respectively. The primers that were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA), were the following: *VvACO1*(f) 5′-GAAAGAAAAGGAGACAAGCGAAG-3′, *VvACO1*(r) 5′-TGGGACCCAAATTAACAGTAGGT-3′; *VvACO2*(f) 5′-TACCTGTCTCAAACGTCTCTGAT-3′, *VvACO2*(r) 5′-AAGTTTGGA CCCTTGGAGCCAT-3′; *VvACO3*(f) 5′-GCCCTGGAGGATAAAGAAACTG-3′, *VvACO3*(r) 5′-TTGCCACTTTTGTCCCTACTGAA-3′. To characterize the putative forms of *VvACO2* and *VvACO3*, we obtained full-length cDNAs through RACE-PCR assays using the procedures from the GeneRacer kit (Invitrogen, Breda, Netherlands). The isolated 3′ and 5′ RACE fragments were purified using the QIAquick gel extraction kit (Qiagen, MD, USA). DNA fragments were cloned into the pGEM T-Easy vector (Promega, Madison, WI, USA) according to the manufacturer's protocols, and both strands were sequenced (Macrogen Corp, Seoul, Korea). The resulting full-length cDNAs were compared to sequences deposited at the National Center for Biotechnology Information (NCBI) using the BLAST alignment program (Altshult et al., 1997). The nucleotide sequences of *VvACO2* and *VvACO3* were translated and the ORFs were identified using ORF Finder (Wheeler et al., 2003) and Swiss-Model Tools (Arnold et al., 2006). Similarity analyses were performed using ClustalW analyses with ClustalX 2 software (Larkin et al., 2007).

2.6. Real-time quantitative PCR assays (qPCR)

The expression of *VvACO* genes was analyzed by real-time PCR with the LightCycler Real-Time PCR system (Roche Diagnostics, Mannheim, Germany), using the above primers and specific primers for *eEF-1alpha* (elongation factor 1 alpha, GenBank accession ID XM_002284888): *eEF-1alpha*

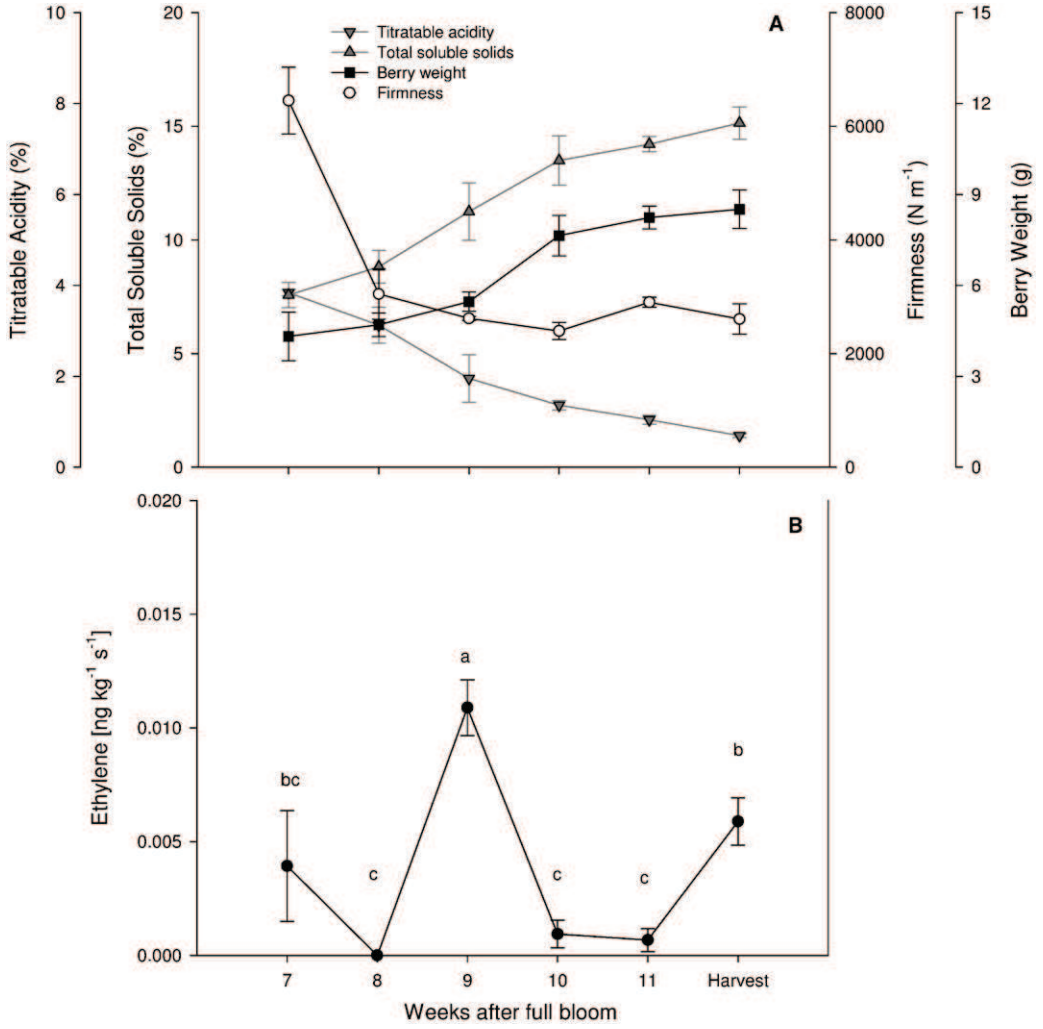

Fig. 1. Quality parameters during table grape development were (A) total soluble solids (%), titratable acidity (%), berry weight (g) and firmness ($N\,m^{-1}$) and (B) ethylene production rate ($ng\,kg^{-1}\,s^{-1}$) of the "Thompson Seedless" grape. Data are represented as the means ± SD from nine replicates.

(f) 5′-AGGATGGACAAACCCGTGAG-3′ and *eEF-1alpha* (r) 5′-AAGCCAGAGATGGGGACAAA-3′. Conditions, procedures and analyses for qPCR were performed on four biological replicates for each sample, as described by González-Agüero et al. (2008). The resulting expression values were normalized against *eEF-1alpha* abundance. The abundance of *eEF-1alpha* mRNA remained stable among the different samples (data not shown). Finally, the data were subjected to variance analyses, and the means were separated by Tukey test at the 5% level of significance using Statgraphics Centurion XVI (Manugistics, Inc., Rockville, MD, USA).

3. Results and discussion

3.1. Maturity parameters and ethylene production rate

To identify the veraison stage in the variety studied, profile changes for TSS, TA and berry firmness were followed during berry development. During the first trial year, the grapes showed a marked increase in TSS content between 8 and 10 WAFB, increasing slightly thereafter (Fig. 1A). Concomitantly with the TSS increase, a reduction in TA of close to 4% was observed during berry development, with a main decrease occurring between 7 and 9 WAFB, and then the TA level dropped slightly until harvest (Fig. 1A). Concomitant changes in the total sugar and organic acid profile showed by Muñoz-Robredo et al. (2011) confirmed these results. In relation to berry firmness, there was a sharp increase in softening rate between 7 and 8 WAFB, reaching almost steady levels after this period until harvest (Fig. 1A). The set of all previously mentioned changes led to definition of the veraison stage at approximately 9 WAFB during the first trial year. The measured berry ethylene production rate was very low during fruit development, reaching levels below $0.02\,ng\,kg^{-1}\,s^{-1}$ (Fig. 1B). However, the static system method used in this work was able to detect a weak but significant peak around veraison (9 WAFB) (Fig. 1B). Similar observations were made in wine grapes by Chervin et al. (2004) and recently by Sun et al. (2010), who obtained a small but a clear ethylene peak before the veraison stage.

During the second trial year, the veraison stage was identified at close to 8 WAFB in all orchards on the basis of their TSS and TA profiles (data not shown). Given that veraison occurred one week earlier than in the first year, this change could be explained by agroclimatic differences between seasons that would affect berry growth and development. During this trial, ethephon-treated berries showed a remarkable increase in endogenous ethylene production from 8 WAFB until harvest, inducing significantly the production of ethylene up to 60-fold changes at veraison in comparison to non-treated berries (Table 1), and then declined at harvest. Similar behavior was detected by Dal Ri et al. (2010) in wine grapes. The fact that ethylene production lasts for more than two weeks

Table 1
Fold-change of ethylene production rate and mRNA expression levels of *VvACO1*, *VvACO2* and *VvACO3* during berry development in grapes treated with ethephon and control. *The fold-change of ethylene production rate was calculated against each corresponding control group. **VvACO genes transcript concentration was determined by qPCR in quadruplicate using cDNAs from six developmental stages for each gene form. The relative abundance of each mRNA was normalized to the eEF-1alpha gene in the corresponding samples. The expression data was log2 transformed and the fold-change of mRNA expression levels was calculated against each corresponding control group. *,**Different letters indicate statistical differences by Tukey analysis ($P < 0.05$).

WAFB	Ethylene*	Gene expression**		
		VvACO1	*VvACO2*	*VvACO3*
4	1	0	0	0
6	1	0	0	0
8	61.8^{a}	0.01^{c}	−1.97^{c}	−1.01^{c}
9	35.9^{ab}	2.54^{a}	−0.66^{bc}	1.28^{ab}
10	49.0^{a}	1.91^{b}	0.60^{bc}	1.52^{ab}
12	48.1^{a}	2.83^{a}	1.52^{b}	0.22^{b}
Harvest	11.3^{bc}	2.38^{ab}	2.81^{a}	2.46^{a}

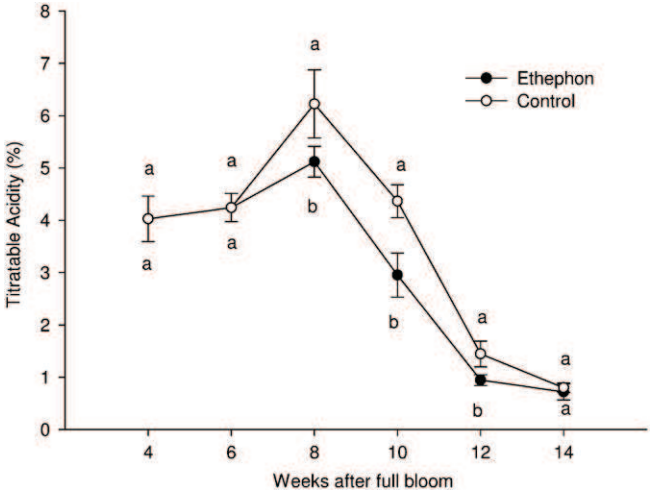

Fig. 2. Titratable acidity (TA) in "Thompson Seedless" grapes after ethephon application and in controls. TA is shown as the percentage of acidity (%). Data are presented as the means ± SD from nine replicates. Different letters indicates significant differences between treatments by Tukey analysis ($P < 0.05$).

in treated berries is not surprising because ethephon's long-term effects on grape berries have already been observed in relation to anthocyanin synthesis pathway transcripts (Abeles et al., 1992; El-Kereamy et al., 2003). Coombe and Hale (1973) showed that ethylene production that follows an application of exogenous ethylene can last for several weeks after treatment, but depending on the timing of application, it can either delay ripening when applied too early before veraison or enhance it when applied at veraison (Hale et al., 1970). In our experiments, TA showed an ethylene-dependent pattern, whereas TA values in treated berries were significantly reduced from veraison until 12 WAFB, reaching similar levels at harvest to those that were untreated (Fig. 2). Therefore, the TA drop that is induced by ethephon application, which is typical of an enhanced ripening, is proof that exogenous ethylene was applied to grapes that were close to veraison.

3.2. Identification of VvACO genes

An exhaustive in silico search of new *ACO* putative genes was carried out by aligning *VvACO1* amino acid sequences (GenBank accession ID AY211549) against the *Vitis vinifera* genome sequence (Jaillon et al., 2007). As a result, two new overlapping expressed sequence tags that shared structural characteristics associated with functional ACO proteins were identified. Based on their homology to *VvACO1*, the novel sequences were annotated as *VvACO2* (GenBank accession ID JX446622) and *VvACO3* (GenBank accession ID JX446623) with 1418 and 1117 bps in length, respectively. The predicted amino acid sequences of each of the three *VvACO* open reading frames (ORFs) were aligned and compared (Fig. 3). *VvACO1* and *VvACO2* share a high nucleotide sequence identity (83.2%) within their ORFs. In contrast, *VvACO3* shares a lower sequence identity with both *VvACO1* (53.2%) and *VvACO2* (52.6%) (Supplemental Table A1). All of the deduced amino acid sequences of VvACO proteins formed a 2OG-Fe (II) oxygenase domain (highlighted in gray in Fig. 3) with conserved Fe^{2+}-binding site motifs represented by residues H^{177}, D^{179} and H^{234} (black boxes in Fig. 3), which is characteristic of the ACO family. The slight differences found in the 2OG-Fe (II) oxygenase domain between VvACO sequences (Supplemental Fig. A2) suggest that they may act with different kinetics, as well as different occasions, as observed in other species such as tomato (Cara and Giovannoni, 2008).

Multiple alignments of full-length predicted VvACO1, 2 and 3 protein sequences revealed a high sequence homology to other characterized ACOs, including tomato, strawberry and apple (*Malus domestica*) (Supplemental Table A1). Interestingly, strawberry ACO genes were highly similar at the amino acid level to VvACOs, where FaACO1 shared a higher similarity with VvACO1 and 2 than FaACO2 (Supplemental Table A1). This finding would suggest a similar role of FaACO1 and VvACO1 in ethylene biosynthesis during strawberry

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VvACO1    MESFPVINMENLNGEERAATMENIKDACENWGFFELVNHGISHELMDTVEKLTKEHYKKCMEQRFKEMVASKGLEAVQSEINDLDWESTFFLRHLPTSNM
VvACO2    MEAFPVINMEMLNGEERGATMEMIKDACENWGFFELVNHGISHEQMDAVEKLTKGHYRKCMEQRFKELVAAKALEGVQTEIKDMDWESTFFLRHLPVSNV
VvACO3    -MEVPMIDFDKLDGEKRAETKALLHQACEKWGFFQIDNHGVDKKLMEKVKHLVNTHYEEIMKRSFYESEIAKALEDKETASN-IDWESSFFLWHRPTSNI
             .*:*::: *:**:*. *   :::***:****:: ***:.:: *: *::*.: **.: *:: * *   :*.**  ::  : :****:*** * *.**:

VvACO1    SEIPDLEEDYRKAMKEFTAHLEKLAELLLDLLCENLGLEKGYLKKAFYGSQGPSFGTKVSNYPPCPQPELIKGLRAHTDAGGIILLFQDDKVSGLQLLKD
VvACO2    SDFPDLDEEYRKVMKDFALKLEKLAEELLDLLCENLGLEKGYLKKAFHGSKGPNFGTKVSNYPPCPKPDLIKGLRAHTDAGGIILLFQDDTVSGLQLLKD
VvACO3    DEFTNISEDLRKTMDEYIAQLFKLAEKLSELMCENLGLEKGYIREAFSGNAGPSVGTKVAKYPQCPRPELVRGLRBHTDAGGIILLLQDDQVPGLEFYKD
          .::.::.*: **.*.::  :* **** * :*:*********:::** *. **..****::** **:*:*::*** ********** *:*** *.**:: **

VvACO1    GQWIDVPPMR-HSIVINLGDQLEVITNGKYKSVMHRVIAQTDGNRMSLASFYNPGSDAVIYPAPALVEKEKETSEVYPKFVFEDYMKLYAGLKFQAKEPR
VvACO2    GQWVDVPPMR-HSIVVNLGDQLEVITNGKYKSVLHRVVAQTDGNRMSIASFYNPGNDAVIYPAPALLEKEAEKDQVYPKFVFDDYMKLYAGLKFQAKEPR
VvACO3    GEWVVIPPSKNNTIFVNTGDQIEVLSNGRYKSTLHRVMADKNGSRLSIATFYNPSGDAIISPAPKLLYPD--------HFRFQDYLKLYATTKFADKGPR
          *:*: :** : ::*.:* ***:**::**:***.:***:*:.:*.*:*:*:****..**:* *** *:  :        :* *:**:****  **  * **

VvACO1    FEAMKAMEPTVNLGPIATV
VvACO2    FEAMKNVEASVNMGPIATA
VvACO3    FESMKNMAIGHHS--LHA-
          **:** :    :   : :
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Fig. 3. Sequence analysis of the three genes *VvACO1*, *VvACO2* and *VvACO3* identified in *V. vinifera*. Amino acid sequence comparisons were made using a ClustalX multiple sequence alignment. The gray box indicates the 2OG-Fe (II) oxygenase domain for the ACO sequences and the black boxes (with white letter) show positions of amino acids involved in metal chelation of ACO protein. The GenBank IDs for *VvACO1* (AY211549), *VvACO2* (JX446622), and *VvACO3* (JX446623).

and grape ripening, respectively, as both of them are examples of non-climacteric fruit.

3.3. Expression of ACO genes

The mRNA expression levels of *VvACO1*, *VvACO2* and *VvACO3* were analyzed by real-time quantitative PCR throughout table grape berry development. During the first trial year, high expression levels of *VvACO1* were observed around the veraison stage (9–10 WAFB), where the transcript accumulation of *VvACO1* was 4-fold higher than at the harvest stage (Fig. 4). Interestingly, the maximum transcript levels of *VvACO1* coincided with the ethylene production peak around veraison and had the highest rates of change in TSS and TA content (Fig. 1). As in *VvACO1*, *VvACO2* transcript accumulation increased around veraison, showing a significant maximum expression level at week 10 after full bloom, while transcripts of *VvACO3* accumulated at constant levels throughout the sampling period (Fig. 4).

To study the expression pattern of *VvACO1* in table grapes under different environmental and cultural management conditions, expression analyses were carried out to determine *VvACO1* transcript accumulation throughout berry development in grapes from three different orchards. As in the first trial year, the accumulation of *VvACO1* transcripts around veraison represented the highest transcript abundance during the sampling period and showed the same trend at all locations (Fig. 5). Similar expression patterns of *VvACO1* have previously been seen in Cabernet Sauvignon berries grown in France (Chervin et al., 2004), Italy (Dal Ri et al., 2010) and Australia (Wheeler, 2006).

The expression of *VvACO1* was responsive to ethylene because a significant up-regulation of this gene at the transcriptional level was observed around two weeks after the first application of ethephon and until harvest (Table 1). *VvACO2* and *VvACO3* expressions were also induced by exogenous ethylene, with both experiencing up-regulation two weeks after ethephon application. However, their maximum increase was reached at harvest (Table 1). The differential response of *VvACO* genes to exogenous ethylene application during berry development suggests that *VvACOs* are involved in different steps of the ethylene biosynthesis regulatory mechanism, or may have different roles during grape development, as observed in other fruit species (Cara and Giovannoni, 2008). To better understand the mechanisms that control the expression of ethylene responsive genes during grape ripening, the promoter regions of *VvACO* genes were isolated and analyzed with the aim to identify functional regulatory motifs. In our work, a search of sequences of putative ethylene-responsive element (ERE) in the promoter region from *VvACO*s revealed a DNA motif (ATTTCAAA) that was present in one and two copies of *VvACO1* and *VvACO2*, respectively; but it was absent in *VvACO3* (data not shown), which could be explaining the constitutive expression in *VvACO3*. An induction of *ACO* gene expression by ethephon has already been observed over several days following exogenous ethylene application in non-climacteric fruits such as citrus (Yuan et al., 2005) and strawberries (Trainotti et al., 2005). In model fruit species, such as tomato, five *ACO* genes and nine *ACS* genes have been characterized with a differential expression pattern during fruit maturity and ripening, suggesting a different role on ethylene-regulated process during fruit development (Cara and Giovannoni, 2008). On the other hand, in other fruits such as apricot (*Prunus armeniaca*), only one ACO encoding gene has been identified during fruit maturity and ripening, suggesting the variability of ethylene regulation among species (Muñoz-Robredo et al., 2012).

The increase in ethylene production and the ethylene up-regulated expression of *VvACO* genes were concomitant with the reduction in TA level of ethephon-treated berries (Fig. 2). These trends most likely resulted from the enhancement of fruit

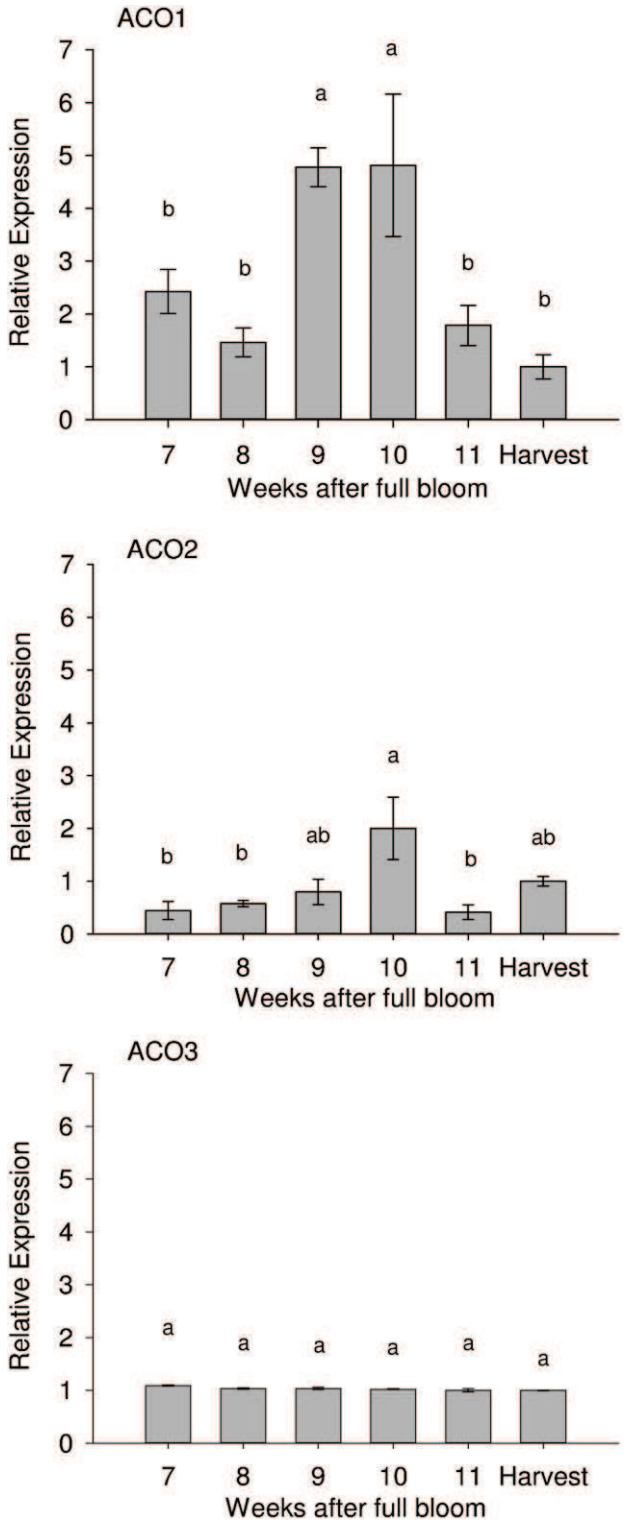

Fig. 4. Gene expression analysis of *VvACO1*, *VvACO2* and *VvACO3* during berry development of "Thompson Seedless" grape. Transcript accumulation was assayed by qPCR in quadruplicate using cDNAs from six developmental stages for each gene form. The relative abundance of each mRNA was normalized to the *eEF-1alpha* gene in the corresponding samples. The results are presented as the relative change in gene expression with respect to the value measured at harvest with a nominal value of 1 in each graph. Data are presented as the means ± SE from four replicates. Different letters indicates statistical significant differences by Tukey analysis ($P < 0.05$).

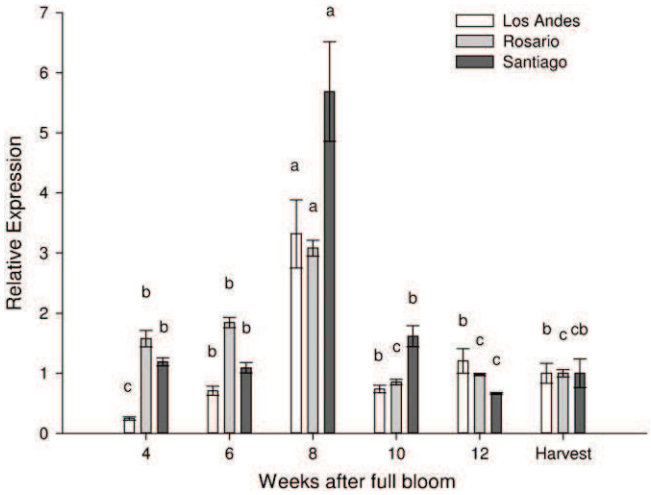

Fig. 5. Gene expression analysis for *VvACO1* during berry development of "Thompson Seedless" grapes from three different orchards. Transcript accumulation was assayed by qPCR in quadruplicate using cDNAs from six developmental stages for each location. The relative abundance of each mRNA was normalized to the *eEF-1alpha* gene in the corresponding samples. The results are presented as the relative change in gene expression with respect to the value measured at harvest with a nominal value of 1 for each location. Data are represented as the means ± SE from four replicates. Different letters indicate statistical differences within each location by Tukey analysis ($P < 0.05$).

metabolism already shown in other species (Jeffery et al., 1983; Defilippi et al., 2004), or from the effect of ethylene in increasing the enzyme activity involved in malate degradation (Knee and Finger, 1992).

4. Conclusions

In this work, we describe three homologous *VvACO* genes, and we report their expression over the berry ripening period. The gene expression profile, response to ethylene, and phenotypic changes observed suggest that these genes might have different physiological roles. In particular, *VvACO1* and *VvACO2* may have a role in the grape berry ripening phase. Despite the consistency of the *ACO* peak in veraison in this report and in previous studies, further investigations with other cultivars are necessary to confirm that some *ACO* homolog could be used as a quantitative indicator of the veraison stage. The transcript accumulation that follows an ethephon treatment of all *VvACO* genes is typical of the long-lasting effects observed in non-climacteric fruit. Additional studies could also be performed to understand why long-lasting ethylene production (triggered by ethephon) is leading to a type of feedback at the ACO transcript level.

Acknowledgment

This study was funded by project FONDECYT 1100273.

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.postharvbio.2012.10.006.

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