MOLECULAR ANALYSIS OF THE IMPROVEMENT IN RACHIS QUALITY BY HIGH CO_2 LEVELS IN TABLE GRAPES STORED AT LOW TEMPERATURE

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ABSTRACT

Rachis browning is one of the main factors reducing the quality of table grapes during storage at low temperature. To better understand the effect of a 3-day CO_2 pretreatment (20% CO_2 plus 20% O_2) on maintaining the rachis quality of table grapes (*Vitis vinifera* cv. Cardinal) at 0 ºC, we analyzed the expression of genes codifying enzymes related to the synthesis and oxidation of phenolic compounds (phenylalanine ammonia-lyase, *VcPAL*; and polyphenol oxidase, *GPO*) and the detoxification of reactive oxygen species (catalase, *GCAT*; and ascorbate peroxidase, *VcAPX*) in rachis of treated and non-treated bunches. Furthermore, due to their role in senescence, the implication of ethylene and abscisic acid (ABA) was also investigated by studying the expression pattern of key regulatory genes for these hormones such as ACC synthase (*ACS1*) and oxidase (*ACO1*), *VvNCED1* and *2*. To determine whether these changes in gene expression were specifically related to rachis deterioration, their expression pattern in pulp and skin of treated and non-treated grapes were evaluated. The appearance of browning in non-treated rachis was associated with an increase in *GPO* and *VcPAL* mRNA levels, whereas high CO_2 levels arrested this accumulation. In pulp, even though browning was not evident, a slight increase in *GPO* accumulation in non-treated bunches was detected. Moreover, lipid peroxidation level revealed lower oxidative stress in rachis of CO_2-treated bunches than in non-treated ones, which seemed to be regulated by *VcAPX* and *GCAT* gene expression induction. Interestingly, this regulation was specific to rachis, showing a different pattern in pulp and skin. Regarding phytohormones, our results pointed to the participation of the ethylene biosynthesis pathway in rachis browning. On the other hand, neither *VvNCED1* nor *VvNCED2* expression levels were altered in rachis, but *NCED1* was induced specifically by low temperature in pulp. Overall, our results suggest a specific response of rachis to high levels of CO_2 that could be related to the mitigation of rachis browning.

Keywords: Table grapes; carbon dioxide; low temperature; rachis browning; gene expression

Introduction

Table grape (*Vitis vinifera* L.) is a non-climacteric fruit with a relatively low rate of physiological activity. Storage at low temperature, around 0 ºC, is recommended for the maintenance of postharvest quality of mature table grape. However, the length of storage is limited by their high susceptibility to fungal decay and the sensitivity of rachis to water loss and browning. Rachis lacks the thick epidermis with cuticular wax depositions that protect berries against dehydration and, although the rachis only represent about 4% of cluster fresh weight (Carvajal-Millán et al., 2001), such disadvantage reduces the market where the condition of rachis in terms of color and turgor is an excellent indicator of postharvest quality. Different postharvest treatments have been used to maintain table grape quality. The application of controlled atmospheres (CA) under a continuous flow has been reported to be beneficial for controlling postharvest diseases in table grapes for prolonged cold storage (Yahia et al., 1983) but not to avoid rachis browning (Crisosto et al., 2002). In previous works, we have shown the efficacy of a 3-day pretreatment with high CO_2 levels maintaining the quality of table grapes and reducing rachis browning during storage at 0 ºC (Sanchez-Ballesta et al., 2006). So far, our studies indicated that the beneficial effect of the high CO_2 pretreatment in the rachis appearance was linked to an increase in the content of unfreezable water (Goñi et al., 2011) as well as to the induction of *VvCBF4* gene expression (Fernandez-Caballero et al., 2012), nonetheless further work is needed to understand the molecular basis of its favorable effect.

Phenolic compounds play an important role in fruit visual appearance. Phenylalanine ammonia-lyase (PAL) is the enzyme at the entry-point of the phenylpropanoid pathway producing a variety of phenolic compounds. In the oxidative degradation of these compounds the enzyme polyphenol oxidase (PPO) plays a relevant role in terms of quality. PPO participates in browning by oxidizing phenolic substrates into quinones which subsequently form brown, black and red color pigments (revised by Tomas-Barberan and Espin, 2001). It has been reported that development of rachis browning during table grape storage is associated

with polyphenol oxidase activity (Carvajal-Millán et al., 2001). The storage of Thompson Seedless berries at 0 ºC induced internal browning and PPO activity (Pool and Weaver, 1970). In reference to gaseous treatments, CA with high O_2 levels reduced PPO activity in the flesh of Kyoho grapes as well as rachis browning during storage at 0 ºC (Deng et al., 2006). However, the molecular regulation of the PPO enzyme in table grape during storage at low temperature as well as how the application of high CO_2 levels could modulate its gene expression in relation to the reduction of rachis browning is still unknown.

Browning and senescence under fruit stress conditions are associated with the production of reactive oxygen species (ROS). During storage of litchi fruit the development of pericarp browning was associated with the rapid increase of hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) contents (Ruenroengklin et al., 2009). Moreover, in these fruits treatment with adenosine triphosphate prevented the accumulation of ROS and slowed the pericarp browning (Yang et al., 2009). During the stress response, plants cells exhibit defense mechanisms to detoxify the synthesized ROS including enzymes such as catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD) and glutathione reductase (GR). In grapes, prolonged cold storage of mature Red Globe clusters reduced CAT activity in the rachis whereas SOD and APX activities were not affected (Campos-Vargas et al., 2012). However, these authors did not show any information about rachis deterioration. In previous work, we have analysed the *APX* gene expression in the skin of Cardinal grapes and the results pointed out the ability of high CO_2 levels to prevent the generation of ROS in this tissue rather than their inactivation once formed (Romero et al., 2008). Nonetheless, little is known about the role of antioxidant enzymes in rachis deterioration during cold storage as well as in the beneficial effect of high CO_2 levels.

Among the physiological changes that take place during postharvest fruit storage, those related to hormone biosynthesis and action are very important considering their role in senescence. Despite the fact that grape berries are classified as non-climacteric, different works have shown that a transient increase of endogenous ethylene production occurred just before

veraison (Chervin et al., 2004; Sun et al., 2010). Chervin et al. (2004) also reported that treatment of berries with an inhibitor of ethylene perception, 1-methylcyclopropene (1-MCP), inhibited grape berry ripening. It is well established that ethylene is synthesized from methionine via S-adenosyl-L-methionine (AdoMet) and the cyclic non-protein amino acid 1-aminocyclopropane-1-carboxylic acid (ACC). ACC is formed from AdoMet by the action of ACC synthase (ACS) and the conversion of ACC to ethylene is carried out by ACC oxidase (ACO) (Kende, 1993). Nevertheless, there are few reports about the regulation of ethylene biosynthesis during postharvest storage of table grapes. In addition to ethylene, abscisic acid (ABA) has been implicated in the control of grape berry ripening and stress response. NCED, 9-*cis*-epoxycarotenoid dioxygenase, is a key enzyme in ABA biosynthetic pathway that catalyzes the cleavage of the double bond of 9-*cis* neoxanthin and/or -violaxanthin to produce xanthoxin, the direct precursor of ABA (Cutler and Krochko, 1999). It has been indicated that the carotenoid cleavage reaction is a key regulation step in the stress-induced ABA biosynthesis (Tan et al., 2003). With regards to berry ripening, trace endogenous ethylene induces the expression of *VvNCED1*a, then the generation of ABA followed (Sun et al., 2010). Furthermore, these authors indicated that ABA appears to trigger the onset of senescence in detached grape berries after harvest. However, treatment of Crimson Seedless clusters with ABA improved rachis quality during storage at 0 ºC (Cantin et al., 2007).

The objective of the present work was to explore the effectiveness of high CO_2 levels reducing rachis deterioration during cold storage of table grapes by studying changes in the expression of genes that codified enzymes related to the synthesis and oxidation of phenols *(PAL* and *PPO)*, the antioxidant system (*CAT* and *APX)*, as well as ethylene (*ACO* and *ACS)* and ABA (*NCED1* and *NCED2*) biosynthesis. In order to distinguish from the changes observed which ones were specifically linked to the effect of gaseous treatment on rachis browning, we have also analyzed the expression of the genes mentioned above in the skin and pulp of berries treated and non-treated with high levels of CO_2.

2. Materials and Methods

2.1 Plant material

Table grape clusters (*V. vinifera* L. cv. Cardinal) were harvested from a commercial orchard in Sevilla (Spain) at early-harvesting stage (12.7% total soluble solids; 0.81% tartaric acid). The field-packaged bunches were transported to the laboratory and immediately forced-air precooled for 14 h at -1 ºC (time 0). After cooling, bunches free from physical and pathological defects were randomly divided into two lots and stored at 0±0.5 ºC and 95% relative humidity (RH) in two sealed neoprene containers of 1 m^3 capacity. Ten plastic boxes containing about 3 kg of table grapes per box were stored in each container. One lot was stored under normal atmosphere for up to 33 days (non-treated) and the other one under a gas mixture containing 20% CO_2 + 20% O_2 + 60% N_2 (CO_2-treated) for 3 days and then transferred to air under the same conditions as the non-treated for 30 days. Five clusters of grapes (approximately 300 g from each cluster) were sampled periodically and the skin, pulp, and rachis were collected, frozen in liquid nitrogen, ground to a fine powder and stored at - 80 ºC until analysis.

2.2 Quality assessment

Browning indexes and relative water content (RWC) of the rachis were determined for each bunch using at least 3 replicates per sample. Browning index was measured using the following subjective scale: (0) none (entire rachis including the pedicels green and healthy), (1) slight (rachis in good condition, but noticeable browning of pedicels), (2) moderate (browning of pedicels and secondary rachis), (3) severe (pedicels, secondary, and primary rachis were brown), and (4) extreme (pedicels, secondary and primary rachis were black). The water status of the rachis was followed by measuring the RWC. One centimeter of rachis, cut with a razor blade was weighed fresh, again after 24 h rehydration with distilled water at room temperature, and finally after drying at 85 ºC to give the fresh (FW), turgid (TW) and dry (DW) weights,

respectively. The RWC expresses in percentage the water content at a given time and tissue as related to the water content at full turgor (Sanchez-Ballesta et al., 2006):

$$RWC\ (\%) = (FW - DW)/(TW - DW) \times 100$$

2.3 Determination of lipid peroxidation

Quantification of the end product of lipid peroxidation, malondialdehyde (MDA), was assayed using a thiobarbituric acid method (Ederli et al., 1997) with some modifications depending on the tissue analyzed. Briefly; 0.1, 0.15, and 0.5 g of rachis, skin, and pulp respectively, were homogenized with 1.5 mL 1% trichloroacetic acid (TCA) and centrifuged at 10,000 x g for 5 min. After centrifugation, 250 µL (for skin and raquis) or 500 µL (pulp) were mixed with 1 mL of 0.5% thiobarbituric acid in 20% TCA. This mixture was incubated at 100 ºC for 30 min and then cooled at room temperature. Absorbance was determined at 532 nm and adjusted for non-specific absorbance at 600 nm. Three independent extractions were made for each sample and extracts were analyzed in duplicate. MDA content was estimated by using a molar extinction coefficient of 155 mmol L^{-1} cm^{-1}.

2.4 Relative gene expression by quantitative RT-PCR

Relative expression of all studied genes was assayed using quantitative RT-PCR (RT-qPCR) with samples of skin, pulp, and rachis from CO_2-treated and non-treated bunches stored for 0, 3, 15, and 33 days at 0 ºC. Total RNA was extracted three times from each sample (Zeng and Yang, 2002) and treated with DNase I recombinant-RNase free (Roche) for genomic DNA removal. Then, 1 µg of each extraction was used to synthesize cDNA by using the iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad). Amplifications were run in a 96 well-plates iCycler iQ thermal cycler (Bio-Rad) and quantification was performed with the iCycler iQTM associated software (Real Time Detection System Software, version 2.0). Each gene was evaluated at least in two independent runs. Primer pairs used in the RT-qPCR are shown in Supplementary Table 1. In order to calculate the efficiency of the reaction (optimal

range 90-110%) and to establish the most suitable template concentration, cDNAs synthesized from serial dilutions (from 40 ng to 2.5 ng) of total RNA were amplified. Standard curves and linear equations were determined by plotting cycle threshold (*Ct*) values (*y*-axis) against logs of total RNA (*x*-axis). The efficiency of each individual run was calculated based on the raw fluorescence data (ΔR_n) exported as output file and subsequently imported into the LinReg PCR program. The specificity of products was validated by dissociation curve analysis and by agarose gel; and its sequences confirmed at the Genomic Department of the CIB-CSIC. *Actin1* gene from *V. vinifera* (*ACT1*: XM_002282480) was used as the internal reference gene for normalizing the transcript profiles following the $2^{-\Delta\Delta Ct}$ method, relative to the calibrator sample (time 0).

2.5 Statistical analysis

All statistics were performed using Statistical Analysis System for PC (SAS Institute Inc., Cary, NC). Data were analyzed using ANOVA and means were separated by Duncan's multiple-range test ($p<0.05$).

3. Results

3.1. Changes in rachis relative water content and development of rachis browning

We analyzed changes in RWC and rachis browning as a measure of two of the main factors contributing to rachis deterioration in table grapes; water loss and tissue browning. As shown in Table 1, although rachis RWC decreased considerably throughout storage at 0 ºC, high levels of CO_2 were able to reduce this effect. Consistent with this, rachis from non-treated bunches stored for 3 days at 0 ºC experienced a reduction of 22% in RWC compared with time 0, whereas application of a 3-day CO_2 pretreatment caused a decrease of 15% in RWC. When CO_2-treated bunches were transferred to air, the loss of RWC was not significant even after 33 days of storage, whereas the reduction of RWC in non-treated rachis was significant (about

36%). Rachis browning increased markedly in non-treated bunches from only slight browning after 3 days to almost severe at 15 and 33 days of storage. In contrast, rachis browning in treated bunches was significant only when they were transferred to air, but even then only showing slight browning towards the end of low-temperature storage (Table 1).

3.2. Changes in VcPAL and GPO1 gene expression in rachis, pulp and skin of CO_2-treated and non-treated bunches stored at 0 ºC

To better understand the relationship between phenolic compound oxidation and rachis browning in *V. vinifera*, we analyzed the expression of phenylalanine ammonia-lyase (*VcPAL*; DQ887093) and polyphenol oxidase (*GPO1*; A27657) in Cardinal bunches. Regarding *VcPAL*, the first changes in its expression levels appeared in non-treated rachis at 15 days of cold storage, being over two-fold higher than in time 0 and in CO_2-treated rachis (Fig. 1A). The application of 3-day CO_2 pretreatment delayed the increase in *VcPAL* transcript levels caused by low temperature. *VcPAL* accumulation was only induced in CO_2-treated rachis after 33 days (about 3.5-fold compared to time 0), reaching values similar to those found in non-treated rachis. In the case of *GPO1*, 3 days of storage at 0 ºC significantly increased the transcript levels in non-treated rachis, but they then decreased to levels lower than at time 0 by the end of storage (Fig. 1A). The application of high CO_2 levels for 3 days caused a significant decrease in the accumulation of *GPO1* mRNA in comparison with non-treated rachis. When treated bunches were transferred to air a decrease in *GPO1* gene expression was observed in rachis after 33 days at 0 ºC reaching values lower than in non-treated rachis.

The expression of *VcPAL* and *GPO1* was also measured in pulp and skin of CO_2-treated and non-treated table grapes stored during 33 days at 0 ºC in order to study the behavior of both genes in fruit tissues where browning was not evident. The *VcPAL* expression pattern in pulp was similar to that found in rachis, with the highest induction at 15 days of treatment in non-treated fruit pulp which was subsequently maintained until the end of storage (Fig. 1B). In the case of CO_2-treated grapes, an increase in the accumulation of *VcPAL* mRNA was also

observed in the pulp after 15 days, although it was significantly lower than that of the non-treated pulp. At the end of storage *VcPAL* transcript levels reached values similar to those obtained in non-treated pulp. On the other hand, *GPO1* expression in pulp was induced by cold from day 3 to the end of low temperature storage, though CO_2 delayed and reduced this induction, i.e. in CO_2-treated pulp *GPO1* transcript levels only increased transitorily after 15 days of storage, going back to basal levels at 33 days (Fig. 1B). However, neither the storage at 0 ºC nor CO_2 treatment induced the accumulation of *GPO1* transcripts in fruit skin (Fig. 1C).

3.3 Oxidative stress in rachis, pulp, and skin of CO_2-treated and non-treated table grapes stored at 0 ºC

In order to determine the role of oxidative stress in the deterioration of rachis at 0 ºC and ascertain whether this response was tissue specific, we analyzed the formation of MDA and changes in ascorbate peroxidase (*VcAPX:* DQ887095) and catalase (*GCAT:* XM_003695412) gene expression in rachis, pulp and skin of CO_2-treated and non-treated bunches. The MDA levels were, in general, lower in tissues from CO_2-treated bunches when compared with non-treated tissues (Table 2). In rachis of non-treated bunches, the MDA content increased during storage at 0 ºC and reached higher levels than at time 0 and in samples from treated clusters. In the latter, application of 3-day high CO_2 levels did not change the MDA content in rachis compared to time 0, though there was a transitory increase at 15 days followed by a fall after 33 days of storage at 0 ºC (Table 2). The lowest lipid peroxidation in CO_2-treated rachises coincided with the highest expression of the genes that codify for ascorbate peroxidase (*VcAPX:* DQ887095) and/or catalase (*GCAT:* XM_003695412), even if the temporal patterns were not identical. As shown in Figure 2A, *VcAPX* gene expression in rachis from CO_2-treated clusters after 15 and 33 days of storage was over two-fold higher than at time 0. By contrast, *VcAPX* mRNA accumulation in non-treated rachis did not change until the end of the storage period when an almost two-fold increase was observed. As regards the *GCAT* expression level, an increase was observed after 3 days at 0 ºC and was significantly higher in CO_2-treated

rachis. Although there was a subsequent decrease in *GCAT* mRNA accumulation in both treated and non-treated rachis, it remained higher in CO_2-treated than at time 0 and in non-treated rachis (Fig. 2A).

In pulp, low temperature increased the MDA content in both CO_2-treated and non-treated grapes, although this was only significant after 15 days in non-treated grapes, decreasing to levels similar to those reached at time 0 after 33 days (Table 2). The *VcAPX* expression in pulp from CO_2-treated bunches was not significantly different ($p<0.05$) from that of fruit at time 0. However, in pulp of non-treated fruit, *VcAPX* expression increased slightly during the first 3 days of storage and then gradually decreased to a significantly lower level of expression at the end of storage compared with fruit at time 0 (Fig. 2B). Similarly, the *GCAT* expression level at 15 and 33 days of storage was significantly lower in pulp of non-treated fruit compared with time 0 fruit, and lower than treated fruit at the same time points, but these differences were not significant (Fig. 2B).

In skin, MDA levels increased transitorily in response to low temperature storage in non-treated grapes, although these changes were not significant. However, in the skin of CO_2-treated grapes the MDA content decreased significantly after 3 and 15 days in comparison with time 0 and non-treated samples. *GCAT* expression in treated and non-treated fruit skin did not differ significantly compared with time 0, except at the end of the conservation period when a 1.5-fold decrease was observed (Fig. 2C).

3.4. Response of ethylene biosynthesis genes to low temperature and high CO_2 levels in rachis, pulp and skin of table grapes

To evaluate ethylene involvement in rachis browning as well as in table grape response to low temperature, we analyzed changes in the expression pattern of *ACS1* (XM_002263552) and *ACO1* (AY211549) genes that codify for key the regulatory enzymes of ethylene biosynthesis in rachis, pulp and skin of *V. vinifera* bunches stored at 0 ºC, both non-treated and treated with high levels of CO_2. A sharp increase in *ACS1* gene expression was observed in

rachis from non-treated bunches after 33 days of storage (Fig. 3A), which was concomitant with severe rachis browning (Table 1). Interestingly, a 2.5-fold increase in *ACO1* transcript levels was also detected in rachis of non-treated fruit after 3 days of storage, which subsequently decreased to levels below time 0 samples (Fig. 3A). Rachis from bunches treated with high levels of CO_2 did not show any change in *ACS1* or *ACO1* expression, with the only significant exception of *ACO1* which was down-regulated after 33 days (Fig. 3A). In contrast, in pulp and skin *ACS1* and *ACO1* showed a different expression pattern to that described for rachis. In pulp, the *ACS1* relative gene expression was induced in response to low temperature in treated and non-treated fruit from 15 days of storage, with a further increase at 33 days which was significantly higher in the CO_2-treated fruit than in the non-treated ones. The *ACO1* mRNA accumulation, on the other hand, was not promoted by either cold or high CO_2 levels. Finally, in the skin, the application of high CO_2 levels for 3 days at 0 ºC significantly induced the *ACS1* and *ACO1* gene expression compared with time 0 and non-treated samples. Subsequently, mRNAs levels of both genes decreased reaching values similar to those for non-treated skin (Fig. 3C). However, in non-treated fruit, the induction of *ACS1* and *ACO1* gene expression by low temperature was delayed, being 3-fold higher at the end of storage in the case of *ACS1* and 1.8-fold higher at 15 days of storage for *ACO1*.

3.5. Response of ABA biosynthesis genes to low temperature and high CO_2 levels in rachis, pulp and skin of table grapes

To help us understand the role of ABA in rachis deterioration and in the response of fruit pulp and skin to low temperature storage and high levels of CO_2, we studied the expression profiles of *VvNCED1* (AY337613) and *VvNCED2* (AY337614). With regard to the *VvNCED2* relative gene expression, no significant differences were detected in either treated or non-treated rachis, pulp, or skin compared to time 0 samples (data not shown). On the other hand, the *VvNCED1* gene expression was regulated differentially by low temperature and high CO_2 levels in rachis, pulp and skin. As depicted in Figure 4A, there was a down-regulation of

the *VvNCED1* relative gene expression in rachis during cold storage that was more pronounced in CO_2-treated bunches. Similarly, fruit skin also showed an important decline in *VvNCED1* transcript levels, beginning at 3 days of storage in treated fruit and later (15 days) in non-treated grapes (Fig. 4C). In fruit pulp, however, 3 days of cold storage transitorily induced *VvNCED1* expression, but this effect did not last long and pulp of non-treated fruit had much lower transcript levels at 15 and 33 days of storage than at time 0. Interestingly, the application of high levels of CO_2 prevented the induction of *VvNCED1* expression observed in non-treated pulp at 3 days of storage (Fig. 4B).

4. Discussion

Table grapes undergo changes during postharvest storage involving accelerated softening and fungal attack. Additionally, postharvest quality deterioration in grapes is also attributed to rachis browning, where an important contributing factor is water loss. Crisosto et al. (2001) observed that the development of visual stem browning symptoms in five table grape cultivars was closely linked to cluster water loss. However, Litchter et al. (2011) argued that weight loss did not always correlate directly with rachis browning, because many clusters developed browning with weight losses lower than other clusters that remained green with greater losses. We have used RWC as an indicator of water balance status in rachis, because it reflects the metabolic activity of tissues by expressing the absolute amount of water that is required to reach artificial full saturation (González and González-Vilar, 2001). RWC decreased in plants as a response to low temperature stress, possibly due to a reduction in the amount of metabolites and osmotica available to hold the water within the cells (Farooq et al., 2008). In cotton leaf, RWC decreased during chilling stress, whereas overexpression of the *betA* gene markedly improved tolerance to chilling in transgenic seedlings as well as maintaining a higher RWC (Zhang et al., 2012). In a previous work we observed that rachis from CO_2-treated clusters stored for 33 days at 0 ºC showed a lower browning index and higher RWC than non-treated ones (Sanchez-Ballesta et al., 2006). Likewise, our results indicated that

high CO_2 levels modified water status in fruit and non-fruit tissues of table grapes, increasing the content of unfreezable water while it remained stable in non-treated bunches (Goñi et al., 2011). In the present work, we have shown that the improvement in rachis RWC in response to high CO_2 levels was also evident at the end of 3-day pretreatment. Moreover, this effect was mainly observed when treated table grapes were transferred to air, indicating that the gaseous treatment could promote an osmotic adjustment, which prevents cell damage caused by low temperature. In this sense, we observed that treatment of strawberries with 20% of CO_2 led to an increase in cellular water retention that was associated with an accumulation of osmolytes, some of which exerted cell protection and regulated ion homeostasis, in addition to mediating osmotic adjustments (Blanch et al., 2012).

Storage of non-treated bunches at low temperature during 3 days was enough to induce a slight rachis browning index parallel to a sharp increase in *PPO* gene expression, whereas transcript levels of *VcPAL* did not change in comparison to time 0. Development of rachis browning during postharvest storage of table grapes has been associated with PPO activity (Carvajal-Millán et al., 2001). Litcher et al. (2011) proposed that this enzyme, which is normally localized in the chloroplast, is in contact with the substrates in the vacuole due to loss of compartmentalization caused by desiccation. However, the fact that rachis browning and *VcPAL* gene expression continued increasing throughout the storage at 0 ºC in non-treated bunches, while *PPO* transcript levels decreased to reach values lower than time 0 at the end of storage, would seem to indicate that other factors are involved in the development of rachis browning. It has been noted that the application of high CO_2 levels as CA induced accelerated rachis browning during low temperature storage of table grapes (Crisosto et al., 2002; Deng et al., 2006), whereas high O_2 levels reduced it (Deng et al., 2006). By contrast, we have observed that application of 20% of CO_2 pretreatment during 3 days at 0 ºC caused a lower increase in rachis browning index, decreased *VcPAL* gene expression and modulated *PPO* transcript accumulation to reach values significantly lower than in non-treated rachis. Murr and Morris (1974) also reported that a high concentration of CO_2 irreversibly inhibited the oxidation of

monophenols by PPO, since CO_2 is a competitive inhibitor of the enzyme. Furthermore, our results indicated that the modulating effect of high CO_2 pretreatment on the table grape response to low temperature was still evident when CO_2-treated bunches were transferred to air, as shown by less rachis browning and *VcPAL* and *PPO* transcript accumulation. All the above findings support that CO_2 pretreatment has the effect of either maintaining constant or restricting the increase in the levels of total phenolic compounds in both early and late harvested Cardinal table grapes (Romero et al., 2009). This could lead to reduced rachis browning due to lower substrate availability. Siriphanich and Kader, (1985) argued that high CO_2 levels prevented the browning of wounded plant tissues by both blocking the production of new phenolic compounds as well as by inhibiting PPO activity. As in the case of rachis, a sharp increase in *PAL* mRNA accumulation in pulp of non-treated fruit was observed after 15 days, but in CO_2-treated grapes it was significantly lower. This seems to support a previous study done by our group where it was shown that table grapes of Cardinal cultivar are sensitive to temperature shifts at 0 ºC by activating phenylpropanoid gene expression in the skin, whereas 3-day high CO_2 pretreatment at 0 ºC avoids and/or modifies these changes (Sanchez-Ballesta et al., 2007). Likewise, the induction of *PPO* gene expression was not a specific response of rachis to low temperature because transcript levels also increased in the pulp of non-treated grapes, although with a different pattern of expression, since they were activated throughout the storage period. However, it is important to note that no visual browning was observed in the pulp of non-treated grapes, so this induction might be associated with the activation of defense responses in non-treated grapes exposed to low temperature.

It is known that fruit browning and senescence are associated with ROS production (Ruenroengklin et al., 2009). ROS accumulation may cause oxidative damage to lipids, forming toxic products such as MDA, a secondary end product of polyunsaturated fatty acid oxidation. The degree of lipid peroxidation represented by the MDA content reflects the state and integrity of plant cell membranes, and has been extensively used as an indicator of oxidative injury. In this respect, an increase in lipid peroxidation, and the concomitant production of MDA, was

reported in different plants as a response to environmental stresses (Cakmak and Horst, 1991; Xu et al., 2012) and has been associated with plant tissue senescence. Our results indicated that although increases in MDA content in skin were not significant, there was a significant increase in rachis and pulp of non-treated table grapes as a response to low temperature storage, though different trends of accumulation were observed between both tissues. Thus, in rachis, the increase was evident throughout the storage period, while in pulp it was transitory, increasing only after 15 days. These results, together with the fact that high CO_2 levels reduced or maintained the MDA content in all the tissues analyzed, would seem to indicate that the gaseous treatment reduced cell injury caused by oxidative stress at 0 ºC and supported our previous results, where differences in the perception of low temperature were observed between treated and non-treated table grapes (Sanchez-Ballesta et al., 2006; Romero et al., 2008; Fernandez-Caballero et al., 2012). Karnosky (2003) proposed that elevated levels of atmospheric CO_2 might reduce the basal rate of O_2 activation and ROS formation in plant cells by enhancing the CO_2/O_2 ratio in the photosynthetic apparatus, and as a consequence, possibly cause a decline in lipid peroxidation levels (Vurro et al., 2009). In spinach leaves, Hodges and Forney (2000) indicated that CA with 10% of CO_2 might inhibit the production of ROS during the latter stages of storage at 10 ºC by retarding mitochondrial oxidative respiration rates. Similarly, our results indicated that the reduction in MDA content observed in rachis of CO_2-treated bunches was linked to the activation of the enzymatic antioxidant system, with significant increases in both *APX* and *CAT* gene expression detected throughout the storage of treated table grapes. On the other hand, it seems that although *GCAT* and *VcAPX* transcript levels increased in non-treated rachis after 3 days and 33 days of low temperature storage, respectively, it was not sufficient to reduce MDA content. In peach fruit, storage at 0 ºC induced the development of irreversible chilling injury (flesh browning) and a gradual increase in MDA content, whereas SOD, CAT and peroxidase activity decreased significantly and there was a loss of membrane integrity. By contrast, CA (5% O_2 plus 5% CO_2) reduced chilling injury, and delayed the reduction of antioxidant enzymes activity compared with the control

(Wang et al., 2005). It is important to note that although high CO_2 levels maintained or restrained MDA content in pulp and skin, no significant differences were observed in the accumulation of *VcAPX* transcript levels in pulp or of *GCAT* mRNA levels in both tissues. In the case of skin, we had previously suggested that APX participate in removing the putative high levels of H_2O_2 in the skin of non-treated grapes (Romero et al., 2008). The overall results might indicate that the beneficial effect of the pretreatment with high CO_2 levels for controlling oxidative stress by the induction of the enzymatic antioxidant system depends on the type of tissue and could be more closely related with the reduction of rachis browning rather than with a general response in table grapes.

Ethylene is considered a major hormonal regulator of senescence in most plant organs (Abeles et al, 1992). Our results revealed that the development of rachis browning in non-treated table grapes was linked to an induction of the expression of *ACS1* and *ACO1* genes, which are responsible for ethylene synthesis. However, the pattern of expression of both transcripts was different, as *ACS1* increased at the end of storage, while *ACO1* was transitorily induced after 3 days at 0 ºC. Likewise, the application of 3-day high CO_2 treatment avoided the accumulation of *ACS1* and *ACO1* transcript levels in rachis. In climacteric fruit such as tomato, treatment with 20% of CO_2 concomitantly prevented an increase in *ACS* and *ACO* mRNA during ripening (Rothan et al., 1997). In non-climacteric fruit, high CO_2 treatment has been described as possibly affecting ethylene action. Thus, in strawberries, treatment with 20 kPa of CO_2 for 48 hours down-regulated three ethylene receptors (Ponce-Valadez et al., 2009). CO_2 may act both as an inducer and as a suppressor of ethylene biosynthesis depending on the commodity, plant tissue, CO_2 concentration and time of exposure (Mathooko, 1996). On the one hand, it is known that CO_2 is an essential cofactor for ACO (Dong et al., 1992; Escribano et al., 1996), and it has been shown to induce ethylene production by enhancing ACS activity and synthesis (reviewed by Mathooko, 1996). In this sense, Becatti et al., (2010) observed that high CO_2 applied to detached wine grapes for 3 days at 20 ºC induced *ACO* and an *ACS*-like gene expression in skin and pulp. On the other hand, elevated levels of CO_2 can reduce

ethylene biosynthesis mainly by inhibiting *ACS* gene expression and affecting ACO action (de Wild et al., 2003). We observed that in table grapes the regulation of *ACS1* and *ACO1* gene expression by high CO_2 levels was dependent on the type of tissue. Thus, *ACS1* transcript accumulation increased in pulp of both treated and non-treated grapes stored at 0 ºC, whereas *ACO1* did not change. By contrast, both transcript levels were markedly induced in grape skin treated with high CO_2 levels, while in non-treated skin the induction was lower or appeared later during storage. These results seem to indicate that *ACS* and *ACO* genes in non-climacteric table grapes showed different transcriptional behavior in response to high CO_2 levels and low temperature in fruit and non-fruit tissues, which may indicate differences in gene structure and regulatory elements.

ABA accumulation plays a key role in the regulation of ripeness and senescence in fruit, including grapes (Zhang et al., 2009; Sun et al., 2010), and even appears to trigger the onset of senescence in detached grape berries after harvest (Sun et al., 2010). By contrast, application of ABA at veraison improved the rachis quality of Crimson grapes during storage at 0 ºC (Cantin et al., 2007). With reference to gene expression, we observed a down-regulation of *VvNCED1* transcript levels produced by both, low temperature and high CO_2 levels in rachis and skin, so we cannot establish a link with rachis deterioration, but rather with a more general response. Becatti et al., (2010) also observed a down-regulation of *NCED* in the skin of wine grapes treated with high CO_2 levels. Conversely, our results showed that 3 days of storage at 0 ºC induced a sharp increase in *NCED1* accumulation in pulp, whereas high CO_2 levels clearly restrained this induction. These results, together with the hypothesis of reduced ABA synthesis as a response to high CO_2 levels in table grape pulp, are also consistent with our previous results indicating that the gaseous treatment has been positively correlated with high tolerance to temperature shifts at 0 ºC (Sanchez-Ballesta et al., 2007; Fernandez-Caballero et al., 2012).

In conclusion, 3-day high CO_2 pretreatment on table grape activated specific responses in the rachis that could lead to the control of browning, avoiding the activation of ethylene biosynthesis and promoting an osmotic adjustment as well as the maintenance of the membrane

integrity. Furthermore, these results reinforce our previous studies in which we reported that the gaseous treatment minimized or modified the activation of defense mechanisms observed in non-treated grapes as a response to temperature shifts at 0 ºC.

Acknowledgements

This work was supported by CICYT projects AGL2008-02949 and AGL2011-26742. R.R, C.F.-C. and I. R. were supported by a postdoctoral JAE contract from the CSIC, a predoctoral contract from the MEC and a postdoctoral Juan de la Cierva contract from the MICINN, respectively.

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FIGURE CAPTIONS

Fig. 1. Effect of low temperature and 3-day high CO_2 pretreatment on *VcPAL* and *GPO1* gene expression in rachis (A), pulp (B), and skin (C) of *V. vinifera* cv. Cardinal bunches stored at 0 ºC. Transcript levels of each gene were assessed by quantitative RT-PCR and normalized using *actin* (*ACT1*) as reference gene. Results were calculated relative to a calibrator sample (time 0, before storage) using the formula $2^{-\Delta\Delta Ct}$. Values are the mean ± SD, n=3. Different letters on bars indicate means are statistically different using Duncan's test (p<0.05).

Fig. 2. Effect of low temperature and 3-day high CO_2 pretreatment on *VcAPX* and *GCAT* expression in rachis (A), pulp (B), and skin (C) of *V. vinifera* cv. Cardinal bunches stored at 0 ºC. Transcript levels of each gene were assessed by quantitative RT-PCR and normalized using *actin* (*ACT1*) as reference gene. Results were calculated relative to a calibrator sample (time 0, before storage) using the formula $2^{-\Delta\Delta Ct}$. Values are the mean ± SD, n=3. Different letters on bars indicate means are statistically different using Duncan's test (p<0.05).

Fig. 3. Effect of low temperature and 3-day high CO_2 pretreatment on *ACS1* and *ACO1* expression in rachis (A), pulp (B), and skin (C) of *V. vinifera* cv. Cardinal bunches stored at 0 ºC. Transcript levels of each gene were assessed by quantitative RT-PCR and normalized using *actin* (*ACT1*) as reference gene. Results were calculated relative to a calibrator sample (time 0, before storage) using the formula $2^{-\Delta\Delta Ct}$. Values are the mean ± SD, n=3. Different letters on bars indicate means are statistically different using Duncan's test (p<0.05).

Fig. 4. Effect of low temperature and 3-day high CO_2 pretreatment on *NCED1* expression in rachis (A), pulp (B), and skin (C) of *V. vinifera* cv. Cardinal bunches stored at 0 ºC. Transcript levels of each gene were assessed by quantitative RT-PCR and normalized using *actin* (*ACT1*) as reference gene. Results were calculated relative to a calibrator sample (time 0, before storage) using the formula $2^{-\Delta\Delta Ct}$. Values are the mean ± SD, n=3. Different letters on bars indicate means are statistically different using Duncan's test (p<0.05).