UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS BIOLÓGICAS

TESIS DOCTORAL

Mecanismos moleculares implicados en la adaptación a elevadas concentraciones de CO_2 en uva de mesa (*Vitis vinifera L. cv. Cardinal*) con diferentes grados de madurez conservada a bajas temperatura

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Carlos Fernández-Caballero Aguilar

Directoras

Carmen Merodio Moreno
María Teresa Sánchez Ballesta

Madrid, 2015

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MEMORIA PARA OPTAR AL GRADO DE DOCTOR
PRESENTADA POR:

Carlos Fernández-Caballero Aguilar

Bajo la dirección de:
Dra. Carmen Merodio Moreno
Dra. María Teresa Sánchez Ballesta
(ICTAN-CSIC)

Madrid, 2015

Carmen Merodio Moreno, Doctora en Biología, Investigador Científico del Consejo Superior de Investigaciones Científicas adscrito al Instituto de Ciencia y Tecnología de los Alimentos y Nutrición de Madrid

CERTIFICA que:

D. Carlos Fernández-Caballero Aguilar, Licenciado en Biología por la Universidad Complutense de Madrid, ha realizado bajo su codirección el trabajo que, con el título: **"Mecanismos moleculares implicados en la adaptación a elevadas concentraciones de CO_2 en uva de mesa (*Vitis vinifera* L. cv. Cardinal) con diferentes grados de madurez conservada a bajas temperaturas"**, presenta para optar al grado de Doctor en Biología por la Universidad Complutense de Madrid.

Para que conste a los efectos oportunos, firma el presente certificado en Madrid, a 11 de mayo de 2015.

Carmen Merodio Moreno

SEDE CIUDAD UNIVERSITARIA:

C/ JOSÉ ANTONIO NOVÁIS, 10
CIUDAD UNIVERSITARIA
28040 MADRID, ESPAÑA
TELS.: 91 544 56 07 – 91 549 23 00
FAX: 91 549 36 27

SEDE JUAN DE LA CIERVA:

C/JUAN DE LA CIERVA, 3
28006 MADRID, ESPAÑA
TELS.: 91 562 29 00
FAX: 91 564 48 53

María Teresa Sánchez Ballesta, Doctora en Farmacia, Científico Titular del Consejo Superior de Investigaciones Científicas adscrito al Instituto de Ciencia y Tecnología de los Alimentos y Nutrición de Madrid

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SEDE JUAN DE LA CIERVA:

C/JUAN DE LA CIERVA, 3
28006 MADRID, ESPAÑA
TELS.: 91 562 29 00
FAX: 91 564 48 53

“Antes de ser hombres de ciencia, deberíamos ser hombres”

(Albert Einstein)

“Actuar sobre la realidad y cambiarla, aunque sea un poquito, es la única manera de probar que la realidad es transformable"

(Eduardo Galeano)

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“Ha pasado el tiempo y sigo sin creer que ya no estés aquí. Se supone que el tiempo lo va curando todo, pero sigo sintiendo el mismo dolor en mi corazón por tu ausencia. Quizá sea un sentimiento para algunos en exceso intenso, especialmente tras el tiempo pasado, pero no entiendo otra manera de querer, no entiendo otra manera de quererte que no sea desde la intensidad de los afectos. Porque si algo que tengo claro es que te quiero, y siento que cada día más. Por ello, no hay noche que en el silencio de mi cama no vengan a mí tus recuerdos, y en esos momentos mis deseos buscan encontrarte de nuevo, sentirte. Aunque parezca una tontería, cuando ya estabas muy malita y sabía perfectamente que te ibas, te cogía de la mano y, además de parecer relajar el sufimiento que tenías, quería egoístamente no olvidarme de lo que yo sentía. Por eso, cada vez que te añoro, cierro los ojos e intento acordarme de ese momento, de lo que sentía, de tu piel, de tu caricia. Pero me duele a veces no conseguirlo. Lo veo en mi mente, pero no lo siento. En este punto, y a pesar de la tristeza, lo que me queda es tu legado, un legado lleno de amor del que me siento un privilegiado. Pero también me quedan tus recuerdos, cada momento que me inundaste de tu amor, generosidad y valentía. Es más, no sólo me queda el aprendizaje vital, sino el aprendizaje de tu marcha, porque ahora más que nunca siento la necesidad de vivir este privilegio, el privilegio de la vida y, sobre todo, ser feliz. Por eso, GRACIAS. Pero gracias también por darme las fuerzas de poder seguir y terminar este proyecto, que tanto esfuerzo me ha costado y el que tanto confiabas. Por todo ello, y como bien sabes, seguiré poniendo cada día en casa un clavel al lado de tu foto, porque me reconforta saber lo mucho que te gustaban, y seguiré también cerrando los ojos para poder verte ahí acurrucadita, y me acercaré a susurrarte una vez más al oído: TE QUIERO, y así sentirte un poco, aunque sea un poco, más cerca de mí”.

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RESUMEN / ABSTRACT

Mecanismos moleculares implicados en la adaptación a elevadas concentraciones de CO_2 en uva de mesa (*Vitis vinifera* L. cv. Cardinal) con diferentes grados de madurez conservada a bajas temperaturas.

La pérdida de agua y el ataque fúngico son factores clave en la pérdida de calidad de la uva de mesa y de su valor en el mercado. De ahí, la necesidad de utilizar tecnologías no contaminantes que garanticen su control durante la conservación. En trabajos previos, verificamos en el cultivar Cardinal que pretratamientos cortos (3 días) con altas concentraciones de CO_2 (20% CO_2) reducían significativamente las pérdidas de peso y el marchitamiento del raquis después de 33 días a 0ºC. Estos interesantes resultados nos llevaron a proponer el presente estudio centrado en el análisis integral de las respuestas metabólicas y de los mecanismos moleculares inducidos por cortos tratamientos de CO_2 que permitan superar la fase crítica de conservación a 0ºC (3 días), de forma que se eviten las posteriores manifestaciones de pérdida de calidad en uva de mesa Cardinal. Este objetivo general se ha desarrollado según los siguientes objetivos parciales: 1) Análisis e identificación de marcadores metabólicos determinantes de la fase crítica de conservación a 0ºC y de la efectividad del tratamiento con altos niveles de CO_2, en función del tipo de tejido y del grado de madurez del fruto; 2) Caracterización y regulación de los mecanismos moleculares implicados en la tolerancia a las bajas temperaturas en los diferentes tejidos del racimo tratados con altas concentraciones de CO_2; 3) Análisis transcriptómico de los mecanismos implicados en la adaptación a elevadas concentraciones de CO_2 de uva de mesa de dos estados de madurez diferente durante la fase crítica de conservación a 0ºC.

Nuestros resultados mostraron mayores niveles de agua ligada o no congelable en los frutos tratados con CO_2, tanto al finalizar el tratamiento como durante la transferencia al aire, especialmente en pulpa y raquis. Los tejidos de pulpa que tenían un mayor contenido en agua ligada exhibían una mayor capacidad de retención de agua y una mejor estructura celular. En la piel, el tratamiento gaseoso evitó el desequilibrio metabólico provocado por el salto térmico al inicio de la conservación a 0ºC y previno el descenso en el contenido de agua no congelable, la salida de flujo extracelular de agua y de potasio y la consiguiente pérdida de volumen de las células más superficiales de la epidermis. Más aún, la importancia del estado del agua y su distribución en la piel se confirmó en frutos procedentes de diferentes campañas. Asimismo, la influencia del

grado de madurez en la efectividad de diferentes tratamientos gaseosos (3 y 6 días) se evidenció en la expresión de la *PAL,* en el contenido en antocianos, fenoles y en la actividad antioxidante. Mientras que en los frutos con menor grado de madurez ni el CO_2 ni la baja temperatura afectaron al metabolismo fenólico, en los recolectados tardíamente únicamente el tratamiento gaseoso de 3 días evitó su activación en la fase crítica de conservación.

En el raquis, el tratamiento gaseoso tuvo un efecto directo sobre el estado del agua, previniendo el descenso en el contenido de agua no congelable lo que podría asociarse a un efecto protector frente al marchitamiento y pardeamiento del mismo, causado por las bajas temperaturas. Asimismo, el sistema antioxidante enzimático desempeñó un papel importante en el control del desarrollo del pardeamiento. En este sentido, observamos en los racimos tratados con CO_2 una buena correlación negativa entre el nivel de pardeamiento y la expresión de *GCAT.* Estas respuestas, posiblemente dependientes de la señalización por etileno en base a la acumulación transitoria en los niveles de transcritos de la síntesis de etileno ACC sintasa (*ACS1*) y oxidasa (*ACO1*), se evitaron por el tratamiento gaseoso.

La activación de la expresión de *CBFs* en pulpa y raquis por la combinación de altos niveles de CO_2 y bajas temperaturas podría indicar una mayor regulación de la expresión de estos tejidos por altas concentraciones de CO_2 en la superación de la fase crítica de conservación a 0ºC. En referencia al análisis de dehidrinas, nuestros resultados mostraron por primera vez la retención del intrón en los transcritos de *VvcDHN1a*, no sólo en respuesta a las bajas temperaturas sino también a los elevados niveles de CO_2. Por otro lado, mientras que el binomio bajas temperaturas-altos niveles de CO_2 indujeron los transcritos de *DHN1a* en la piel, pulpa y semillas, se observó una expresión constitutiva en el raquis, sugiriendo que la regulación de *DHN1a* en uva de mesa podría atribuirse a otras vías de señalización diferentes a *CBF1* y *CBF4.*

El análisis transcripcional llevado a cabo utilizando el GrapeGen GeneChip® mostró que en la fase crítica de conservación a 0ºC tiene lugar un marcado cambio en el transcriptoma de la piel de uva independientemente del grado de madurez del fruto. Por el contrario, ligeras diferencias se observaron en los tejidos de frutos tratados con CO_2 al compararse con los frutos recién recolectados. El análisis funcional de los cambios moleculares, mostró que las respuestas transcripcionales a las bajas temperaturas en la piel de uva en ambos estados de madurez coincidían con procesos biológicos de

respuesta a distintos estreses bióticos y abióticos. Sin embargo, los términos más representados durante la conservación a 0ºC de los frutos con menor madurez estaban relacionados con la 'gluconeogénesis', con 'procesos catabólicos de quitina' y 'fotosíntesis'. Entre las respuestas transcripcionales específicas a la conservación a 0ºC de los frutos con mayor grado de madurez, se observó la inducción en los niveles de expresión de genes relacionados con el término 'plegamiento de proteínas', cuyo mantenimiento es uno de los retos más importantes de los organismos sometidos a distintas condiciones de estrés. Nuestros resultados permiten concluir que el efecto del tratamiento con altos niveles de CO_2 en la piel de uva Cardinal es dependiente del grado de madurez del fruto, activando principalmente factores de transcripción en los frutos recolectados tempranamente y genes relacionados con el metabolismo energético en los frutos en estado de madurez más avanzado.

Molecular mechanisms involved in the adaptation to high CO_2 in table grapes (*Vitis vinifera* L. cv. Cardinal) with different degrees of ripeness stored at low temperatures.

Water loss and fungal attack are the main factors responsible for loss in quality and commercial value in table grapes. Hence, they should be cooled as soon as possible after harvesting at low temperatures, close to 0ºC, but even under these conditions there is a detrimental effect on the quality and appearance of the bunches. Moreover, the rapid shift from warm harvested conditions to this low temperature led to a pronounced structural damage that it has been also associated with a strong activation of stress responses. Therefore, there is a need to employ co-adjuvant and non-contaminating technologies during low temperature storage. In previous studies we verified that short-term high CO_2 concentrations (20% CO_2 for 3 days) reduced markedly weight loss and withering in bunches after 33 days at 0ºC. These interesting results led us to undertake the main objective of the present study focused on metabolic responses and molecular mechanisms induced early by high CO_2 concentrations, thus permitting the forecasting of later changes in quality. The following specific objectives were carried out: 1) Analysis and identification of metabolic markers linked to the initial stage of storage at 0ºC and to the effectiveness of the gaseous treatment in tissues of tables grapes at different degrees of ripeness; 2) Characterization and regulation of molecular mechanisms implicated in low temperature tolerance induced by high CO_2 in different tissues of table grapes; 3) Transcriptomic analysis of the mechanisms involved in the adaptation to high CO_2 levels in grapes harvested with different degrees of ripeness.

Our results showed a higher level of unfreezable or bound water fraction in high CO_2-treated fruit than in non-treated ones, at the end of treatment as well as after transferring to air. Moreover, the pulp tissues that had the highest amounts of bound water also had a higher capacity of water retention and a better cellular structure as revealed by cryo-microscopy. In skin tissues, storage at 0ºC caused a decrease in the bound water fraction correlated with a larger water-soluble K^+ pool. Potassium presented a non-uniform distribution in the cells as determined by energy dispersive X-ray microanalysis. In contrast, high CO_2 treatment prevented the decrease in the bound water fraction, and the water-soluble K^+ accumulation associated with cellular water stress was not triggered. The importance of the status and distribution of water in peel

tissues was confirmed in early-harvested fruit from different years. The present work brings new insights about the great influence of ripeness stage on L-phenylalanine ammonia-lyase (*PAL)* gene expression, anthocyanins, total phenolics and antioxidant activity in the peel of table grapes treated with two different high CO_2 treatments. In early harvested grapes neither exposure to CO_2 nor low temperature storage affected the total anthocyanin levels. In non-treated late harvested grapes there was an increase in the content of anthocyanins, total phenolics and antioxidant activity during the first days parallel with an increase in *VcPAL* mRNA levels. Whereas the 3- days CO_2 pretreatment restrained this initial phenolic response in a similar manner to *PAL* expression, 6-days CO_2 treatment maintained a high phenolic content, antioxidant activity and *VcPAL* expression level, even at the end of the storage period at 0ºC.

In the rachis, high CO_2 treatment also had a direct effect on tissue water status, preventing the decrease in the bound water fraction that could determine the level of protection against withering and browning caused by low temperatures. Likewise, the antioxidant enzymatic system seems to play a role in the control of rachis browning development. Thus, we observed in CO_2-treated bunches a good negative correlation between rachis browning and *GCAT* gene expression. The development of rachis browning was linked to the induction of *ACS1* and *ACO1* ethylene biosynthetic genes, while the gaseous treatment avoided such accumulation.

Taking into account our results, we might hypothesize that the *CBFs* expression in pulp and rachis activated by high CO_2 levels could help table grapes to face temperature shifts at 0ºC. In reference to the dehydrin analysis, our results showed for the first time intron retention in *VvcDHN1a* transcripts not only in response to low temperature, as indicated previously by other authors, but also to high levels of CO_2. On the other hand, whereas low temperature and high CO_2 levels induced spliced transcripts of *DHN1a* in skin, pulp and seeds, a constitutive expression was observed in rachis, suggesting that *DHN1a* regulation in table grapes could be attributed to other cold-activated pathways different from *CBF1* and *CBF4.*

The comparative large-scale transcriptional analysis using the custom-made GrapeGen GeneChip® showed that low temperature, in the first stage of storage, led to an intense change in grape skin transcriptome independently of fruit ripeness stage but with different changes in each one. By contrast, slightly differences were observed in CO_2 treated samples in comparison to freshly-harvested fruit. Major modifications in

the transcriptome profile of early- and late-harvested grapes storage at 0ºC are linked to biotic and abiotic stress-responsive terms, indicating the cross-talk between stresses. However, specific transcriptional modifications were observed depending on ripeness stage. In both cases there is a reprogramming of the transcriptome during the course storage in order to withstand the stress imposed by low temperatures mainly associated to gluconeogenesis, photosynthesis, mRNA translation and lipid transport, in early-harvested grapes; whereas the maintenance of protein folding stability and intracellular membrane trafficking seems to play an important role in late-harvested grapes. The effect of the high CO_2 pretreatment maintaining quality seems to be an active process requiring the activation of a great number of transcription factors. Likewise, although only few genes were significantly regulated by high CO_2 levels in late harvested grapes, functional annotation chart indicated that its effectiveness also seems to be linked to active processes for controlling energy metabolism in the fruit.

ABREVIATURAS

1-MCP:	1-metilciclopropeno
ABA:	Ácido abscísico
ABTS:	Ácido 2,2'-azinobis (3etilbenzotiazolín)-6-sulfónico
AP2	'APETALA 2'
ACC:	Ácido 1-aminociclopropano-1-carboxílico
ACO:	ACC oxidasa
ACS:	ACC sintasa
AFP:	Proteína anticongelante o 'antifreeze protein'
APX:	Ascorbato peroxidasa
bZIP:	'Basic domain leucine-zipper'
C:*	Croma
CA:	Atmósfera controlada o 'controlled atmosphere'
CAT:	Catalasa
CBF:	'C-repeat binding factor'
CBS:	Cistationina beta sintasa
CHS:	Chalcona sintasa
COR:	Regulado por frío o 'cold regulated'
CRT:	'C-repeat element'
DAVID:	'Database for Annotation, Visualization and Integrated Discovery'
DHN:	Dehidrina o 'dehydrin"
DMSO:	Dimetilsulfóxido
DRE:	Elemento de respuesta a deshidratación o 'dehydration responsive element'
DREB:	Proteína de unión a DRE o 'DRE binding protein'
DSC:	Calorimetría diferencial de barrido
eIF:	Factor de iniciación de eucariotas o 'eukaryotic initation factor'
ERF:	Factor de transcripción de respuesta a etileno o 'ethylene responsive factor'
FAD:	Desaturasa de ácido graso o 'fatty acid dasaturase'
FDR:	'False Discovery Rate'
GAPC:	Gliceraldehído-3-fosfato deshidrogenasa
GO:	Ontología Génica o 'Gene Ontology'
GR:	Glutatión reductasa
HCA:	Análisis de grupos hidrofóbicos Análisis de conglomerados jerárquicos o 'Hierarchical Cluster Analysis'
HPLC-MS:	Cromatografía líquida acoplada a espectroscopía de masas
HSP:	Proteína de choque térmico o 'heat shock protein'

ICP-OES:	Espectroscopía de emisión óptica de plasma de acoplamiento inductivo
L^*:	Luminosidad
LDH:	Lactato deshidrogenasa
LEA:	'Late embryogenesis abundant'
LT-SEM:	Microscopía electrónica de barrido de baja temperatura
MA:	Atmósfera modificada o 'modified atmosphere'
MAP:	Envasado en atmósferas modificadas o 'modified atmospheres packaging'
Mb:	Millones de pares bases
MDA:	Malondialdehído
NCED:	9-*cis*-epoxicarotenoide dioxigenasa
nsLTP:	Proteína de transferencia de lípidos no específica o 'non-specific lipid transfer protein'
$OH^{\bullet}$:	Radical hidroxilo
PAL:	L-fenilalanina amonio-liasa
PCA:	Análisis de componentes principales o 'Principal Component Analysis'
PCK1:	Fosfoenolpiruvato carboxiquinasa 1
POD:	Peroxidasa
PPIase:	Peptidil-propil cis-trans isomerasa
PPO:	Polifenol oxidasa
PR:	Proteína relacionada con patogénesis o 'pathogenesis-related protein'
RMN:	Resonancia magnética nuclear
ROS:	Especies reactivas de oxígeno o 'reactive oxygen species'
RWC:	Contenido relativo de agua o 'relative water content'
SAM:	S-adenosil metionina
	'Significance Analysis Microarray'
SEM-EDX:	Microscopía electrónica de barrido a bajas temperaturas-microanálisis por dispersión de energía de rayos-X
SOD:	Superóxido dismutasa
STS:	Estilbeno sintasa
SSC:	Contenido en sólidos solubles
TAC:	Capacidad antioxidante total
TEAC:	Capacidad antioxidante equivalente de Trolox
T_{onset}	Temperatura de inicio de congelación
UFW:	Agua no congelable o 'unfreezable water'
V-ATPase:	ATPasa translocadora de protones vacuolares o 'vacuolar proton ATPase'
ΔH_f:	Entalpía o calor de fusión o 'heat of fusion'
^{o}h:	Ángulo hue

INTRODUCCIÓN

En las últimas décadas, los cambios globales en la industria agroalimentaria han afectado de manera drástica a las técnicas de conservación postcosecha. La estructura de la industria se ha concentrado y los patrones de demanda se han desplazado hacia el mayor valor añadido de los productos. El sistema multilateral de negocio consolida un comercio cada vez más liberalizado, y existe una mayor orientación de los países en desarrollo hacia los mercados de exportación como fuente de crecimiento económico. Asimismo, está aumentando la preocupación por temas relacionados con el medioambiente y el desarrollo sostenible, de manera que los consumidores exigen una mayor reducción del uso de productos químicos. Como consecuencia, la incorporación de tecnologías postcosecha no contaminantes, que permitan reducir el uso de compuestos agroquímicos manteniendo la calidad, es vital para dinamizar la oferta de productos vegetales frescos.

La uva de mesa (*Vitis vinifera* L.) es un fruto no climatérico, no madura después de ser cosechada, alcanzando el óptimo de aceptabilidad en apariencia, sabor y textura mientras está en la vid. La composición de la uva varía según se trate de uvas blancas o tintas. En ambas destacan dos tipos de nutrientes: los azúcares (siendo la glucosa y la fructosa más del 99% de los hidratos de carbono en el zumo de uva y constituyendo del 12 al 27% del peso fresco de la baya madura) y las vitaminas (principalmente ácido fólico y vitamina B6). La fracción ácida de las uvas está formada principalmente por los ácidos tartárico y málico, constituyendo alrededor del 90% de la acidez total (Winkler *et al*., 1974). El comercio y abastecimiento de uva de mesa ha sufrido una expansión en los últimos años debido a un mayor consumo. De las 947.096 ha cultivadas en España (Anuario de Estadística 2013), el 1,53% se dedica a la producción de uva de mesa, siendo España el duodécimo productor a nivel mundial y el segundo productor en Europa, por detrás de Italia.

La solución idónea, desde el ámbito de la postcosecha, para preservar la buena calidad de los frutos y satisfacer las exigencias de los mercados nacionales e internacionales consiste en utilizar técnicas no invasivas, no contaminantes y de fácil manejo evitando el uso de grandes instalaciones y largos periodos de aplicación.

1.- Parámetros de calidad de uva de mesa.

Una vez recolectada, la uva de mesa, es altamente perecedera al estar sujeta a importantes pérdidas de vapor de agua, que causan desecamiento y oscurecimiento del raquis, roturas e inclusos marchitamiento de las bayas y pérdidas de peso (Nelson, 1979). Por ello, en el caso de uva de mesa, se emplean bajas temperaturas de conservación cercanas a 0ºC y una humedad relativa del 90-95% para una mayor eficacia en el control de la pérdida de agua, manteniendo su calidad y prolongando su periodo de vida útil postcosecha. Sin embargo, aunque la uva de mesa es tolerante a las bajas temperaturas, y no desarrolla los tradicionales "daños por frío", su conservación frigorífica se ve limitada debido a su susceptibilidad a la **podredumbre o enfermedad del moho gris producida por *Botrytis cinerea*** (Pearson & Goheen, 1988; Snowdon, 1990). Este hongo es un patógeno necrotrófico que coloniza los tejidos senescentes o muertos, pero debido a que es capaz de infectar también a bajas temperaturas, puede producir importantes pérdidas económicas durante la conservación en frío (Mansfield & Hutson, 1980). En estudios previos de nuestro grupo, hemos observado un aumento gradual de la podredumbre en uva de mesa cv. Cardinal durante su periodo de conservación a 0ºC, alcanzando niveles del 25% al final del mismo, con respecto a los de los frutos recién recolectados después de 33 días (Romero *et al*., 2006; Sanchez-Ballesta *et al*., 2006). Resultados similares se han obtenido en distintas variedades de uva, en los que el desarrollo de *Botrytis* fue aumentando durante su conservación a 0ºC (Crisosto *et al*., 2002; Artés-Hernández *et al*., 2004, 2007; Candir *et al*., 2012). Estos resultados se deben en parte a la capacidad de crecimiento y actividad patogénica de *B. cinerea* a temperaturas incluso por debajo de -0,5ºC (Gindro & Pezet, 2001; Chervin *et al*., 2012). Además, hay que considerar la posible influencia de las bajas temperaturas en el desarrollo fúngico a través del efecto de la elevada humedad relativa (Darras *et al*., 2006). Para evitar o reducir las pérdidas de calidad de uva de mesa durante su conservación a bajas temperaturas son numerosos los estudios realizados para desarrollar tratamientos gaseosos no contaminantes coadyuvantes a las bajas temperaturas que controlen el desarrollo del hongo, manteniendo su calidad (Thomposon, 2001; Retamales *et al*., 2003; Artés-Hernández *et al*., 2006; Romero *et al*., 2006; Sanchez-Ballesta *et al*., 2006; Guillen *et al*., 2007; Candir *et al*., 2012). Por su carácter fungistático, el empleo de altas concentraciones de CO_2 se presenta como una

alternativa al uso de SO_2 ya que sus residuos son dañinos para la población alérgica a sulfitos, siendo 10 µL/L el umbral máximo tolerado para estos residuos en frutos según la Administración de Drogas y Alimentos de los Estados Unidos (Crisosto *et al*., 1994); mientras que la Unión Europea ha prohibido su uso (Directiva 95/2 CE). Además, el SO_2 no puede ser utilizado en uvas certificadas de obtención orgánica (Gabler & Smilanick, 2001). En trabajos previos, nuestro grupo ha observado la eficacia de un pretratamiento gaseoso con 20% de CO_2 aplicado durante 3 días a 0ºC, sin cambios en los niveles atmosféricos de O_2, en el mantenimiento de la calidad de la uva de mesa, reduciendo el porcentaje de podredumbre por *B. cinerea y* el pardeamiento de raquis (Romero *et al.,* 2006; Sanchez-Ballesta *et al*., 2006). Además, aunque la uva es tolerante a las bajas temperaturas, trabajos previos también indican su sensibilidad a los cambios de temperatura en la fase inicial de conservación a 0ºC, provocando una serie de desajustes metabólicos que se vieron controlados en parte por el pretratamiento gaseoso con altos niveles de CO_2 (Sanchez-Ballesta *et al*., 2007; Romero *et al*., 2008a).

Otras de las disfunciones fisiológicas que conducen a la pérdida de calidad postcosecha de la uva de mesa durante la conservación a bajas temperaturas son **la pérdida de agua**, que producen diferentes alteraciones como el marchitamiento del raquis y el arrugamiento de la baya, **y el pardeamiento de sus estructuras.** El agua, al ser el componente mayoritario en uva, así como en todos los frutos frescos, y estar implicada en sus principales procesos fisiológicos, cualquier modificación en su contenido y propiedades van a afectar a su calidad y, por extensión, van a definir el periodo de vida útil del fruto y su capacidad de adaptación a las diferentes condiciones de conservación (Hills & Remigereau, 1997; Moraga *et al*., 2006; Alferez *et al*., 2010). En general, uno de los efectos de la pérdida de agua durante el manejo y conservación postcosecha de uva de mesa resulta en el oscurecimiento o pardeamiento del raquis (Crisosto *et al*., 2001; Lichter *et al*., 2011; Valverde *et al*., 2005a; Sanchez-Ballesta *et al*., 2006; Balic *et al*., 2012). En consecuencia, tanto el oscurecimiento como el marchitamiento del raquis influyen en la apariencia visual de los racimos y, aunque éste solo representa alrededor del 4% de su peso fresco (Carvajal-Millán *et al*., 2001), ambos parámetros pueden ayudar a determinar el período óptimo de conservación de los racimos. El raquis es, en líneas generales, susceptible a estas pérdidas debido a su elevada proporción superficie-volumen y a su alta tasa de respiración con respecto a las bayas (Chervin *et al*., 2012). Además, a diferencia del fruto, carece de una delgada

epidermis con depósitos de cutícula que le hace ser más susceptible a la deshidratación (Nelson, 1985; Carvajal-Millán *et al*., 2001), lo que supone una importante desventaja comercial ya que el color y turgencia del raquis son índices excelentes de la calidad postcosecha. Ngcobo *et al*. (2013) observaron que durante la conservación de uva de mesa a bajas temperaturas, el raquis perdía agua más rápidamente que los frutos. Asimismo, cuanto menor tamaño tenía el raquis mayor era la pérdida de agua. No obstante, aunque una elevada humedad relativa pueda ser un factor importante a la hora de prevenir o retardar el pardeamiento del raquis (Raban *et al*., 2013), la calidad del mismo ha presentado numerosas variaciones en función de los distintos cultivares estudiados (Valverde *et al*., 2005b; Chen *et al*., 2011; Lichter *et al*., 2011). En el caso de uva de mesa Cardinal, en anteriores trabajos, se utilizó el contenido relativo de agua (RWC) como un indicador del estatus del agua en el raquis después de 33 días de conservación a 0ºC, observándose una disminución significativa de este parámetro al mismo tiempo que aumentaba su índice de pardeamiento, que fue menor en los racimos pretratados con 20% de CO_2 durante 3 días. Este trabajo también mostró una mayor pérdida de peso en los racimos no tratados en ese mismo período de conservación (Sanchez-Ballesta *et al*., 2006). En cambio, Lichter *et al*. (2011) plantearon que la pérdida de peso no siempre se correlaciona directamente con el pardeamiento del raquis, ya que obtuvieron un mayor índice de pardeamiento en aquellos racimos que presentaron menores pérdidas de peso. Asimismo, Raban *et al*. (2013) comparando variedades de uva de mesa apirena (Mystery, Superior y Crimson) y con semilla (Red Globe), corroboraron que el pardeamiento del raquis no podía ser atribuido únicamente a la pérdida de peso, ni del racimo entero ni del propio raquis, por lo que la susceptibilidad al pardeamiento necesita de nuevas investigaciones en distintas variedades de uva de mesa. Para entender el papel del contenido de agua en las alteraciones del raquis durante su conservación es importante tener en cuenta no sólo sus posibles pérdidas, sino que también otros factores, ya que el agua está presente en distintos estados en los tejidos vegetales, influyendo en distintos procesos (Ruan & Chen, 1998; Goñi *et al*., 2007; Agüero *et al*., 2008, Wright *et al*., 2009; Blanch *et al.,* 2012a). Por ello, determinar el estado en el que se encuentra el agua en los tejidos de uva de mesa y cómo la variación de sus propiedades afecta a su calidad, podría también proporcionar una buena aproximación de los cambios metabólicos y bioquímicos que participan en dicho pardeamiento. Así, en este trabajo planteamos un estudio completo

del estado de agua en los distintos tejidos del racimo de uva de mesa como reflejo de los cambios metabólicos y fisiológicos producidos durante su conservación a bajas temperaturas.

Por otro lado, distintos trabajos sugieren que el deterioro del raquis es debido a la combinación de otros factores, como son los procesos de oxidación de compuestos fenólicos, el estrés oxidativo y la senescencia. La asociación bioquímica más común del pardeamiento en frutos y vegetales es con la enzima polifenol oxidasa (PPO), que oxida compuestos fenólicos de la ruta de los fenilpropanoides a quinonas, originándose pigmentos de color marrón, negro y rojo (Tomás-Barberan & Espin, 2001; Lei *et al*., 2004; Lichter *et al*., 2011). De igual manera, el desarrollo del pardeamiento del raquis durante la conservación postcosecha de uva de mesa se ha asociado con la actividad de la PPO (Pool & Weaver, 1970; Deng *et al*., 2006; Carvajal-Millán *et al.,* 2001). Así, Lichter *et al*. (2011) propusieron que esta enzima, que normalmente se encuentra localizada en el cloroplasto, podría entrar en contacto con los sustratos en la vacuola debido a la pérdida de compartimentalización causada por la desecación. Por su parte, Rizzini *et al*. (2009) previamente vincularon la pérdida de agua postcosecha con importantes cambios en la transcripción de genes, como la inducción de la expresión del gen que codifica la L-fenilalanina amonio-liasa (PAL), enzima clave en el metabolismo de los fenilpropanoides.

Asimismo, se sabe que tanto el pardeamiento de frutos como los procesos de senescencia están asociados con la producción de especies reactivas de oxígeno (ROS). Durante el almacenamiento de frutos de lichi se observó que el desarrollo de pardeamiento en su pericarpo estaba relacionado con un rápido incremento en el contenido de H_2O_2 y radical hidroxilo ($OH^{\bullet}$) (Ruenroengklin *et al*., 2009). Además, en este fruto, el tratamiento con adenosín trifosfato previno la acumulación de ROS y retrasó el pardeamiento (Yang *et al*., 2009). En uva de mesa, Campos-Vargas *et al*. (2012) estudiaron algunos de los procesos fisiológicos relacionados con el estrés oxidativo que se desencadenaban durante la conservación postcosecha a bajas temperaturas. Concretamente, observaron una disminución de la actividad catalasa (CAT) en el raquis de uva cv. Red Globe conservada a bajas temperaturas, mientras que las actividades superóxido dismutasa (SOD) y ascorbato peroxidasa (APX) no mostraron cambios. No obstante, estos autores no aportan ninguna información sobre el deterioro del raquis. Sin embargo, los resultados de Balic *et al*. (2012) proporcionaron

evidencias de la correlación entre el pardeamiento del raquis de uva Red Globe almacenada a 0ºC y los cambios a nivel transcripcional de determinados genes como el que codifica la cistationina beta-sintasa (*CBS*). Aunque su papel no es muy conocido en plantas, proteínas CBS han sido asociadas con reguladores redox que controlan la actividad de tiorredoxinas, que a su vez regulan la actividad de diferentes antioxidantes y enzimas que protegen frente el daño causado por radicales libres o ROS (Buchanan *et al*., 2002; Yoo *et al*., 2011).

Entre los cambios fisiológicos que tienen lugar durante la conservación postcosecha de frutos, aquellos relacionados con la biosíntesis y acción de hormonas son clave teniendo en cuenta su papel en la senescencia. En el caso de la uva, a pesar de que es un fruto no climatérico, se ha sugerido que el etileno podría estar implicado en la expresión de sus atributos de calidad, en las etapas más tempranas de su desarrollo (Chervin *et al*., 2004; Sun *et al*., 2010). Si bien se ha demostrado que existe un incremento transitorio en la producción endógena del etileno justo antes de dicha etapa, así como de la expresión de genes claves en su biosíntesis, tales como los que codifican las enzimas ACC oxidasa (ACO) (Chervin *et al*., 2004, 2008; Sun *et al*., 2010; Muñoz-Robredo *et al*., 2013), los estudios sobre la regulación de la biosíntesis del etileno durante la conservación de uva de mesa son muy limitados. Palou *et al.* (2003), observaron que la exposición continua a distintas concentraciones de etileno durante la conservación de uva de mesa a 0 o 5ºC no afectaba a la capacidad de infección de *B. cinerea* ni al pardeamiento del raquis. Además del etileno, el ácido abscísico (ABA) parece jugar un papel en el control de la maduración de los frutos, incluida la uva (Zhang *et al*., 2009a; Sun *et al*., 2010), participando en los mecanismos que conducen a la senescencia de las bayas después de la cosecha de los racimos (Sun *et al*., 2010). Sin embargo, la aplicación de ABA durante el envero en uva Crimson Seedless mejoró la calidad del raquis durante la conservación a 0ºC (Cantin *et al*., 2007). Campos-Vargas *et al*. (2012) sugirieron que el hecho de que el ABA puede inducir la actividad de la lipoxigenasa implicada en la oxidación de lípidos en uva (Costantini *et al*., 2006), podría indicar en parte el mayor nivel de peroxidación lipídica de la membrana que encontraron en el raquis de racimos maduros.

2.- Importancia del estado de madurez durante la conservación de la uva de mesa.

La fase de maduración en la uva se inicia con el envero, del término francés 'véraison', que es la etapa de transición que se utiliza para describir los cambios fisiológicos que se producen en la uva, indicativos del inicio de la maduración y culmina con la recogida de los racimos, de manera que el inicio del envero va a determinar de forma crítica la fecha de la recolección. Estos cambios incluyen el ablandamiento del tejido, un crecimiento basado en la expansión celular, descenso en la acidez, acumulación de azúcares y compuestos relacionados con el sabor y el aroma, pérdida de clorofila en la piel, y acumulación de antocianinas en las variedades de color (Robinson & Davies, 2000; Conde *et al*., 2007). Si se produce un retraso en la fecha de recolección, tiene lugar la sobremaduración de las bayas, produciéndose una concentración de sus componentes y la pérdida de peso debido al agua evaporada.

El estado de madurez del fruto en el momento de la recolección va determinar su calidad comestible, su susceptibilidad a daños mecánicos y en general su potencial vida útil de comercialización (Crisosto, 1994). De ahí, la importancia y necesidad de profundizar en los procesos bioquímicos y moleculares que gobiernan estos cambios fisiológicos y, en especial, en los cambios que tienen lugar durante la conservación postcosecha según el índice de madurez con el que se recolecten las bayas. En este sentido, puesto que la resistencia natural a la infección por *B. cinerea* disminuye a medida que aumenta la madurez de la uva (Jeandet *et al*., 1991), se podría explicar que uva de mesa Thompson Seedles recolectadas en el estado de madurez más avanzado mostrasen después de 8 semanas a -0,5ºC niveles de podredumbre por *Botrytis* superiores a los de uvas menos maduras (Burger *et al*., 2005). Igualmente, el grado de madurez no sólo afecta a la podredumbre. Así, la susceptibilidad al pardeamiento de la piel de uva de mesa cv. Princess durante la conservación a bajas temperaturas aumentó cuando las bayas se cosecharon en un estado avanzado de madurez (≥18,0% SSC) (Vial *et al*., 2005).

En el caso del raquis, puesto que a medida que avanza el estado de madurez del racimo se observa un incremento en lignina y suberina y una disminución en el contenido del agua, cabe la posibilidad de que sea menor su susceptibilidad a la deshidratación después de la recolección (Nelson, 1985). Carvajal-Millán *et al*. (2001) observaron que tanto el pardeamiento del raquis como la pérdida de peso de racimos de

uva de mesa cv. Flame Seedless incrementaban significativamente durante la conservación a bajas temperaturas en los dos estados de madurez analizados, uno de madurez comercial y otro, tres semanas más tarde, cercano a la madurez fisiológica. Sin embargo, aunque la actividad PPO incrementó en el raquis durante la conservación, independientemente del estado de madurez, el aumento fue mayor en el raquis de los racimos de madurez comercial. Durante la maduración de uva de mesa Crimson Seedless, López-Miranda *et al*. (2011) observaron una correlación positiva y significativa entre la actividad PPO y la evolución de los sólidos solubles. A pesar de que se han observado variaciones en la actividad de la PPO durante la maduración de variedades blancas y tintas, la literatura no menciona una evolución sistemática de la misma. En otro trabajo con uva Red Globe, Campos-Vargas *et al*. (2012) analizaron el efecto del almacenamiento a bajas temperaturas en el estrés oxidativo de raquis de racimos con dos estados de madurez diferentes. Sus resultados mostraron un mayor nivel de lipoperoxidación de membrana y capacidad antioxidante en los raquis maduros que en los inmaduros, independientemente del tiempo de exposición a 0ºC.

El efecto de los tratamientos postcosecha con altos niveles de CO_2 sobre la podredumbre y al marchitamiento del raquis también se ha relacionado con el estado de madurez. Crisosto *et al*. (2002) observaron que en uvas cv. Red Globe recolectadas tardíamente, la aplicación de atmósferas controladas (CA) con 10 kPa de CO_2 combinado con 3, 6 ó 12 kPa de O_2 era efectiva en el control del ataque fúngico y del pardeamiento del raquis durante 12 semanas de conservación a bajas temperaturas. Sin embargo, estos autores observaron que en uvas recolectadas tempranamente la combinación de 10 kPa CO_2 + 6 kPa O_2 sólo fue efectivo durante 4 semanas.

Numerosos estudios llevados a cabo durante la maduración de distintas variedades de uva de mesa han tenido en cuenta otros aspectos que influyen en su calidad, como son parámetros químicos y la composición fenólica (Jayasena & Cameron, 2008; Singh Brar *et al*., 2008; Muñoz-Robredo *et al*., 2011; Crupi *et al*., 2012), o el impacto del estado de madurez en propiedades de textura de las bayas (Río Segade *et al*., 2013). Aunque su finalidad comercial es distinta, tales procesos también son aspectos a tener en cuenta en uva de vino, ya que pueden llegar a definir la calidad del caldo que se obtiene de ellas. En consecuencia, distintos autores han analizado la relación entre el estado de madurez de la uva y los cambios químicos que en ella tienen lugar, y que determinan la química y calidad final del vino (Pérez-Magariño &

González-San José, 2006; Ivanona *et al*., 2011; Cadot *et al*., 2012; Bindon *et al*., 2013). Un análisis transcriptómico reciente, en el que se analizaron los cambios en la piel y pulpa de uva cv. Muscat Hamburg durante su maduración, indicó que una gran parte del programa de maduración es compartido por ambos tejidos, aunque algunos componentes están retrasados en la piel. Además, diferencias importantes entre ambos tejidos estaban presentes desde las etapas tempranas, antes del inicio de la maduración, incluyendo reguladores específicos para cada tejido (Lijavetzky *et al*., 2012).

3.- Bases fisiológicas, bioquímicas y moleculares asociadas al efecto beneficioso del empleo de altos niveles de CO_2 durante la conservación postcosecha a bajas temperaturas.

La conservación a bajas temperaturas, por encima de las de congelación, ha sido la principal estrategia para aumentar el tiempo de vida útil de frutos. Sin embargo, debido a la alta susceptibilidad de determinadas especies, la incidencia de alteraciones fisiológicas limita su uso. Además, el efecto de las bajas temperaturas y las respuestas generadas para contrarrestarlas son heterogéneas, especialmente si se tiene en cuenta las diferencias que existen en la capacidad de resistir o adaptarse entre especies sensibles y tolerantes al frío (Sevillano *et al*., 2009). Por ello, aunque se han hecho importantes esfuerzos para conocer los cambios fisiológicos y metabólicos que experimentan los frutos durante su conservación en frío (Maul *et al*., 2008; Wang & Wang, 2009; Liu *et al*., 2011; Costa *et al*., 2012; Sanchez-Bel *et al*., 2012; Tonutti & Bonghi, 2014), al ser un proceso complejo, es difícil obtener un perfil global de sus efectos fundamentado en el análisis de genes o metabolitos específicos. De ahí, la necesidad de emplear un amplio espectro de métodos que nos ayuden a conocer los procesos más relevantes durante la conservación a bajas temperaturas.

Por otro lado, se han desarrollado diferentes tecnologías postcosecha con el fin de retrasar o evitar las alteraciones causadas por las bajas temperaturas, y así mantener la calidad del producto. Algunas de ellas tienen naturaleza física, y consisten principalmente en cambios de temperatura, humedad relativa o composición gaseosa de la atmósfera que rodea al fruto. En concreto, el uso de atmósferas modificadas (MA) o controladas, así como el empleo de cortos pretratamientos gaseosos, fundamentan generalmente su efectividad en el establecimiento de una atmósfera con bajas

concentraciones de O_2 y/o altas concentraciones de CO_2 (Romero *et al*., 2006; Sanchez-Ballesta *et al*., 2006; Sevillano *et al*., 2009; Yahia, 2009). No obstante, hay que tener en cuenta que la exposición a elevados niveles de CO_2 afecta al metabolismo general de estos productos, y puede llegar a ser perjudicial dependiendo de la concentración del gas, la temperatura, la duración del tratamiento y el genotipo (Becatti *et al*., 2010). Por tanto, es importante analizar las bases fisiológicas, bioquímicas y moleculares implicadas en los mecanismos de respuesta de los frutos a altas concentraciones de CO_2 durante su conservación frigorífica.

3.1.- Efecto sobre la membrana plasmática.

Aunque hasta el momento no se han identificado los sensores de la plantas a las bajas temperaturas, los descubrimientos de los últimos años apuntan a que múltiples factores podrían participar en la detección del estrés (revisado por Miura & Furomoto, 2013). Uno de los efectos primarios de las bajas temperaturas en las células es la alteración de las propiedades de fluidez de la membrana y la composición de los ácidos grasos debido a un incremento en el contenido de lípidos poliinsaturados (Murata & Los1997; Beck *et al*., 2007; revisado por Sevillano *et al.*, 2009). El incremento en el grado de insaturación de los lípidos de membrana se ha descrito como un mecanismo de aclimatación a las bajas temperaturas durante la conservación postcosecha (Lurie & Ben-Arie, 1987; Mirdehghan *et al*., 2007; Zhang & Tian, 2010; Cao *et al*., 2011) que puede conducir al mantenimiento de la fluidez de la membrana. Concretamente, Zhang & Tian (2009) indicaron que los frutos de melocotón almacenados a 0ºC mostraban mayor tolerancia a las bajas temperaturas que los almacenados a 5ºC, encontrando una relación entre la mayor acumulación de ácido linoleico (C18:3) y el incremento en los niveles de los transcritos de una desaturasa del ácido graso omega-3 en los frutos más tolerantes. Asimismo, los resultados obtenidos por Hernández *et al*. (2011) mostraron que las bajas temperaturas regulaban a nivel transcripcional genes de desaturasas de ácidos grasos en el mesocarpo de aceitunas de las variedades Picual y Arbequina.

Por otro lado, se ha observado que la exposición de las plantas a bajas temperaturas incrementa los niveles de calcio citosólico, que actúa como un mensajero secundario en la señal de estrés por frío (Knight, 2002). Este incremento de Ca^{2+} en el citosol puede también estar mediado por los canales de calcio activados por ligando o

mecano-sensitivos inducidos por la rigidificación de la membrana. En *Medicago sativa* y *Brassica napus*, la rigidificación de la membrana plasmática inducida por las bajas temperaturas conduce a la reorganización del citoesqueleto, a la inducción de los canales de Ca^{2+}, y al incremento de los niveles de Ca^{2+} citosólico. Todo ello induce la expresión de genes regulados por frío (*COR*) y la aclimatación al frío. Además, el tratamiento con dimetilsulfóxido (DMSO), un rigidificador de la membrana, puede inducir la expresión de genes *COR* incluso a 25ºC, mientras que la aplicación de alcohol bencílico, que fluidifica la membrana, previene la inducción de la expresión de estos genes incluso a 0ºC (Ovar *et al*., 2000; Sangwan *et al*., 2001). La evidencia genética de que las plantas sienten las bajas temperaturas a través de la rigidificación de la membrana viene dado por el mutante *fad2* de *Arabidopsis*, deficiente en oleato desaturasa. Vaultier *et al*. (2006) mostraron que el mutante presentaba rigidificación de la membrana y activación de diacilglicerol quinasa a temperaturas elevadas (18ºC) en comparación con el tipo silvestre (14ºC) y plantas transgénicas de *Arabidopsis* que sobreexpresan linoleato desaturasa (12ºC).

El efecto de tratamientos postcosecha en la integridad de la membrana y en la estructura general de las células durante la conservación a bajas temperaturas ha sido analizado. En estudios previos, nuestro grupo ha observado mediante microscopía electrónica de barrido (LT-SEM) que el pretratamiento gaseoso de 3 días con altas concentraciones de CO_2 (20% CO_2 + 20% O_2) mantenía la estructura celular del mesocarpo de chirimoya (*Annona cherimola* Mill. cv. Fino de Jete) y del parénquima de fresa (*Fragaria vesca* L. cv. Mara de Bois), en comparación con los frutos no tratados conservados 0ºC (Maldonado *et al*., 2002; Blanch *et al*., 2012a,b). Zhang & Tian (2010) describieron que melocotones almacenados en atmósferas controladas (5% O_2 + 5% CO_2) a 0ºC, presentaban una mayor fluidez e integridad de sus membranas que los conservados en aire, en parte debido a la presencia de mayor grado de insaturación de sus lípidos de membrana y a la reorganización de los mismos, relacionada ésta con menores niveles de los mensajeros de la fosfolipasa D. De hecho, el empleo de atmósferas controladas indujo la acumulación de una clase de fosfolípidos inusuales, N-acil-fosfatidiletanolamina, implicados en la protección y estabilización de la membrana en respuesta a condiciones de estrés que implican cambios degenerativos de la misma (Schmid *et al*., 1990; Rawyler & Braendle, 2001). Asimismo, el almacenamiento de uva de mesa en atmósferas controladas con altos niveles de O_2 (80%) mejoró la calidad de

las bayas, manteniendo la integridad de las membranas al retrasar el incremento en la permeabilidad de las mismas que tuvo lugar en los frutos almacenados en aire (Deng *et al*., 2005a,b).

Otro factor importante en el estudio de las biomembranas es el efecto específico del agua en ellas. El grado de disponibilidad de agua determina las propiedades de los lípidos de la membrana y, por tanto, puede afectar a las propias propiedades biofísicas de las membranas. Bendel *et al*. (2001), utilizando técnicas de imagen de resonancia magnética, mostraron que los procesos de almacenamiento a bajas temperaturas estaban acompañados por la conversión de agua ligada a agua libre. En línea con estos resultados, Vertucci & Stushnuff (1992) indicaron que la aclimatación al frío de las yemas vegetativas de manzana implicaba distintos procesos, entre los que se incluye un incremento en los niveles de agua no congelable. En fresa, nuestro grupo ha observado que el pretratamiento con 20% de CO_2 durante 3 días a 0ºC, conducía a un aumento en la retención de agua celular que se asoció con una acumulación de compuestos osmoprotectores (Blanch *et al*., 2012b).

3.2.- Efecto sobre el estrés oxidativo.

Además del efecto directo de las bajas temperaturas en la organización molecular de los lípidos de membrana, su pérdida de integridad se ve potenciada por los procesos oxidativos, ya que las bajas temperaturas pueden incrementar los niveles de ROS (revisado por Sevillano *et al*., 2009). El balance entre la formación y detoxificación de ROS es crítico para la supervivencia de la célula durante la exposición a bajas temperaturas (Mittler, 2002; Blokhina *et al*., 2003; Jaspers & Kangasjärvi, 2010). Con esta finalidad, las plantas han desarrollado un complejo sistema antioxidante que incluye antioxidantes liposolubles (α-tocoferol y ß-caroteno), reductores hidrosolubles (ascorbato y glutatión) y enzimas antioxidantes, como superóxido dismutasa (SOD), catalasa (CAT), ascorbato peroxidasa (APX) y glutatión reductasa (GR) (Zhang *et al*., 1995; Prasad, 1996; Moller, 2001; Mittler, 2002; Kuk *et al*., 2003). No obstante, distintos trabajos muestran evidencias de que incluso durante la situación de estrés la producción de ROS no es necesariamente un síntoma de disfunción celular sino que podría representar una señal que conduciría al ajuste de la maquinaria celular ante situaciones adversas (revisado por Jaspers & Kangasjärvi, 2010). Baek & Skinner

(2012) revisaron distintos trabajos en los que se proponían a las especies reactivas de oxígeno, especialmente el peróxido de hidrógeno (H_2O_2), como transductores de señales en los mecanismos de defensa de las plantas frente estreses bióticos y abióticos, entre los que se incluyen las bajas temperaturas.

Se sabe que los productos vegetales modulan sus defensas antioxidantes cuando se exponen a bajas temperaturas de conservación y a distintos tratamientos postcosecha. Wang *et al*. (2005a) mostraron que la sobreexpresión de una *APX* citosólica de *Pisum sativum* L. en plantas de tomate les proporcionaba una protección significativa frente a las bajas temperaturas. Por otro lado, Kerdnaimongol & Woodson (1999), observaron que tomates transgénicos que expresaban el gen antisentido *CAT1* incrementaban su susceptibilidad al estrés oxidativo impuesto por el tratamiento con H_2O_2, así como a los daños por frío, no siendo viables después de la exposición a 4ºC. En un análisis transcriptómico del efecto de la conservación a bajas temperaturas en melocotón, se observó que frutos almacenados 21 días a 4ºC mantienen la capacidad de detoxificar ROS mediante la inducción de la expresión de genes relacionados con elementos fundamentales del sistema antioxidante, tales como *GR*, *CAT* y *SOD*, así como los que codifican proteínas de choque térmico (HSPs) (Pavez *et al*., 2013). Recientemente, Yuan *et al*. (2014) realizaron un análisis bioquímico y proteómico en uva de mesa Kyoho durante su conservación postcosecha a 2ºC y observaron una inducción de distintas enzimas antioxidantes, que incluyen APX, SOD y glutatión S-transferasa. Similares resultados se observaron en pimiento almacenados en frío (Sánchez-Bel *et al*., 2012). Un estudio transcriptómico del efecto del almacenamiento con elevadas concentraciones de CO_2 en dos cultivares de fresa (Cavendish y Jewel), sugirió que el tratamiento gaseoso influye en el metabolismo oxidativo del fruto ya que se detectaron cambios en la expresión de genes implicados en los sistemas antioxidantes en ambos cultivares (Ponce-Valadez *et al*., 2009). Asimismo, el empleo de atmósferas modificadas en melocotones almacenados a 0ºC, redujo los daños por frío y retrasó la disminución de las actividades enzimáticas de la SOD y CAT observados en los frutos control (Wang *et al*., 2005b). En el caso de uva de mesa, se ha sugerido que la APX podría participar en la eliminación de H_2O_2 inducido por las bajas temperaturas, y que el pretratamiento gaseoso de 3 días con CO_2 podría mitigarlo (Romero *et al*., 2008b).

3.3.- Efecto sobre el metabolismo de los fenilpropanoides.

Los compuestos fenólicos son un grupo heterogéneo de metabolitos secundarios de las plantas que están implicados en la calidad de los frutos, ya que juegan un papel relevante en su aspecto y características organolépticas (Tomas-Barberán & Spin, 2001; Kalt, 2005; Jaakola, 2013), siendo las uvas una de las mayores fuentes de compuestos fenólicos dentro de las distintas especies de frutos (Macheix *et al*., 1990). El interés por estos compuestos se centra en su efecto beneficioso para la salud, debido en general a sus propiedades antioxidantes (Hertog *et al*., 1992). Además, como ya se ha comentado, su degradación oxidativa, catalizada por acción de las enzimas PPO y peroxidasa (POD), es uno de los principales problemas que tiene la industria alimentaria ya que da lugar al pardeamiento enzimático de frutos y vegetales frescos (Carvajal-Millán *et al*., 2001; Tomás-Barberán & Espin, 2001; Lei *et al*., 2004; Deng *et al*., 2006; Lichter *et al*., 2011; López-Miranda *et al*., 2011). Por otro lado, se ha demostrado que distintos compuestos fenólicos están implicados en la defensa de las plantas frente a estreses bióticos y abióticos (Dixon & Pavia, 1995). Por tanto, puesto que la conservación postcosecha de los frutos, puede tener un importante impacto sobre los compuestos fenólicos y enzimas involucradas en su metabolismo, el estudio de su biosíntesis y regulación es de especial interés. La enzima L-fenilalanina amonio-liasa (PAL; EC 4.3.1.5), es clave al catalizar el primer paso metabólico de la vía de los fenilpropanoides, que da lugar a distintos compuestos fenólicos con funciones estructurales y relacionadas con la defensa, incluyendo antocianos, flavonoides, furanocumarinas, fitoalexinas, ligninas y ésteres fenólicos (Hahlbroock & Scheel, 1989; Dixon & Pavia, 1995). Las fitoalexinas de las especies de *Vitis*, que juegan un papel importante en los mecanismos de defensa frente a los patógenos fúngicos (Jeandet *et al*., 2002), constituyen un grupo bastante restringido de moléculas pertenecientes a la familia del estilbeno (Langcake & Pryce, 1977), cuyo esqueleto se basa en la estructura de *trans*-resveratrol. La síntesis de *trans*-resveratrol está catalizada por la enzima estilbeno sintasa (STS), estrechamente relacionada con la chalcona sintasa (CHS), enzima que cataliza el primer paso de la biosíntesis de flavonoides entre los que se incluyen los antocianos, ya que ambas enzimas actúan sobre el mismo sustrato, el *p*-cumaril-CoA, pero con reacciones de ciclación distintas. Los antocianos son responsables del color rojo, violeta y azul de flores y frutos (Harbone & Grayer, 1988).

Sus principales funciones parecen que están relacionadas con atraer insectos y pájaros para la polinización de las flores, y animales para la diseminación de las semillas de los frutos (Timberlake & Bridle, 1982). Además, se ha demostrado que algunos antocianos, junto con otros flavonoides, presentan actividades antivirales, antibacterianas y fungicidas (revisado por Lev-Yadun & Gould, 2009). Asimismo, debido a su conocida capacidad antioxidante, se han publicado distintos trabajos donde se describen ciertos beneficios terapéuticos asociados a los antocianos, como propiedades antiinflamatorias y vasoprotectoras (Garcia-Alonso *et al*., 2004; Oak *et al*., 2006; He & Giusti, 2010).

La activación del metabolismo de los fenilpropanoides puede jugar un importante papel en el desarrollo de barreras en las células dañadas. Por ejemplo, plantas transgénicas donde la *PAL* fue silenciada mostraron necrosis espontáneas (Tamagnone *et al*., 1998) o el desarrollo más rápido de lesiones más extensas que las plantas salvajes después de la infección por patógenos (Maher *et al*., 1994), sugiriendo que la inhibición de la biosíntesis de fenilpropanoides podría comprometer la salud de las plantas. Asimismo, se ha descrito la acumulación de los transcritos de la *PAL*, *STS* y *CHS* en respuesta a estreses bióticos y abióticos (Leyva *et al*., 1995; Sanchez-Ballesta *et al*., 2000; Versari *et al*., 2001; Balic *et al*., 2012; Crifó *et al*., 2012). Distintos estudios indican que las bajas temperaturas inducen la actividad de enzimas del metabolismo de los fenilpropanoides y la acumulación de fenoles totales en distintos frutos (revisado por Sevillano *et al*., 2009). Estos cambios no sólo constituyen un mecanismo de defensa a las bajas temperaturas (Wang *et al*., 2007; Rinaldo *et al*., 2010), sino que también pueden estar vinculados con el desarrollo de los daños por frío (Sanchez-Ballesta *et al*., 2000; Sala *et al*., 2005; Gálvez *et al*., 2010). Un estudio transcriptómico realizado en uvas rojas cv. Raboso Piave, sometidas a distintas condiciones postcosecha de deshidratación, que incluían temperaturas de 5ºC y humedad relativa de 96%, mostró que junto con la inducción en los niveles de expresión de un gen *PAL* también tenía lugar la disminución en la acumulación de los transcritos de otros dos genes *PAL* (Bonghi *et al.*, 2012). Estos resultados sugieren que la regulación de la expresión de los numerosos miembros de la familia multigénica *PAL* es compleja y puede representar un paso clave en las múltiples respuestas fisiológicas a los estreses ambientales. Asimismo, el empleo de tratamientos postcosecha coadyuvantes con las bajas temperaturas afectan al metabolismo de los fenilpropanoides. La utilización de CA con un 30% de CO_2 retrasó el ablandamiento en aguacate, indujo los niveles de los mensajeros de la *PAL* así

como su actividad, e incrementó el contenido del polifenol epicatequina (Prusky *et al*., 1993). Sin embargo, no se detectaron diferencias significativas en la expresión de *PAL4* entre uvas de mesa Red Globe almacenadas en aire y las almacenadas en CA (15% de CO_2 y 5% de O_2) durante 90 días de almacenamiento a 0ºC (Balic *et al*., 2012). Estudios previos de nuestro grupo, mostraron un fuerte incremento de los niveles de los transcritos de *PAL*, *STS* y *CHS* en la piel de uvas almacenadas 3 días a 0ºC, que fue menor en los frutos tratados con 20% de CO_2 durante 3 días a la misma temperatura (Sanchez-Ballesta *et al*., 2007). Christie *et al*. (1994) indicaron que distintos genes de la ruta biosintética de los antocianos, tales como la *PAL* y *CHS,* pueden ser considerados genes *COR*. Aunque la uva es tolerante a las bajas temperaturas, la activación de la expresión de genes de los fenilpropanoides en la primera etapa de almacenamiento a 0ºC puede estar relacionada con la percepción del cambio de temperatura por los frutos, que podría ser menos evidente en los frutos tratados con CO_2 (Sanchez-Ballesta *et al*., 2007). A este respecto, distintos trabajos han mostrado la existencia de una adaptación cruzada en las plantas, de manera que la exposición a un estrés moderado no sólo induce resistencia a ese estrés sino que también puede mejorar la tolerancia a otros (Bowle & Fluhr, 2000; Wang *et al*., 2003). En consecuencia, la aplicación del pretratamiento con altos niveles de CO_2 (20%) en uva de mesa podría mejorar la tolerancia a los cambios de temperatura.

Es conocido que la síntesis de antocianos continúa después de la recolección de frutos, e incluso durante la conservación postcosecha a bajas temperaturas, pero es inhibida en los frutos almacenados con altas concentraciones de CO_2. El almacenamiento a bajas temperaturas incrementó el contenido de antocianos en distintos frutos, tales como fresa (Gil *et al*., 1997; Kalt *et al.*, 1999), arándanos (Connor *et al*., 2002), cerezas (Gonçalves *et al*., 2005), naranjas (Lo Piero *et al*., 2005) y uva de mesa (Sanchez-Ballesta *et al*., 2007). Diferentes estudios han mostrado que el tratamiento con altos niveles de CO_2 aplicado como CA o MAP inhibe el incremento en el contenido de antocianos, al afectar a su síntesis, degradación o a ambas (Gil *et al*., 1997; Holcroft *et al*., 1998; Remón *et al*., 2004). Sin embargo, Veazie & Collins (2002) mostraron un incremento en el contenido total de antocianos monoméricos de moras almacenadas a 2ºC en CA durante los 3 primeros días, disminuyendo posteriormente. Nuestro grupo observó que la eficacia del tratamiento gaseoso controlando la podredumbre en uva de mesa no estaba mediado por el incremento en los niveles del mensajero de la *STS* ni el

contenido de *trans*-resveratrol en la piel, que sí incrementaron en los frutos no tratados (Sanchez-Ballesta *et al*., 2007). Por otro lado, Wang *et al*. (2007) observaron un aumento en el contenido de resveratrol en fresas que crecieron en atmósferas enriquecidas en CO_2 en comparación con las que crecieron en aire.

3.4.- Efecto en los niveles hormonales.

El papel de las hormonas vegetales en la maduración y senescencia de los frutos, hace que sean un factor relevante a tener en cuenta durante su conservación postcosecha, ya que pueden producir cambios que afecten a la calidad de los productos. Por ello, el efecto de las tecnologías de conservación sobre la síntesis y acción del etileno y del ABA, entre otras hormonas, han sido centro de numerosas investigaciones bioquímicas y moleculares.

3.4.1.- Efecto sobre el etileno.

El etileno, como simple fitohormona gaseosa, tiene numerosas funciones en los procesos de desarrollo de la planta y en la reacción de ésta frente a factores ambientales (Zhao & Guo, 2011). Se ha descrito ampliamente su función en la modulación de la maduración de frutos climatéricos y en la regulación de su calidad (Pesis *et al*., 2002; Pech *et al*., 2008; Villalobos-Acuña *et al*., 2010). Sin embargo, nuevas investigaciones apuntan a que algunos aspectos de la maduración de frutos no climatéricos también podrían ser regulados por etileno. Así, el análisis de la biosíntesis de etileno durante el desarrollo reproductivo de naranjas ha mostrado una producción autocatalítica de esta hormona en frutos inmaduros (Katz *et al*., 2004). En el caso de la uva, como ya se ha indicado anteriormente, el contenido de etileno y la expresión de genes claves en su biosíntesis y regulación aumentan justo antes del envero (Chervin *et al*., 2004, 2008; Sun *et al*., 2010; Balic *et al*., 2012; Muñoz-Robredo *et al*., 2013). De hecho, se ha sugerido su participación en los atributos de calidad del fruto en esa etapa (Chervin *et al*., 2004; Amiri *et al*., 2009; Sun *et al*., 2010). Además, también se ha observado que el tratamiento con 1-MCP, inhibía la maduración de las bayas (Chervin *et al*., 2004).

Además de su papel a nivel fisiológico en diferentes estados del desarrollo vegetal, el etileno es una hormona cuya síntesis está claramente regulada en respuesta a

estímulos ambientales de estreses bióticos, como el ataque por patógenos, o abióticos, como lesiones, hipoxia, ozono o bajas temperaturas, entre otros (Wang *et al*., 2002). El hecho de que algunas de estas respuestas puedan ser inducidas por la aplicación exógena de etileno, sugiere que esta hormona puede actuar como una señal que coordina las distintas respuestas de las plantas ante situaciones adversas (Wang *et al*., 1990). Se ha descrito que las bajas temperaturas estimulan cambios tanto en la respuesta al etileno como en su biosíntesis (Knee, 1993; Gerasopoulos & Richardson, 1997).

En plantas superiores, el etileno se sintetiza a partir de la metionina via S-adenosil metionina (SAM) y el ácido1-aminociclopropano-1-carboxílico (ACC). El ACC se forma a partir de SAM por la acción de la enzima ACC sintasa (ACS), y su conversión a etileno la lleva a cabo la enzima ACC oxidasa (ACO) (Kende, 1993). Se ha descrito que la producción de etileno inducido por distintos estreses está controlada, normalmente, por una aceleración en la conversión de SAM a ACC, lo que sugiere que la expresión de la ACS es una diana importante en la regulación de dicho proceso, y en consecuencia, la ACO (Johnson & Ecker, 1998; Wang *et al*., 2002). En peras Bartlett conservadas en frío se detectó un aumento en la producción de etileno y de las actividades enzimáticas ACS y ACO, así como una inducción moderada de la expresión de *ACO*, *ACS1*, *ACS4* y *ACS5*. Sin embargo, no existía correlación significativa entre sus niveles de expresión y su actividad enzimática (Villalobos-Acuña *et al*., 2010). Dentro de los frutos no climatéricos también se han realizado diferentes aproximaciones. Así, en muestras de piel de uvas sometidas a deshidratación se observó una regulación positiva de los transcritos de *ACO*, sugiriendo que el etileno podría estar implicado en las respuestas de este fruto al estrés hídrico postcosecha (Rizzini *et al*., 2009). En el mismo sentido, Grimplet *et al*. (2007) observaron previamente en vides sometidas a déficit de agua un incremento significativo en la acumulación de los transcritos de una *ACO* en la piel de las bayas. Por otro lado, en un estudio reciente con otro fruto no climatérico como es el calabacín, se demostró que *ACS1* y *ACO1* participan en la biosíntesis de etileno durante el almacenamiento a 4ºC (Megías *et al*., 2014). Sin embargo, no hay trabajos sobre la regulación de la biosíntesis de etileno durante el almacenamiento postcosecha a bajas temperaturas en uva de mesa.

El CO_2 puede actuar tanto como inductor o supresor de la biosíntesis de etileno, dependiendo del producto, del tejido, de la concentración de CO_2 y del tiempo de exposición (Mathooko, 1996). Por un lado, se sabe que el CO_2 es un cofactor esencial

de la ACO (Dong *et al*., 1992; Escribano *et al*., 1996), y se ha observado que induce la producción de etileno mediante la activación de la síntesis y la actividad ACS (revisado por Mathooko, 1996). Por otro lado, concentraciones elevadas de CO_2 pueden reducir la biosíntesis de etileno principalmente mediante la inhibición de la expresión génica de *ACS*, que afecta a la acción de ACO (de Wild *et al*., 2003). Así, en fresas, el tratamiento con 20 kPa de CO_2 durante 2 días produjo una disminución en la expresión de tres receptores del etileno (Ponce-Valadez *et al*., 2009). Sin embargo, Becatti *et al*. (2010) observaron que altos niveles de CO_2 aplicados a uvas de vino durante 3 días a 20ºC inducían la acumulación de los transcritos de *ACO* y *ACS* en piel y pulpa. Por lo tanto, la diferente regulación de la biosíntesis de etileno en los distintos frutos tras su exposición a altas concentraciones de CO_2 refleja diferencias en sus estructuras génicas y elementos de regulación.

3.4.2.- Efecto sobre el ácido abscísico.

La acumulación de ABA juega un papel clave en la maduración y senescencia de los frutos (Galpaz *et al*., 2008; Finkelstein, 2013; Zhang *et al*., 2009b). En frutos climatéricos, se ha encontrado que el contenido máximo de ABA precede a la producción de etileno (Zhang *et al*., 2009a,b), y en frutos no climatéricos, se detectó el pico en la producción de esta hormona al comienzo de la maduración (Wheeler *et al*., 2009; Jia *et al*., 2011; Zifkin *et al*., 2012; Karppinen *et al*., 2013). Además, se ha observado que hay una correlación entre el incremento en ABA, la acumulación de azúcares y la reducción de ácidos (Manning, 1994; Jiang & Joyce, 2003; Lijavetzky *et al*., 2012), así como con la producción de diferentes pigmentos en los frutos (Kato *et al*., 2006; Rodrigo *et al*., 2006; Lund *et al*., 2008; Koyama *et al*., 2010; Jia *et al*., 2011). Por otro lado, también se ha visto que existe una interacción funcional entre el ABA y el etileno durante la maduración tanto de frutos climatéricos como no climatéricos. Así, Zhang *et al*. (2009b) observaron que, en tomate, la expresión de un gen biosintético del ABA (*LeNCED1*) incrementaba antes que la de los genes de la biosíntesis del etileno, y que también el ABA podía inducir la síntesis de etileno mediante la regulación de la expresión de *ACS* y *ACO*. En cambio, en el caso de un fruto no climatérico como la uva, se ha sugerido que el etileno podría estar implicado en la inducción de la biosíntesis del ABA (Deluc *et al*., 2009). De hecho, Sun *et al*. (2010) propusieron en *Vitis* que la

interconexión entre las dos hormonas podía ser necesaria para iniciar el proceso de maduración de la baya, ya que las trazas de etileno endógeno inducen la transcripción de *VvNCED1*, teniendo después lugar la acumulación de ABA.

Aunque la regulación de los genes implicados en su síntesis podría variar no solo entre las diferentes partes de la planta, estados de desarrollo y especies de planta (Xiong & Zhu, 2003; Finkelstein *et al*., 2013), es ampliamente aceptado que la enzima NCED, 9-*cis*-epoxicarotenoide dioxigenasa, es clave en la ruta de síntesis de esta fitohormona (Iuchi *et al*., 2001; Nambara & Marion-Pool, 2005; Wheeler *et al*., 2009), al catalizar la ruptura del doble enlace de la 9-*cis* neoxantina y/o -violaxantina a xantoxina, precursor directo del ABA (Cutler & Krochko, 1999). De hecho, se considera que la inducción de *NCED* es un primer paso comprometido en la regulación de la biosíntesis del ABA inducida por estrés (Tan *et al*., 2003; Frey *et al*., 2012). En uva, cereza, fresa o manzana, se ha descrito que la inducción en la expresión de *NCED* en respuesta a condiciones de estrés (Zhang *et al*., 2009a; Ren *et al*., 2010; Ji *et al*., 2012; Xia *et al*., 2014). Sun *et al*. (2010) observaron que tras la recolección de la uva, estreses abióticos como la deshidratación, podrían inducir la transcripción de *VvNECD1* y la acumulación de ABA, desencadenándose los procesos de senescencia. En cambio, se ha observado que el tratamiento con ABA de racimos de uva cv. Crimson Seedless mejoraba la calidad del raquis durante su conservación (Cantin *et al*., 2007). Por tanto, el ABA se presenta como un factor a tener en cuenta en los cambios en la calidad de los frutos durante su conservación postcosecha. En diferentes investigaciones se ha observado un incremento en el contenido de ABA (Yoshikawa *et al*., 2007) y en los niveles de los mensajeros de *NCED* en respuesta al estrés por frío (Maul *et al*., 2008; Xia *et al*., 2014). Igualmente, Sevillano *et al*. (2009) revisaron cómo en distintos trabajos se observaba que el ABA se acumulaba en respuesta a las bajas temperaturas, y que este regulador era capaz de inducir la expresión de varios genes *COR* mediante factores de transcripción CBF, elementos cuya expresión fue debida únicamente a las bajas temperaturas. Además, también se ha descrito que el ABA sirve como una señal secundaria que juega algún papel en la transducción de señales por frío mediante mensajeros secundarios, tales como H_2O_2 y Ca^{2+} (revisado por Theocharis *et al*., 2012).

Por otro lado, debido al papel del ABA en los procesos de senescencia, también se ha estudiado los cambios en la síntesis de esta hormona en respuesta a altas concentraciones de CO_2. Así, Caprioli *et al*. (2009) detectaron que la síntesis del ABA

en melocotones maduros estaba afectada por los altos niveles de CO_2, ya que el tratamiento con un 100% durante 48 horas resultaba en una marcada disminución en el contenido de ABA y en una reducción en la 9-*cis* violaxantina. Esta alteración en la síntesis del ABA es coherente con la disminución en la expresión de *NCED* observada por Becatti *et al*. (2010) en piel de uva de vino tratada con altos niveles de CO_2. Los datos de expresión de *NCED* y la hipótesis de una síntesis de ABA reducida son consistentes con los resultados anteriores de nuestro grupo que indican que los niveles altos de CO_2 pueden retrasar la senescencia y extender la vida postcosecha en uva de mesa (Sanchez-Ballesta *et al*., 2006).

3.5.- Efecto sobre las respuestas moleculares.

En los últimos años, se han identificado genes y vías metabólicas implicadas en la percepción y transducción de la señal de las repuestas de las plantas a bajas temperaturas (revisado por Sung *et al*., 2003; revisado por Miura & Furumoto, 2013), utilizando *Arabidopsis thaliana* como planta modelo. Sin embargo, poco se conoce a cerca de los mecanismos moleculares de la respuesta a las bajas temperaturas en plantas de interés agronómico, y aún menos en respuesta a tratamientos postcosecha coadyuvantes con la conservación en frío. El análisis transcriptómico de la respuesta a las bajas temperaturas ha mostrado que las plantas inducen la expresión de genes que forman parte de la superfamilia *COR*, que codifican, entre otras, un conjunto de proteínas hidrofílicas denominadas LEA (Close *et al*., 1997), familia a la que pertenecen las dehidrinas (DHNs), así como proteínas con función crioprotectora y/o anticongelante (AFPs) (Hon *et al*., 1994) y genes relacionados con la defensa de las plantas frente al estrés oxidativo (Sung *et al*., 2003).

Las DHNs, también conocidas como LEA D-11 o LEA de tipo 2 se inducen durante periodos de déficit de agua impuesto por sequía, salinidad, bajas temperaturas o congelación (Close, 1997). Las DHNs presentan características comunes como son una hidrofilicidad extrema, solubilidad a elevadas temperaturas y la presencia de un dominio muy conservado de 15 aminoácidos rico en lisinas (consenso EKKGIMDKIKEKLPG) llamado dominio K, presente en una o más copias y que es el único dominio de repetición conservado que se encuentra en todas las DHNs (Close, 1997). Otros dominios estructurales presentes en la mayoría de las dehidrinas incluyen el segmento

S, rico en serinas, el segmento Y (T/VDEYGNP) que normalmente se encuentra en 1-3 copias en el extremo N-terminal y el segmento Φ, rico en aminoácidos polares y glicina o una combinación de prolina y alanina. Aunque la función específica de las DHNs sigue siendo especulativa, diferentes trabajos apoyan su papel en la protección de macromoléculas o estructuras celulares de las plantas frente al daño inducido por el déficit de agua o la congelación (Close, 1997; Thomashow, 1999). Se ha observado que DHNs inducidas por las bajas temperaturas pueden proteger *in vitro* enzimas lábiles a la congelación como la lactato deshidrogenasa (LDH) (Sanchez-Ballesta *et al*., 2004; Hughes & Graether, 2011) y prevenir el crecimiento de cristales de hielo en una manera similar a las AFPs (Wisniewski *et al*., 1999). Un trabajo reciente con DHN-5 de trigo sugirió que los segmentos K son indispensables para las funciones protectoras de las DHNs (Drira *et al*., 2013). Además, se ha documentado que la región flexible Φ junto con la presencia de los segmentos K son requeridos para asegurar que DHN es lo suficientemente grande para evitar la desnaturalización de las enzimas (Hughes & Graether, 2011).

La sobreexpresión heteróloga de DHNs confiere tolerancia a las bajas temperaturas en plantas (Hara *et al*., 2003; Yin *et al*., 2006). Asimismo, se ha relacionado la expresión de genes que codifican distintas dehidrinas en el flavedo de pomelo con la tolerancia a los daños por frío inducida por tratamientos postcosecha con altas temperaturas (Porat 2002, 2004; Sapitnitskaya *et al*., 2006). Por otro lado, Weiss & Egea Cortines (2009), indicaron que un gen homologo a una dehidrina de tomate podía ser utilizado como un marcador transcripcional del estrés por bajas temperaturas en hojas y tomates maduros. En el caso de *Vitis*, Xiao & Nassuth (2006) observaron que la exposición de plántulas a 4ºC inducía mayoritariamente los transcritos "unspliced" de *DHN1a,* que codifica una dehidrina de tipo YSK_2, en hojas y yemas de *V. riparia* mientras que en *V. vinifera* se observó un incremento en la acumulación tanto de los transcritos "spliced" como "unspliced". Además, en este trabajo se sugiere que la mayor inducción temprana de *DHN1a* en *V. riparia* en comparación con *V. vinifera* podría conferirle mayor tolerancia a la congelación. Igualmente, Yang *et al*. (2012) observaron que junto con la inducción de *DHN1* otro miembro de la familia de las dehidrinas de *Vitis*, *DHN2*, también incrementaba los niveles de los transcritos por las bajas temperaturas en hojas, aunque *DHN1* parecía ser más sensible. Curiosamente, la

inducción fue mayor en *V. vinifera* que en *V. yeshanensis*, lo cual es contrario a los niveles de sensibilidad a la temperatura entre las dos especies.

Distintos genes que codifican DHNs contienen en su promotor el motivo CCGAC, que es la secuencia central del elemento de regulación en *cis* CRT/DRE (Gilmour *et al*., 2004). Este dominio es suficiente para mediar la respuesta de la expresión génica a las bajas temperaturas y la deshidratación (Yamaguchi-Shinizaki & Shinozaki, 1994) e interacciona específicamente con la familia de factores de transcripción denominados CBF/DREB (Stockinger *et al*., 1997) pertenecientes a la familia de factores de transcripción AP2/ERF. La sobreexpresión constitutiva de *CBFs* en *Arabidopsis* activó la expresión de genes que contenían el elemento CRT/DRE, incluyendo proteínas LEA tales como *COR47*, *ERD10* y *COR15a* en condiciones normales de crecimiento y mejoró la tolerancia a la congelación en plantas no aclimatadas (Gilmour *et al*., 2004). Resultados similares han sido obtenidos más recientemente en *Arabidopsis* transformadas con *CBF1* y *CBF4* de *V. riparia* (Siddiqua & Nassuth, 2011). Además, en plántulas de arroz transgénicas que sobreexpresaron CBF1 se observó un incrementó en la expresión de una dehidrina (*OsDhn1*), indicando que *OsDhn1* es un gen diana en la vía CBF/DREB1 (Lee *et al*., 2005). Los componentes CBFs de la vía de respuesta al frío están altamente conservados en las plantas. Normalmente, los *CBFs* muestran una inducción muy rápida a nivel transcripcional después de la exposición a bajas temperaturas. Sin embargo, en hojas de *Capsicum annuum* los transcritos de *CaCBF1A* se acumularon en respuesta a la exposición a 4ºC y se mantuvieron estables al menos durante 4 días (Kim *et al*., 2004). Asimismo, en hojas de *V. riparia* y *V. vinifera* la acumulación de los transcritos de *CBF3* y *CBF4* se observaron después de 1-2 días a bajas temperaturas (Xiao *et al*., 2006, 2008) en contraste con la rápida inducción de *CBF1* y *CBF2* (Xiao *et al*., 2006). Por su parte, Takuhara *et al.* (2011) describieron también que los transcritos de *CBF4* se inducían en hojas, tallos y flores de *V. vinifera* cv. Koshu tras su exposición a 4ºC durante 4 horas. Sin embargo, el papel de los *CBFs* en la respuesta de los frutos a la conservación postcosecha a bajas temperaturas o a tratamientos gaseosos no es del todo conocido. En tomate, Zhao *et al*. (2009a), detectaron una rápida expresión de *LeCBF1* durante la conservación postcosecha a 2ºC, siendo correlacionada positivamente con la tolerancia a frío de los dos cultivares analizados (Lichun y Santiam). Sin embargo, cuando se analizaba el patrón de expresión de *LeCBF1* en períodos más largos de conservación a

bajas temperaturas, éste variaba según el estado de madurez al que fueron recolectados los frutos (Zhao *et al*., 2009b). En el caso de melocotones almacenados a bajas temperaturas, se ha descrito la regulación transcripcional de tres genes que codifican CBFs (*PpCBF1/5/6*), siendo mayor la acumulación de los transcritos cuando los frutos se almacenaban a 0ºC, temperatura que retrasa los daños por frío, en comparación con la temperatura (5ºC) que los induce (Liang *et al*., 2013). Igualmente, Ma *et al*. (2014) han relacionado recientemente la tolerancia a las bajas temperaturas de almacenamiento de frutos de kiwi con la expresión de un gen que codifica un CBF, *AcCBF*.

Estudios realizados *in vitro* con extractos intercelulares de plantas de centeno aclimatadas a las bajas temperaturas permitió detectar la presencia de AFPs con una elevada similitud con proteínas relacionadas con patogénesis (PRs), concretamente con endoquitinasa, β-1,3-glucanasa y taumatina (Hon *et al*., 1995; Yeh *et al*., 2000; Yaish *et al*., 2006). La actividad anticongelante de estas proteínas se tradujo en un retraso del proceso de congelación, aumentando las probabilidades de supervivencia de las plantas durante el invierno (Griffith & Yaish, 2004). En el caso de uva de mesa conservada a bajas temperaturas, nuestro grupo observó una acumulación de los transcritos de una quitinasa y β-1,3-glucanasa de clase I (Romero *et al*., 2006). El estudio de la funcionalidad de estas dos proteínas mediante su expresión heteróloga en *E. coli* mostró su efecto crioprotector sobre la LDH tras varios ciclos de congelación-descongelación, sugiriendo la participación de ambas enzimas en la respuesta de tolerancia de la uva a las bajas temperaturas (Romero *et al*., 2008c; Fernandez-Caballero *et al*., 2009). Hasta el momento hay poca información concerniente al efecto de tratamientos postcosecha en los cambios observados en PRs. En chirimoya Fino de Jete, nuestro grupo ha establecido que la mejora en la tolerancia a las bajas temperaturas inducida por un pretramiento con altos niveles de CO_2 está asociado al incremento en la acumulación de dos isoenzimas quitinasa y una β-1,3-glucanasa (Merodio *et al*., 1998). Asimismo, este pretratamiento gaseoso indujo la acumulación de una β-1,3-glucanasa ácida y una quitinasa básica de bajo peso molecular (Goñi *et al*., 2009), que presentaron una significativa actividad hidrolítica a bajas temperaturas, clasificando a la β-1,3-glucanasa como una enzima adaptada al frío por su comportamiento termodinámico (Goñi *et al*., 2011). Asimismo, ambas proteínas presentaron una potente actividad crioprotectora que las define como endohidrolasas bifuncionales, estando asociadas al mantenimiento de la

estructura celular y formando parte del mecanismo de tolerancia a las bajas temperaturas activado por (Goñi *et al*., 2010, 2011).

Los factores de transcripción ERFs pertenecientes, junto con los CBFs, a la familia AP2/ERF se unen a los elementos *cis* reguladores, denominados caja GCC, presentes en las regiones promotoras de las PRs regulando su expresión en respuesta al etileno y a estreses ambientales como las bajas temperaturas (Fujimoto *et al*., 2000; revisado por Singh *et al*., 2002). En los último años hay un interés particular en esta familia de factores de transcripción por el papel del etileno en la regulación de la maduración y porque los frutos se enfrentan a condiciones ambientales desfavorables durante el desarrollo y la conservación postcosecha. Sin embargo, a diferencia de los componentes aguas arriba de señalización del etileno, los genes *ERFs* han sido estudiados sólo en algunos frutos en relación con la maduración, incluyendo el tomate (Gu *et al*., 2002; Tournier *et al*., 2003), ciruela (El-Sharkawy*et al*., 2009), kiwi (Yin *et al*., 2010), manzana (Tacken *et al*., 2010) y uva (Licausi *et al*., 2010). Sólo algunos trabajos recogen informaciones acerca de su papel en la repuesta a estreses ambientales en frutos. En tomate, se ha demostrado que la mayoría de los *ERFs* (*LeERF*, *Pti* o *JERF*) responden a estreses tales como las bajas temperaturas, herida y salinidad (Chen *et al*., 2008; Sharma *et al*., 2010). La expresión heteróloga de *ERFs* de tomate en *Arabidopsis* o tabaco activó genes de defensa o relacionados con el estrés e incrementó la tolerancia de las plantas (Wang *et al*., 2004; Zhang *et al*., 2004a). Sin embargo, ninguno de estos trabajos se realizó directamente en los frutos llevándose a cabo en tejidos vegetativos, tales como hojas, tallos o raíces. Un trabajo reciente en pomelo sugiere que *ERF2* podría estar implicado en la cascada de eventos inducidos por las bajas temperaturas y que está regulado negativamente por etileno, ya que el 1-MCP estimula su expresión (Lado *et al*., 2014). En kiwi, se observó la expresión diferencial de 13 *ERFs* en respuesta a distintas condiciones postcosecha como las bajas y altas temperaturas, altos niveles de CO_2 y elevadas pérdidas de agua (Yin *et al*., 2012). En uva de mesa, si bien Balic *et al*. (2012) detectaron, en un estudio molecular y fisiológico del pardeamiento postcosecha del raquis de uva de mesa cv. Red Globe, una disminución en la transcripción de un *ERF* cuando los racimos fueron almacenados a 0ºC durante 90 días, poco es conocido sobre su papel en las bayas durante la conservación postcosecha a bajas temperaturas y con altos niveles de CO_2.

La mayor parte de lo que se conoce hasta el momento sobre los mecanismos moleculares asociados a la conservación a bajas temperaturas y altos niveles de CO_2 en uva de mesa deriva de estudios moleculares de cambios de expresión génica llevados a cabo de forma individual con determinados genes. Los estudios moleculares que emplean microarrays permiten evaluar cambios de expresión de grandes conjuntos de genes de forma simultánea en respuesta a estrés o asociados con diversos procesos del desarrollo en numerosos sistemas vegetales, tales como *Arabidopsis*, tomate o arroz, entre otros (Kreps *et al*., 2002; Seki *et al*., 2002; Rabbani *et al*., 2003; Zhang *et al*., 2004b). Asimismo, en los últimos años han empezado a realizarse en frutos aproximaciones al estudio global de los cambios de expresión mediante el uso de microarrays, especialmente, en respuesta a la conservación a bajas temperaturas. Utilizando el microarray de Affymetrix Citrus GeneChip, Maul *et al*. (2008) observaron en el flavedo de pomelos que la exposición a bajas temperaturas, además de conducir a la detención de la expresión de genes implicados en la actividad metabólica celular, como mecanismo de adaptación aumentó los niveles de transcritos de genes relacionados con membranas, con el metabolismo de lípidos, esteroles y carbohidratos, estímulos a estrés, biosíntesis de hormonas y modificaciones de las uniones de DNA y los factores de transcripción. Con esta misma plataforma, Zhu *et al*. (2011) estudiaron los cambios transcripcionales producidos en pulpa de mandarina conservada durante tres meses a bajas temperaturas. El análisis de los resultados obtenidos sugirió que el etileno podría tener un papel vital en la respuesta de cítricos a largos períodos de almacenamiento a bajas temperaturas. Además, se produjo una inducción rápida de PRs, que podría estar relacionada con la aclimatación del fruto a estas condiciones de temperatura, así como alteraciones en varias rutas metabólicas, como el aumento de la acumulación de azúcares solubles.

El efecto de la aplicación de altos niveles de CO_2 ha sido analizada utilizando microarrays en dos frutos no climatéricos como la fresa (Ponce-Valadez *et al*., 2009) y la uva de vino (Becatti *et al*., 2010). Para los análisis en fresa, se utilizó un microarray de cDNA de tomate (TOM1) que contenía 8,700 unigenes para evaluar el efecto de la aplicación de un 20% CO_2 durante 2 días en de dos cultivares de fresa, Jewel que acumula grandes cantidades de etanol y acetaldehído en respuesta a elevadas concentraciones de CO_2, y Cavendish que no acumula estos productos de fermentación. Los autores observaron que el diferente comportamiento de ambos cultivares al

tratamiento gaseoso se relacionaba con diferencias en términos de genes con expresión diferencial, 168 en Jewel y 51 en Cavendish, sugiriendo la presencia de diferentes mecanismos moleculares regulatorios activados en relación con la presencia de CO_2. Entre los cDNAs que mostraron una expresión diferencial entre ambos cultivares, los más representados mostraron homología con genes implicados en la síntesis de proteínas y el metabolismo de ácidos nucleicos. En uva de vino blanca cv. Trebbiano, mantenida durante 3 días con un 30% de CO_2 y después transferida a aire durante 9 días para alcanzar una deshidratación parcial con fines de vinificación, el análisis transcriptómico utilizando un microarray de *Vitis* (Grape AROS V1.0) reveló que en comparación con la pulpa, la piel albergó los cambios más pronunciados en el perfil transcriptómico (Becatti *et al*., 2010). El análisis de enriquecimiento funcional mostró que en la piel las categorías más representadas fueron la fermentación, el metabolismo de carbohidratos y la regulación redox, mientras que las categorías relacionadas con proteínas, estrés, transcripción, RNA y metabolismo de hormonas (etileno y ABA) estuvieron muy representadas tanto en la piel como en la pulpa.

En el caso de *V. vinifera*, la secuenciación del genoma ha facilitado la integración de los diferentes 'ómicas' para investigar los procesos que intervienen en las distintas etapas de desarrollo, y en la calidad del vino, así como la respuesta a estreses bióticos y abióticos. Su genoma relativamente pequeño, así como su importancia económica, ha atraído la atención de los investigadores en los últimos años. El Consorcio Público Italo-Francés para la Caracterización del Genoma de la Uva secuenció la variedad Pinot Noir PN40024 con un tamaño de genoma de 467,5 Mb conteniendo 30,434 genes, 149,351 exones y 118,917 intrones (Jaillon *et al*., 2007). El consorcio internacional, Grapevine Genome Initiative, publicó la secuenciación del genoma del clon Pinot Noir ENTAV 115.5 y, en este caso, predicen un tamaño de genoma de 504,6 Mb y un total de 29,585 genes (Velasco *et al*., 2007). La conclusión de estos datos genómicos ha permitido determinar que *Vitis vinifera* posee un genoma de entre 475 y 500 Mb, detectar genes ligados a propiedades físico-químicas, organolépticas o nutricionales, y deducir que el genoma actual de la uva es un genoma ancestral paleohexaploide. Es una planta cuyos ancestros lejanos aparecieron gracias a la triplicación del genoma de una especie anterior (Jaillon *et al*., 2007).

Diferentes tejidos de *Vitis* como hojas (Ablett *et al*., 2000; Kobayashi *et al*., 2009), bayas enteras (Ablett *et al*., 2000), piel (Kobayashi *et al*., 2009; Ali *et al*., 2011;

Lijavetzky *et al*., 2012) y pulpa (Lijavetzky *et al*., 2012), así como diferentes estados de crecimiento de las bayas durante el desarrollo (Terrier *et al*., 2005; Fortes *et al*., 2011) y la maduración (Davies & Robinson 2000; Kobayashi *et al*., 2009; Guillaumie *et al*., 2011), han sido utilizados para realizar estudios transcriptómicos, utilizando microarrays. Asimismo, se han analizado las respuestas transcripcionales de *V. vinifera* a distintos estreses abióticos como déficit de agua (Castellarin *et al*., 2007; Cramer *et al*., 2007), salinidad (Cramer *et al*., 2007; Tattersall *et al*., 2007), estrés osmótico (Tattersall *et al*., 2007), altas temperaturas (Liu *et al*., 2012; Carbonell-Bejerano *et al*., 2013), frío (Tattersall *et al*., 2007) y radiación UV-B (Pontin *et al*., 2010).

La presente revisión bibliográfica pone de manifiesto que hasta el momento en uva de mesa no se dispone de información global de los mecanismos moleculares implicados en la respuesta de los frutos a las bajas temperaturas, y que puedan explicar el efecto beneficioso de la aplicación de altos niveles de CO_2 que mantiene la calidad de los mismos. Por ello en este trabajo, se abordará un estudio transcriptómico que permita entender la respuesta de la uva de mesa al tratamiento gaseoso en dos estados de madurez diferentes.

OBJETIVOS

Esta Tesis Doctoral se plantea como una solución científico-tecnológica a la pérdida de calidad de uva de mesa que tiene lugar durante la conservación a 0ºC y al conocimiento *sub iúdice* de la tolerancia de estos frutos a elevadas concentraciones de CO_2. Por ello, el objetivo general de este trabajo ha sido el análisis integral de las respuestas metabólicas y de los mecanismos moleculares inducidos por cortos tratamientos de CO_2 que permiten superar la fase crítica de conservación a 0ºC (3 días), de forma que se eviten las posteriores manifestaciones de pérdida de calidad en uva de mesa cv. Cardinal.

Este objetivo general se ha desarrollado según los siguientes objetivos parciales:

1. Análisis e identificación de marcadores metabólicos determinantes de la fase crítica de conservación a 0ºC y de la efectividad del tratamiento con altos niveles de CO_2, en función del tipo de tejido y del grado de madurez del fruto.

2. Caracterización y regulación de los mecanismos moleculares implicados en la tolerancia a las bajas temperaturas en los diferentes tejidos del racimo tratados con altas concentraciones de CO_2.

3. Análisis transcriptómico de los mecanismos de adaptación a elevadas concentraciones de CO_2 en uva de mesa en dos estados de madurez diferente durante la fase crítica de conservación a 0ºC.

RESULTADOS

CAPÍTULO 1

Water status and quality improvement in high-CO_2 treated table grapes

Oscar Goñi[1], Carlos Fernandez-Caballero[1], María T. Sanchez-Ballesta, María I. Escribano and Carmen Merodio*

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[1] These authors contributed equally to this work

* Corresponding author

ABSTRACT

Unfreezable water (UFW) content in berry tissues (pulp, skin, seed) and rachis of table grape clusters stored at 0 ºC have been studied using differential scanning calorimetry (DSC). The effect of short exposure to high CO_2 (20% CO_2 for 3 days) and the transfer to air were also studied. Water status of pulp tissues was related to the thawing behaviour and the structural characteristics, using low-temperature scanning electron microscopy (LT-SEM). The UFW content in all tissues increased rapidly in response to high CO_2 while it remained stable or decreased in untreated clusters. The strong potential of this beneficial gaseous treatment for increasing the UFW content was also evident after transfer to air. The metabolic adjustment caused by exposure to high CO_2 which reduced the amount of freezable water content available to be frozen, improved stored fruit quality, thus minimizing structural damage and reducing water leakage associated with freezing-thawing process.

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1. Introduction

The quality of table grapes (*Vitis vinifera* L.*)* is affected by their high sensitivity to water loss and fungal attack. Low temperatures, close to 0 ºC and 90-95% relative humidity is a technology used to extend table grape postharvest life (Ginsburg, Combrink & Trute, 1978) but even under these conditions there is a detrimental effect on the quality and appearance of the bunches by storage at this low temperature. Previous studies have indicated that Cardinal table grapes are sensitive when the temperature is lowered to 0 ºC (Romero, Sanchez-Ballesta, Escribano & Merodio, 2008). Owing to the fact that severe low temperatures can cause structural damage and fruit quality loss, many efforts have been made to develop effective non-damaging gaseous treatments that could maintain the quality of table grapes (Guillen, Zapata, Martinez-Romero, Castillo, Serrano & Valero, 2007; Thompson, 2001).

Water is a very important component in fruit affecting quality, adaptation to environmental storage, shelf-life and processing (Alferez, Alquezar, Burns & Zacarias, 2010; Hills & Remigereau, 1997; Moraga, Martinez-Navarrete & Chiralt, 2006). Thus, moisture content has been a parameter widely determined in fruits. However, water in biological materials exists in different states that have strong impact on different processes (Ruan & Chen, 1998). Therefore it is of great interest to quantify the amount of free or freezable water and of the unfreezable water fractions in table grapes that exhibit a large quantity of water (Glidewell, Williamson, Goodman, Chudek, & Hunter, 1997). Our working hypothesis is that variations in water properties could affect table grape quality associated with cellular structural damage and deterioration during storage at severe low temperatures. There are several traditional methods for determining the water status of fresh produce. An approach to the study of water characterization in plant tissues is the application of Magnetic Resonance Imaging (MRI) (Clark, Hockings, Joyce, & Mazzuco, 1997) or using differential scanning calorimetry (DSC) (Biliaderis, 1983). We previously reported the suitability of DSC to monitor the changes in water status in fruits (Goñi, Muñoz, Ruiz-Cabello, Escribano, & Merodio, 2007) and we found a good correlation between a drop in the T_1 values of whole fruit, as measured by MRI, and the increase in the UFW content determined by DSC. Moreover, it has been reported that T_1 is a better parameter for describing plant water status than the traditional water relation indices (Nagarajan, Chahal, Gambhir & Tiwari, 1993).

With respect to structural dysfunctions caused by non-freezing and freezing temperatures most of the studies have been focused on rigidification of membrane, loss of membrane integrity and changes in cell wall properties (Kratsch & Wise, 2000; Yamada, Kuroda, Jitsuyama, Takezawa, Arakawa & Fujikawa, 2002). Bauchot, Hallett, Redgwell & Lallu (1999) noted modifications of cell wall composition and properties associated with storage at low temperature in fruits. Rajashekar & Lafta (1996) reported the impact of both the strength and pore size of cell-wall on freezing behavior of leaves and cell cultures of grapes and apples.

The aim of this work is to determine whether the freezable and unfreezable water content of pulp, skin, seed and rachis of table grape clusters undergo modifications during cold storage and how protective short-term high CO_2 treatment such as 20% CO_2 for 3 days modify fruit water status, using DSC methodology. Changes in water status in pulp tissues were related to water loss after thawing and structural modifications, using low-temperature scanning electron microscopy (LT-SEM) which allows for the direct observation of frozen tissues.

2. Materials and methods

2.1. Plant material

Table grapes (*Vitis vinifera* L. cv. Cardinal) were harvested from orchards in Camas (Sevilla, Spain) when they have reached the commercial maturity stage in June. Selected clusters were forced-air precooled at -1 ºC shortly afterwards and then randomly divided into two lots and stored in two sealed neoprene containers of $1m^3$ capacity at 0 ± 0.5 ºC and 95% relative humidity. One lot was stored in air (untreated fruit) and the other under a gas mixture containing 20% CO_2 + 20% O_2 + 60% N_2 (CO_2-treated fruit) for 3 days. After 3 days, CO_2-treated clusters were transferred to air under the same conditions as the untreated fruit until the end of the 22-day storage period. Five clusters were collected randomly before storage (freshly harvested fruit) and for every subsequent sampling period during storage at 0 ºC (3, 15 and 22 days). The rachis obtained from five clusters were frozen in liquid nitrogen and stored at -80 ºC. From these five clusters, 45 berries were removed at random, peeled and the skin, pulp and seeds were frozen in liquid nitrogen and stored at -80 ºC. Another 15 whole berries were

frozen and packed in polyethylene bags, sealed and stored at -80 ºC until frozen quality analysis.

2.2. DSC measurements

A differential scanning calorimeter (DSC822e, Mettler-Toledo Inc., USA) equipped with a liquid nitrogen cooling accessory was used to study water fusion. Indium, water deionized and zinc were used for calibrarion. Frozen pulverized tissues (10-20 mg) from the clusters of table grapes (skin, pulp, seed, and rachis) were placed in 100 µL Mettler-Toledo aluminium pans, and they were hermetically sealed and then cooled from 25 ºC to -80 ºC at 10 ºC min^{-1}. The tissues were left at this temperature for 5 min and then heated to 25 ºC at 10 ºC min^{-1}. A method based on the heat of fusion was used to calculate the amount of UFW (Goñi et al., 2007). Samples for DSC analysis were taken from different areas of the bunch in order to obtain a representative population, and at least five measurements were made on each sampling day. The ice-melting enthalpy of deionized water was measured three times and its value 322.85 ± 1.14 J/g was used in the UFW determination. The total water content (g/100 g fresh weight) was determined after a stable weight had been obtained after drying at 105 ºC.

2.3. Measurement of drip and thawing losses

Frozen pulp tissues of table grapes were stored at -20 ºC for 4 and 9 days and thereafter the tissues were thawed at 8 ºC for 1 hour before measurement of drip loss. Drip loss was determined by a method adapted from Lowithun & Charoenrein (2009). The amount of water released from the sample was measured directly weighing the filter paper.

2.4. Analysis of microstructure

Microscopic observations were carried out using a cold stage Cryotrans CT 1500 linked to a Zeiss DSN-960 electron scanning microscopy (Oxford Instruments). Frozen samples were cryofractured at -180 ºC and then placed in the microscope stage for

etching at -90 ºC, for 2 min. They were then gold-coated and subsequently transferred to the microscope, where observations were carried out at 5-10 kV and -150 to -160 ºC.

2.5. Statistical Analyses

Data from at least three replicates per sampling period were subjected to an analysis of variance (ANOVA) at $p < 0.05$ (Statgraphics program, STSC, Rockville, MD). Two-way analysis of variance was performed using the LSD test procedure with type III sums of squares and a confidence level of 95%. The main effects of CO_2 treatment, time of storage at 0 ºC, and treatment x time interaction on table grape clusters were analysed.

3. Results and discussion

The moisture content in berry tissues (pulp, skin, seed) and also in the rachis of untreated and CO_2-treated table grape clusters is shown in Table 1. Regarding the effect of the different factors analysed (CO_2 treatment, time, and CO_2-time interaction) ANOVA confirmed that the factor CO_2 treatment significantly ($p < 0.05$) affected the moisture content of all tissues of the bunch, except for pulp tissues. With respect to the time factor, this did not significantly affect the moisture content of rachis tissues. However, statistical analysis confirmed that the factor CO_2-time interaction influenced all variables studied. At the end of low temperature storage, although the moisture content in seeds and the skin of CO_2-treated tissues significantly decreased, they remained markedly higher (81.53 ± 0.30 and 48.62 ± 2.46 g/100 g fresh weight) than their respective values in the untreated tissues (78.57 ± 0.79 and 42.04 ± 2.46 g/100 g fresh weight, respectively). Also a higher moisture content was quantified in the rachis of CO_2-treated clusters after 22 days of storage with values similar to those of freshly harvested samples. Several authors (Barth, Kerbel, Broussard & Schmidt, 1993; Serrano, Martinez-Romero, Guillen, Castillo & Valero, 2006) reported a higher moisture retention in broccoli spears stored under elevated CO_2 environments developed during modified atmosphere packaging.

Table 1

Changes in moisture content (g/100 g fresh weight) in the pulp, skin, seed and rachis of untreated and CO_2-treated table grape clusters stored for 22 days at 0 ºC.

	Pulp		Skin		Seed		Rachis	
Days	Untreated	CO_2-treated	Untreated	CO_2-treated	Untreated	CO_2-treated	Untreated	CO_2-treated
	91.18±0.30	91.18±0.30	83.12±0.43	83.12±0.43	54.56±0.66	54.56±0.66	76.86±1.01	76.86±1.01
	91.10±0.31	89.96±0.05	81.83±0.53	82.21±0.34	54.20±1.20	57.00±0.91	72.67±1.57	76.71±1.12
	90.21±0.45	90.54±0.04	78.37±1.10	83.07±0.44	42.71±0.60	49.72±1.74	72.20±0.60	77.80±2.29
	89.95±0.76	90.67±0.19	78.57±0.79	81.53±0.30	42.04±0.42	48.62±0.54	73.12±2.12	77.28±1.22
	D*, DxT*		D*, T*, DxT*		D*, T*, DxT*		T*, DxT*	

Values are the mean of at least three replicate samples ± SE.

[Y] Significant at $p \leq 0.05$ and LSD test, where D = days and T = CO_2-treatment.

In Fig. 1, we can see the absolute values (g/g dry weight) of freezable and unfreezable water content in pulp tissues from untreated and CO_2-treated clusters of Cardinal table grape, which includes shaded areas showing the percentage of unfrozen water relative to total water content. Freshly harvested pulp tissue had the highest UFW content (3.45 ± 0.03 g/g dry weight) when compared with the other tissues (Fig. 2). Despite the high absolute levels of UFW in pulp tissues, the percentage of UFW with respect to the total water content was 33.3% (Fig. 1). The UFW content in pulp tissues of untreated grapes remained stable during the storage period, and the variations in the percentage of UFW seems to be associated with the decrease in the amount of total water content quantified in this tissue after 15 days of storage at 0 ºC. On the contrary, the UFW content in the pulp of CO_2-treated clusters sharply increased at the end of treatment (3 days). The strong potential for increasing the UFW content was also evident after transfer to air, with a value of 5.61 ± 0.13 g/g dry weight and with only slight changes in total water content. After 22 of storage, although a significant decrease in UFW content was quantified, the percentage was much higher (52.1%) than the 42.9% found in untreated grapes. Thus, our results indicate that in CO_2-treated grapes stored at 0 ºC the unfreezable water content increased rapidly, while it remained constant in untreated samples.

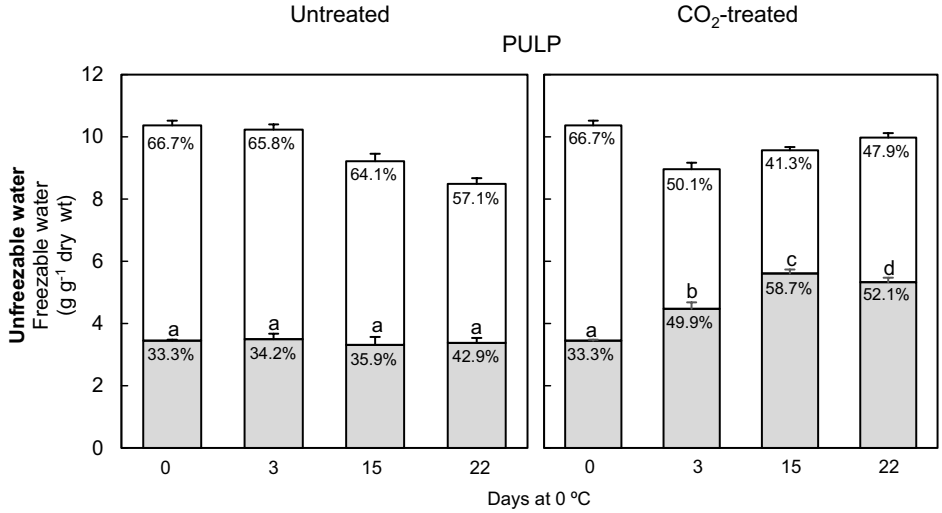

Fig. 1. Changes in the content of unfreezable and freezable water fractions (g/g dry weight) in the pulp of berries from untreated and CO_2-treated clusters of Cardinal table grapes stored at 0 ºC for 22 days. Dark bars indicate unfreezable and white bars freezable water content respectively. The percentage of each water fraction with regard to total water content is also shown. Error bar ± S.E.

In the skin (Fig. 2A) , the storage at 0 ºC for 3 days caused a significant decrease in the UFW content from 1.52 ± 0.10 to 1.12 ± 0.10 g/g dry weight, associated with a significant decrease in total water content. At this time, the percentage of freezable water content increased from 69.1 to 75.1%. Bendel, Zemah, Kamenetsky, Vergeldt & van As (2001) using parameter sensitive magnetic resonance imaging experiments, reported that cold storage processes were accompanied by conversion of bound water to free water. After 15 days of storage, an unchanging UFW value (1.12 ± 0.05 g/g dry weight) was quantified and its percentage increased associated with the decrease in total water content. In the skin of CO_2-treated clusters the initial decrease in UFW content was less pronounced than in untreated tissues, from 1.52 ± 0.1 to 1.34 ± 0.05 g/g dry weight and increased to 1.86 ± 0.07 g/g dry weight after transfer to air by day 15, without much change in total water content. After 22 days of storage, the UFW fraction significantly decreased to 1.71 ± 0.08 g/g dry weight, although it remained significantly higher than it respective value in untreated tissue (1.21 ± 0.05 g/g dry weight). Moreover, at this time the decrease in the total water content in the skin of CO_2-treated grapes was significantly lower than in the untreated grapes. Considering that in previous work we reported that by lowering the storage temperature to 0 ºC, the stress responses in the skin of table grapes were prevented by high CO_2 treatment (Romero et al., 2008),

we suggest that the observed water status change in CO_2-treated skin tissue is a result of the protective mechanism induced by this treatment.

In seed tissues (Fig. 2B), that exhibited the highest proportion of UFW among the berry tissues, a marked drop in UFW content was recorded after 3 days at 0ºC from 0.58 ± 0.04 to 0.37 ± 0.08 g/g dry weight but without change in total water content. After 15 days of storage, although the UFW content did not change, the sharp decrease in the total water content resulted in a significant increase in the percentage of UFW from 31.5 to 48.5%. The UFW content in the seeds of CO_2-treated grapes remained stable during the 15 days of storage although the transitorial changes in total water content caused the variation in the percentage of UFW. Even in seed tissues in which a sharp decrease in UFW content was recorded by day 22 of storage, the amount was higher than in untreated tissues.

In terms of the water status in the rachis (Fig. 2C), the high UFW content in CO_2-treated tissues was associated with a high water content that did not fall with respect to the values in freshly harvested rachis. In the rachis of CO_2-treated clusters the increase in the amount of UFW was evident at the end of treatment and the transfer to air caused a sharp rise in this content from 0.94 ± 0.06 to 1.42 ± 0.01 g/100 g fresh weight), remaining constant thereafter. We previously reported the improved appearance and modified relative water content in the rachis of CO_2-treated clusters (Sanchez-Ballesta et al., 2006). According to our results, it is possible that the beneficial effects of high CO_2 atmosphere storage involved a slowing of senescence-like changes that might be attributed to metabolic adjustment, in a similar way to that proposed by several authors (Agüero, Barg, Yommi, Camelo & Roura, 2008).

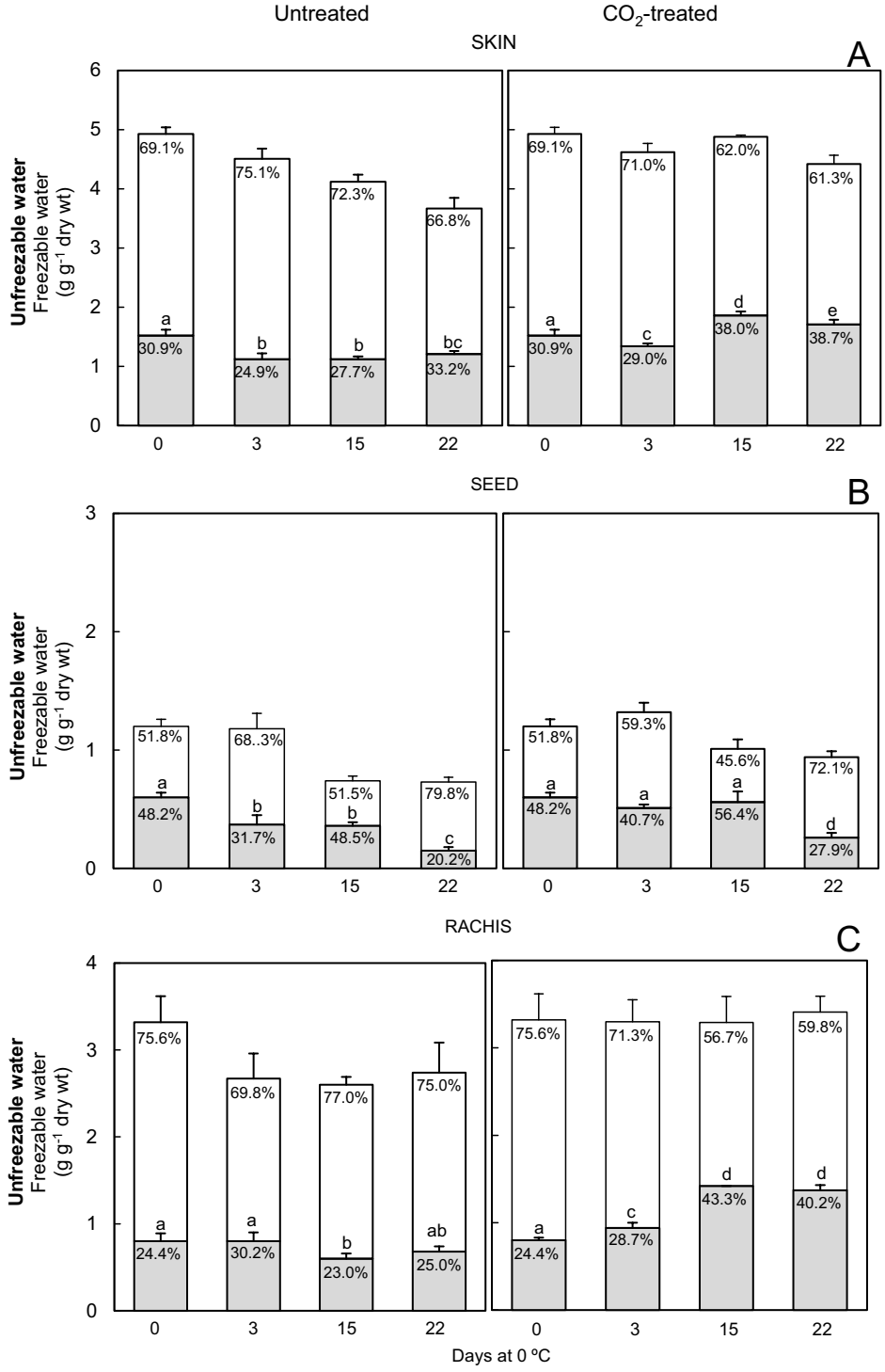

Fig. 2. Changes in the content of unfreezable and freezable water fractions (g/g dry weight) in the skin, seed and rachis from untreated and CO_2-treated clusters of Cardinal table grapes stored at 0 ºC for 22 days. Dark and white bars indicate unfreezable and freezable water content respectively. The percentage of each water fraction with regards to total water content is also shown. Error bar ± S.E.

Taking into account the changes in water status of all tissues, our DSC results indicate that the UFW levels increased rapidly in response to high CO_2 while it remained stable or decreased in the tissues of clusters stored in air. The increase in the UFW content which is associated with membranes, proteins and macromolecules (Wolfe, Bryant & Koster, 2002), might constitute a sensory parameter that reflects metabolic adaptations in CO_2-treated tissues due to the alterations caused by storage in air at severe low temperature. In line with these results, Vertucci & Stushnuff (1992) indicated that cold acclimation of vegetative apple buds involves several processes including an increase in the levels of unfreezable water.

In order to analyse the alterations caused by prolonged storage at low temperature, LT-SEM analysis of ultrastructure of untreated and CO_2-treated berry pulp cells after 22 days at 0 ºC has been carried out (Fig. 3). Compared with untreated grapes (Fig. 3A-1), the morphology of CO_2-treated fruit tissues (Fig. 3B-1) is well defined, in the same way as freshly harvested samples (Fig. 3C-1). The untreated fruit cells exhibited the highest degree of tissue disorganization. A detailed histological characterization revealed that the shape of cells of freshly harvested grapes (Fig. 3A-2) appears well-rounded while a polygonal form is observed in the cells of CO_2-treated fruit (Fig. 3B-2) after 22 days of storage. At this time, the cells of untreated grapes showed a loss of cell integrity (Fig. 3C-2). In cherimoya fruit, LT-SEM micrographs revealed (Maldonado, Molina-García, Sanchez-Ballesta, Escribano & Merodio, 2002) that storage in air at chilling temperature caused severe structural disruption of mesocarp cells and alterations in the strength of cell adhesion compared to high CO_2 atmosphere storage. Rajashekar & Lafta (1996) reported that cell tensions increased in response to cold acclimation in leaves of broadleaf evergreen species during extracellular freezing. On the contrary, decreases in peak tensions were generally associated with lethal freezing injury. It has also been reported that not only the plasma membrane but also the cell wall greatly influences the freezing behavior of plant cells (Yamada et al., 2002).

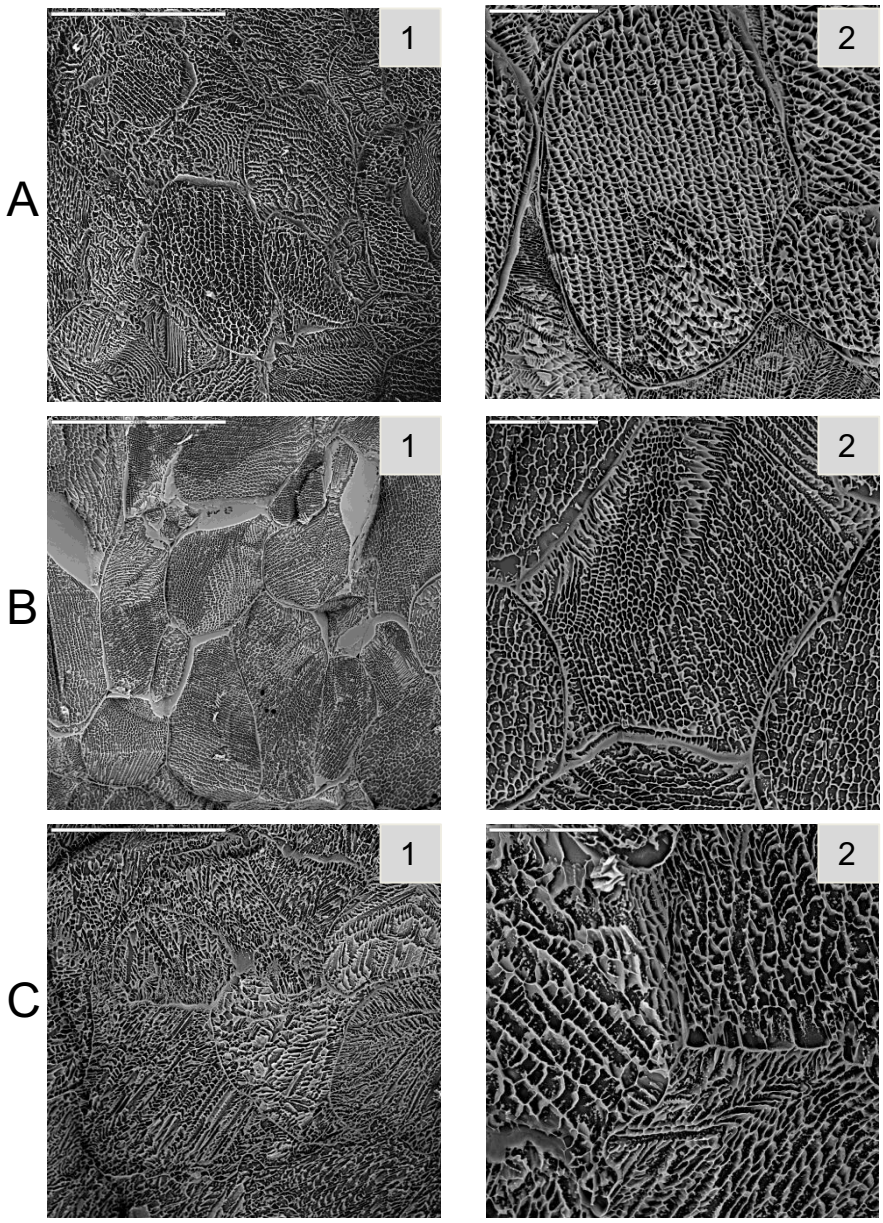

Fig. 3. LT-SEM micrographs showing both general (1) and detailed (2) views of cell tissues of fractured pulp tissue from freshly harvested (A), CO_2-treated (B) and untreated (C) table grapes stored for 22 days at 0 ºC.

In the present work we also try to determine whether the lower levels of freezable water content in CO_2-treated grapes might affect the thawing behaviour of fruit tissue. The water-holding capacity of pulp tissues after thawing was determined analyzing the drip loss of the pulp of CO_2-treated and untreated samples (Fig. 4). It is interesting to note that drip loss was higher in the frozen pulp tissues of untreated grapes

than in the CO_2-treated ones. Moreover, although the longer storage time increased the drip loss value of all samples, the increase rate was much pronounced in the untreated samples. After 4 days of frozen storage, the drip loss of thawed frozen untreated samples was significantly higher than that of the others and it had the highest loss of water. By contrast, the CO_2-treated sample had the lowest value and did not change with respect to freshly harvested frozen samples. With a frozen storage time of 9 days, the drip loss of the CO_2-treated samples was also the lowest. The better thawing behaviour of the CO_2-treated tissues resulted in a lower quantity of water being released from the sample, and this higher water-holding capacity could play an important role in determining the juiciness and overall acceptance of the product (Torreggiani & Maestrelli, 2006).

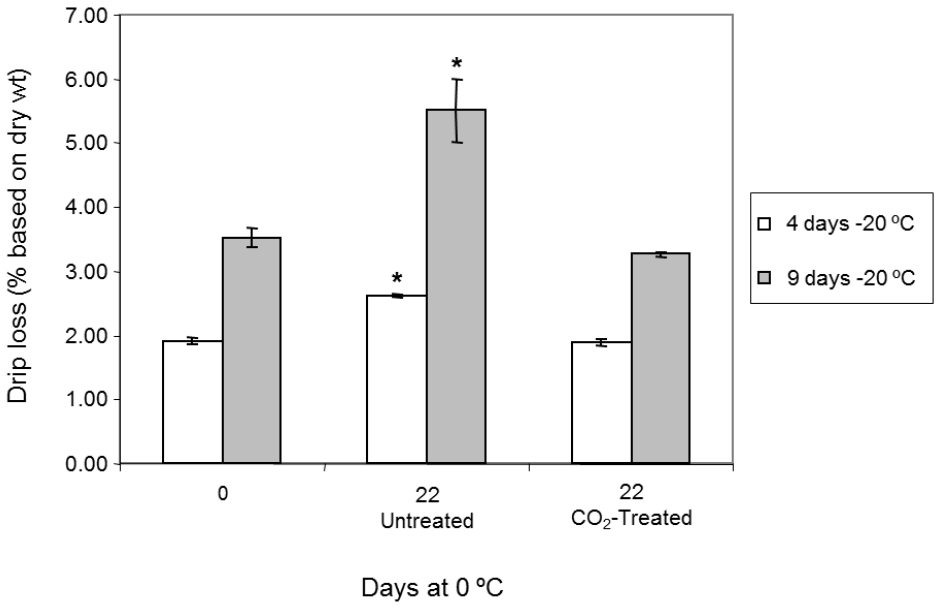

Fig. 4. The effect of the frozen period of 4 and 8 days at -20 ºC on drip loss was assessed in thawed pulp from freshly harvested grapes or grapes stored for 22 days at 0 ºC, with and without CO_2 treatment. The mean values within the same period of frozen storage at -20 ºC indicated with an asterisk (*) were significantly different ($p \leq 0.05$).

In conclusion, this study revealed that short high CO_2 treatment (20% CO_2 for 3 days) has a direct effect on fruit tissue water status, increasing the fraction of UFW, or preventing its decrease that could determine the level of protection against cellular damage caused by low temperatures. These results provide the background for further

development of efficient technologies for increasing the steady-state levels of UFW in fruit tissues to facilitate processing and freshness preservation. Thus further approaches are needed to elucidate the metabolic adjustments caused by high CO_2 treatment that might involve the synthesis of hydrophilic compounds playing a primary role in facilitating water retention, thereby improving the quality of chilled and frozen fruits.

Acknowledgements

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CAPÍTULO 2

Accumulation and distribution of potassium and its association with water balance in the skin of Cardinal table grapes during storage

Maria Blanch, Carlos Fernandez-Caballero, María T. Sanchez-Ballesta, María I. Escribano, Carmen Merodio*

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* Corresponding author

ABSTRACT

Although potassium participates in distinct mechanisms that influence grape growth and development, including osmoregulation, little is known about the association between water and potassium in grape during storage at low temperature. We analyzed the relationship between potassium and the bound water fraction in the skin of early-harvested Cardinal table grapes (*Vitis vinifera* L.) from two different harvest years, both of which were stored at 0 ºC for 3 days in air (20% O_2 + 0.03% CO_2) or in air + CO_2 (20% O_2 + 20% CO_2). The relative K^+ content and distribution in the skin cells was determined by energy dispersive X-ray microanalysis, revealing a non-uniform accumulation of K^+ in grape skin cells. Storage at 0 ºC in air causes a significant decrease in bound water levels and greater soluble-water K^+ accumulation, irrespective of the harvest year. Furthermore, low temperature-scanning electron microscopy images revealed that the epidermal and the first hypodermal layers of the cells were compressed in the skin of fruit stored in air. However, when exposed to air plus 20% CO_2, there was no decrease in the bound water content or in the associated K^+ accumulation, nor were the outer skin cells compressed.

1. Introduction

Table grapes are very sensitive to fungal decay and water loss, both causing substantial postharvest losses. Susceptibility to disease and water loss is particularly dependent on the cuticular barrier and the condition of the underlying epidermal cells in the skin (Boyer et al., 1997; Comménil et al., 1997). The central vacuole of grape berry cells plays an important role in maintaining their volume, and in controlling the gradients of vacuolar ion concentrations that are essential for acid and sugar balance in the berry. Potassium is perhaps the most important ion in grapes, playing an important role in controlling the vacuolar ion concentration.

The post-harvest quality of table grapes can be enhanced by short-term high CO_2 treatments during low temperature storage (Retamales et al., 2003; Sanchez-Ballesta et al., 2006). The effectiveness of high CO_2 treatment is influenced by the stage of ripeness (Romero et al., 2009) and it also varies according to the temperature at which the commodity can be stored without producing damage (Ahumada et al., 1996; Prange and Lidster, 1992). In grapes, sugar accumulation in the flesh and anthocyanin accumulation in the skin have traditionally been studied to discriminate the harvest maturity. Berry development and ripening also affect the concentration of potassium and its transport by channels (Pratelli et al., 2002), transporters (Davies et al., 2006) and cation/proton antiporters (Hanana et al., 2007). In tomato fruit, changes in pH and K^+ levels have been described in the apoplastic fluid during ripening (Almeida and Huber, 1999; 2007). However, little is known about the relationship between potassium and water fractions during post-harvest storage of table grapes at low temperature.

Different stresses are known to alter a plant's water status (Karen et al., 1992; Ashraf and Foolad, 2007), and bound water is the fraction that probably plays the most important role in tolerance to abiotic stress given that it is responsible for maintaining the structural integrity and cell wall extensibility of living tissues (Sing et al., 2006). In harvested fruits, the ability to cope with changes in internal water content by varying the water fractions seems to be fundamental to maintain quality during storage. We previously demonstrated the benefits of short-term exposure to high concentrations of CO_2 on water status, highlighting the general increase produced in the bound water fraction in the different cluster tissues, and a decrease in the drip loss during freeze-thaw cycles (Goñi et al., 2011). Indeed, among the natural osmoprotective mechanisms

induced when high CO_2 treatment is associated with low temperature storage, grapes accumulates water-soluble fructo-oligosaccharides (Blanch et al., 2011).

Besides organic solutes, ions also play key roles in the osmoregulation of cells, and they are involved in a range of other important biological phenomena (Salt, 2004). Specifically, the strong correlation between potassium and sugar in the skin and flesh of grape berries, respectively, suggests that potassium acts as an osmoticum in skin cells, as sugars does in the flesh. Indeed, potassium may play different roles depending on the stage of berry development, and as well as contributing to the charge balance it may also be involved in sugar transport (Lang, 1983). However, excess potassium in berries at harvest has negative effects, reducing wine quality by increasing the pH value, particularly for red wines. Considering that we are working with detached grapes and the mechanism for potassium loading is disrupted, it is important to understand the functional implications of the subcellular localization and the dynamics of water-soluble potassium. Moreover, the effect of this ion on low temperature storage and the response to high CO_2 levels should also be defined.

The aim of this study was to investigate whether potassium accumulation is associated with changes in water balance caused by storage of table grapes at low temperature (0 ºC) in air (20% O_2 + 0.03% CO_2). Moreover, we studied whether these changes are dependent on the harvest date and if they are preserved by storage in air plus high CO_2 (20% O_2 + 20% CO_2). Accordingly, changes in the different water fractions were assessed by differential scanning calorimeter (DSC). Moreover, single-cell measurements of K^+ by energy dispersive X-ray microanalysis (EDX) of the different types of skin cells were accompanied by ultrastructural analysis of skin tissues by low temperature-scanning electron microscopy (LT-SEM). In addition, the mineral content was evaluated using inductively coupled plasma optical emission spectrometer (ICP-OES).

2. Materials and methods

2.1. Plant material

Table grapes (*Vitis vinifera* L. cv. Cardinal) were sampled from field-grown vines cultivated in Camas (Sevilla, Spain). Clusters were collected randomly at the beginning

of the commercial harvest on two different years. In the first harvest, (G1) grapes were picked with 11.9 ºBrix and in the second, (G2) grapes were picked with 12.8 ºBrix. At each harvest (G1 and G2), field-packaged bunches were transported to the laboratory, and those free from physical and pathological defects were randomly divided into two lots, storing both at 0 ± 0.5 ºC and 95% relative humidity (RH) in two sealed neoprene $1m^3$ containers. Ten plastic boxes containing about 3 kg of table grapes per box were stored in each container. One lot was stored in air for 3 days (air) and the other lot in air with 20% CO_2 (air + CO_2) for 3 days. At the end of this 3-day period, five clusters from each treatment were sampled, and 45 berries were randomly removed and distributed among the three replicates of 15 berries each. For (G1) and (G2), quality parameters were analysed in three replicates samples containing five-berries. Furthermore, three replicates of one-berry samples were immediately frozen in liquid nitrogen and the skin's micro-structure was analysed. Finally, another three replicates of nine-berries were deseeded and peeled, and the skin and flesh was ground into a fine power, and stored at -80 ºC.

2.2. Unfreezable water fraction content determination by differential scanning calorimetry

A method based on the heat of fusion was used to calculate the amount of unfreezable water (UFW) or bound water, expressed in g per g of dry weight. It was assumed that the heat of fusion of freezable water in the tissue studied was equal to the heat of fusion of pure water at 0 ºC. This analysis was performed using a DSC822e Mettler-Toledo differential scanning calorimeter (Mettler-Toledo Inc., Columbus, OH, USA) equipped with a liquid nitrogen cooling accessory (as described by Goñi et al., 2007), and calibrated using indium, n-octane and pure water. Frozen pulverized tissue was placed in 100 µL coated aluminum pans, which were immediately hermetically sealed and weighed. Samples were cooled from 25 ºC to -80 ºC at a rate of 10 ºC/min, left at -80 ºC for 5 min and then warmed to 25 ºC at 10 ºC/min. The total water content (%) in skin tissues was determined by heating to 65 ºC until the skin tissue reaches a minimum constant weight. All the results are the means of at least three measurements.

2.3. Cellular distribution of K^+ in the skin of table grapes at harvest

LT-SEM studies were performed using a Zeiss DSN- 960 electron scanning microscope equipped with a cold stage (Cryostrans CT-1500, Oxford Instruments). Frozen tissue sections were cryofractured at −180 ºC, etched at −90 ºC, gold-coated and subsequently transferred to the microscope where they were analyzed at −150 to −160 ºC. Samples were observed with both secondary and retro-dispersed electrons, and the best images were selected in each case. Through LT-SEM, the components of the samples, including water, are physically stabilized by freezing *in situ*. A combination of scanning electron microscopy and energy dispersive X-ray microanalysis (SEM-EDX) was used on frozen skin sections to measure the K^+ content in the epidermal and hypodermal skin cells. Freshly harvested fully-flattened skin was analyzed by EDX using the method described previously (Leidi et al., 2010), and in the same cryopreparation chamber (CT1500; Oxford Instruments) attached to the SEM (DSM 960; Zeiss). The SEM was fitted with an ATW detector that interfaced with a Link ISIS analyzer. Measurements were taken after focusing on epidermal cells from the top and continuing to penetrate through to the deepest hypodermal layers of the skin tissues.

2.4. Water-soluble Ca^{2+}, Mg^{2+}, K^+, Na^+ and P content.

A sample of frozen fruit (1 g) was homogenized for 5 min in 10 mL of ultra-pure water (for Na^+ and K^+) or 10 mL of ultra-pure water slightly acidified with 5 mM hydrochloric acid (for Ca^{2+} and Mg^{2+}), and this homogenate was analyzed as described previously (Blanch et al., 2012). Samples were centrifuged at 2000 × g for 20 min, after which the levels of soluble ions were determined in the supernatants. The samples were digested with HNO_3 and H_2O_2 in a Microwave Digestion Labstation (Milestone, mod. Ethos 1: Milestone, Shelton, CT – USA) and the digested samples were then diluted with ultrapure deionized water. The mineral content was evaluated on an Optima 4300 DV ICP-OES (inductively coupled plasma optical emission spectroscope: Perkin-Elmer, Norwalk, CT, USA). The data represent the means of three replicates, with two different measurements taken from each. The ICP-MS values obtained were used to calculate the ion content (mg/100g FW) based on the mass and dilution of each sample.

2.5. Color, pH, titratable acidity and total soluble solid analyses

Homogenized skin tissue color was assessed with a Konica Minolta CM-3500d and the chromatic analyses according to the CIE (Commission International de l'Eclairage) system. L^*, a^* and b^* values were measured to describe a three-dimensional color space and interpreted as follows: L* indicates lightness, from 0 (completely opaque or black) to 100 completely transparent or white. A positive a^* value indicates redness ($-a^*$ is greenness) and a positive b* value yellowness ($-b^*$ is blueness) in the hue-circle. The L^*, a^* and b^* values were used to calculate the hue angle ^{o}h = arctg (b^*/a^*) and the chroma (C^*) = $(a^{*2} + b^{*2})^{1/2}$, which indicates the intensity or color saturation.

The frozen skin from three berries from each replicate was ground and the powdered tissues were used to analyze the total soluble solids (ºBrix), pH and titratable acidity (TA). A sample of 1g was diluted with 3 mL of ultrapure deionized water and the initial pH of each sample was measured using a pH meter. The TA was measured by titration with sodium hydroxide (NaOH 1M) to pH 8.1.

2.6. Statistical analysis

One-way ANOVA and a correlational analysis were performed using SPSS v. 19.0. Multiple comparison of the means was performed using the Tukey's test, with the level of significance at $P < 0.05$. In this study the main effects of CO_2 treatment, storage time and the treatment × time interaction were analysed.

3. Results

3.1. Analysis of the DSC data

The changes in the unfreezable or bound water fraction were assessed in skin tissue from G1 and G2 grapes (Table 1). The bound water content was quite similar in the skin of both groups (1.9 and 1.7 g/g dry weight in G1 and G2, respectively). Interestingly, there was a significant decrease of around 20% in the bound water content in the skin of grapes stored in air, irrespective of the harvest date. By contrast, high CO_2

treatment prevented this decrease in the bound water fraction, with higher values in both G1 and G2 CO_2-treated fruit than in fruit stored in air. Although CO_2 had a positive effect on both G1 and G2 grapes, this effect was more moderate in G1 and more pronounced in the G2 samples with respect to the grapes stored in air. The onset of freezing temperature was significantly lower in grapes harvested at G2 (-5.67 ºC) than at G1 (-4.78 ºC) (Table 1). Moreover, there appeared to be a slight decrease in the freezing onset temperature during storage at low temperature that although not significant, was greater in CO_2-treated fruit in which it fell from -4.78 to -5.16 ºC in G1 berries and from -5.67 to -6.14 ºC in the G2 fruit.

Table 1

Changes in unfreezable water content (UFW) (g/g DW) and freezing onset temperature (T_{onset}) (ºC) in the skin of Cardinal table grapes over two different harvest years (G1 and G2). Skin tissues were analyzed from fruit at harvest or when stored for 3 days at 0 ºC in air or in air + 20% CO_2.

		at harvest	**air**	**air + 20% CO_2**
UFW	G1	1.98 ± 0.33b	1.55 ± 0.11a	1.80 ± 0.27b
	G2	1.70 ± 0.27b	1.40 ± 0.10a	1.86 ± 0.14b
T_{onset}	G1	-4.78 ± 0.19a	-5.10 ± 0.20a	-5.16 ± 0.29a
	G2	-5.67 ± 0.15a	-6.03 ± 0.24a	-6.14 ± 0.40a

The data are presented as the means ± SE of the three replicates (n = 6). Values followed by same letter within a column are not significantly different $P < 0.05$.

3.2. *Color, pH, titratable acidity and total soluble solid analyses.*

The chromatic characteristic of the homogenized skin of G1 and G2 berries indicated that storage in air at 0 ºC induced a loss of lightness (reflected by a decrease in L^*), and a reduction in the hue angle ^{o}h and chroma C^* values compared to those at harvest (Table 2). The decreases in L^* and C^* were independent of the harvesting year and they were smaller in CO_2 treated grapes. Moreover, and as expected, the chromatic characteristics of the skin indicated that there were differences in L^*, C^* and ^{o}h between G1 and G2 grapes at harvest. The less intense red color of the skin of G1 and the lower total soluble solid (Table 2) values were consistent with their less advanced stage of

maturity. CO_2-treated G1 and G2 grapes had significantly lower ºBrix values than the fruit at harvest and the pH value of macerated skin from G1 and G2 grapes displayed a similar trend during storage at low temperature (Table 2), as did the titratable acidity (data not shown).

Table 2

Changes in color, ºBrix and pH value in the skin of Cardinal table grapes over two different harvest years (G1 and G2). Skin tissues were analyzed from fruit at harvest or when stored for 3 days at 0 ºC in air or in air + 20% CO_2.

		at harvest	air	air + 20% CO_2
L*	G1	37.45 ± 0.05c	36.34 ± 0.01a	37.11 ± 0.01b
	G2	30.44 ± 0.29c	28.64 ± 0.01a	29.94 ± 0.08b
a*	G1	13.71 ± 0.02c	13.19 ± 0.01b	12.68 ± 0.20a
	G2	9.57 ± 0.44b	7.26 ± 0.04a	7.24 ± 0.03a
b*	G1	11.03 ± 0.00c	8.87 ± 0.03a	9.64 ± 0.33b
	G2	2.10 ± 0.07b	1.37 ± 0.03a	1.35 ± 0.02a
ºh	G1	38.83 ± 0.05c	33.93 ± 0.11a	37.25 ± 0.03b
	G2	12.37 ± 0.15b	10.68 ± 0.22a	10.58 ± 0.12a
C*	G1	17.60 ± 0.02b	15.90 ± 0.02a	15.93 ± 0.03a
	G2	9.79 ± 0.45b	7.39 ± 0.04a	7.37 ± 0.03a
ºBrix	G1	3.35 ± 0.12b	3.10 ± 0.11ab	3.05 ± 0.12a
	G2	4.28 ± 0.24b	3.75 ± 0.13a	3.67 ± 0.34a
pH	G1	4.36 ± 0.05a	4.33 ± 0.01a	4.31 ± 0.01a
	G2	4.45 ± 0.01a	4.53 ± 0.01a	4.52 ± 0.01a

The data are presented as the means ± SE of the three replicates (n = 6). Values followed by same letter within a column are not significantly different $P < 0.05$.

3.3. Micro-structural characteristics of skin cells and K^+ distribution.

LT-SEM was used to analyze the microstructure of Cardinal table grape skin cells. The section (Fig. 1) shows cells fully ruptured with membranous structures clearly visible and those fractured through the plane of the plasma membrane are without perpendicular membrane fractures. The K^+ distribution in the skin cells of fruit at harvest was also determined by SEM-EDX (Fig. 1). Although this latter technique does

not quantified the absolute ion concentrations, changes in the relative abundance of mineral elements can be assessed. As this technique uses silver, the percentage of K^+ percentage was expressed relative to silver as an external reference (100%). In these analyses, the outer surface consisted of a cuticle (c) covering the outer cell wall of the epidermal cells (e), and the epidermal cell layer of Cardinal table grapes was composed of small elongated and oval cells with a thick cell wall. There was a large separation between these cells, such that the cuticle enters into the epidermis. The hypodermis (h) in this variety of grape contains at least 10 layers of cells. The first hypodermal layer of the skin is irregular and some of the cells are triangular in shape, with the apex situated between the outer oval epidermal cells. The second layer is also made up of elongated oval cells similar to those in the first but larger while the next two hypodermal layers the cells are globular in shape and they become progressively larger towards the centre (nearer the pulp). From the fifth layer onwards, the cells have weaker walls, their outline is less irregular and the cells have a different internal aspect. Together, these cells occupy a depth of 0.4-0.7mm. When we quantified the K^+ content in at least 6 cells from each layer, a differential deposition of K^+ was evident in the different layers of skin cells (see Fig 1). Accordingly, the outer hypodermal layers seem to contain less K^+, while the subsequent hypodermal layers have a higher concentration of K^+. Less K^+ was also found in the layers deeper than the sixth hypodermal layer.

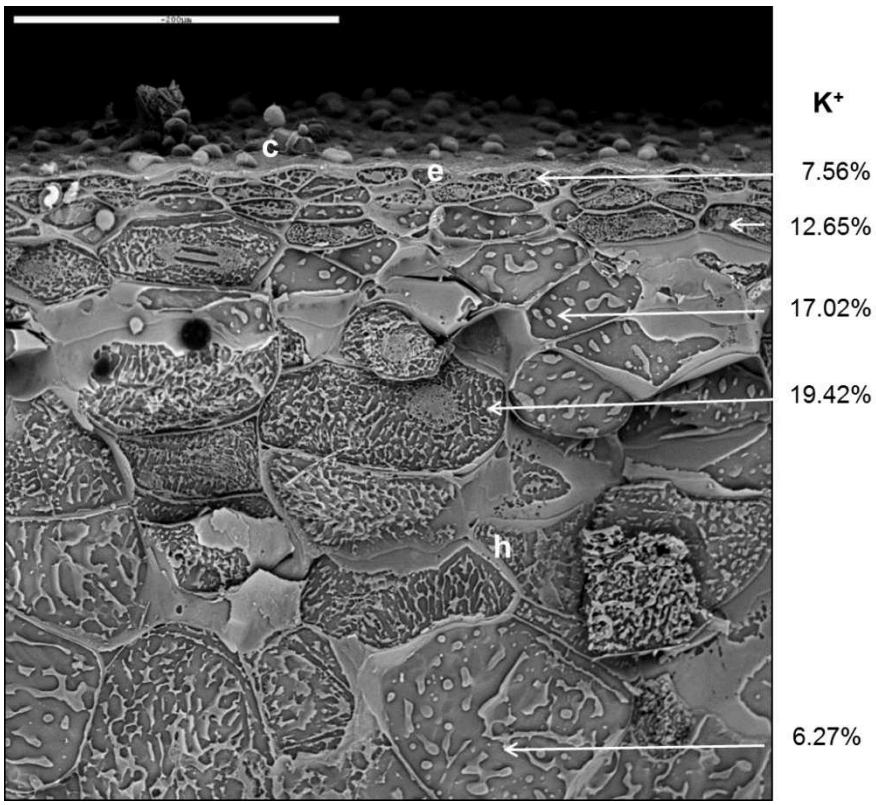

Fig. 1. LT-SEM micrograph showing skin cells of freshly harvest Cardinal table grape. The relative K^+ content of epidermal and hypodermal cells was determined by EDX analysis, performing the SEM-EDX analysis and quantification on six cells from the epidermis and following hypodermal layers. The values shown are the percentages of the total counts. *Abbreviations used*: (e = epidermal cells; c = cuticle; h = hypodermal cells).

Fig. 2 gave us an overall view of the effect of storage at low temperature with and without 20% CO_2 in terms of the morphology and ultrastructure of the distinct cell types in the skin of table grapes. Thus, when we compared the micro-structure of the epidermal (e), and subsequent hypodermal (h) cells in the skin of grapes stored at low temperature in air with cells from grapes stored in air plus 20% CO_2 and control fruit (at harvest), the most striking feature of the fruit stored air at 0 ºC was the compression of these cells.

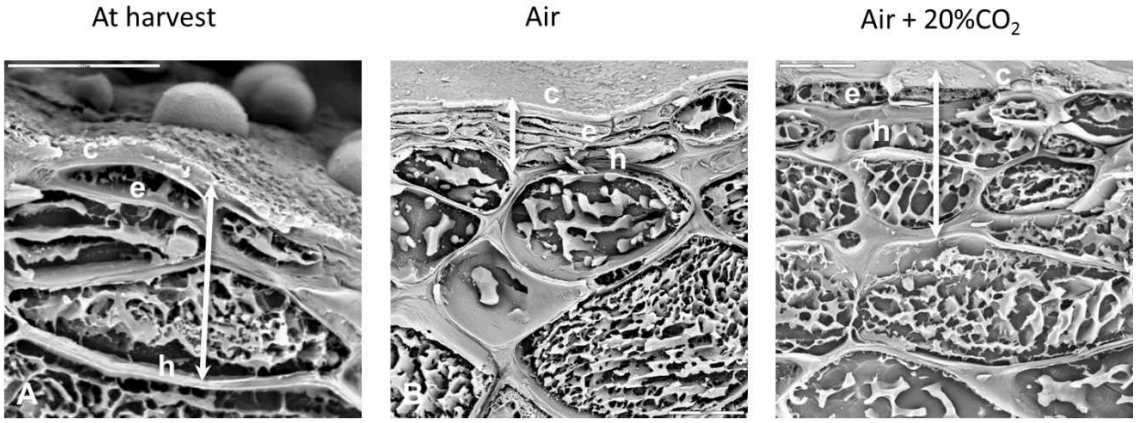

Fig. 2. Micro-structural characteristics observed by LT-SEM of the epidermal and adjacent hypodermal cells in Cardinal table grape skin at harvest (A), and after storage at 0 ºC in air (B) or in air + 20% CO_2 (C). *Abbreviations used*: (e = epidermal cells; c = cuticle; h = hypodermal cells). Epidermal and adjacent hypodermal cell space is indicated by double arrow and the scale bars represent 20 μm.

3.4. Changes in K^+, Na^+, Ca^{2+}, Mg^{2+} and P levels

K^+ is the most important ion in the skin of Cardinal table grapes (Table 3). Here, the highest levels of water-soluble K^+ were found in the skin cells of G1 and G2 fruit stored in air. Indeed, there was a significant ca. 20% accumulation of K^+ after 3 days of low temperature storage in air, increasing from 393.7 to 473.4 mg/100 g FW in G1 grapes and from 408.9 to 484.0 mg/100 g FW in G2 fruit. By contrast, when grapes were exposed to high CO_2 their K^+ content was no different from that in freshly harvested fruit, irrespective of the harvest date. With regards the other less abundant cations (Table 3), G2 fruit at harvest had less free water-soluble ions than G1 grapes. The skin tissues of fruit stored in air exhibited the highest Na^+ values, which accompanied the larger pool of K^+. In terms of divalent cations, the main differences were associated with the harvest date rather than an effect of the storage conditions, and high levels of soluble Ca^{2+} were quantified in the skin of G1 fruit. In the case of P, CO_2-treated fruit from G1 and G2 displayed different trends during storage at low temperature.

Table 3

Water-soluble ions (K^+, Na^+, Ca^{2+}, Mg^{2+}, P) in the skin of Cardinal table grapes over two different harvest years (G1 and G2). Skin tissues were analyzed from fruit at harvest, or after storage for 3 days at 0 ºC in air or in air + 20% CO_2. Data are expressed per (mg/100g fresh weight).

	G1			G2		
	at harvest	**air**	**air + 20% CO_2**	**at harvest**	**air**	**air + 20% CO_2**
K^+	393.70±10.7a	473.42±14.3b	333.95±23.8a	408.92±17.5a	484.07±7.8b	406.55±11.9a
Na^+	3.65±0.2b	4.77±0.3c	0.35±0.4a	0.88±0.1a	3.07±1.1b	2.32±0.4a
Ca^{2+}	22.27±1.1a	20.52±1.1a	22.42±1.1a	14.84±0.2b	12.64±0.3a	14.44±1.2ab
Mg^{2+}	10.09±0.1b	9.54±0.2ab	8.88±0.5a	9.35±0.2a	10.20±0.2a	8.88±0.8a
P	7.59±0.4a	10.93±0.1b	7.60±0.4a	5.28±0.1a	9.43±0.2b	10.42±0.4b

The data are presented as the means ± SE of the three replicates (n = 6) and the different letters within rows (from each experiment) indicate significant differences at $P < 0.05$

4. Discussion

Given that no changes in total water content were detected, our DSC data indicate that storage in air at low temperature is associated with the conversion of bound water to free water, as has been observed in tulip bulbs stored at low temperature through magnetic resonance imaging (Bendel et al., 2001). These data raise important questions, namely, whether the modifications of skin cell water status by storage at low temperature could affect water vapour exchange. Indeed, the hydration of the cuticle itself may even affect its conductance properties (van Gardingen and Grace, 1992). By contrast, the decrease in the bound water fraction provoked by storage at low temperature in air can be prevented by exposure to 20% CO_2. Interestingly, CO_2-treated tissues maintained their water-soluble K^+ pool while it increased significantly in skin tissues of grapes stored in air. Owing to the enlarged K^+ pool in fruit after 3 days storage at 0 ºC in air, we suggest that this may reflect the cellular stress associated with storage at a non-optimal temperature. This is consistent with our previous findings where table grapes, although a chilling tolerant fruit, could be sensitive to temperature shifts during the first phase of storage at 0 ºC (Sanchez-Ballesta et al., 2007). The implications of chilling damage, cytoplasmic acidosis, increased vacuolar pH and K^+ accumulation have been studied previously (Yoshida, 1994). Although macerated pH values did not differ significantly from the pH value determined with a pH-meter, it

remains possible that changes in the cytoplasmic pH in skin tissues stored at low temperature in air could be linked to the accumulation of K^+. Determination of the cytoplasmic and vacuolar pH in intact fruit tissues by ^{31}P nuclear magnetic resonance spectroscopy revealed an upward displacement in the chemical shift and changes in the intensity of the cytoplasmic Pi pool when stored at chilling temperatures, reflecting cytoplasmic acidification with an average decrease in pH of 0.72 units (Muñoz et al., 2001). The higher water-soluble K^+ concentration in the skin cells of fruit stored in air could imply a greater release of K^+ that better follows the flow of free water. Taking into account the shrinkage observed in the two outer layers in fruit stored in air (Fig. 2), the free water fraction may have been rapidly withdrawn from the cytosol, producing the consequent visible effect on cell volume. Therefore, these structural changes can be considered as a feature of the response to low temperature, and they are associated with the disrupted water balance and possibly perturbation of the membrane, facilitating the flow of potassium and water movement. Structural changes and alterations to the pools of water have been reported elsewhere in cotton leaves (Canny et al., 2012). Notably, this shrinking was not evident in the skin tissues from CO_2-treated grapes, evidence that high CO_2 treatment influences the regulation of skin water balance. Finally, it should not be overlooked that excess production of reactive oxygen species is another detrimental consequence of chilling stress (Hodges et al.; 2004; Aghdam et al., 2013).

Besides, the specific distribution of K^+ in the epidermal cells and the cells in the hypodermal layers of the skin may also play an important role in the water relationships in this variety of table grapes. Similar differences in inorganic ion accumulation have been identified in different root zones (Rodríguez et al., 1997; Hajibagheri et al., 1987) as well as in the K^+ distribution in mesophyll cells from non-acclimatized and cold-acclimatized rye leaves (Pihakaski-Maunsbach and Harvey, 1992). Since the vacuole was not clearly visible, we cannot determine the vacuolar compartmentalization of K^+ in skin cells. Potassium channels and other transporters have been characterized in grape berries (Pratelli et al., 2002; Davies et. al., 2006). In addition, NHX proteins (Leidi et al., 2010) may also participate in the proton-linked K^+ transport that is thought to facilitate active K^+ uptake at the tonoplast. Our results indicate a non-uniform accumulation and localization of K^+ in skin cells, results that are consistent with earlier data (Storey, 1987), although the reasons underlying this differential distribution of K^+ between different cells remain unclear.

Furthermore, the increase in the amount of water-soluble K^+ in grape tissues stored at low temperature in air also implies that K^+ ions are not retained. Conversely, CO_2-treated tissues maintained their water-soluble K^+ pool, indicative of a sequestering of K^+ ions, which is thought to be accompanied by the concurrent synthesis and accumulation of compatible solutes (Hasegawa et al., 2000). The slight decrease in the freezing onset temperature in CO_2-treated tissues suggested that exposure to high 20% CO_2 probably induced the accumulation of compatible solutes.

In relation to water, strong correlations have been reported between the % of leaf P, leaf water (total, free and bound water) and the leaf expansion rate under water stress conditions in severely drying soils (Singh et al., 2006). It was suggested that water-soluble Ca^{2+}, rather than total Ca^{2+}, could be a more meaningful index to measure changes that reflect the involvement of Ca^{2+} in the development of disorders, like senescent breakdown in apples (Saks et al., 1990). It has also been suggested that as well as proton transport, calcium transport at the tonoplast also fulfills an important role in the temperature acclimatization of plants (Yoshida, and Matsuura-Endo, 1991). In addition to the aforementioned effects, Ca^{2+} and some monovalent cations influence cell wall structure. Indeed, the pH and mineral composition of the fruit apoplast, mainly K^+ levels, provides a means for the biochemical regulation of cell wall metabolism (Almeida and Huber, 1999; 2007).

5. Conclusions

In this study, skin tissues of early-harvested grapes stored at 0 ºC in air consistently contained significantly less bound water than freshly harvested fruit. Moreover, compression and shrinkage of epidermal cells and of the adjacent hypodermal cells was also evident. Furthermore, the decrease in the bound water content in skin tissues of fruit stored in air was correlated with a larger water-soluble pool of K^+. These differences in K^+ levels presumably reflect the facility of potassium flow and that of free water in the skin of grapes stored in air. By contrast, storage at 0 ºC in air plus 20% CO_2 levels prevented bound water loss as well as avoiding water-soluble K^+ accumulation, and the shrinkage and compression of outer cell layers. Some of the beneficial effects of this high CO_2 treatment could be explained by restricting water mobility, and its influence on ion and volume homeostasis.

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Appendix A. Supplementary data

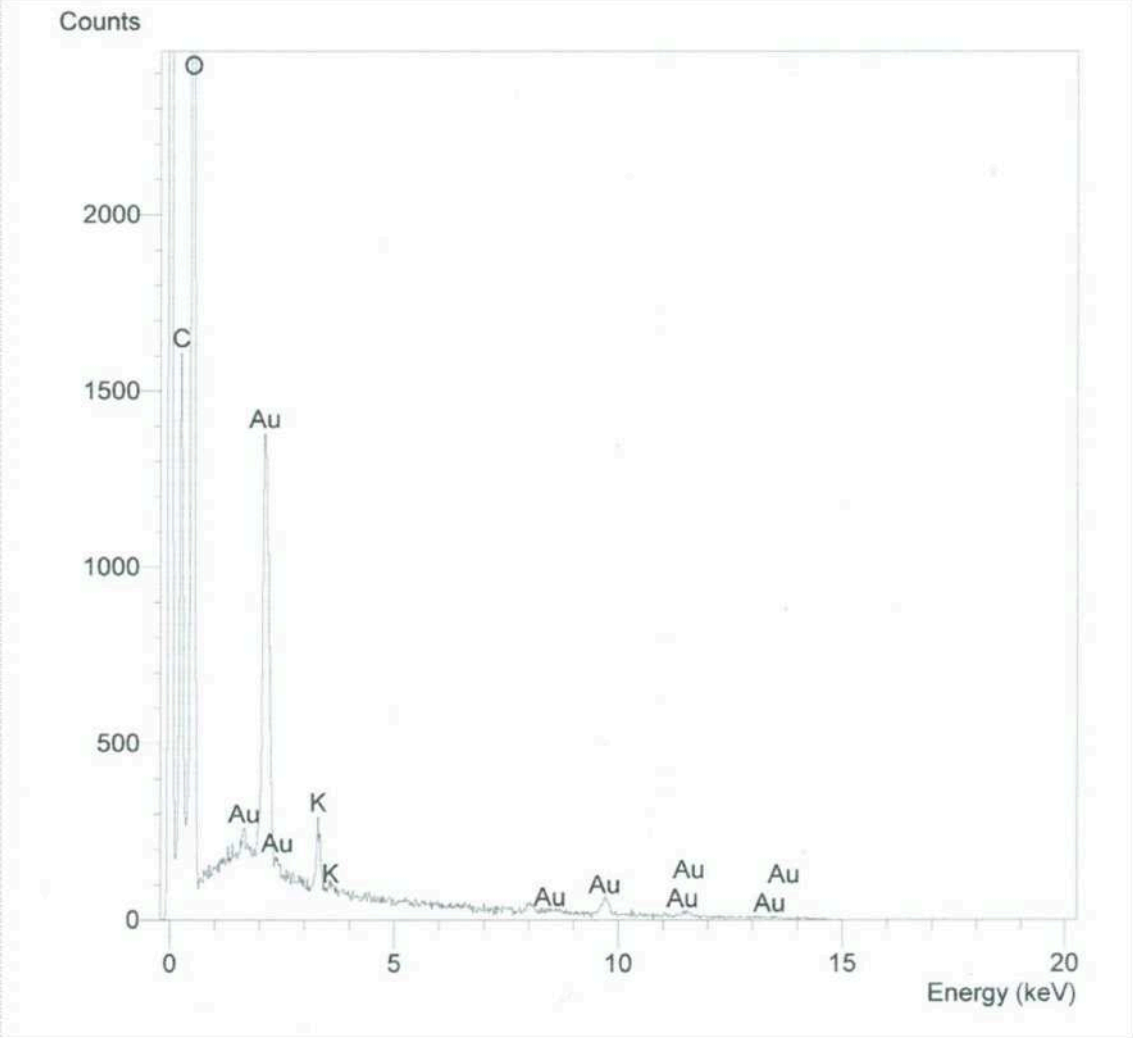

Fig. S1. Graph which shows that potassium is clearly the most abundant of the minor elements detected by SEM-EDAX in the skin of table grapes.

CAPÍTULO 3

Influence of the stage of ripeness on phenolic metabolism and antioxidant activity in table grapes exposed to different CO_2 treatments

Irene Romero, Carlos Fernandez Caballero, María T. Sanchez-Ballesta, María I. Escribano, Carmen Merodio*

Postharvest Biology and Technology (2009) 54: 118-121

* Corresponding author

ABSTRACT

We have analyzed the influence of the stage of ripeness on L-phenylalanine ammonia-lyase (PAL*)* gene expression, on the accumulation of anthocyanins and total phenolics, and on antioxidant activity in the skin of table grapes treated with 20% CO_2 + 20% O_2+ 60 % N_2 for 3 or 6 days at low temperature (0 ºC). The residual effect of high CO_2 treatment after transfer to air was also studied. In early harvested grapes, neither the anthocyanin content nor the accumulation of *VcPAL* mRNA was affected by any of the CO_2 treatments applied. However, in late harvested grapes, the length of high CO_2 treatment determined its effect and a 6-day treatment with CO_2 sustained the higher levels of total phenolics and anthocyanin accumulation, and of *VcPAL* expression observed in untreated late harvested grapes. Indeed, their increased antioxidant capacity was correlated with the total amount of phenolics and anthocyanins. Conversely, in grapes treated for 3-days with CO_2 the phenylpropanoid pathway does not appear to be induced and a relationship between antioxidant activity and anthocyanins was not observed. Thus, further studies are needed to identify the most important antioxidants in these treated fruit.

1. Introduction

Table grapes are highly perishable and their quality deteriorates rapidly due mainly to water loss and sensitivity to fungal decay. Hence, they should be cooled as soon as possible after harvesting. It is well known that postharvest storage at low temperature affects anthocyanin accumulation in grapes and many other fruits (Awad and de Jager, 2002; Romero et al., 2008). Agronomic and environmental factors also have a strong influence on the accumulation of anthocyanins and polyphenols in grapes. Moreover, induction of phenylalanine ammonia-lyase (PAL) gene expression, as well as that of other genes involved in anthocyanin biosynthesis, has been reported in response to low temperature stress (Christie et al., 1994). Furthermore, the accumulation of anthocyanins and phenolic compounds is known to be developmentally regulated in grapes (Romeyer et al., 1983; Mateus et al., 2002). Phenolic compounds determine the characteristics of color and taste in fruit, such as bitterness and astringency. Moreover, the total phenolics and anthocyanin content has positively been correlated with antioxidant capacity, and these compounds are considered to contribute significantly to the health benefits of consuming fruit and vegetables. The degree of maturity and harvest date also affect the quality and antioxidant contents of stored fruit (Shin et al., 2008). However, there is little information about the influence of the stage of ripeness of table grapes on PAL gene expression, and on the amount of anthocyanins and total phenolics, as well as their relationship with the overall antioxidant capacity of table grapes exposed to different postharvest treatments. Short-term exposure to high CO_2 concentrations is an effective treatment to maintain quality and to control fungal decay in grapes (Retamales et al., 2003; Sanchez-Ballesta et al., 2007). However, the mode of action of such treatments on health-related compounds is still not understood. The aim of this work was to investigate whether the degree of ripening had any influence on the effect of exposure to high CO_2 during low temperature storage at 0ºC on (1) the expression of the gene encoding the PAL enzyme (2) the accumulation of anthocyanins and total phenolics and (3) the antioxidant activity in the berry skins. Moreover, since gas treatment is affected by concentration and exposure time, we also analyzed the effect of exposing both early and late harvested table grapes to 20% CO_2 for 3 or 6 days.

2. Materials and methods

Table grapes (*Vitis vinifera* L. cv. 'Cardinal') were harvested at a vineyard in Southern Spain (Sevilla) twice over 3 weeks from 10 vines. The first harvest began on the 13th June 2005 (early harvested, maturity index of 12.45 ± 0.01 and the second on the 5th July 2005 at commercial ripeness (late harvested, maturity index of 41.08 ± 0.30). Two lots of 15 replicate bunches were kept in air at 0 ± 0.5 ºC for up to 27 days (untreated) and another four lots were stored under a gas mixture of 20% CO_2 + 20% O_2 + 60% N_2 (CO_2-treated fruit). After 3 or 6 days in 20% CO_2, a single lot was transferred to continuous humidified air flow to analyze the residual effect of the CO_2. Ten clusters were sampled at the end of the CO_2 treatments and at the 27th day of storage at 0 ºC.

The berries from five clusters (approx. 300 g/cluster) were peeled and the skin was frozen in liquid nitrogen, ground into a fine powder and stored at –80 ºC until analysis. Total RNA was extracted from the skin of grapes and denatured samples (10 µg) were blotted and hybridized with the *VcPAL* cDNA (Sanchez-Ballesta, 2007). Results obtained by Northern blot analysis were representative of at least two biological replicates. Autoradiographs were digitally scanned and band densities were quantified by image densitometry using Scion Image software (Scion Corporation, Frederick, MD) and a value of 100% was assigned to the maximum optical density value achieved in each northern blot and the rest of optical densities were normalized to the maximum value and expressed as percentage of relative accumulation (RA).

The total phenolics were quantified using the Folin-Ciocalteau colorimetric reagent method, and using gallic acid as a reference standard. The concentration of total phenolics was expressed as the mg gallic acid equivalents per gram of fresh weight (FW). Total anthocyanin content was determined by the differential pH method and the results were expressed as mg of malvidin-3-glucoside equivalent per gram FW. The antioxidant capacity of grape skin extracts was calculated as mM Trolox equivalents (TE) per gram (FW), using ABTS radical anion scavenging activity assay as described by Re et al. (1999).

The experimental data is expressed as the mean ± S.E. of three replicates of each sample. A variance analysis (one-way ANOVA) using the Fisher's least significant difference (LSD) test (Statgraphics 5.1 Plus program, STSC, Rockville, MD) was

performed to determine whether the differences between CO_2-treated and untreated grapes stored at 0 ºC were significant ($P \leq 0.05$).

3. Results and discussion

3.1. Effect of CO_2 treatment and storage at 0 ºC on PAL expression in the skin of table grapes harvested at different stages of ripeness

Irrespective of the stage of ripeness, PAL expression was minimal in both early and late harvested grapes (Fig. 1). Low temperature storage increased *VcPAL* mRNA accumulation both in untreated early and late harvested grapes although in early harvested fruit, *VcPAL* mRNA only increased at the end of storage, whereas in late harvested fruit a sharp accumulation was detected after 3 days at 0 ºC and it was maintained throughout the storage period. Likewise, the effect of the CO_2 treatment on *VcPAL* gene expression depended on the length of treatment and the ripeness. In early harvested fruit the duration of CO_2 treatment (3 or 6 days) did not affect *VcPAL* gene expression, whereas in late harvested grapes, *VcPAL* gene expression closely reflected the length of exposure to high CO_2 concentration. Thus, whereas 3 days of CO_2 treatment limited the increase in *VcPAL* transcripts compared with that observed in untreated fruit, these transcripts accumulated intensely after 6 days of CO_2 treatment. It has been reported that PAL may be induced during development, ripening and in response to several stress factors, including low temperature storage (Dixon and Paiva, 1995). In conjunction with previous observations (Sanchez-Ballesta et al., 2007), these results provide new evidence indicating that storage at 0 ºC sharply increased the abundance of *PAL* transcripts in untreated grapes and in those exposed to 20% CO_2 for 6 days. By contrast, a shorter 3 day CO_2 treatment did not enhance their accumulation.

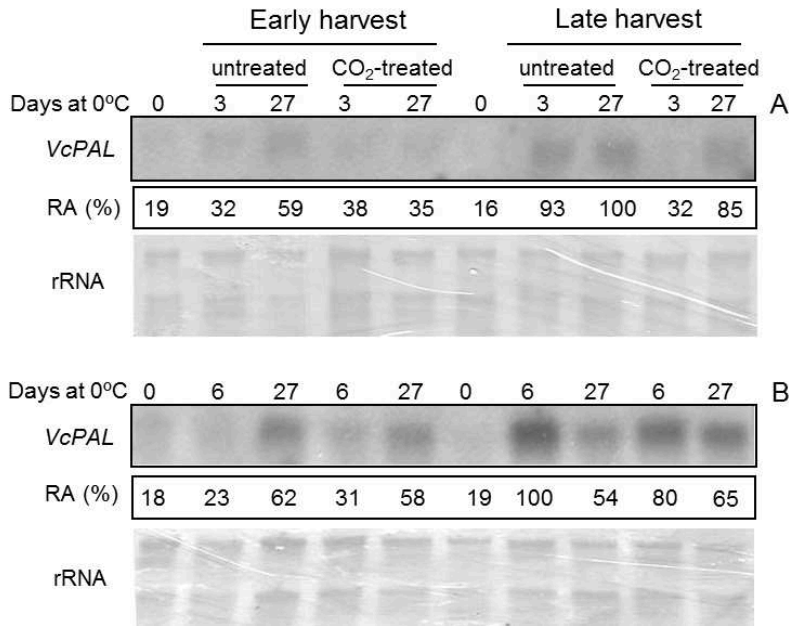

Fig. 1. Accumulation of *VcPAL* mRNA in the skin of early and late harvested grapes treated with high levels of CO_2 for 3 (A) or 6 days (B) and then transferred to air for up to 27 days at 0ºC. Total RNA (10 μg) from the skin was fractionated by gel electrophoresis, blotted and hybridized with the *VcPAL* probe. The intensity of the bands was quantified by scanning densitometry of the autoradiographs. Optical density values were normalized to the maximum value and expressed as percentage of relative accumulation (RA). The equivalence of RNA loading of the lanes was demonstrated by methylene blue staining.

3.2. Effect of CO_2 treatment and storage at 0 ºC on anthocyanin and polyphenol content, and on antioxidant activity in the skin of table grapes harvested at different stages of ripeness

In early harvested grapes, CO_2 treatment had no significant effect on the index of maturity, while the application of 20% CO_2 for 3 or 6 days was effective in delaying the evolution of maturity index in late harvested (data not shown).

At harvest, the anthocyanin content was about 5.7 times lower in early harvested grapes (0.35 mg/g FW) than in late harvested fruit (1.99 mg/g FW), and these levels only changed slightly during storage at 0 ºC irrespective of whether the fruit received CO_2 or not (Fig. 2). By contrast, there was a significant increase in anthocyanin content in untreated late harvested grapes after 3 and 6 days storage at 0 ºC. It is already known that anthocyanin synthesis in grapes begins during veraison and that anthocyanins

gradually accumulate in berry skin throughout ripening (Mateus et al., 2002) and after harvest during low temperature storage (Romero et al., 2008) This increase in anthocyanin content was not observed in late harvested fruit exposed to CO_2 for 3 days while in grapes treated with CO_2 for 6 days a significant increase in total anthocyanins was evident.

According with our results, low temperature storage led to less pronounced increases in total phenolic content in early harvested grapes than in late harvest ones (Fig. 2). The length of the gas treatment also affected the accumulation of phenolic compounds in late harvested grapes. Thus, while the 3-day CO_2 treatment maintained or restricted the increase in the levels of total phenolics in both early and late harvested grapes, the 6-day treatment with CO_2 increased their content. Accumulation of phenolics is a response to a range of biotic and abiotic stresses (Dixon and Paiva, 1995) and the role played by phenolics in low temperature-affected cells varies depending on the tissue studied, involving cell wall reinforcement and cellular protection against radiation or oxidative stress (Chalker-Scott et al., 1989). However, less attention has been paid to the role of phenolic compounds in freshly harvested fruit exposed to low temperature stress. It is well documented that the structural chemistry of polyphenols predicts their potential role as free radical scavengers. Here we found that harvest time also significantly affected antioxidant activity (Fig 2). Indeed, the antioxidant activity of grapes picked at the earlier stage was higher (31.12 ± 2.35 mM TE/g FW) than that of late harvested fruit (13.39 ± 2.10 mM TE/g FW), and it remained similar or decreased during storage. Moreover, in early harvested grapes no correlation was evident between total antioxidant capacity, total phenolics and anthocyanins. The fact that early harvested grapes had similar or higher amounts of phenolic compounds than late harvested grapes, while their anthocyanin content was significantly lower, could suggest that other phenolic compounds rather than anthocyanins may be responsible for the high antioxidant capacity of these grapes. It is well known that each phenolic compound has a specific antioxidant capacity and that polyphenol composition in grapes is developmentally regulated (Romeyer et al., 1983). Thus, additional research is required to study the specific phenolic compounds with high antioxidant capacity in early harvested grapes.

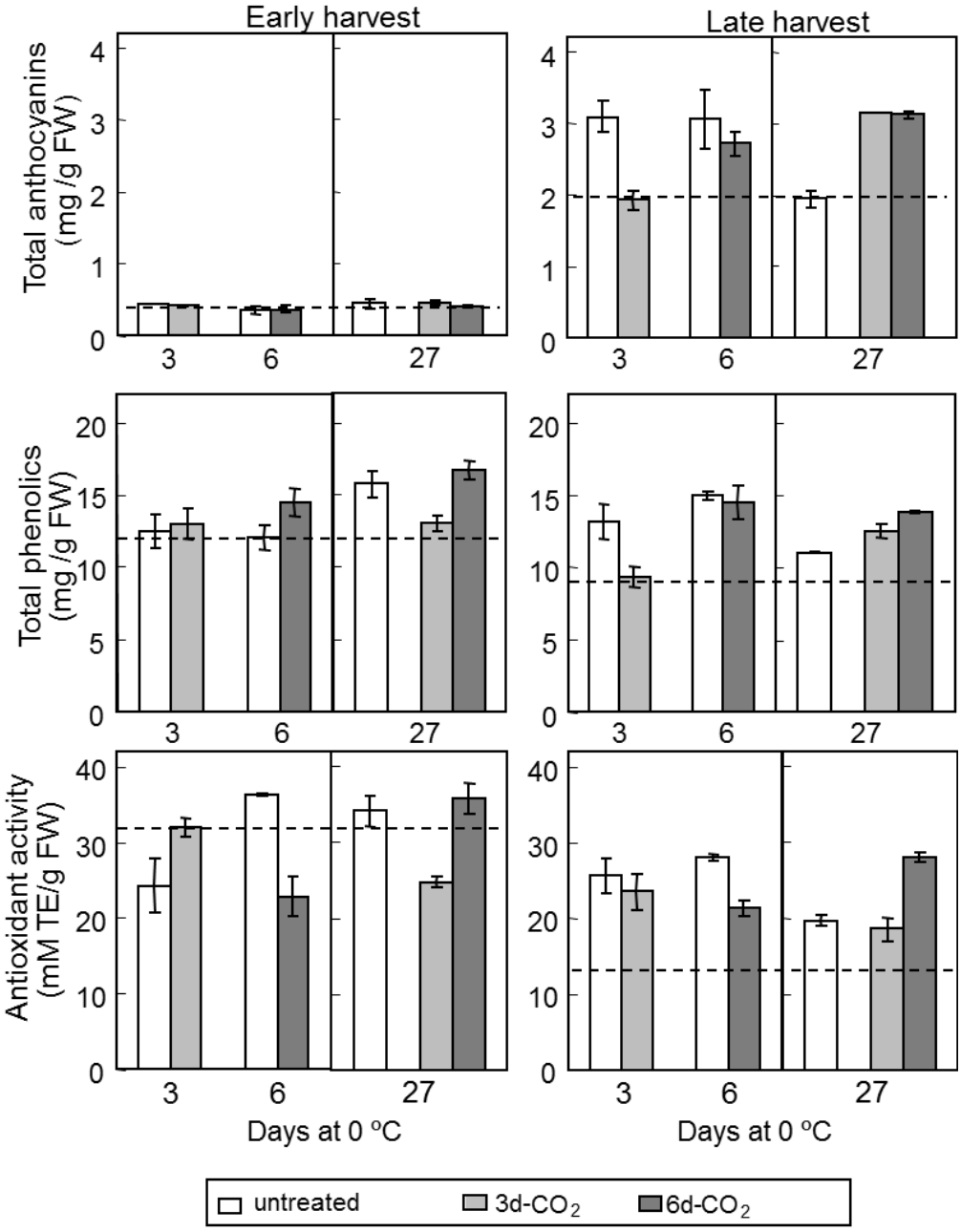

Fig. 2. Changes in the total anthocyanin and total phenolics content, and in antioxidant activity in the skin of early and late harvested grapes treated with high levels of CO_2 for 3 or 6 days and then transferred to air for up to 27 days at 0 ºC. The dotted lines indicate the values for freshly harvested fruit and all values are the means of three replicates ± S.E.

In late harvested grapes, the antioxidant activity of both untreated and high CO_2-treated grapes increased significantly at low temperature, although the differences observed depended on the duration of the treatment. The strongest correlation between

antioxidant capacity and anthocyanins was that in grapes treated for 6-days in CO_2 (r^2 = 0.993), probably reflecting the greater contribution of these compounds to the total antioxidant capacity of this fruit. In grapes treated for 6-days with CO_2 the correlation between antioxidant activity and phenols was high (r^2 = 0.836), albeit lower than that in untreated grapes (r^2 = 0.988). By contrast, in grapes treated for 3-days with CO_2 the correlation between antioxidant capacity and anthocyanins was negligible (r^2 = -0.049), unlike the good correlation observed in untreated grapes (r^2 = 0.892).

These results provide new evidence that the increased antioxidant capacity in late harvested grapes treated for 3-days with CO_2 does not appear to be due to changes in the concentration of anthocyanins. Flavonols are the most abundant phenolic compounds in grape skin although proanthocyanidins are also evident and developmentally regulated (Kennedy et al., 2001). Thus, additional studies are necessary to determine the effect of high CO_2 treatment on the concentration of non-anthocyanin polyphenolic compounds. By contrast, the results obtained in both untreated grapes and those treated with 20% CO_2 for 6 days suggest that the activation of phenolic metabolism is a defense mechanism involved in the accumulation of phenols and anthocyanins, and that it is closely connected with their increased antioxidant capacity. Moreover, these results indicate that when compared to a 3 day treatment, a 6 day exposure to high CO_2 concentrations is too long, since there is a sharp accumulation of total phenolics and anthocyanins, and an increase in *VcPAL* expression at the end of treatment and even when the fruit was transferred to air. The overall results suggest that a delay in the harvest date is decisive to increase antioxidant activity by inducing phenolic metabolism in stressed untreated and 6 days CO_2-treated grapes stored at 0 ºC.

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CAPÍTULO 4

Molecular analysis of the improvement in rachis quality by high CO_2 levels in table grapes stored at low temperature

Raquel Rosales, Carlos Fernandez-Caballero, Irene Romero, Mª Isabel Escribano, Carmen Merodio, Mª Teresa Sanchez-Ballesta*

Postharvest Biology and Technology (2013) 77: 50-58

* Corresponding author

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 *GPO1* mRNA 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, the effect of high CO_2 levels reducing rachis browning seems to be linked to the modulation of ethylene biosynthesis genes. 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.

1. 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 beneficial 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 VvNCED1a, 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 *(VcPAL* and *GPO1)*, the antioxidant system (*GCAT* and *VcAPX)*, as well as ethylene (*ACO1* and *ACS1)* and ABA (*VvNCED1* and *VvNCED2*) 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 rachis) 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, except for *VcPAL* and *VcAPX* previously determined in the skin (Sanchez-Ballesta et al., 2007; Romero et al., 2008), 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 ($p < 0.05$). The relationship between rachis browning index, MDA content and gene expression was described as Pearson product-moment correlation coefficient (r), $p < 0.05$.

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).

Table 1

Relative water content (RWC) and browning index of rachis of non-treated and CO_2-treated table grapes cv. Cardinal stored for 33 days at 0 ºC.

	RWC (%)		Rachis browning index[a]	
Days at 0 ºC	Non-treated	CO_2-treated	Non-treated	CO_2-treated
0	88.78 ± 1.48 a		0 d	
3	69.09 ± 2.76 bc	75.24 ± 4.35 b	0.93 ± 0.16 c	0.53 ± 0.21 cd
15	61.23 ± 2.41 cd	77.46 ± 2.17 b	2.27 ± 0.30 a	1.00 ± 0.23 bc
33[b]	56.52 ± 2.46 d	76.97 ± 1.33 b	2.83 ± 0.36 a	1.67 ± 0.25 b

[a] Scale of browning: 0, none; 1, slight; 2, moderate; 3, severe; 4, extreme.

[b] Data previously published in Sanchez-Ballesta et al. (2006).

Values are the mean of three replicate samples ± SE

Different letters (a-d) indicate that means are statistically different (Duncan test, $p < 0.05$)

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).

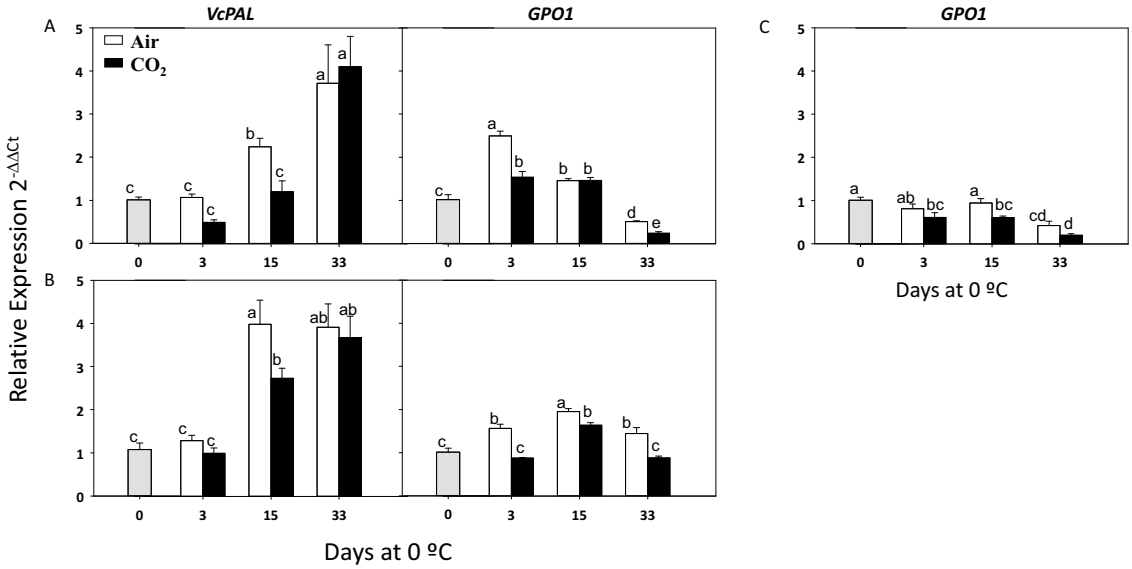

Fig. 1. Effect of low temperature and 3-day high CO_2 pretreatment on *VcPAL* and *GPO1* gene expression in rachis (A), pulp (B), and skin (C) of *V. vinifera* cv. Cardinal bunches stored at 0 ºC. Transcript levels of each gene were assessed by quantitative RT-PCR and normalized using *actin* (*ACT1*) 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 ($p < 0.05$).

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 Fig. 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).

Table 2

Malondialdehyde (MDA) content (nmol/g FW) in rachis, pulp and skin of non-treated and CO_2-treated table grapes cv. Cardinal stored for 33 days at 0 ºC.

	Rachis		Pulp		Skin	
Days at 0 ºC	Non-treated	CO_2-treated	Non-treated	CO_2-treated	Non-treated	CO_2-treated
0	55.60 ± 1.20 c		26.21 ± 0.59 bc		333.22 ± 9.45 ab	
3	62.78 ± 0.17 b	52.35 ± 0.68 cd	31.42 ± 2.36 abc	29.54 ± 0.62 ab	341.01 ± 16.72 a	263.83 ± 26.77 cd
15	67.91 ± 5.30 a	64.49 ± 4.96 ab	33.06 ± 1.44 a	30.08 ± 1.90 ab	381.73 ± 9.85 a	211.41 ± 22.20 d
33	62.78 ± 1.54 b	46.02 ± 0.11 c	25.67 ± 0.82 bc	24.78 ± 0.54 c	262.69 ± 19.03 cd	277.40 ± 8.63 bc

Values are the mean of three replicate samples ± SE

Different letters (a-d) within each tissue indicate that means are statistically different (Duncan test, $p < 0.05$)

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).

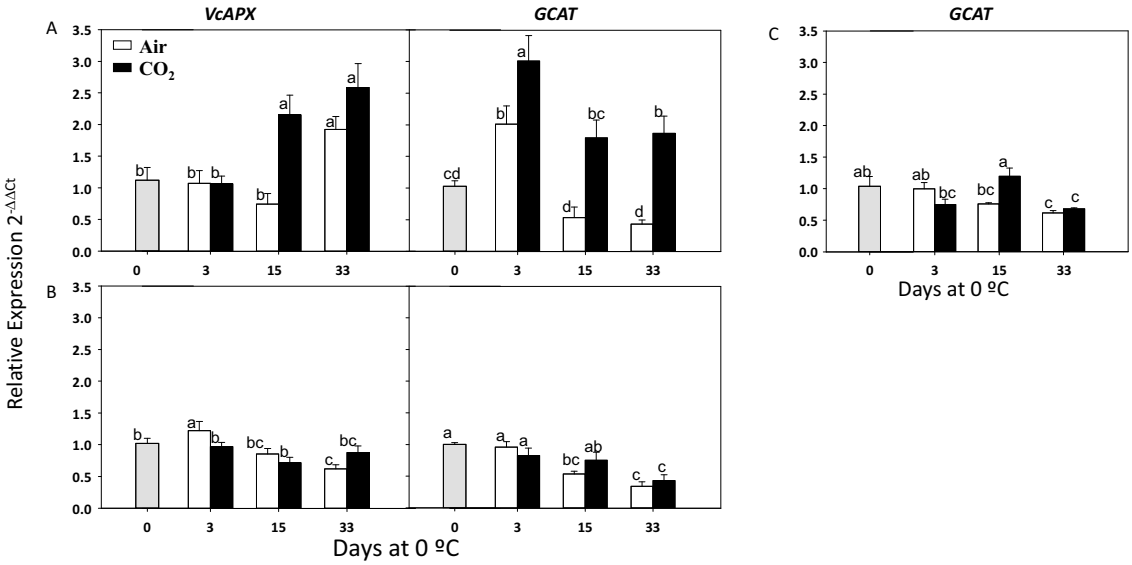

Fig. 2. Effect of low temperature and 3-day high CO_2 pretreatment on *VcAPX* and *GCAT* expression in rachis (A), pulp (B), and skin (C) of *V. vinifera* cv. Cardinal bunches stored at 0 ºC. Transcript levels of each gene were assessed by quantitative RT-PCR and normalized using *actin* (*ACT1*) as 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 ($p < 0.05$).

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*.

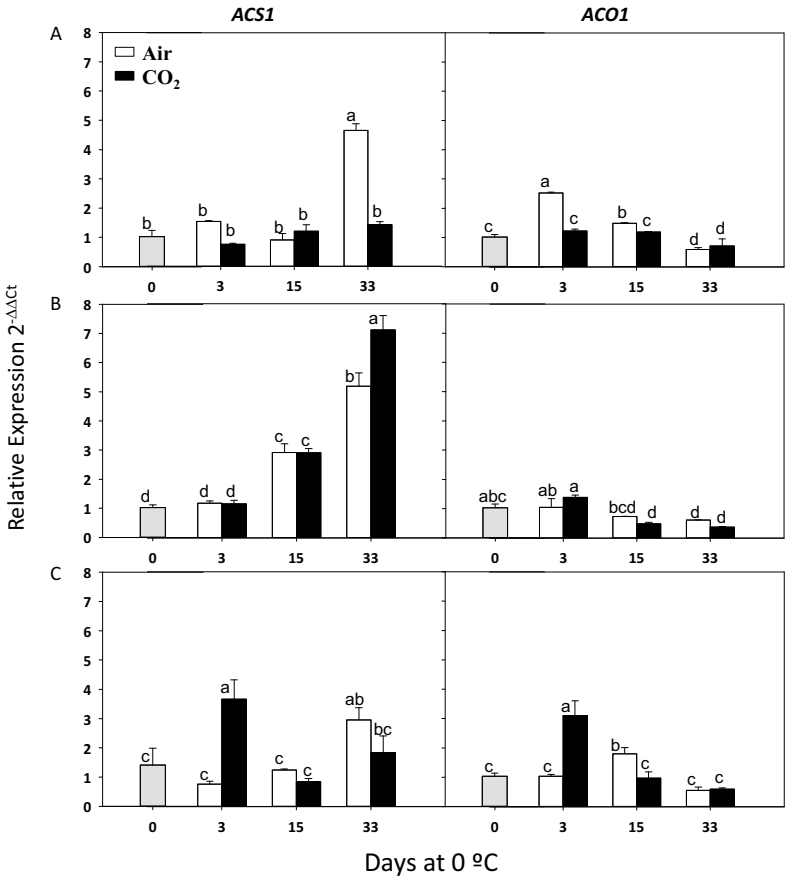

Fig. 3. Effect of low temperature and 3-day high CO_2 pretreatment on *ACS1* and *ACO1* expression in rachis (A), pulp (B), and skin (C) of *V. vinifera* cv. Cardinal bunches stored at 0 ºC. Transcript levels of each gene were assessed by quantitative RT-PCR and normalized using *actin* (*ACT1*) as reference 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 ($p < 0.05$).

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 Fig. 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).

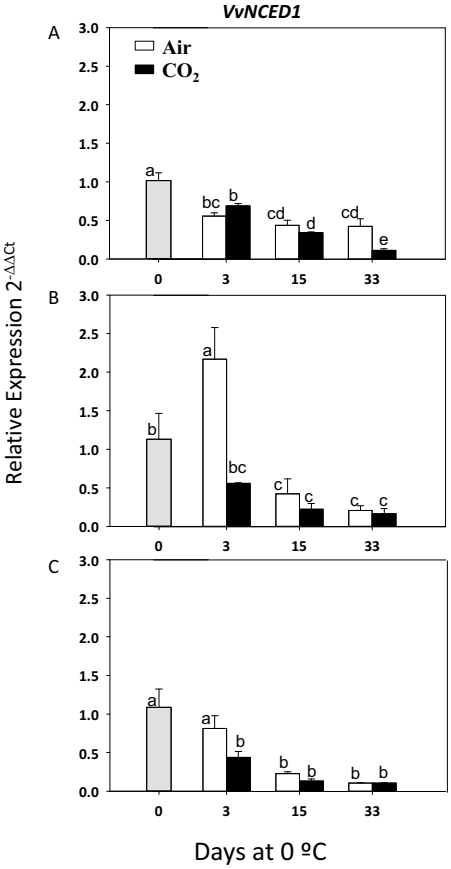

Fig. 4. Effect of low temperature and 3-day high CO_2 pretreatment on *NCED1* expression in rachis (A), pulp (B), and skin (C) of *V. vinifera* cv. Cardinal bunches stored at 0 ºC. Transcript levels of each gene were assessed by quantitative RT-PCR and normalized using *actin* (*ACT1*) as reference gene. 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 ($p < 0.05$).

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). Our results showed a good negative Pearson correlation coefficient between RWC and rachis browning (r = -0.88) during cold storage. Likewise, we have previously indicated that high CO_2 levels modified water status in fruit (skin, pulp and seeds) and non-fruit tissues (rachis) 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).

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. 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 reduced rachis browning index and the sharp increase observed in *GPO1* gene expression in non-treated bunches, whereas *VcPAL* transcript accumulation did not change in treated and non-treated samples in comparison to time 0. However, the fact that rachis browning and *VcPAL* gene expression continued increasing throughout the

storage at 0 ºC in non-treated bunches being lower in CO_2-treated samples, while *GPO1* transcript levels decreased both in treated and non-treated rachis independently of rachis browning until reaching values lower than time 0 at the end of storage, seem to indicate that other factors are involved in the development of rachis browning. Murr and Morris (1974) 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* transcript accumulation. These findings seem to 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 *GPO1* gene expression was not a specific response of rachis to low temperature. Thus, transcript levels also increased in the pulp of non-treated grapes, although with a different pattern of expression, since it was not only activated after 3 days but 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 at 15 days of storage and decreasing thereafter. 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). We want to point out that although no correlation between rachis browning and MDA content (r = 0.35) was observed, there was a trend in which rachis of non-treated bunches showed higher browning index as well as MDA content during storage at 0 ºC compared to time 0 and CO_2-treated samples. 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 *APX* and *CAT* gene expression, although only a moderate negative correlation was observed with *CAT* transcript accumulation (r = -0.53). It was especially evident in CO_2-treated rachis at 33 days of storage, albeit browning was moderate the MDA content was low, what could be related to their higher *VcAPX* and *GCAT* gene expression from 15 and 3 days of storage, respectively. On the other hand, it seems that the increase in *VcAPX* and *GCAT* transcript levels observed in non-treated rachis either occurred too late or was not sufficient to reduce their MDA content. In this sense,

VcAPX expression in non-treated rachis was only induced after 33 days, while the induction of *GCAT* transcription was transitory after 3 days and lower than that of CO_2-treated samples. In reference to the role of antioxidant enzymatic system in rachis browning we have only observed a good negative correlation in the case of *GCAT* gene expression (r = -0.93), indicating that the accumulation of this transcript could be related to a reduction of rachis browning development. 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 level 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.

Our results revealed that the development of rachis browning in non-treated table grapes was linked to an induction of the expression of ethylene synthesis genes. However, while *ACS1* gene expression increased at the end of storage, *ACO1* transcription was transitorily induced after 3 days at 0 ºC. On the other hand, the application of 3-day high CO_2 pretetreatment avoided the accumulation of *ACS1* and *ACO1* transcript levels in rachis, existing a positive correlation between rachis browning indexes and *ACS1* gene expression (r = 0.70). Likewise, it is interesting to note that we observed a moderate correlation between *ACS1* and *PAL* gene expression (r = 0.57), whereas *ACO1 t*ranscript levels were correlated with *PPO* (r = 0.94). The ethylene-induced increase in PAL and PPO activities has been related to the development of brown necrotic tissue areas in iceberg lettuce and mesocarp discoloration in avocado (Hyodo et al., 1978; Pesis et al., 2002). In non-climacteric fruit, high CO_2 pretreatment 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). 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. 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. 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 during low temperature storage, avoiding the activation of ethylene biosynthesis genes and promoting an osmotic adjustment as well as the induction of enzymatic antioxidant system. 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.

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Supplementary Table 1

Name	GeneBank Acc. number	Primers	Size (bp)	Reference
GCAT	XM_003635412	**CAT-Fw**: 5'-AGGCTGGGAAGGCAAATTAT-3' **CAT-Rv**: 5'-CGTCTGGATGAAAAGCTTCC-3'	176	
VcAPX	DQ887095	**APXiQ-5**: 5'-TGCTGTTGAGGTCACTGGAG-3' **APXiQ-3**: 5'-CCCCAGAGAGAGCTACGATG-3'	176	
VcPAL	DQ887093	**PAL_RT-Fw**: 5'-CTGTGACCAACCATGTCCAG-3' **PAL_RT-Rv**: 5'-GCAAGTCTTTTTCGCAGACC-3'	276	
GPO1	A27657	**PPO_RT_Fw:** 5'-GGAGCCGAAGATGATGAGAG-3' **PPO_RT_Rv:** 5'-ATGGGATCAATCGGAAACAA-3'	108	
ACS1	XM_002278453	**ACS-5'**: 5'-GTTCCCGTGGGTTTGCTTTA -3' **ACS-3'**: 5'-GTTGCATCATCCTCCATGTG-3'	115	
ACO1	AY211549	**ACO1-Fw**: 5'-GCATCATTCTACAACCCAGG-3' **ACO1-Rv**: 5'-GGAACTTCAAACCGGCATAA-3'	139	
VvNCED1	AY337613	**NCED1-Fw**: 5'-GCAGAGGACGAGAGTGTAAAGGA-3' **NCED1-Rv**: 5'-GCAGAGTAAAAACACATGAAGCTAGTG-3'	130	Lund et al., 2008
VvNCED2	AY337614	**NCED2-Fw**: 5'-ATGCTCAAACCGCCTCTGAT-3' **NCED2-Rv**: 5'-TCCCAAGCATTCCAGAGGTG-3'	76	Lund et al., 2008
ACT1	XM_002282480	**Actin1-Fw**: 5'-CTTGCATCCCTCAGCACCTT-3' **Actin2-Rv**: 5'-TCCTGTGGACAATGGATGGA-3'	82	Reid et al., 2006

CAPÍTULO 5

Unraveling the roles of *CBF1*, *CBF4* and dehydrin 1 genes in the response of table grapes to high CO_2 levels and low temperature

Carlos Fernandez-Caballero[1], Raquel Rosales[1], Irene Romero, Mª Isabel Escribano, Carmen Merodio, Mª Teresa Sanchez-Ballesta*

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[1] These authors contributed equally to this work

* Corresponding author

ABSTRACT

CBFs (C-repeat binding factors) are transcription factors that are rapidly induced by low temperature and recognize the CRT/DRE element in the promoter of a set of cold regulated genes, the CBF regulon. Dehydrins are proteins that accumulate in plants under stress conditions, such as low temperature, and some of them form part of the CBF regulon. In order to investigate their role in the response of table grapes clusters to 0 ºC long storage as well as to 3-day high CO_2 postharvest treatment, we isolated two partial *CBF* genes (*VvcCBF1* and *VvcCBF4*) and a full-length dehydrin (*VvcDHN1a*) from *Vitis vinifera* cv. Cardinal. Hydrophobic cluster analysis (HCA) identified differences in the secondary and tertiary structure between *Vitis* CBF4s and CBF1s. Overall, our results showed that in table grapes the expression of *CBF* genes is induced mainly in response to CO_2 treatment, suggesting that the response of *DHN1a* in this fruit could be attributed to a cold-inducible CBF-independent pathway.

Introduction

In recent years, transcriptome analysis of cold response have shown that plants induce the expression of genes that encode proteins such as COR (cold-regulated) and dehydrins, which are thought to participate in stress tolerance (Yamaguchi-Shinozaki and Shinozaki, 2006). Dehydrins are induced during periods of water deficit imposed by drought, salinity, and low temperature (Close, 1997). Cold-induced dehydrins have been shown to cryoprotect freezing-labile macromolecules *in vitro* (Hughes and Graether, 2011). In *Vitis*, spliced (*DHN1a* and *DHN1b*) genes encoding YSK_2-type dehydrins as well as unspliced transcripts were induced during exposure to 4 ºC for up to 48 h (Xiao and Nassuth 2006).

Several dehydrin genes contain in their promoter a DNA regulatory element, the C-repeat (CRT) dehydration responsive element (DRE), that imparts responsiveness to low temperature and dehydration (Gilmour et al., 2004). Transcriptional activators that bind to the CRT/DRE, designated as CBF/DREB are highly conserved in plants and normally showed a very quick induction at transcriptional level after exposure to cold. However, in leaves of *Vitis* the accumulation of *CBF3* and *CBF4* transcripts was observed after 1-2 days (Xiao et al., 2006; 2008). Constitutive overexpression of *CBF* in *Arabidopsis* activated the expression of CRT/DRE-containing target genes, including dehydrins under normal growing conditions and enhanced freezing tolerance in nonacclimated plants (Gilmour et al., 2004). Similar results were obtained recently in *Arabidopsis* transformed with *V. riparia CBF1* and *CBF4* (Siddiqua and Nassuth, 2011).

Low temperature is the most important mechanism for maintaining postharvest fruit quality. Table grapes are classified as chilling tolerant but their storage life at low temperature is limited by their high susceptibility to fungal decay and sensitivity to serious water loss after harvest. In previous works, we have shown the efficacy of a 3-day CO_2 pretreatment in maintaining table grapes quality (Romero et al., 2006; Sanchez-Ballesta et al., 2006). Likewise, the activation of cold-defense responses in the first stage of grape storage at 0 ºC seems to be related to the perception by the fruit of temperature shifts, which was less noticeable in CO_2-treated grapes (Sanchez-Ballesta et al., 2007). Different works have shown the importance of CBFs and dehydrins in the response of *Vitis* plants to cold stress, however their role in fruit under postharvest cold

long storage as well as their relationship with cold tolerance induced by high CO_2 levels is still unknown. In order to investigate whether CBFs and DHNs would also be involved in these processes, we present the characterization and expression pattern analysis of *CBF1*, *CBF4* and *DHN1a* in different fruit- and non-fruit tissues of CO_2-treated and non-treated table grapes stored at 0 ºC.

Materials and methods

Plant material

Early-harvesting mature table grapes (*V. vinifera* L. cv. Cardinal) from Sevilla (Spain) were used (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 in two sealed neoprene containers of 1 m^3 capacity. 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 up to 30 days. Five clusters of grapes (approximately 300 g from each cluster) were sampled periodically and the skin, pulp, seeds and rachis were collected, frozen in liquid nitrogen, ground to a fine powder and stored at -80 ºC until analysis.

Cloning of CBF1 and CBF4 partial cDNAs

Total RNA was extracted from different tissues of table grapes (Sanchez-Ballesta et al., 2007) and partial cDNA clones were obtained by RT-PCR as described by Romero et al. (2008). A 527 bp fragment of *VvcCBF1* and a 409 bp fragment of *VvcCBF4* were amplified using degenerate oligonucleotides previously described by Xiao et al. (2006) (Supplementary Table 1). PCR products were cloned into the pGEMT vector (Promega) and sequenced at the Genomic Department of the CIB-CSIC.

Isolation of DHN1a and DHN1b

DNA from leaves of *V. vinifera* cv. Cardinal was extracted as described by Martín-Platero et al. (2007). Genomic *DHN1a* and *DHN1b* sequences were amplified by PCR combining oligonucleotides based on reported *V. vinifera* genomic sequences (Xiao and Nassuth, 2006). *DHN1a* cDNA was amplified on RNA isolated from pulp of non-treated and CO_2-treated grapes stored 3 days at 0 ºC by RT-PCR. Primer pairs used are shown in Supplementary Table 1. Amplified products were cloned and sequenced as described above.

Hydrophobic cluster analysis

Hydrophobic cluster analysis (HCA) program was conducted using the web-based interface at: http://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py?form=HCA. Hydrophobic amino acids [valine (V); isoleucine (I); leucine (L); phenylalanine (F)] and moderately hydrophobic amino acids [methionine (M); tryptophan (W); tyrosine (Y)] separated by four or more nonhydrophobic residues, or by a proline, were placed into distinct clusters, which were grouped by outlines.

Quantitative real-time RT-PCR

Total RNA was extracted three times from each sample as described above. After treatment with DNase I recombinant, RNase free (Roche), cDNA was synthesized from 1 µg of total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). 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). Primer pairs used in the real-time PCR are shown in Supplementary Table 1. The specificity of products was validated by dissociation curve analysis and by agarose gel. *Actin1* 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).

Semi-quantitative RT- PCR

Total RNA was extracted, treated with DNase and used as a template for DNA synthesis as described above. Primers described by Xiao and Nassuth (2006) were used to differentiate spliced and unspliced mRNAs (Supplementary Table 1). The number of cycles was chosen so as not to reach the maximum amplification. Control reactions with *actin1* were performed to analyze that equal amount of template were used in each reaction set. Following amplification, products were visualized by electrophoresis in a 2% agarose gel. The identification of each PCR product was confirmed by sequencing.

Results

Isolation and structural characterization of CBF genes

Two partial cDNA clones, *VvcCBF1* (GenBank accession no. JN566062) and *VvcCBF4* (GenBank accession no. JN566061), were isolated from table grapes. The deduced amino acid sequences of VvcCBF1 and VvcCBF4 were 98% and 100% identical with VvCBF1 (GenBank accession no. AY390372) and VvCBF4 (GenBank accession no. AY706986) from *V. vinifera* cv. Chardonnay. As it has been indicated previously by Xiao et al. (2006; 2008), both genes contained typical features of CBF proteins including an AP2 DNA-binding domain, DSAWR and a putative acidic C-terminal domain (data not shown). The HCA of the acidic C-terminal region from AtCBF1 showed five hydrophobic clusters (HC2-HC6), which are known to be responsible for conferring trans-activation (Wang et al., 2005). When the full-length amino acid sequence of the close homologues CBF1 and CBF4 from *Vitis* were compared by HCA, the five clusters were also observed in the *Vitis* CBF4s (Fig. 1). By contrast, the analysis of the different *Vitis* CBF1s revealed the presence of only four clusters since HC3 and HC4 become a single cluster due to the absence of a proline. This amino acid is present in AtCBF1 and CBF4s from Vitaceae between both clusters, and as it often breaks secondary structures it is currently considered a cluster breaker (Fig. 1).

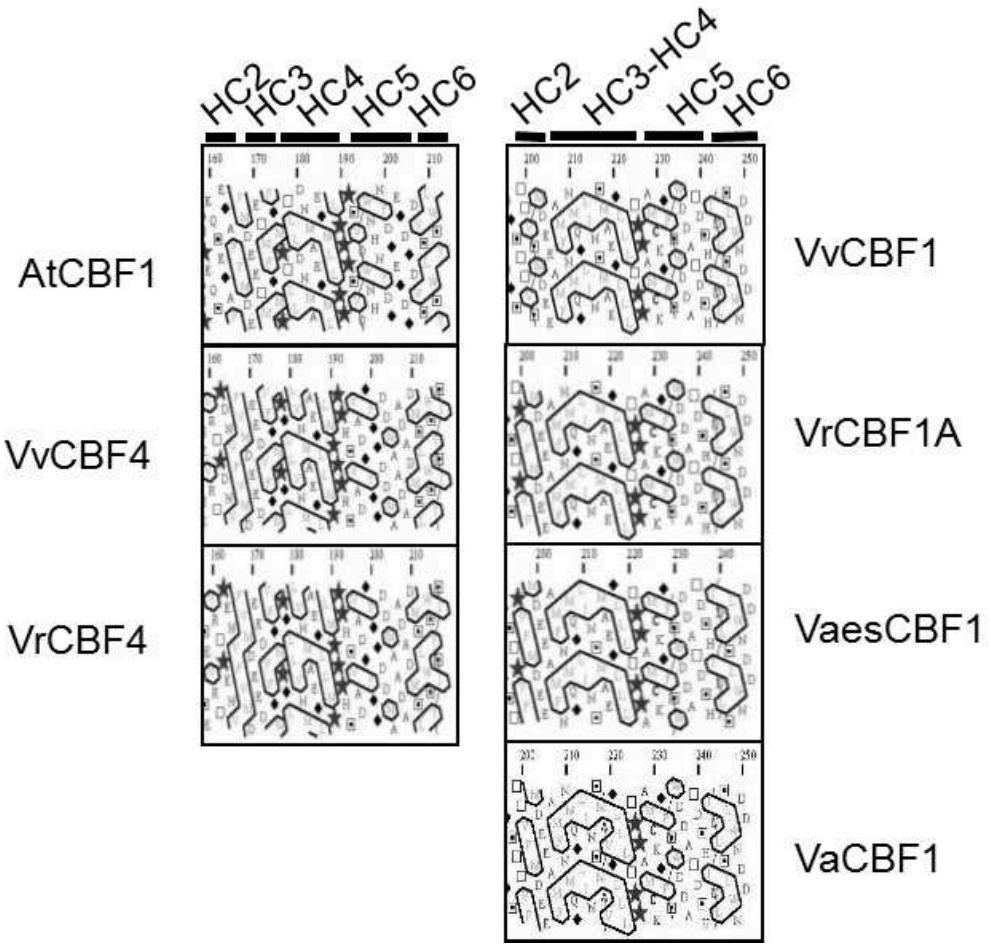

Fig. 1. Hydrophobic cluster analysis (HCA) of AtCBF1 (AY667247) from *A. thaliana*; VaesCBF1 (EF150888) from *V. aestivalis*; VaCBF1 (DQ517296) from *V. amurensis*; VvCBF1 (AY390372), VvCBF4 (DQ497624) from *V. vinifera* cv. Chardonnay and VrCBF1A (AY390370) and VrCBF4 (AY706986) from *V. riparia* COOH-terminal 50 amino acid residues. Each hydrophobic cluster is identified above the HCA output. Classic symbols were used for amino acids except for proline (★), glycine (♦), theronine (□) and serine (■).

Cloning and sequence analysis of DHN1 genes

Two fragments of dehydrins were isolated by PCR using genomic DNA derived from leaves of *V. vinifera* cv. Cardinal as template. Sequence analysis indicated that the 503 bp fragment (*VvcDHN1a*; GenBank accession no. JN689936) shared 99.4% identity with the dehydrin from *V. vinifera VvDHN1a* (GenBank accession no. AY706989), previously described by Xiao and Nassuth (2006), whereas the 489 bp sequence

(*VvcDHN1b*; GenBank accession no. JN689937) was 99.6% identical to *VvDHN1b* (GenBank accession no. AY706990). Sequence analysis by using the software program NetPlantGene available online revealed that the coding region of *VvcDHN1a* and *VvcDHN1b* was comprised of 2 exons and 1 intron as has been indicated previously by Xiao and Nassuth (2006).

RT-PCR strategy was used to isolate two cDNAs containing 390 bp and 474 bp, respectively. Sequence analysis of the predicted protein of the smaller cDNA showed that it shared 100% identity with dehydrin from *V. vinifera* VvDHN1a (AY706989), which belongs to the dehydrin type 'YSK_2'. The 474 bp cDNA corresponded to the *VvcDHN1a* unspliced isoform with the retained intron that coded for a truncated YS protein. It is important to note that, whereas a genomic fragment identical to *VvDHN1b* was isolated; no cDNA homologous to *VvDHN1b* was amplified by RT-PCR.

Response of VvcCBF1, VvcCBF4 and VvcDHN1a genes to low temperature and high CO_2 levels in different tissues of table grape bunches.

To analyze the mechanisms associated with the response of table grape bunches at 0 ºC and to determine whether high CO_2 levels could modulate them, we investigated changes in *VvcCBF1* and *VvcCBF4* gene expression using the quantitative RT-PCR method while *VvcDHN1a* spliced and unspliced transcript levels were analyzed by semi-quantitative RT-PCR. As shown in Fig. 2A, neither storage at 0 ºC nor exposure to high CO_2 levels induced any *VvcCBF1* or *VvcCBF4* gene expression in table grape skin. By contrast, whereas exposure to 0 ºC did not affect the expression of either gene in the pulp, the application of high CO_2 levels for 3 days at 0 ºC did induce the accumulation of *VvcCBF1* and *VvcCBF4* (3.6-fold and 3.4-fold, respectively), which decreased when treated fruit were transferred to air for 12 days (Fig. 2B). In seeds, a slight accumulation of *VvcCBF1* was observed in response to 0 ºC and 3-day CO_2 treatment (Fig. 2C). However, a sharp induction of *VvcCBF4* gene expression was observed in response to both 0 ºC and CO_2 after 3 days, although it was 1.45 times higher in the seeds of non-treated fruit. This response was transitory and prolonged storage reduced *VvcCBF4* gene induction both in non-treated and CO_2-treated fruit. When we analyzed gene expression in a non-fruit tissue such as rachis we observed a sharp induction of *VvcCBF4* gene expression but only in response to CO_2 after 3 days (6.2-fold), decreasing 1.8 times

when treated bunches were transferred to air for up to 12 days (Fig. 2D). It is important to note that no expression was detected in any tissues analyzed after 33 days at 0 ºC (data not shown).

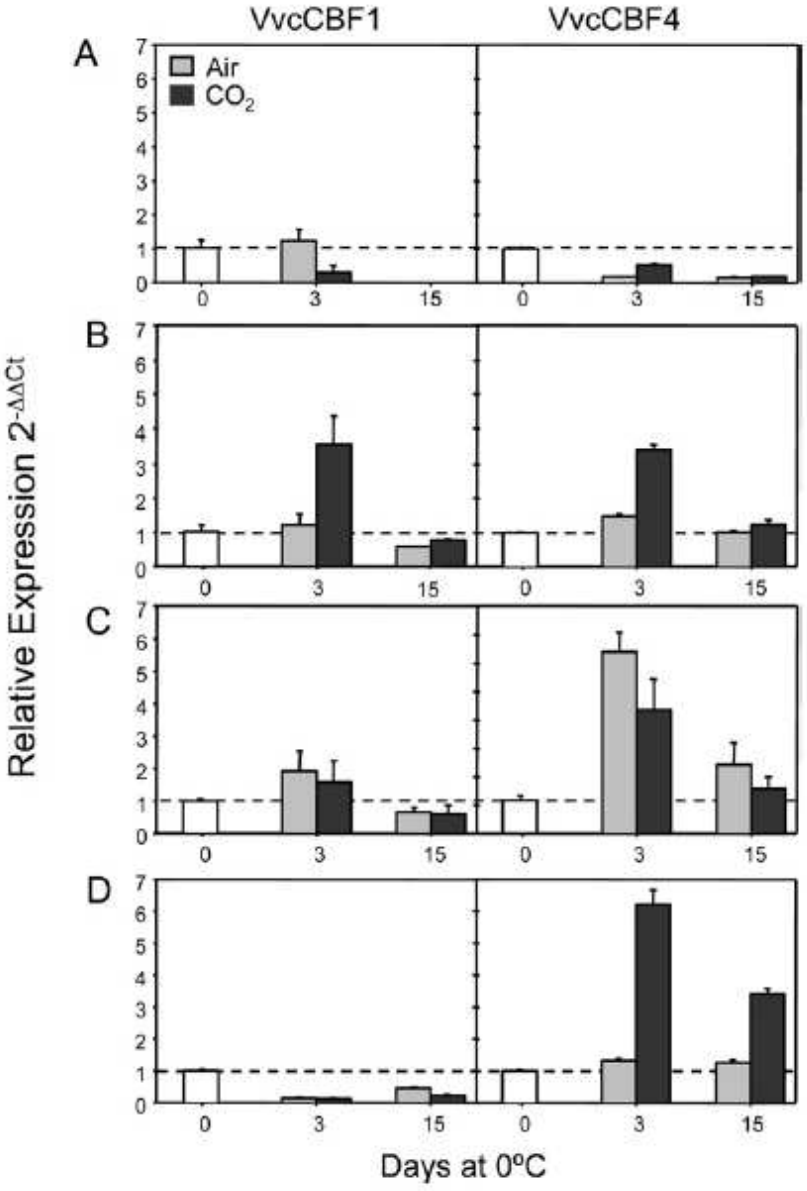

Fig. 2. Effect of low temperature and 3-day high CO_2 pretreatment on *VvcCBF1* and *VvcCBF4* expression in the skin (A), pulp (B), seeds (C) and rachis (D) of Cardinal bunches stored at 0 ºC. Quantitative RT-PCR results were normalized to *actin1* and the results were calculated relative to a calibrator sample (time 0). Data represent means ± SD, n = 3.

To perform the semi-quantitative RT-PCR analysis we used different pairs of primers so that we would be able to distinguish between spliced and unspliced *VvcDHN1a* and *VvcDHN1b* (see Supplementary Table 1). Our results indicated that

only *VvcDHN1a* was expressed in the different tissues analyzed as was shown by the fragments size and confirmed by cloning and sequencing of the PCR products. *VvcDHN1a* spliced transcripts were predominant in all the different tissues analyzed, although the pattern of expression varied depending on the tissue and storage conditions. Thus, in the skin of treated and non-treated fruit a progressive increase in the levels of spliced transcript was observed from day 3 to 33 days, reaching higher values in CO_2-treated samples (Fig. 3A). On the other hand, only high CO_2 levels modulated the accumulation of unspliced transcripts, increasing slightly after 15 days and then decreasing after 33 (Fig. 3A). In the pulp, *VvcDHN1a* expression was also detected in response to 0 ºC and high CO_2 levels, both in spliced and unspliced transcripts but the levels reached were different (Fig. 3B). Low temperature increased progressively the accumulation of the *DHN1a* spliced form after 3 days, whereas the accumulation of unspliced transcript was detected after 15 days. Likewise, 3 days of CO_2 treatment at 0 ºC slightly increased both spliced and unspliced transcript levels in pulp, increasing the accumulation when treated fruit were transferred to air for up to 33 days (Fig. 3B). In the case of seeds, the results revealed that *DHN1a* spliced transcripts were detected in time 0 samples, increasing in response to long exposure to 0 ºC (33 days; Fig. 3C). By contrast, 3 days of CO_2 pretreatment was enough to increase *VvcDHN1a* spliced gene accumulation in this tissue, being maintained when grapes were transferred to air (Fig, 3C). However, no expression of the *DHN1a* unspliced transcripts was detected in seeds samples at time 0 although 0 ºC or high CO_2 levels did induce their accumulation but with a different pattern. Thus, while storage at 0 ºC induced *DHN1a* unspliced transcripts after 15 days until the end of the storage, the 3-day high CO_2 treatment increased the levels of *DHN1a* unspliced transcripts, maintaining this accumulation only after 12 days in air and being undetectable at the end of the storage (Fig. 3C).

We also analyzed the expression pattern of *DHN1a* spliced and unspliced transcripts in the rachis. The results indicated that spliced transcripts accumulated constitutively in treated and non-treated rachis, while unspliced transcripts were induced both in treated and non-treated rachis after 15 days at 0 ºC, maintaining these levels until the end of storage (Fig. 3D).

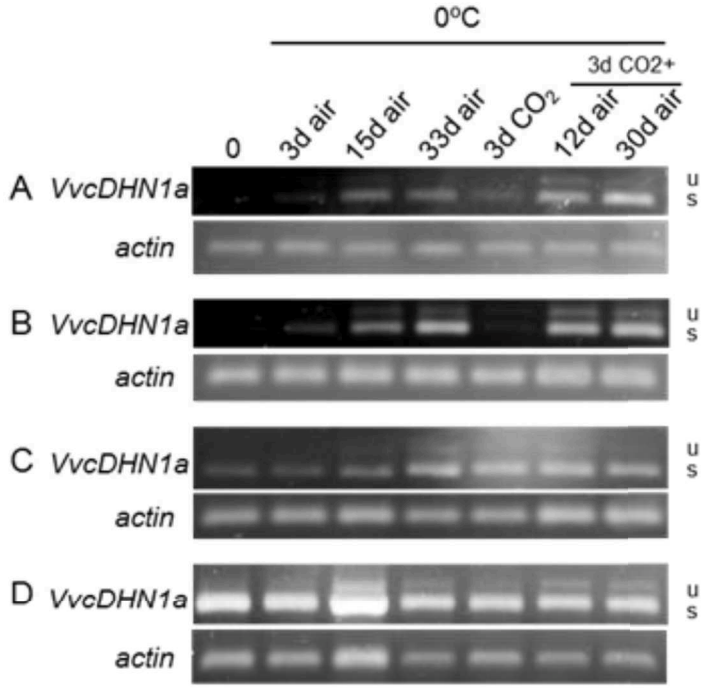

Fig. 3. Effect of low temperature and 3-day high CO_2 pretreatment on *VvcDHN1a* spliced and unspliced mRNAs accumulation in the skin (A), pulp (B), seeds (C) and rachis (D) of Cardinal bunches stored at 0 ºC. The levels of *VvcDHN1a* spliced and unspliced transcripts were determined by semi-quantitative RT-PCR analysis. u: fragments produced on unspliced transcript; s: fragments produced on spliced transcripts. RT-PCR products of *actin1* mRNA are provided as a standard for normalization.

Discussion

The CBF gene family plays a prominent role in the cold resistant process by triggering the cold response transcriptional pathway. We have isolated partial *CBF1* and *CBF4* cDNAs from *V. vinifera* cv. Cardinal that shared a 98% and 100% identity with *CBF1* and *CBF4* from *V. vinifera* cv. Chardonnay, respectively (Xiao et al., 2006; 2008). Differences between both CBFs affected the predicted p*I* value, which was acidic in VvCBF4 (5.3), as is the case of most dicot CBFs, whereas in VvCBF1 the predicted p*I* was basic (8.9) (Xiao et al., 2006; 2008) as was observed in CBF1s from Vitaceae. Nonetheless, the putative activation domain at the C-terminal maintained a strong acidic character in both CBFs. According to Wang et al. (2005), the presence of numerous hydrophobic residues in this acidic C-terminal domain makes it a strong candidate for possessing trans-activation properties. To identify these residues the full-length closely

homologous protein sequences of CBF1 and CBF4 from *Vitis,* as well as AtCBF1 from *Arabidopsis* were compared by HCA. This analysis identified five clusters (HC2-HC6) in *Vitis* CBF4s as was observed in AtCBF1 (Wang et al., 2005), in comparison with the four cluster (HC2-HC5) indentified previously by Xiao et al. (2008). However, our analysis indicated for the first time that only four clusters were identified in *Vitis* CBF1s as had already been detected in monocot CBFs. This is due to the fact that HC3 and HC4 formed just one cluster, because a glutamine (Q) replaces the proline residue (P), which is considered a cluster breaker and is present between HC3 and HC4 in the majority of the dicot CBFs. The effect that this change would have on the trans-activating properties of CBFs remains unknown. However, the fact that Xiao et al. (2008) observed that VrCBF4 was a more effective activator than VrCBF1 could be related to these differences. Wang et al. (2005) concluded that only when HC2, HC3 and HC4 were eliminated the trans-activation was compromised to levels below the sensitivity of northern analysis. Our HCA analysis also showed for the first time that *Vitis* CBF4s failed to generate a reliable HC5 since it lacks the tryptophan residue (W) present in AtCBF1 and *Vitis* CBF1s, what interestingly, it is another aspect that Vitis CBFs share with most of the monocot CBFs (Wang et al., 2005). These authors suggested the importance of this residue because AtCBF1 HC5 mutants, in which tryptophan was substituted by an alanine, showed one of the most severe reduction in trans-activation as well as the degeneration of HC5 structure.

Low temperature induces the expression of most CBFs analyzed. In leaves, apical tips and buds of *V. riparia* and *V. vinifera,* the expression of CBF 1-4 was enhanced upon exposure to low temperature (Xiao et al., 2006; 2008). However, we observed that with the exception of seeds, storage at 0 ºC by itself was not sufficient to activate *VvcCBF1* and *VvcCBF4* gene expression in the skin, pulp and rachis of table grapes cv. Cardinal. By contrast, a combination of 3-day high CO_2 levels and low temperature induced the expression of both CBFs in the pulp and in the case of *VvcCBF4* also in the rachis. Moreover, it should be noted that *VvcCBF1* and *VvcCBF4* expression was dependent on tissue. Thus, neither of the CBFs showed expression in skin and *VvcCBF1* was not accumulated in rachis. Another feature of both CBFs is that by combining high CO_2 and low temperature, gene expression remained stable after a relatively long period of time (3 days) compared with the few hours reported in most of the CBFs studied. Despite the fact that further analyses are needed to determine how the

differences observed in the CBF activating region could affect the output necessary for plant survival under stress conditions, given the overall results, we might hypothesize that the *CBF* expression activated by high CO_2 levels in the pulp could help table grapes to face temperature shifts at 0 ºC. On the other hand, the high *CBF4* gene expression observed in rachis of treated clusters stresses the fact that high CO_2 treatment modulates the response of table grapes to low temperature not only in fruit tissues but in rachis, as is evident from the improvement in appearance (Sanchez-Ballesta et al., 2006) and the increase in unfreezable-water content (Goñi et al., 2011).

Genomic DNA analysis from *V. vinifera* cv. Cardinal revealed the presence of two *DHN1* genes, *VvcDHN1a* and *VvcDHN1b*, identical to those previously described by Xiao et al. (2006). However, by RT-PCR using total RNA from pulp of CO_2-treated and non-treated table grapes, we were only able to isolate *VvcDHN1a* that encoded for a typical YSK_2-type dehydrin and that shared a 100% identity with VvDHN1a from *V. vinifera* (Xiao et al. 2006). Our results showed intron retention in dehydrin transcripts not only in response to low temperature as indicated previously Xiao et al. (2006), but also to high CO_2 levels applied at 0 ºC in skin, pulp, seeds and rachis of table grapes. This is, to our knowledge, the first time that this fact has been described for a gaseous treatment in plants. On the other hand, whereas exposure to 0 ºC and high CO_2 levels induced spliced transcripts of *VvcDHN1a* in skin, pulp and seeds, a constitutive expression was observed in rachis. Cold induced mainly unspliced *VvDHN1a* transcripts in leaves and buds of *V. riparia*, whereas induction of both spliced and unspliced transcript levels were observed in these tissues in *V. vinifera* (Xiao and Nassuth 2006). Furthermore, these authors analyzed spliced and unspliced gene expression for up to 48 hours at 4 ºC, while in our study cold transcript regulation remained after a long period of storage (33 days). Another difference with the above mentioned work is that we did not observe any accumulation of spliced and unspliced *VvcDHN1b* transcripts in the different tissues analyzed in response to low temperature and/or high CO_2 levels. By contrast, cold treatment increased unspliced *VvDHN1b* transcript in leaves as well as spliced and unspliced *VvDHN1b* in buds (Xiao and Nassuth 2006).

Considering our overall results, the fact that the skin, seed and rachis of table grapes did not show any induction of *CBF1* gene expression in response to cold and/or high CO_2 levels, and that *CBF4* accumulation was observed mainly in response to CO_2 treatment, suggest that the response of *DHN1a* in table grapes could be attributed to

other cold-activated pathways different from *CBF1* and *CBF4* which have yet to be determined. Another remarkable aspect of this study is that, with the exception of seeds, *CBF1* and *CBF4* gene expression was not induced at 0 ºC. Moreover, for the first time, high CO_2 levels at low temperature have been seen to induce the expression of *CBF1* and *CBF4*, and that this fact has been positively correlated with the different response to temperature shifts at 0 ºC and the maintenance of table grape quality observed previously in CO_2-treated clusters.

Acknowledgements

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Appendix A. Supplementary data

Supplementary Table 1

Materials and Methods Section	Use	Name	Sequence	Reference
Cloning of *CBF1* and *CBF4* partial cDNAs		CBFd1-H	**5'-TTYMRDGAGACDMGDCACCC-3'**	Xiao et al., 2006
		CBFd4-C	**5'-ARRAGMADNCCYTCNGCCAT-3'**	Xiao et al., 2006
Isolation of *DHN1a* and *DHN1b*	Cloning of *DHN1a* and *DHN1b* DNA sequence	DHN1_Fw_a	**5'-ATGGCATATCAGCAAGATCCA-3'**	
		DHN1-C578	**5'-CATGTCCCCTTCATTTCCAG-3'**	Xiao and Nassuth 2006
	Cloning of *DHN1a* cDNA sequence	DHN1_Fw_a	**5'-ATGGCATATCAGCAAGATCCA-3'**	
		DHN1_Rv_a	**5'-CTAGTGGCCCCCAGGCAGCTTCTC-3'**	
Quantitative Real-Time PCR	*CBF1*	VvcCBF1_Fw	**5'-TGGAAGTTTCTCCGGTGGAT-3'**	
		VvcCBF1_Rv	**5'-TGAACACTGTCGTTGAACCATCT-3'**	
	CBF4	VvcCBF4_Fw	**5'-GACGCCAAGGACATTCAGAAG-3'**	
		VvcCBF4_Rv	**5'-CATAACCCCATCATTCTCCATTG-3'**	
	Actin (Accesion #: XM_002282480)	Actin1_Fw	**5'-CTTGCATCCCTCAGCACCTT-3'**	
		Actin1_Rv	**5'-TCCTGTGGACAATGGATGGA-3'**	
Semi-quantitative RT-PCR	Amplification of spliced and unspliced *DHN1a*	DHN1_H225	**5'-CGACCAGTATGGAAACCCAG-3'**	Xiao and Nassuth 2006
		DHN1-C578	**5'-CATGTCCCCTTCATTTCCAG-3'**	Xiao and Nassuth 2006
	Amplification of unspliced *DHN1a* and *DHN1b*	DHN1_Fw_a	**5'-ATGGCATATCAGCAAGATCCA-3'**	
		DHN1-C383	**5'-CATTTGGCATGGCAACTTAC-3'**	Xiao and Nassuth 2006

CAPÍTULO 6

Changes in table grapes transcriptome at two ripening stages in response to low temperature and high CO_2 levels

Carlos Fernandez-Caballero, Raquel Rosales, Irene Romero, Mª Isabel Escribano, Carmen Merodio, Mª Teresa Sanchez-Ballesta*

Plant Science (Submitted)

* Corresponding author

ABSTRACT

Table grapes (*Vitis vinifera* cv. Cardinal) are highly perishable and their quality deteriorates during postharvest storage at low temperature due mainly to sensitivity to fungal decay and senescence of rachis. The application of a 3-day CO_2 treatment (20% CO_2 + 20% O_2 + 60% N_2) at 0ºC reduced total decay and retained fruit quality in early and late-harvested table grapes during postharvest storage. To study the transcriptional responsiveness of table grapes to low temperature and high CO_2 levels in the first stage of storage and how the ripeness stage affect these changes, we have performed a comparative large-scale transcriptional analysis using the custom-made GrapeGen GeneChip®. Low temperature, in the first stage of storage, led to an intense change in grape skin transcriptome independently of fruit ripeness stage but with different changes in each one; whereas slightly differences were observed in CO_2 treated samples in comparison to freshly-harvested fruit. Functional enrichment analysis revealed that major modifications in the transcriptome profile of early- and late-harvested grapes storage at 0ºC are linked to biotic and abiotic stress-responsive terms. However, in both cases there is a specific reprogramming of the transcriptome during the course of low temperature storage in order to withstand the stress linked to gluconeogenesis, photosynthesis, mRNA translation and lipid transport, in the case of early-harvested grapes, and to protein folding stability and intracellular membrane trafficking in late-harvested grapes. The high CO_2 pretreatment, which maintains table grapes quality, seems to be an active process requiring the activation of transcription factors and the regulation of protein function by kinases in early-harvested grapes, and the activation of process associated to the maintenance of energy in late-harvested grapes.

Introduction

The maintenance and improvement of fruit quality during postharvest life is becoming increasingly important as a response to the market situation where consumers demand products of high quality throughout the year. Low temperature storage has been used as the main method to extend the postharvest life of horticultural commodities but sometimes their use is limited depending on susceptibility to chilling injury and/or fungal decay. The use of atmospheres modified in O_2 and CO_2 concentrations may extend the storage life at low temperature of different fruit and vegetables by reducing respiration, maintaining firmness and controlling decay. Although there has been much information on the use of high CO_2 levels as modified and controlled atmospheres to maintain fruit quality (reviewed by Watkins 2000; Yahia, 2009), little is known about the application of short-term high-CO_2 treatments at low temperature. In previous work, we have shown the efficacy of a 3 day pretreatment with high CO_2 levels at 0 ºC in maintaining the quality of table grapes and controlling total decay (Romero et al., 2006; Sanchez-Ballesta et al., 2006). This gaseous pretreatment minimized or modified, at transcriptional level, the activation of cold-response mechanisms observed in non-treated grapes related to the perception of temperature shifts at 0 ºC by the fruit (Sanchez-Ballesta et al., 2007; Romero et al., 2008; Rosales et al., 2013). The majority of previous work in the molecular biology field related with the fruit response to high CO_2 treatments has been confined to the level of individual genes or small groups of genes. However, recently, by using microarray, Becatti et al. (2010) reported that in detached wine grapes a treatment with 30 KPa of CO_2 during 3 days at 20 ºC was effective in altering the general metabolism, being functional categories related to protein and hormone metabolism, transport and stress highly represented in both skin and pulp tissues. Moreover, these authors also observed that the skin cells appeared to undergo more pronounced changes in transcriptome profiling in response to high CO_2 than the pulp. Thus, fermentation, CHO metabolism, and redox regulation functional categories were represented only in the skin. In addition, short-term high-CO_2 treatment in strawberry was effective in altering the expression of genes with homologies to enzymes involved in cell wall metabolism, ethylene action and stress when a heterologous microarray approach was used (Ponce-Valadez et al., 2009).

Beside this, it is important to mention that the degree of maturity and harvest date affect the quality of stored fruit (Shin et al., 2008). Likewise, the effect of high CO_2 levels depends on cultivar, storage length and maturity (Terry et al., 2009). Thus, Nunes et al. (2002) reported that three-quarter colored strawberries responded better to CA storage at low temperature, maintaining greater firmness and better color, and developing much less decay than fully red fruit . In table grapes, a delay in the harvest date was decisive to increase antioxidant activity by inducing anthocyanin accumulation in stressed non-treated fruit stored at 0 ºC but not in CO_2-treated ones (Romero et al., 2009). However, to our knowledge no transcriptome profiling analysis on the response of fruit to low temperature and high CO_2 levels at different ripeness stages have been performed. Thus, in the present work we used a custom made Affymetrix GrapeGen GeneChip™ (Lijavetzky et al., 2012) to investigate gene expression responses of table grape skin to low temperature and high CO_2 levels and to understand how the ripeness state could modify these changes.

Material and methods

Plant Material

Table grapes (*Vitis vinifera* L. cv. Cardinal) were harvested from a commercial orchard in Sevilla (Spain) twice over 3 weeks. The first harvest began on the 13th June 2005 (early harvested, maturity index of 12.45±0.01) and the second on the 5th July 2005 at commercial ripeness (late harvested, maturity index of 41.08±0.30). The field-packaged bunches were transported to the laboratory (freshly-harvested, FH) and those free from physical and pathological defects were randomly divided into two lots and stored during 3 days at 0±0.5 ºC and 95% relative humidity (RH) in two sealed neoprene containers of 1 m^3 capacity. One lot of 6 replicate bunches were kept under normal atmosphere (non-treated fruit) and the other one under a gas mixture of 20% CO_2 + 20% O_2 + 60% N_2 (CO_2-treated fruit). At time 0 (FH) and after 3 days of storage under air or CO_2 conditions, the skin of three biological replicates, each consisting of 2 bunches, were collected, frozen in liquid nitrogen, ground to a fine powder and stored at - 80 ºC until analysis.

RNA isolation and GeneChip® hybridization

Total RNA was extracted from skin samples according to the procedures described by Zeng and Yang (2002). RNA samples were treated with DNase I recombinant-RNase free (Roche) for removing possible genomic DNA contamination. Thereafter, final RNA purification was performed using RNeasy Moni Kit (Qiagen) following the manufacturer's instructions. Samples were analyzed at the Genomics Unit of the Spanish National Centre for Biotechnology (CNB-CSIC, Madrid). RNA integrity analyses were performed with an Agilent's Bioanalyzer 2100. A custom Affymetrix GrapeGen GeneChip® (Lijaveztky et al., 2012) containing 23,096 probe sets corresponding to 18,711 non redundant transcripts was used. Probe synthesis, microarrays hybridization, washing, staining and scanning with the GeneChip™ Scanner 3000 were performed according to the Affymetrix GeneChip® Expression.

Microarray data processing

Three biological replicates per experiment were processed to evaluate intra-specific variability. Signal values from all the microarray hybridizations were normalized together by applying the RMA (Robust Multiarray Average) algorithm using RMAExpress (Bolstad et al., 2003). Average of expression values for redundant probe sets was performed using GEPAS (Gene Expression Pattern Analysis Suite) software v4.0 (Herrero et al., 2003). Multivariate analysis as Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) (ANOVA test, $p < 0.01$ after Bonferroni adjusted correction) over the normalized dataset was performed by using the MeV (MultiExperiment Viewer) TM4 Microarray Software Suite (Saeed et al., 2003).

Microarray data analysis

To identify differentially expressed genes in response to low temperature storage in air or high CO_2 levels, SAM (Significance Analysis Microarray) algorithm (Tusher et al., 2001) implemented in TIGR MEV 4.5.1 (Saeed et al., 2003) was used. SAM analyses were computed using two-class unpaired comparison on 1000 permutations. Genes that satisfied the statistical threshold FDR (false discovery rate) lower than 0.001

and fold differences of at least 1.5 were considered to be differentially expressed. Area-proportional Venn diagrams were generated in order to compare and visualize selected lists by using BioVenn software (Hulsen et al., 2008).

The Database for Annotation, Visualization and Integrated Discovery (David 6.7; Huang et al., 2009, http://david.abcc.ncifcrf.gov) was used to classify the genes into functional groups based on Gene Ontology. Medium stringency was applied for the analyses. DAVID analysis identifies significantly enriched biological themes by examining for enrichment in over 40 different publicly available annotation categories, analyzing up- and down-regulated sets separately. Significance was determined using a modified Fisher's exact statistic (EASE score), and significantly enriched biological themes were identified as clusters of annotated terms (Huang et al., 2009). Annotation terms with EASE scores of ≤ 0.05 were taken as significantly enriched in a group of related genes and used to assign functional annotation to the group.

A cluster enrichment score of 1.3 for an annotation cluster is equivalent to non-log scale 0.05, and therefore scores of 1.3 or greater are considered enriched (Huang et al., 2009). Fold-enrichment scores were also used to indicate the magnitude of enrichment for individual terms and fold-enrichment scores greater than 1.4 are suggestive of an informative change (Huang et al., 2009).

Quantitative Real-time RT-PCR (RT-qPCR)

Total RNA extraction was extracted as described above and treated with DNAse I recombinant-RNase free (Roche) to avoid DNA contamination. Then, 1 µg of RNA was used to synthesize cDNA by using the iScript™ Reverse Transcription Supermix (Bio-Rad). Transcript levels were determined by qRT-PCR using a iCycler iQ thermal cycler (Bio-Rad) and SYBR Green dye (Bio-Rad). Gene-specific primers pairs for qRT-PCR (Table 1) were designed with Primer 3 software (Koressaar and Remm, 2007) using as templates the gene sequence from the V. vinifera 12X genomic sequence assembly (Adam-Blondon et al., 2011; available at http://bioinfogp.cnb.csic.es/tools/GrapeGendb/). Each gene was evaluated at least in two independent runs. 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 (ΔRn) exported as output file and subsequently imported into the LinReg PCR program. The transcription levels were calculated relative to the calibrator sample (time 0) after normalization to the reference gene *Actin1* from *V. vinifera* using the Pfaffl method (Pfaffl et al., 2001). Correlation coefficients (r^2) between the log_2-transformed expression values as measured by microarray and RT-qPCR were calculated. 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.

Results and discussion

Main variation components in the grape skin transcriptome in response to low temperature and high CO_2 in the first stage of storage

To obtain an overview of how low temperature and high CO_2 levels affect the transcriptome of table grape skin in the first stage of storage and to analyze whether the ripeness state affect these changes we have used the GrapeGen GeneChip®. As a first approach to analyze the complexity of the gene expression dataset and to cluster samples according to their global gene expression profile, a PCA and HCA over the expression data of the 18 analyzed samples, corresponding to 3 biological replicates of FH plus, non-treated and CO_2-treated samples at two ripeness stages, was performed (Figure 1). In all conditions, the transcriptional profiles of the 3 separate RNA replicate samples were tightly clustered and, therefore, the experiment was considered highly reliable for further analysis. Principal component 1 (PC1) explained 53% of the expression variation and separated FH and CO_2-treated samples in relation to the ripeness state (early vs. late harvested), being each CO_2-treated sample close to their respective FH. However, non-treated fruit at both ripeness states clustered far from their respective FH. PC2 accounted for 16% of the variance in the data set and separated non-treated samples according to the ripeness stage. Interestingly, samples from non-treated fruit harvested at early stage grouped near to samples from FH and CO_2-treated late harvested grapes, far from their respective FH samples. HCA confirmed results obtained

by PCA, where FH and 3-days CO_2 treated samples at each ripeness state were clustered together but in independent branches, being samples from non-treated fruit at early stage close to FH and treated samples from late harvested fruit. Curiously, although 3-days non-treated samples are in the same branch of their respective FH, are clustered into an independent group. These results indicated that low temperature in the first stage of storage led to an intense change in grape skin transcriptome independently of fruit ripeness stage but with different changes in each one, whereas slightly differences were observed in CO_2 treated samples in comparison to FH fruit. These findings are in concordance with our previous work where analyzing individual genes, the activation of cold-defense responses in the first stage of grape storage at 0 ºC seems to be related to the perception of temperature shifts by the fruit, which was less noticeable in CO_2-treated grapes (Sanchez-Ballesta et al., 2007).

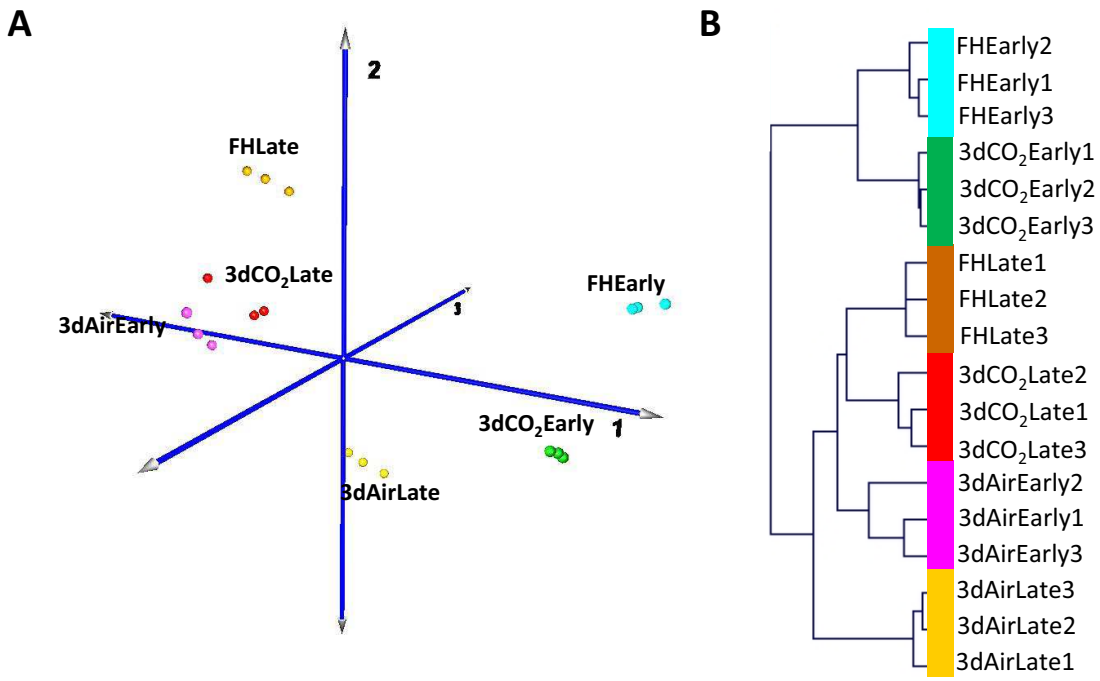

Figure 1. (A) Principal Component Analysis (PCA), (B) Hierarchical Cluster Analysis (HCA) of transcriptome data from the skin of 3-days CO_2-treated and non-treated grapes at early and late stages. Colors in PCA for each condition are consistent with those in HCA. Three independent biological replicates of each condition were used and the analysis was carried out from expression data previously RMA normalized between samples and after the average of expression values among redundant probesets.

Transcriptional bases for the response of grapes to low temperature and high levels of CO_2 at two ripeness stages

Venn diagrams summarize the number of differentially expressed genes (SAM, FDR 0.001 and ≥ 1.5 or ≤ -1.5 fold change) in grape skin for early (Figure 2A) or late (Figure 2B) harvested CO_2-treated and non-treated fruit respect to each respective FH fruit. Major changes in the number of differentially expressed genes occurred in non-treated samples at early stage where a total of 4,990 non-redundant accumulated transcripts were identified, representing 26.6% of the total analyzed. Among them, 61% were down-regulated and 39% up-regulated by storage at low temperature. In late-harvested fruit, exposure to 3 days at 0 ºC also prompted major changes in gene expression (3,257), with 2,131 non-redundant transcripts (66%) up-regulated and 1,126 (34%) down-regulated. The number of genes with a modified expression in response to 3-days high CO_2 treated samples, in comparison to FH, was less remarkable in both ripeness stages respect to non-treated fruit, but major changes also occurred in the early stage (830) compared to the late one (632). In both cases, the number of genes up-regulated was higher than the down-regulated.

In order to validate the microarray data, 10 genes were randomly selected among the up- and down-regulated differentially expressed genes and were analyzed by qRT-PCR in the same 3 biological replicates of FH, 3-days non-treated and CO_2-treated samples at two ripeness stages used for microarray analysis (Table 1). Linear regression analyses displayed reliable r^2 correlation coefficients between 0.73 and 0.99, confirming the validity of the microarray results.

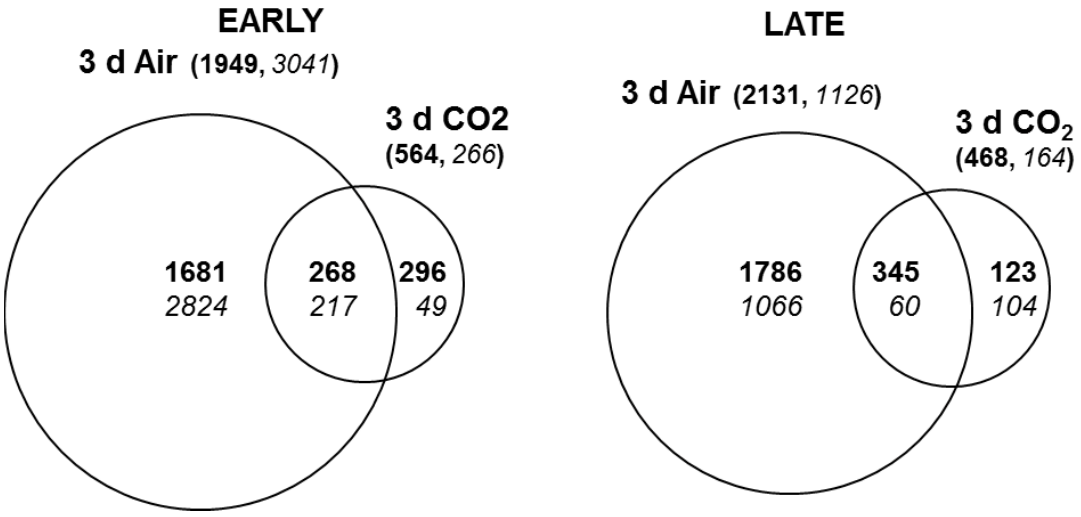

Figure 2. Venn diagrams showing differentially expressed genes (SAM analysis, FDR<0.001) in the skin of CO_2-treated and non-treated grapes at the early and late ripeness stage. Expression levels for up- (bold) and down-(italics) genes in these table grapes were compared to those of FH fruit at each ripeness stage. Numbers in brackets are the sum of all induced or repressed genes in the early or late stage. The sizes of the circles are consistent with the total number of differentially expressed genes for each condition.

Table 1. Selected genes and primers used for quantitative RT-PCR and comparison between GrapeGen GeneChip® microarray and RT-qPCR gene expression data. Multiple linear regression analysis (r^2) was performed for each reported gene including samples from all comparisons.

Unique transcript	Gene	Most similar protein	Homology *Arabidopsis*	Fw/Rv	Primer sequence 5'-3'	r^2
VIT_08s0040g00470	CAM7	Calmodulin 7	AT3G43810	F	CCAAGGAGCTAGGGACAGTG	0.91
				R	CTCGGAATGCTTCTTTCAGC	
VIT_04s0079g00690	GSTF11	glutathione S-transferase F11	AT3G03190	F	TCCTACCTCGAATGGGTGAG	0.94
				R	TTCGACAGCCTCTGCTCATA	
VIT_03s0038g02110	DNAJ	Chaperon protein DNAJ 11	AT2G17880	F	GCAGCCTACTCCACCTTGTC	0.98
				R	ACCAGCACTGGTCAGTCTCC	
VIT_06s0004g08190	CRF1	Ethylene-responsive transcription factor cytokinin response factor 1	AT4G11140	F	CCTCCTCCATTCCAACAAGA	0.93
				R	TCCCTCCACTCACCATTAGG	
VIT_09s0018g00240	WRKY40	Putative WRKY Transcription Factor 40	AT1G80840	F	GAAGACGGGGAAGAAAAAGG	0.98
				R	CTTGGGTGGGTCAGTCAGAT	
VIT_02s0012g01040	NAC071	NAC domain-containing protein 71	AT4G17980	F	CCATGGCTTATTGCAGGACT	0.99
				R	CAAATTCAACTTCCCCAGGA	
VIT_03s0088g00710	PRP1	Pathogenesis-related protein 1 precursor	AT2G14580	F	GTGGTTCGCACATGCAACT	0.79
				R	CCTTTGTCAACTAAACGCACA	
GSVIVT00015576001	RPS7	ribosomal protein S7	ATCG00900	F	CGATCCGTGAAAAAGATTCAA	0.96
				R	ATGAGTCGATCCGCCTACAC	
VVTU6013_s_at		U-box domain-containing protein 35-like	AT4G25160	F	GTCACTTGATTTCTGTCCCAAT	0.81
				R	CCATTCATTATCCACATCCTCA	
VIT_11s0016g04080	MBF1c	Multiprotein-bridging factor 1c-like	AT3G24500	F	CTTGCGAAGATGGAGAAGGT	0.83
				R	CGAGCGACGGACAAGACAC	
VIT_02s0025g00360	ACS6	1-aminocyclopropane-1-carboxylate synthase-like	AT4G11280	F	GTTCCCATGGGTTTGCTTTA	0.94
				R	GTTGCATCATCCTCCATGTG	
VIT_13s0067g02130	ERD15	Dehydration-induced protein (ERD15)		F	GGAGGAGGAGAAGGAGCATC	0.73
				R	GAGCCTTCTCGAAGTGCCTA	

Functional analysis of the differential gene expression in CO_2-treated and non-treated grapes at both ripeness stages

To understand the biological significance of the molecular changes underlying the effect of 3-days storage at low temperature in table grapes treated and non-treated with high CO_2 levels depending of the harvested time, functional analysis of up- and down-regulated genes using DAVID was performed. The functional annotation chart provides data on over-representation of the GO category terms (Tables 2-4). At the early ripeness stage, transcriptional responses to low temperature in non-treated fruit overlapped broadly with 'response to cold stress' and with 4 other biotic and abiotic stressors and stimuli, including heat, metal ions, bacterium and water deprivation (P<0.05 and fold enrichment (FE) between 2.17 and 1.49) (Table 2). However, the most enriched up-regulated genes affected by low temperature were involved in 'gluconeogenesis' and 'chitin catabolic process' (P<0.05 and FE of 6.62 and 4.54, respectively) (Table 2). Thus, phosphoenolpyruvate carboxykinase 1 (PCK1), two glyceraldehyde-3-phosphate dehydrogenase (GAPC1 and GPAC2) and four chitinases (chitinase 1b, IV, V and POM1 chitinase-like protein 1) were induced (Supplementary Table S1). In this sense, it is important to note that the up-regulation of these chitinases preceded the visual appearance of fungal attack in early-harvested grapes that took place after 12 days at 0ºC (data not shown). Furthermore, in a previous work that expression levels of *chit1b* increased in the skin of table grapes after 3-days storage at 0 ºC and the overexpression of chit1b in *Escherichia coli* showed *in vitro* cryoprotective activity and retained catalytic activity at subzero temperature (Fernandez-Caballero et al., 2009), indicating a putative protective role during storage at low temperature. On the other hand, gluconeogenesis is a fundamental metabolic process that allows organisms to make sugars from non-carbohydrate stores such as lipids and protein. In plants it is widely accepted that PCK plays a pivotal role in gluconeogenesis by catalyzing the conversion of the C4 dicarboxylic acid oxaloacetic acid to phosphoenolpyruvate (PEP) (Benedict and Beevers, 1962; Theodoulou and Eastmond, 2012). Although we have not determined the levels of sugars in the skin, it is important to note that in the pulp, the levels of glucose (100.40 ± 0.48 mg/g FW) were much high than those of sucrose (5.71 ± 0.55 mg/g FW) (Blanch et al., 2011). Furthermore, although it is known that GAPCs act in glycolysis and gluconeogenesis, previous studies have demonstrated that they

play a number of diverse functions in living organisms, including membrane fusion, microtubule bundling, phosphotransferase activity, nuclear RNA export, DNA replication and DNA repair (reviewed by Sirover, 1999). Different works also demonstrated that GAPCs may play roles in plant responses to various stresses, such as cold, drought and hypoxia (reviewed by Kosová et al., 2011; Sanchez-Bel et al., 2012). In plants GAPCs has been localized in the nucleus during cold stress (Bae et al., 2003), and its capacity to bind to DNA has been observed (Holtgrefe et al., 2008), in particular to the coding sequence of the NADP-dependent malate dehydrogenase (MDH) gene (Hameister et al., 2007). This fact seems to indicate that in addition to its role in the gluconeogenesis and glycolysis may be involved in mediating stress signaling and signal transduction to the nucleus.

Another important over-represented GO terms are 'lipid transport' (FE=2.65) with 14 genes, including 6 encoding non-specific lipid transfer proteins (nsLTPs), and 'translational initation' (FE=2.30) (Table 2, Supplementary Table S1). nsLTPs were termed because of their functions transferring phospholipids and fatty acids between membranes *in vitro* (Kader, 1996). Transcript levels of nsLTPs increased in response to drought, salt and cold stresses (Jung et al., 2003). Stabilization of membranes, cuticle deposition and/or changes in cell wall organization, have been claimed as their putative roles in the responses to these stress factors. By other hand, protein synthesis is a key step of gene expression and it is specially regulated at the initiation phase. Translation initiation in eukaryotes depends on many eukaryotic initiation factors (eIFs), playing IF3 a central role in polypeptide chain elongation interacting with many other translation initiation factors and being its expression induced by environmental stress (Kawaguchi and Bailey-Serres, 2002). Between the 9 eIFs induced in the skin of grapes exposed to 0ºC, two eIF3 were identified. In addition, under cold shock in *E. coli*, IF3 is considered to be the most important in terms of the selective translation of cold-shock mRNAs (Giuliodori et al., 2004).

The functional annotation clustering tool, which clusters functionally related annotations into groups and ranks them according to the importance giving them an enrichment score (ES), showed 7 groups with significant ES ≥1.5 (Supplementary table S2). In addition to the terms above mentioned, the second cluster with an enrichment score of 2.12 contained terms associated with 'photosynthetic process', including light reaction and harvesting process. It is well known that cold exposition significantly

affects plant photosynthesis (reviewed by Kosová et al., 2011). A proteomic study comparing the response to 2 ºC of two genotypes of meadow fescue with different frost tolerance showed significant differences for several components of thylakoid-membrane-associated photosynthetic apparatus including light-harvesting complexes (Kosmala et al., 2009).

In late-harvested grapes, the transcriptional response to low temperature were also related to terms associated to stress and stimulus such as 'light intensity', 'nutrient starvation' and 'temperature stimulus' (Table 2). In relation to the storage stressful conditions, genes up-regulated in late-harvested fruit were also associated to 'protein folding' (FE=1.70). Protein folding stability is undoubtedly one of the most challenging problems in organisms undergoing stress conditions. Thus, efficient protein repair systems and general protein stability facilitate survival under sudden changes in the environment. Among the genes involved in this term, it is interesting to note that in addition to heat-shock proteins (Hsps)/chaperones, 7 genes encoding peptidyl-prolyl cis-trans isomerases (PPIases) were up-regulated (Supplementary Table S1). PPIases are enzymes that catalyze the reversible conversion of peptidyl prolyl bond from *cis* to *trans* which is a rate limiting step in the folding proteins (Fischer and Schmid, 1999), suggesting that higher amount of PPIases is required in the skin of late-harvested grapes in order to facilitate the efficient folding of proteins in response to low temperature. The GO term 'small GTPase mediated signal transduction' was also over-represented, including 8 Rab GTPase genes (FE=2.09) (Table 2, Supplementary Table S1) and it is known their function in intracellular membrane trafficking (Zerial and McBride, 2001). Thus, the cold up-regulation of these genes indicates that active membrane trafficking might take place under storage in late-harvested grapes. When functionally related annotations were clustered into groups by using the functional annotation clustering tool, only 1 group was observed contained terms associated with 'response to phosphate starvation' (Supplementary table S3). At the molecular level, recent studies have shown that several proteins carrying the SPX domain are essential for maintaining phosphorous homeostasis in plants (reviewed by Secco et al., 2011). Among genes belonging to this cluster, *SPX1*, *2* and *4* were induced in the skin of late-harvested grapes (Supplementary Table S1). Constitutive overexpression of *OsSPX1* in tobacco plants resulted in decreased total leaf phosphorous concentration and the accumulation of free proline and

sucrose, providing improved cold tolerance compared with the wild-type (Zhao et al., 2009).

Table 2. Functional annotation chart of up-regulated genes from the skin of early- and late-harvested grapes stored 3 days a 0 ºC. Biological Process GO terms, number of gene included (count), *P* value and fold enrichment (FE) are indicated.

Early Harvested

Category	GO Term ID	Go Term	Count	*P* value	FE
GOTERM_BP_FAT	0006094	Gluconeogenesis	4	3.02E-03	6.62
GOTERM_BP_FAT	0006032	chitin catabolic process	4	4.70E-02	4.54
GOTERM_BP_FAT	0009740	gibberellic acid mediated signaling	4	3.16E-02	3.18
GOTERM_BP_FAT	0009765	photosynthesis, light harvesting	8	8.54E-03	3.18
GOTERM_BP_FAT	0043087	regulation of GTPase activity	5	2.48E-02	2.92
GOTERM_BP_FAT	0006739	NADP metabolic process	7	2.44E-02	2.65
GOTERM_BP_FAT	0006869	Lipid transport	14	1.37E-03	2.65
GOTERM_BP_FAT	0006413	translational initiation	10	1.63E-02	2.30
GOTERM_BP_FAT	0009408	response to heat	21	5.04E-04	2.17
GOTERM_BP_FAT	0009808	lignin metabolic process	9	4.76E-02	2.16
GOTERM_BP_FAT	0046686	response to cadmium ion	40	9.22E-06	1.90
GOTERM_BP_FAT	0042742	defense response to bacterium	24	1.10E-02	1.70
GOTERM_BP_FAT	0009414	Response to water deprivation	14	4.09E-02	1.64
GOTERM_BP_FAT	0009409	response to cold	20	4.14E-02	1.49

Late Harvested

Category	GO Term ID	Go Term	Count	*P* value	FE
GOTERM_BP_FAT	0008154	actin polymerization or depolymerization	4	2.56E-02	5.39
GOTERM_BP_FAT	0046777	protein amino acid autophosphorylation	5	2.26E-02	3.37
GOTERM_BP_FAT	0009644	response to high light intensity	12	1.88E-03	2.79
GOTERM_BP_FAT	0016036	cellular response to phosphate starvation	7	3.94E-02	2.62
GOTERM_BP_FAT	0007264	small GTPase mediated signal transduction	13	1.58E-02	2.09
GOTERM_BP_FAT	0010629	negative regulation of gene expression	15	3.85E-02	1.71
GOTERM_BP_FAT	0006457	protein folding	36	8.57E-04	1.70
GOTERM_BP_FAT	0008154	response to temperature stimulus	41	1.26E-02	1.42

The terms over-represented in down-regulated genes in response to low temperature depends on ripeness stage (Table 3). Thus, the term ‘ion transmembrane transport’ and also ‘ATP synthesis coupled proton transport’ processes with a FE of 2.34 and 2.15, respectively, included 6 Vacuolar (V-type) proton ATPases were over-represented in early-harvested grapes (Table 3, Supplementary Table S1). Likewise, the

term 'histone deacetylation' (FE=3), with the repression of 5 histone deacetylases, including *HDA6*, was significantly enriched (Table 3, Supplementary Table S1). Under stress conditions, cell survival depends strongly on maintaining or adjusting the activity of V-ATPase (Dietz et al., 2001). One of the primary events of chilling injury in plants appears to be an inhibition of V-ATPase activity, which leads to an acidification of cytoplasm (Yoshida et al., 1999, Muñoz et al., 2001). Dietz et al. (2001), indicated that a low-temperature induce decline of V-ATPase activity and the concomitant decrease in proton motive force could affect solute compartmentation and possible the hardiness of plants to low temperature. In this sense, in a previous work we have observed that low temperature storage increased significantly the water-soluble K^+ pool in the skin, while high CO_2 levels maintained it (Blanch et al., 2014), suggesting that this may reflect the cellular stress associated with storage at a non-optimal temperature. Likewise, in a proteomic study of cold acclimation of *Arabidopsis*, Minami et al. (2009) suggest that the microdomains in the plasma membrane may function as platforms of membrane transport, trafficking and cytoskeleton interaction; they found many V-ATPases located in these microdomains which were down-regulated after chilling exposure. They hypothesized that these V-ATPases may contribute to cold or freezing tolerance by means of membrane trafficking regulation. On the other hand, gene silencing via histone deacetylation is a universally conserved epigenetic regulation system in eukaryotes (Wolffem, 1996). The involvement of histone deacetylases in plant responses to environmental stresses has recently been reported. In maize, the expression of histone deacetylases was up-regulated during cold acclimation (Hu et al., 2011). HDA6 regulates the expression of several long-term cold stress-responsive genes and plays a role in the acquisition of freezing tolerance in plants. Thus, cold-acclimated *hda6* mutant Arabidopsis plants showed a freezing-sensitive phenotype compared with cold-acclimated wild-type plants (To et al., 2011).

Functional annotation clustering, revealed 2 clusters in down-regulated genes of grapes at the early ripeness stage (Supplementary table S4). The first cluster with an ES of 1.88 contained terms associated with 'ATP synthesis coupled proton transport' and terms included in the second cluster (ES=1.51) are associated with 'GPI anchor biosynthetic' process. Glycosylphosphatidylinositol (GPI) membrane anchors provide an alternative to transmembrane domains for anchoring proteins to the cell surface in eukaryotes. GPI anchoring can confer localized or polarized targeting and therefore can

dramatically alter the functional properties of proteins. GPI biosynthetic pathways are poorly characterized in plants. By contrast, they have been studied extensively in animals and microbes and are a target for the development of parasite-specific therapeutic agents. Deficiencies in GPI biosynthesis lead to embryonic lethality in animals and to conditional lethality in eukaryotic microbes by blocking cell growth, cell division, or morphogenesis (Lalanne et al., 2004).

The analysis of the down-regulated genes by low temperature storage in late-harvested grapes (Table 3) revealed terms which were over-represented in up-regulated genes at the early stage (Table 2), such as 'photosynthesis' and 'response to abiotic stress' (heat, water deprivation, and metal ion). However, terms related to other biotic and abiotic stress such as fungus, red light, wounding, oxidative, salt and osmotic stress, were over-represented in late-harvested grapes. Low temperature storage seems to affect protein synthesis due to the fact that genes associated to 'translational elongation' were down-regulated in the skin of late-harvested grapes. By contrast, the effect of low temperature in early-harvested grapes seems to be the opposite due to the fact that genes associated to 'translational initiation' were up-regulated (Table 2). By regarding the overall results related to the transcriptional responses of table grapes to low temperature storage, we noticed that large proportion of significant differentially expressed genes were specifically regulated depending on the ripeness stage, indicating that some special pathways were correspond to each harvested date, although the others were cross-talked between them.

Table 3. Functional annotation chart of down-regulated genes from the skin of early and late-harvested grapes stored 3 days a 0 ºC. Biological Process GO terms, number of gene included (count), *P* value and fold enrichment (FE) are indicated.

Early harvested

Category	GO Term ID	Biological Process	Count	*P* value	FE
GOTERM_BP_FAT	0007021	tubulin complex assembly	6	2.61E-03	4.50
GOTERM_BP_FAT	0015937	coenzyme A biosynthetic process	5	9.92E-03	4.50
GOTERM_BP_FAT	0016575	histone deacetylation	5	3.00E-02	3.00
GOTERM_BP_FAT	0006506	GPI anchor biosynthetic process	6	3.15E-02	2.63
GOTERM_BP_FAT	0006012	galactose metabolic process	7	4.77E-02	2.43
GOTERM_BP_FAT	0046474	glycerophospholipid biosynthetic process	8	2.02E-02	2.38
GOTERM_BP_FAT	0034220	ion transmembrane transport	13	2.53E-03	2.34
GOTERM_BP_FAT	0015986	ATP synthesis coupled proton transport	10	1.83E-02	2.15

Late harvested

Category	GO Term ID	Biological Process	Count	*P* value	FE
GOTERM_BP_FAT	0009765	photosynthesis, light harvesting	8	1.22E-05	6.04
GOTERM_BP_FAT	0019319	hexose biosynthetic process	5	1.80E-02	4.65
GOTERM_BP_FAT	0006414	translational elongation	4	4.61E-02	3.55
GOTERM_BP_FAT	0010114	response to red light	6	1.40E-02	3.38
GOTERM_BP_FAT	0009808	lignin metabolic process	9	4.45E-03	3.29
GOTERM_BP_FAT	0009414	response to water deprivation	18	1.84E-05	2.92
GOTERM_BP_FAT	0009813	flavonoid biosynthetic process	7	1.90E-02	2.84
GOTERM_BP_FAT	0009620	response to fungus	11	1.19E-02	2.14
GOTERM_BP_FAT	0009408	response to heat	12	1.93E-02	2.01
GOTERM_BP_FAT	0009611	response to wounding	10	2.75E-02	1.99
GOTERM_BP_FAT	0006979	response to oxidative stress	20	2.34E-03	1.95
GOTERM_BP_FAT	0009651	response to salt stress	29	3.69E-04	1.94
GOTERM_BP_FAT	0006970	response to osmotic stress	27	6.25E-04	1.86
GOTERM_BP_FAT	0046686	response to cadmium ion	28	1.31E-03	1.83
GOTERM_BP_FAT	0009737	response to abscisic acid stimulus	15	2.80E-02	1.70

The application of 3-days high CO_2 levels at 0 ºC in early-harvested grapes significantly induced the expression of genes associated to the GO terms 'response to chitin' (FE=10.61), 'ethylene mediated signaling pathway' (FE=4.98) and 'regulation of transcription, DNA-dependent' (FE=2.76) (Table 4) and encoded different transcription factors (Table 5). Stress gene induction occurs primarily at the level of transcription, so transcription factors interact with *cis*-elements in the promoter regions of several stress-related genes and thus up-regulate the expression of many downstream genes resulting

in imparting abiotic stress tolerance (Agarwal and Jha, 2010). Plants devote a large portion of their genome capacity to transcription, with the Arabidopsis genome coding in excess of 1500 transcription factors (Riechmann et al., 2000). In this study, we found 31 transcription factor genes that were differentially up-regulated by high CO_2 levels and belonging to different families such as ERF (Ethylene Responsive Factors) a subfamily of the APETALA2 (AP2)/ERF transcription factor family, as well to the WRKY, MYB, basic-domain leucine-zipper (bZIP), heat stress transcription factors (HSFs) and zinc finger. In this sense, in a previous work we observed that the combination of 3-day high CO_2 treatment and low temperature induced in table grapes the expression of *CBF1* and *CBF4* in the pulp and *CBF4* in the rachis, but not in the skin (Fernandez-Caballero et al., 2012). The fact that CBFs also belong to the AP2/ERF transcription factor family seems to indicate a prominent role of this family in the different response of treated grapes to low temperature. Abundant evidence have illustrated that the transcription network of CBF plays a significant role in cold acclimation of evolutionarily different plant species (Singh et al., 2002). Nevertheless, gene expression analysis revealed several transcription factors besides CBFs that are induced during cold acclimation. However, this is to our knowledge, the first time that this fact has been described for the combination of high CO_2 levels and low temperature in plants.

The gaseous treatment at 0 ºC also induced the expression of genes associated to 'protein amino acid phosphorylation' (FE=1.64) including 15 genes that encoded kinases belonging mainly to the receptor-like kinases (RLKs) family (Table 4, Supplementary Table S1). Phosphorylation is one of the best known post-translational protein modifications affecting conformation, activity, localization and stability of target proteins (Whitmarsh and Davis, 2000). Phosphorylation mediated by kinases is one of the most ordinary mechanisms by which environmental cues are transduced and protein function is regulated in eukaryotic cells. It is known that receptor-like kinases mediate cold acclimation. Thus, a null mutant for a gene encoding a plasma-membrane RLK, namely the Ca^{2+}/CaM-regulated receptor like kinase 1 (CRLK1), shows reduced cold-induced gene expression and reduced capacity to cold acclimate (Yang et al., 2010). Dehydrins are also proteins involved in cold acclimation that are post-translationally modified by phosphorylation. They accumulate in response to diverse abiotic stresses, including low temperature, and act as molecular chaperones, protecting

nucleic acids, enzymes, cytoskeleton and membranes from stress-induced damage (Hara, 2010). In a recent work, we observed that high CO_2 levels induced the accumulation of DHN44 in the skin of table grapes and *in vitro* assays indicated that its isoform was phosphorylated (Navarro et al., 2015).

The most enriched down-regulated genes in CO_2-treated grapes at the early stage codified for 5 transcription factors belonging to the homeobox-leucine zipper, MAD-box and ERF families, all involved in the 'regulation of transcription, DNA-dependent' process (FE=3.84) (Table 4 and 5). This, together with the fact that transcription factors are mainly up-regulated in treated grapes seems to indicate that the gaseous treatment could be an active process requiring the synthesis of transcription factors.

In late-harvested grapes, only 12 up-regulated genes by high CO_2 levels were significantly associated with GO terms. Thus, the terms 'defense response', 'phothosynthesis' and 'generation of precursor metabolites and energy', were over-represented (Table 4). Results of proteomic analyses indicated that tolerant plants can induce several protective mechanisms more efficiently than sensitive ones because they are able to maintain sufficient rates of several metabolic processes, especially those associated with energy metabolism under adverse stress conditions (Ingle et al., 2005; Dumontet al., 2011). Maintenance of sufficient rates of processes associated with energy metabolism is extremely important for an efficient stress acclimation since it is an active process associated with *de novo* biosynthesis of several stress-protective compounds and so it is characterized by enhanced energy requirements (Thomashow, 1999; Bartels and Sunkar, 2005). No significant GO terms were over-represented in down-regulated genes by the gaseous treatment.

Table 4. Functional annotation chart of up- and down-regulated genes from the skin of early and late-harvested grapes treated 3 days with high CO_2 levels at 0 ºC. Biological Process GO terms, number of gene included (count), *P* value and fold enrichment (FE) are indicated.

Early harvested

Up-Regulated

Category	GO Term ID	Biological Process	Count	*P* value	FE
GOTERM_BP_FAT	0010200	response to chitin	14	1.31E-09	10.61
GOTERM_BP_FAT	0009751	response to salicylic acid stimulus	8	2.07E-04	5.42
GOTERM_BP_FAT	0009873	ethylene mediated signaling pathway	8	9.65E-04	4.98
GOTERM_BP_FAT	0000160	two-component signal transduction system (phosphorelay)	9	9.43E-04	4.35
GOTERM_BP_FAT	0009753	response to jasmonic acid stimulus	9	1.12E-03	4.24
GOTERM_BP_FAT	0006355	regulation of transcription, DNA-dependent	28	4.65E-06	2.76
GOTERM_BP_FAT	0009737	response to abscisic acid stimulus	10	1.05E-02	2.72
GOTERM_BP_FAT	0009617	response to bacterium	9	3.15E-02	2.41
GOTERM_BP_FAT	0006468	protein amino acid phosphorylation	15	4.34E-02	1.64

Down-Regulated

Category	GO Term ID	Biological Process	Count	*P* value	FE
GOTERM_BP_FAT	0006355	regulation of transcription, DNA-dependent	6	6.80E-03	3.84
GOTERM_BP_FAT	0048608	reproductive structure development	6	4.70E-02	2.87
GOTERM_BP_FAT	0009628	response to abiotic stimulus	7	1.38E-02	2.60

Late harvested

Up-Regulated

Category	GO Term ID	Biological Process	Count	*P* value	FE
GOTERM_BP_FAT	0042742	defense response to bacterium	7	9.64E-04	5.94
GOTERM_BP_FAT	0015979	photosynthesis	5	1.58E-02	5.05
GOTERM_BP_FAT	0006091	generation of precursor metabolites and energy	6	2.19E-02	3.12

Table 5. Transcription factors up- and down-regulated in the skin of CO_2-treated grapes at early stage.

Up-regulated

Unique transcript	*Vitis vinifera* most similar protein	Homolog in *A. thaliana*	Fold change
VIT_01s0011g03070	AP2-EREBP family, RAVE subfamily protein RAV2	AT1G68840	2.35
VIT_08s0007g05390	ARR9 two-component response regulator ARR9	AT3G57040	2.65
VIT_07s0141g00270	Auxin-responsive protein IAA4	AT5G43700	1.81
VIT_12s0055g00420	BZIP transcription factor	AT5G44080	1.48
GSVIVT00009539001	Ethylene responsive element binding factor 6	AT4G17490	1.60
VIT_02s0234g00130	Ethylene-responsive transcription factor 1A	AT4G17500	2.93
VIT_07s0005g03230	Ethylene-responsive transcription factor 1B	AT3G23240	2.19
VIT_06s0004g08190	Ethylene-responsive transcription factor CRF1	AT4G11140	2.06
VIT_16s0013g01110	Ethylene-responsive transcription factor ERF104	AT5G61600	4.36
VIT_16s0013g00900	Ethylene-responsive transcription factor ERF105	AT5G51190	1.90
VIT_00s0662g00030	Ethylene-responsive transcription factor RAP2-4	AT1G78080	2.54
VIT_01s0011g05810	FKF1 flavin-binding, kelch repeat, f box 1	AT1G68050	1.52
VIT_07s0031g00220	Floral homeotic protein APETALA 2	AT4G36920	1.52
VIT_08s0007g07550	GATA transcription factor 9	AT4G32890	2.01
VIT_10s0003g01770	Heat stress transcription factor A-4a	AT4G18880	1.63
VIT_13s0156g00260	Homeobox-leucine zipper protein HAT14	AT5G06710	2.87
VIT_01s0026g00150	Methyl-CpG-binding domain 9 MBD9	AT3G01460	1.51
VIT_05s0049g01020	myb domain protein 15	AT3G23250	2.25
VIT_18s0001g11170	myb domain protein 70	AT2G23290	2.86
VVTU3179_at	myb-like transcription factor family protein	AT5G47390	1.91
VIT_17s0000g01920	NF-X1-type zinc finger protein NFXL1	AT1G10170	1.64
VIT_07s0005g01450	ocs element-binding factor 1	AT3G62420	3.91
VIT_09s0018g00240	Probable WRKY transcription factor 40	AT1G80840	1.53
VIT_08s0058g01390	Probable WRKY transcription factor 70	AT3G56400	1.94
VIT_03s0180g00210	Transcription factor MYB44	AT5G67300	2.15
VIT_04s0008g05760	WRKY transcription factor 18	AT4G31800	1.74
VIT_15s0046g02190	WRKY transcription factor 22	AT4G01250	2.08
VVTU8929_at	WRKY transcription factor 44	AT2G37260	1.81
VVTU10039_s_at	Multiprotein-bridging factor 1c	AT3G24500	2.17
VIT_18s0001g09230	Zinc finger protein STZ/ZAT10	AT1G27730	1.86
VIT_06s0004g04180	Zinc finger (C2H2 type) protein (ZAT11)	AT2G37430	2.67

Down-regulated

Unique transcript	*Vitis vinifera* most similar protein	Homolog in *A. thaliana*	Fold change
VIT_13s0019g03550	APETALA2 (AP2) floral homeotic protein	AT4G36920	-1.63
VIT_04s0008g06000	Ethylene-responsive transcription factor ERF003	AT5G25190	-1.95
VVTU5223_at	Histone acetyltransferase HAC1	AT1G79000	-1.63
VIT_15s0048g02870	Homeobox-leucine zipper protein HB-7	AT2G46680	-2.57
VIT_06s0004g02800	Homeodomain-leucine zipper protein Revoluta (REV)	AT5G60690	-1.72
VVTU9997_at	MAD-box transcripion factor	AT2G42830	-1.77

Conclusions

Transcriptional responses of table grapes to low temperature and high CO_2 levels are depending on ripeness stage. While it is true that the high CO_2 treatment was effective controlling total decay and maintaining quality at the end of the storage period (data not shown) in both ripeness stages, the modifications in the transcriptome profile seem to be different. Major modifications in the transcriptome profile of early- and late-harvested grapes storage at 0ºC are linked to biotic and abiotic stress-responsive terms, indicating the cross-talked between stress. However, specific transcriptional modifications were observed depending on ripeness stage. The results obtained in this study indicate that in both cases there is a reprogramming of the transcriptome during the course of low temperature storage in order to withstand the stress mainly associated to gluconeogenesis, photosynthesis, mRNA translation and lipid transport, in early-harvested grapes, whereas the maintenance of protein folding stability and intracellular membrane trafficking seems to play an important role in late-harvested grapes. Likewise, the cellular response against low temperature seems to be related to the maintenance of the ion homeostasis, as reflected in the inhibition of key transport proteins such as V-ATPase in early-harvested grapes, which is thought to participate in the very early response to cold stress by means of regulating membrane trafficking.

The effect of the high CO_2 pretreatment maintaining early-harvested table grapes quality seems to be an active process requiring the activation of transcription factors as well as protein kinases implicate in the regulation of protein function. Likewise, although the number of genes significantly regulated in late harvested grapes by high CO_2 levels was low, also seems to be an active process associated to the maintenance of energy in the fruit.

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Supplementary data

Table S1. Genes differentially expressed in the indicated comparisons and belonging to the most relevant biological process.

	Early-Harvested Up-Regulated			
	Unique transcript	*Vitis vinifera* most similar protein	Homolog in *A. thaliana*	Fold change
Gluconeogenesis	VVTU1077_at	GAPC1 glyceraldehyde 3-phosphate dehydrogenase	AT3G04120	1.72
	VVTU18256_at	GAPC2 glyceraldehyde 3-phosphate dehydrogenase	AT1G13440	3.34
	VIT_18s0001g07280	Glucose-6-phosphate isomerase	AT4G24620	2.56
	VIT_00s2576g00010	PCK1 Phosphoenolpyruvate carboxykinase (ATP)	AT4G37870	2.59
Chitin catabolic process	VVTU32073_x_at	Basic endochitinase B class I	AT3G12500	1.59
	VIT_05s0094g00220	Chitinase IV	AT3G54420	4.39
	VIT_11s0206g00030	Chitinase V	AT4G19810	1.53
	VVTU17653_at	POM1 chitinase-like protein 1	AT1G05850	1.61
Lipid transport	VIT_16s0098g01570	acyl-CoA binding protein 2	AT4G27780	1.69
	VIT_05s0020g03750	bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin-like protein	AT3G22620	2.29
	VVTU19197_at	CWLP cell wall-plasma membrane linker protein	AT3G22120	1.87
	VVTU17785_at	glycolipid transfer protein	AT4G39670	1.91
	VVTU19987_at	LP1 non-specific lipid-transfer protein 1	AT2G38540	1.86
	CB349540	LTP11 pathogenesis-related lipid-transfer family protein	AT4G33355	2.23
	VVTU19145_at	LTP3 non-specific lipid-transfer protein 3	AT5G59320	1.82
	GSVIVT00002805001	LTPG1 glycosylphosphatidylinositol-anchored lipid protein transfer 1	AT1G27950	2.61
	VIT_06s0009g01420	non-specific lipid transfer protein GPI-anchored 2	AT3G43720	1.96
	VVTU19234_at	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	AT2G45180	1.60
	VIT_05s0020g03730	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	AT3G22600	3.32
	VIT_13s0019g01550	putative lipid transfer protein	AT4G30880	1.56
	VVTU12351_at	putative lipid-transfer protein DIR1	AT5G48485	1.56
	VIT_16s0039g00010	WBC11 ABC transporter G family member 11	AT1G17840	2.32
Translational initiation	VIT_15s0046g01500	OB-fold nucleic acid binding domain-containing protein	AT2G04520	2.71
	VIT_08s0007g00970	EIF3G1 eukaryotic translation initiation factor 3G1	AT3G11400	1.85
	VIT_00s0880g00020	eukaryotic translation initiation factor 3 subunit 7	AT4G20980	1.65

	VIT_07s0031g02300	eukaryotic translation initiation factor 6A	AT3G55620	1.64
	VIT_11s0037g00490	eukaryotic translation Initiation Factor isoform 4G1 (eIFiso4G1)	AT5G57870	1.68
	VIT_14s0006g01990	probable eukaryotic translation initiation factor 5-1	AT1G36730	1.61
	VIT_05s0062g01080	translation initiation factor 3 subunit H1	AT1G10840	1.54
	VVTU18433_at	translation initiation factor eIF-2 beta subunit	AT5G20920	2.45
	VIT_11s0016g00020	translation initiation factor eIF-5A (eIF5A-1)	AT1G13950	1.67
	VVTU14203_at	translation initiation factor SUI1 family protein	AT5G54940	1.48

Late-Harvested Up-Regulated

	Unique transcript	*Vitis vinifera* most similar protein	Homolog in *A. thaliana*	Fold change
Protein folding	VVTU13893_s_at	PFD6 prefoldin 6	AT1G29990	1.90
	VVTU13987_at	ARL1 ARG1-like 1	AT1G24120	1.67
	VIT_15s0021g02090	ATJ3 chaperone protein dnaJ 3	AT3G44110	1.51
	VIT_01s0011g04820	chaperone DnaJ-domain containing protein	AT1G71000	1.80
	VIT_08s0007g07960	chaperone DnaJ-domain containing protein	AT3G12170	1.71
	VIT_09s0002g00690	chaperone DnaJ-domain containing protein	AT3G14200	16.77
	VIT_06s0004g05140	chaperone DnaJ-domain containing protein	AT5G59610	1.59
	VIT_08s0040g00120	chaperone protein dnaJ 72	AT2G41000	1.61
	VIT_07s0005g01390	CRT1a calreticulin-1	AT1G56340	1.54
	VIT_05s0102g01190	CRT3 calreticulin-3	AT1G08450	1.92
	VIT_13s0084g00620	cyclophilin-like peptidyl-prolyl cis-trans isomerase family protein	AT2G38730	1.51
	VIT_03s0038g02200	cyclophilin-like peptidyl-prolyl cis-trans isomerase family protein	AT4G34960	2.20
	VIT_18s0072g01080	DNAJ heat shock N-terminal domain-containing protein	AT1G65280	1.57
	VIT_03s0038g02110	DNAJ heat shock N-terminal domain-containing protein	AT2G17880	2.96
	VIT_02s0012g02290	DNAJ heat shock N-terminal domain-containing protein	AT2G35540	1.75
	VIT_08s0217g00090	DNAJ heat shock N-terminal domain-containing protein ATERDJ3A	AT3G08970	1.53
	VIT_04s0008g06970	DnaJ/Hsp40 cysteine-rich domain-containing prote	AT2G24860	1.71
	VIT_06s0004g01100	EDA3 embryo sac development arrest 3 protein	AT2G34860	1.87
	VIT_10s0003g01700	ER lumen protein retaining receptor (ERD2)	AT1G29330	1.52
	VIT_01s0011g01650	FKBP-like peptidyl-prolyl cis-trans isomerase family protein	AT4G19830	1.74
	VIT_19s0090g00340	heat shock protein 70 (Hsp 70) family protein	AT1G11660	1.58

	VIT_05s0020g03330	HSP70T-2 heat-shock protein 70T-2	AT2G32120	1.60
	VIT_13s0019g03880	mitochondrial import inner membrane translocase subunit TIM14-1	AT2G35795	1.50
	VIT_08s0032g00960	molecular chaperone HscB	AT5G06410	1.81
	VIT_09s0002g07210	molecular chaperone Hsp40/DnaJ-like protein	AT1G80030	1.65
	VIT_05s0020g03500	PDF2 putative prefoldin subunit 2	AT3G22480	1.57
	VIT_19s0014g03330	peptidyl-prolyl cis-trans isomerase CYP37	AT3G15520	1.67
	VIT_08s0007g02900	peptidyl-prolyl cis-trans isomerase-like 1	AT2G36130	1.62
	VIT_15s0048g01780	peptidyl-prolyl cis-trans isomerase-like 3	AT1G01940	1.63
	VIT_17s0000g02530	putative FKBP-type peptidyl-prolyl cis-trans isomerase 5	AT1G18170	1.84
	VIT_01s0150g00200	putative FKBP-type peptidyl-prolyl cis-trans isomerase 7	AT5G13410	1.57
	VIT_18s0001g08830	ROF2 peptidyl-prolyl cis-trans isomerase FKBP65	AT5G48570	1.68
	VIT_03s0063g01030	SQN peptidyl-prolyl cis-trans isomerase CYP40	AT2G15790	1.61
	VIT_09s0002g03930	TCP-1/cpn60 chaperonin family protein	AT3G13470	1.53
	VIT_07s0005g01290	tetratricopeptide repeat-containing protein	AT2G47440	2.96
	VIT_05s0094g00410	Unknown protein	AT2G30695	1.97
Small GTPAse genes	VIT_04s0008g07360	ADP-ribosylation factor 3 (ARF3)	AT2G24765	1.45
	VIT_07s0005g02000	ARFA1E ADP-ribosylation factor A1E	AT3G62290	1.52
	VIT_05s0062g00840	RAB GTPase ARA4	AT2G43130	1.76
	VIT_12s0035g01150	RAB GTPase FP8	AT3G11730	1.53
	VIT_07s0031g01700	RAB GTPase LIP1	AT5G64813	2.22
	VIT_19s0014g01160	RAB GTPase RAB11	AT1G16920	1.52
	VIT_18s0001g15090	RAB GTPase RAB18	AT1G43890	1.51
	VIT_06s0004g05550	RAB GTPase RABA2B	AT1G07410	1.59
	VIT_18s0001g02250	RAB GTPase RABG3A	AT4G09720	2.07
	VIT_10s0003g01610	RABA1d RAB GTPase homolog A1D	AT4G18800	1.66
	VIT_09s0002g04600	RHA1 Ras-related protein RABF2a	AT5G45130	1.90
	VIT_19s0014g00960	SGP1 monomeric G protein	AT5G54840	1.74
	VIT_04s0023g01820	TTN5 ADP-ribosylation factor-like protein 2	AT2G18390	1.50
Response to phosphate starvation	VVTU10030_at	Catalase-2	AT4G35090	1.65
	VIT_11s0118g00310	MGD2Monogalactosyldiacylglycerol synthase 2	AT5G20410	2.34
	VIT_00s0265g00070	PHO2 putative ubiquitin-conjugating enzyme E2 24	AT2G33770	1.72
	VIT_04s0008g05420	SPX2 (SYG1/Pho81/XPR1) domain-containing protein SPX2	AT2G26660	1.73
	VIT_14s0108g01120	SPX4 SPX domain-containing protein 4	AT5G15330	1.68

	VIT_08s0007g01940	SQD2 sulfoquinovosyldiacylglycerol 2	AT5G01220	2.74

Early-Harvested Down-Regulated

	Unique transcript	*Vitis vinifera* most similar protein	Homolog in *A. thaliana*	Fold change
Ion transmembrane transport	VIT_12s0028g02170	ATP synthase protein I-related protein	AT2G31040	-3.27
	VIT_13s0019g03860	ATPQ ATP synthase subunit D	AT3G52300	-1.54
	VIT_09s0018g01930	ATP synthase subunit epsilon	AT1G51650	-1.86
	VIT_19s0014g04860	ATP synthase subunit G protein	AT4G29480	-1.69
	VIT_04s0008g04760	ATPase, F0/V0 complex, subunit C protein	AT4G32530	-1.51
	VIT_19s0090g01330	ATPase, V0 complex, subunit E	AT5G55290	-1.59
	VIT_01s0011g06550	SOS1 sodium/hydrogen exchanger 7	AT2G01980	-1.60
	VIT_04s0008g02580	V-type proton ATPase catalytic subunit A	AT1G78900	-1.70
	VIT_17s0000g00460	V-type proton ATPase subunit C	AT1G12840	-1.84
	VIT_12s0028g01260	V-type proton ATPase subunit D	AT3G58730	-1.85
	VIT_02s0025g01000	V-type proton ATPase subunit E1	AT4G11150	-1.75
	VIT_02s0025g00960	V-type proton ATPase subunit E3	AT1G64200	-1.82
	VIT_13s0019g04230	V-type proton ATPase subunit G1	AT3G01390	-2.13
Histone deacetylation	VIT_17s0000g09070	Histone deacetylase 6	AT5G63110	-2.19
	VIT_15s0021g00610	Histone deacetylase 9	AT3G44680	-1.61
	VIT_06s0080g00210	Histone deacetylase 8	AT1G08460	-1.91
	VIT_04s0044g01510	Histone deacetylase 14	AT4G33470	-1.52
	VIT_03s0038g04240	Histone deacetylase 19	AT4G38130	-2.20

Table S2. Functional annotation clustering of gene ontology terms associated with genes that are up-regulated in non-treated early-harvested table grapes

Cluster 1	**Enrichment Score: 4.81**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0010038	response to metal ion	45	4.13	5.17E-06	1.86
GOTERM_BP_FAT	GO:0046686	response to cadmium ion	40	3.67	9.22E-06	1.90
GOTERM_BP_FAT	GO:0010035	response to inorganic substance	52	4.78	7.61E-05	1.62
Cluster 2	**Enrichment Score: 2.12**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0019684	photosynthesis, light reaction	15	1.38	2.14E-03	2.43
GOTERM_BP_FAT	GO:0009765	photosynthesis, light harvesting	8	0.73	8.54E-03	3.18
GOTERM_BP_FAT	GO:0015979	photosynthesis	20	1.84	2.31E-02	1.69
Cluster 3	**Enrichment Score: 2.09**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0006094	gluconeogenesis	4	0.37	3.02E-03	6.62
GOTERM_BP_FAT	GO:0046165	alcohol biosynthetic process	8	0.73	3.81E-03	3.25
GOTERM_BP_FAT	GO:0019319	hexose biosynthetic process	5	0.46	1.68E-02	3.66
GOTERM_BP_FAT	GO:0046364	monosaccharide biosynthetic process	5	0.46	2.35E-02	3.40
Cluster 4	**Enrichment Score: 2.02**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0006952	defense response	50	4.59	7.29E-03	1.43
GOTERM_BP_FAT	GO:0009617	response to bacterium	28	2.57	1.08E-02	1.62
GOTERM_BP_FAT	GO:0042742	defense response to bacterium	24	2.20	1.10E-02	1.70
Cluster 5	**Enrichment Score: 1.69**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0009820	alkaloid metabolic process	10	0.92	8.96E-03	2.49
GOTERM_BP_FAT	GO:0019362	pyridine nucleotide metabolic process	9	0.83	1.16E-02	2.56
GOTERM_BP_FAT	GO:0046496	nicotinamide nucleotide metabolic process	8	0.73	1.87E-02	2.55
GOTERM_BP_FAT	GO:0006769	nicotinamide metabolic process	8	0.73	1.87E-02	2.55
GOTERM_BP_FAT	GO:0006739	NADP metabolic process	7	0.64	2.44E-02	2.65
GOTERM_BP_FAT	GO:0043603	cellular amide metabolic process	8	0.73	4.03E-02	2.23
GOTERM_BP_FAT	GO:0006733	oxidoreduction coenzyme metabolic process	9	0.83	4.21E-02	2.09
Cluster 6	**Enrichment Score: 1.59**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0009739	response to gibberellin stimulus	12	1.10	1.66E-02	2.02
GOTERM_BP_FAT	GO:0010476	gibberellin-mediated signaling	4	0.37	3.16E-02	3.18
GOTERM_BP_FAT	GO:0009740	gibberellic acid mediated signaling	4	0.37	3.16E-02	3.18
Cluster 7	**Enrichment Score: 1.50**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0009719	response to endogenous stimulus	55	5.05	1.96E-02	1.29
GOTERM_BP_FAT	GO:0009725	response to hormone stimulus	49	4.50	3.31E-02	1.27
GOTERM_BP_FAT	GO:0010033	response to organic substance	63	5.79	4.94E-02	1.21

Table S3. Functional annotation clustering of gene ontology terms associated with genes that are up-regulated in non-treated late-harvested table grapes

Cluster 1	**Enrichment Score: 1.58**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0031669	cellular response to nutrient levels	10	0.70	1.26E-02	2.50
GOTERM_BP_FAT	GO:0031667	response to nutrient levels	11	0.77	2.18E-02	2.18
GOTERM_BP_FAT	GO:0016036	cellular response to phosphate starvation	7	0.49	3.94E-02	2.62
GOTERM_BP_FAT	GO:0042594	response to starvation	9	0.63	4.51E-02	2.17

Table S4. Functional annotation clustering of gene ontology terms associated with genes that are down-regulated in non-treated early-harvested table grapes

Cluster 1	**Enrichment Score: 1.88**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0034220	ion transmembrane transport	13	0.57	2.53E-03	2.34
GOTERM_BP_FAT	GO:0015672	monovalent inorganic cation transport	27	1.19	9.33E-03	1.60
GOTERM_BP_FAT	GO:0015986	ATP synthesis coupled proton transport	10	0.44	1.83E-02	2.15
GOTERM_BP_FAT	GO:0015985	energy coupled proton transport, down electrochemical gradient	10	0.44	1.83E-02	2.15
GOTERM_BP_FAT	GO:0015992	proton transport	13	0.57	2.48E-02	1.85
GOTERM_BP_FAT	GO:0006818	hydrogen transport	13	0.57	2.48E-02	1.85
Cluster 2	**Enrichment Score: 1.51**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0046474	glycerophospholipid biosynthetic process	8	0.35	2 02E-02	2.38
GOTERM_BP_FAT	GO:0046489	phosphoinositide biosynthetic process	7	0.31	2.03E-02	2.57
GOTERM_BP_FAT	GO:0045017	glycerolipid biosynthetic process	8	0.35	2.94E-02	2.25
GOTERM_BP_FAT	GO:0006506	GPI anchor biosynthetic process	6	0.27	3.15E-02	2.63
GOTERM_BP_FAT	GO:0042158	lipoprotein biosynthetic process	8	0.35	4.12E-02	2.13
GOTERM_BP_FAT	GO:0006497	protein amino acid lipidation	8	0.35	4.12E-02	2.13
GOTERM_BP_FAT	GO:0042157	lipoprotein metabolic process	8	0.35	4.12E-02	2.13

Table S5. Functional annotation clustering of gene ontology terms associated with genes that are down-regulated in non-treated late-harvested table grapes

Cluster 1	**Enrichment Score: 3.47**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0009765	photosynthesis, light harvesting	10	1.54	1.22E-05	6.04
GOTERM_BP_FAT	GO:0019684	photosynthesis, light reaction	13	2.00	4.75E-04	3.21
GOTERM_BP_FAT	GO:0006091	generation of precursor metabolites and energy	33	5.08	5.75E-04	1.87
GOTERM_BP_FAT	GO:0015979	photosynthesis	17	2.62	3.96E-03	2.18
Cluster 2	**Enrichment Score: 3.01**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0010038	response to metal ion	32	4.93	5.26E-04	1.84
GOTERM_BP_FAT	GO:0010035	response to inorganic substance	39	6.01	1.31E-03	1.65
GOTERM_BP_FAT	GO:0046686	response to cadmium ion	28	4.31	1.31E-03	1.83
Cluster 3	**Enrichment Score: 2.43**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0010218	response to far red light	9	1.39	7.01E-05	5.03
GOTERM_BP_FAT	GO:0009639	response to red or far red light	14	2.16	1.36E-02	2.04
GOTERM_BP_FAT	GO:0009637	response to blue light	7	1.08	1.37E-02	3.02
GOTERM_BP_FAT	GO:0010114	response to red light	6	0.92	1.40E-02	3.38
Cluster 4	**Enrichment Score: 2.01**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0019748	secondary metabolic process	29	4.47	3.38E-03	1.76
GOTERM_BP_FAT	GO:0009808	lignin metabolic process	9	1.39	4.45E-03	3.29
GOTERM_BP_FAT	GO:0009698	phenylpropanoid metabolic process	16	2.47	5.71E-03	2.17
GOTERM_BP_FAT	GO:0009699	phenylpropanoid biosynthetic process	13	2.00	8.91E-03	2.31
GOTERM_BP_FAT	GO:0006575	cellular amino acid derivative metabolic process	21	3.24	1.31E-02	1.77
GOTERM_BP_FAT	GO:0042398	cellular amino acid derivative biosynthetic process	16	2.47	1.51E-02	1.95
GOTERM_BP_FAT	GO:0009813	flavonoid biosynthetic process	7	1.23	1.90E-02	2.84
GOTERM_BP_FAT	GO:0009812	flavonoid metabolic process	7	1.23	2.93E-02	2.61
Cluster 5	**Enrichment Score: 1.88**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0010033	response to organic substance	56	8.63	4.22E-03	1.43
GOTERM_BP_FAT	GO:0009719	response to endogenous stimulus	48	7.40	4.98E-03	1.47
GOTERM_BP_FAT	GO:0009725	response to hormone stimulus	43	6.63	1.23E-02	1.43
GOTERM_BP_FAT	GO:0009755	hormone-mediated signaling	22	3.39	2.68E-02	1.58
GOTERM_BP_FAT	GO:0032870	cellular response to hormone stimulus	22	3.39	2.68E-02	1.58
GOTERM_BP_FAT	GO:0009737	response to abscisic acid stimulus	14	2.16	2.80E-02	1.70

Table S6. Functional annotation clustering of gene ontology terms associated with genes that are up-regulated in CO_2-treated early-harvested table grapes

Cluster 1	**Enrichment Score: 5.79**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0010200	response to chitin	14	6.11	1.31E-09	10.61
GOTERM_BP_FAT	GO:0045449	regulation of transcription	42	18.34	7.44E-07	2.23
GOTERM_BP_FAT	GO:0009743	response to carbohydrate stimulus	14	6.11	3.89E-06	5.36
GOTERM_BP_FAT	GO:0006355	regulation of transcription, DNA-dependent	28	11.79	4.65E-06	2.76
GOTERM_BP_FAT	GO:0051252	regulation of RNA metabolic process	28	11.79	5.43E-06	2.74
GOTERM_BP_FAT	GO:0006350	transcription	28	12.23	1.77E-04	2.18
Cluster 2	**Enrichment Score: 3.98**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0010033	response to organic substance	35	15.28	1.72E-07	2.62
GOTERM_BP_FAT	GO:0009719	response to endogenous stimulus	29	12.66	3.82E-06	2.59
GOTERM_BP_FAT	GO:0009725	response to hormone stimulus	27	11.79	7.95E-06	2.61
GOTERM_BP_FAT	GO:0009723	response to ethylene stimulus	13	5.68	1.20E-05	4.82
GOTERM_BP_FAT	GO:0000160	two-component signal transduction system (phosphorelay)	9	3.93	9.43E-04	4.35
GOTERM_BP_FAT	GO:0009873	ethylene mediated signaling pathway	8	3.49	9.65E-04	4.98
GOTERM_BP_FAT	GO:0009755	hormone-mediated signaling	14	6.11	1.25E-03	2.81
GOTERM_BP_FAT	GO:0032870	cellular response to hormone stimulus	14	6.11	1.25E-03	2.81
GOTERM_BP_FAT	GO:0007242	intracellular signaling cascade	17	7.42	1.55E-02	1.90
Cluster 3	**Enrichment Score: 1.81**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0006796	phosphate metabolic process	24	10.48	8.28E-03	1.75
GOTERM_BP_FAT	GO:0006793	phosphorus metabolic process	24	10.48	8.47E-03	1.75
GOTERM_BP_FAT	GO:0016310	phosphorylation	21	9.17	1.87E-02	1.71
GOTERM_BP_FAT	GO:0006468	protein amino acid phosphorylation	15	7.86	4.34E-02	1.64

Table S7. Functional annotation clustering of gene ontology terms associated with genes that are up-regulated in CO_2-treated late-harvested table grapes

Cluster 1	**Enrichment Score: 2.58**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0042742	defense response to bacterium	7	7.29	9.64E-04	5.94
GOTERM_BP_FAT	GO:0009617	response to bacterium	7	7.29	2.71E-03	4.85
GOTERM_BP_FAT	GO:0006952	defense response	9	9.38	7.13E-03	3.09

Table S8. Functional annotation clustering of gene ontology terms associated with genes that are down-regulated in CO_2-treated early-harvested table grapes

Cluster 1	**Enrichment Score: 1.88**					
Category	GO Term ID	GO Term	Count	%	*P* value	FE
GOTERM_BP_FAT	GO:0006355	regulation of transcription, DNA-dependent	6	15.91	6.80E-03	3.84
GOTERM_BP_FAT	GO:0051252	regulation of RNA metabolic process	6	15.91	7.09E-03	3.81
GOTERM_BP_FAT	GO:0045449	regulation of transcription	8	18.18	4.75E-02	2.25

DISCUSIÓN

1.- Análisis e identificación de marcadores relacionados con el estado del agua en los diferentes tejidos del racimo que permitan definir la capacidad intrínseca de conservación y el daño asociado a las bajas temperaturas.

El agua es el componente mayoritario de los frutos frescos, superando incluso el 90% de su peso, y las modificaciones en su contenido y propiedades afectan a la calidad, al periodo de vida útil y a la capacidad de adaptación a diferentes factores ambientales durante su conservación (Hills & Remigereau, 1997; Ruan & Chen, 1998; Moraga *et al*., 2006; Agüero *et al*., 2008; Wright *et al*., 2009; Alferez *et al*., 2010). En el caso de uva de mesa cv. Cardinal, el contenido de agua en la pulpa alcanza valores del 91%. Además, la pérdida de agua en frutos tiene implicaciones económicas ya que disminuye su valor en el mercado. De ahí, la utilidad y necesidad de aplicar tecnologías dirigidas a minimizar su pérdida durante la conservación. En trabajos previos, observamos que el pretratamiento de 3 días con altas concentraciones de CO_2 reducía significativamente las pérdidas de agua después de 33 días de conservación a 0ºC (aproximadamente un 53% menor con respecto a los racimos no tratados), a la vez que minimizaban el crecimiento fúngico y el marchitamiento del raquis en racimos de uva Cardinal (Sanchez-Ballesta *et al*., 2006). Estos resultados nos llevaron a plantear diferentes cuestiones relacionadas con la importancia del estado del agua en los tejidos del racimo. Más aún, considerando que uno de los importantes retos de las tecnología postcosecha consiste en frenar y/o evitar la liberación y pérdida de agua por transpiración durante el periodo de conservación, resulta de especial interés analizar las variaciones en el contenido de agua ligada respecto al agua total. En contraposición al agua libre, el agua ligada interacciona mediante puentes de hidrógeno con solutos, macromoléculas y estructuras celulares lo que reduce su movilidad y limita sus propiedades como solvente. Este tipo de agua se presenta en la bibliografía como agua osmóticamente inactiva o agua no congelable (UFW) (Wang & Kolbe, 1991; Wolfe *et al*., 2002). Este último término se fundamenta en una de sus características más conocidas, que es su permanencia en estado líquido a temperaturas por debajo de la temperatura de congelación en equilibrio con el agua libre. Existen diferentes técnicas para la determinación del contenido de agua no congelable, como son la calorimetría diferencial de barrido (DSC) o la resonancia magnética nuclear (RMN) de protón. En base a los resultados sobre la caracterización del estado del agua en frutos mediante la

aplicación de las técnicas de RMN y DSC realizados previamente en nuestro laboratorio (Goñi *et al*., 2007), y una vez diseñadas y obtenidas las medidas calorimétricas adecuadas, principalmente la entalpía de fusión del hielo (ΔH_f) con respecto al contenido de agua (J/g) y la temperatura de inicio de la fusión del hielo (T_{onset}) (ºC) de la muestra, así como la entalpía de fusión del agua pura, determinamos el contenido de agua no congelable o ligada en los distintos tejidos del racimo, incluyendo pulpa, piel, semillas y raquis. Asimismo, analizamos el efecto de las bajas temperaturas y altas concentraciones de CO_2 en el contenido de agua ligada en los diferentes tejidos. Es interesante señalar que estos análisis son pioneros en el ámbito de estudio de la conservación de frutos. Si bien, se detectaron diferencias en la magnitud de las variaciones entre los diferentes tejidos, los resultados obtenidos mostraron mayores niveles de agua no congelable en los tratados con alto CO_2 que en los mantenidos en aire, llegando a duplicar de forma puntual los niveles descritos en los frutos recién recolectados. Se cuantificaron mayores niveles de agua no congelable en los tejidos de frutos tratados con CO_2, tanto al finalizar el tratamiento como durante la transferencia al aire, especialmente en pulpa y raquis, sugiriendo un marcado efecto residual beneficioso del pretratamiento gaseoso.

En cuanto a los resultados obtenidos sobre la evolución del contenido de agua ligada en los diferentes tejidos en los racimos no tratados mantenidos en aire, destaca el descenso significativo durante la fase inicial de la conservación a 0ºC (3 días) en los tejidos de piel y en las semillas. En relación con las modificaciones sobre el estado del agua, Vertucci & Stushnuff (1992) indicaron que la aclimatación al frío de brotes vegetativos de manzana implicaba un incremento en los niveles de agua no congelable. Wolfe *et al.* (2002) atribuyeron los cambios en el contenido de agua libre a la capacidad de hidratación de las membranas, macromoléculas y otros componentes hidrófilos de la célula. De acuerdo a los resultados experimentales de este trabajo, el mayor contenido de agua ligada en los frutos tratados con altas concentraciones de CO_2 podría ser un reflejo de las respuestas metabólicas generadas en los tejidos al tratamiento gaseoso, dirigidas a la síntesis y acumulación de diferentes solutos que, además de contribuir a los cambios en el contenido de agua ligada, podrían actuar como protectores de las estructuras celulares. En este sentido, también se ha propuesto que la fracción de agua ligada probablemente juegue un papel importante en la tolerancia a determinados estreses abióticos en base al mantenimiento de la integridad estructural de la célula (Singh *et al*., 2006). Por ello, el estudio del estado del agua en la pulpa se complementó

con el análisis microestructural y del grado de deterioro de sus células en respuesta a la combinación de altas concentraciones de CO_2 y bajas temperaturas. Concretamente, investigamos la correlación entre el contenido de agua ligada y la capacidad de retención de agua del tejido tras diferentes procesos de congelación-descongelación. Además, utilizando la microscopía electrónica de barrido de baja temperatura (LT-SEM), visualizamos las modificaciones en la estructura y volumen celular y los cambios en la distribución del agua en los tejidos. Esta técnica permite observar la estructura interna celular sin la necesidad de procesos de fijación y/o deshidratación, y al utilizar congelación por inmersión en N_2, garantiza la estabilidad de la muestra congelada, evitando la salida de agua y gases de los tejidos. Los detalles microestructurales, resultado del proceso de sublimación del hielo, permiten distinguir regiones brillantes, que corresponden a la matriz celular condensada y congelada o las estructuras insolubles de los tejidos, separadas por regiones oscuras, que son las trazas que deja el hielo que sublima. Nuestros resultados mostraron que los tejidos de pulpa de los frutos tratados con altos niveles de CO_2, que tenían un mayor contenido en agua ligada, exhibían una mayor capacidad de retención de agua que los frutos no tratados mantenidos en aire, tanto a los 4 como a los 9 días de ensayos de congelación-descongelación. Esta mayor capacidad para retener agua evidenciaba el mejor mantenimiento de la estructura celular y, además, probablemente podría jugar un papel importante en la jugosidad del fruto (Torreggiani & Maestrelli, 2006). Asimismo, en los frutos tratados con CO_2 tras 22 días a 0ºC se confirmó el mantenimiento de la estructura celular de los frutos mediante las micrografías realizadas por LT-SEM. Las células de estos frutos presentaban una morfología bien definida al igual que la de las muestras recién cosechadas. Por el contrario, las células de los frutos no tratados presentaban pérdida de su integridad y organización. Este efecto beneficioso del tratamiento con altas concentraciones de CO_2 en el mantenimiento de la estructura celular durante la conservación a bajas temperaturas también lo observamos en otros frutos tolerantes al alto CO_2 como chirimoya y fresa (Maldonado *et al*., 2002; Blanch *et al*., 2012a). En el caso de la fresa, el incremento en el contenido de agua no congelable, mantenimiento de la estructura celular y ausencia de fluido extracelular iba asociado a la acumulación de fructanos, compuestos reconocidos como agentes protectores frente a diferentes estreses ambientales y con una elevada capacidad para atrapar moléculas de agua. También en la variedad Cardinal se observó el efecto inductor de las altas concentraciones de CO_2 en

el contenido de fructanos tipo inulina que podrían funcionalmente estar involucrados en incrementar el contenido de agua ligada y evitar los daños estructurales causados por las bajas temperaturas (Blanch *et al*., 2011). Por todo ello, consideramos que la determinación del contenido de agua no congelable puede ser un marcador metabólico idóneo que permite definir la capacidad intrínseca de conservación del fruto. Más aún, el incremento de agua ligada asociado a la menor liberación de líquido extracelular, y complementado con el mantenimiento de la estructura celular, permiten confirmar el efecto beneficioso de altas concentraciones de CO_2 en la mejora de la conservación de la baya.

En relación con el estado del agua en el raquis, el incremento en el contenido en agua no congelable en los racimos tratados con CO_2 a lo largo de su conservación a 0ºC se asoció con un mayor contenido en agua total, en comparación con las muestras no tratadas. Asimismo, analizamos los cambios en el contenido relativo de agua (RWC) y su correlación con el grado de pardeamiento del raquis. El RWC se ha considerado un indicador del estado del balance de agua en los tejidos (González & González-Vilar, 2001). Además, el pardeamiento del raquis durante su conservación en frío se ha asociado comúnmente con la pérdida de agua (Crisosto *et al*., 2001; Valverde *et al*., 2005a; Lichter *et al*., 2011; Balic *et al*., 2012). De hecho, nuestros ensayos mostraron un buen coeficiente de correlación de Pearson negativo entre el RWC y el pardeamiento del raquis (r = -0,88) durante su almacenamiento a 0ºC. Por el contrario, el tratamiento gaseoso con CO_2 produjo una recuperación de los valores de RWC y en los índices de pardeamiento del tejido al comparar con los racimos mantenidos en aire. Por tanto, nuestros resultados corroboran un estudio previo en el que ya describimos en racimos tratados con CO_2 tras 33 días a 0ºC una mejor apariencia y mayores niveles de RWC que en los no tratados (Sanchez-Ballesta *et al*., 2006). Estas observaciones, junto con el incremento observado en el contenido de agua ligada, indican que el posible efecto beneficioso del tratamiento gaseoso implica un retraso en la aparición de senescencia en el raquis, que podría ser atribuido nuevamente a un ajuste metabólico que previene el daño causado por las bajas temperaturas, de manera similar a la propuesta por otros autores (Agüero *et al*., 2008).

Como hemos comentado anteriormente, la piel y las semillas presentaron una rápida disminución de su contenido en agua ligada tras su exposición de 3 días a 0ºC. Al no observarse variaciones significativas en el contenido total de agua, el descenso en el

contenido de agua ligada puede atribuirse a una conversión de agua ligada a libre, tal y como fue descrita por Bendel *et al*. (2001), en bulbos de tulipán en experimentos realizados por RMN. Interesantemente, al finalizar los 3 días de tratamiento con un 20% CO_2 el contenido de agua ligada en ambos tejidos era significativamente mayor. Superada la fase crítica de conservación a 0ºC, los tejidos de la piel presentaron un aumento en la fracción de agua ligada.

Teniendo en cuenta la importancia de la piel como barrera externa en el control de la velocidad de pérdida de agua en el fruto, el estudio de su estructura celular es relevante en la evaluación global de la calidad del racimo durante su conservación. Por ello, en el presente trabajo planteamos el estudio de la dinámica del agua entre las células de la piel en conexión con los cambios en el flujo iónico. Concretamente, hemos investigado las variaciones en el contenido de potasio celular, así como su distribución, como parámetro complementario a la cuantificación del contenido de agua ligada en estos tejidos. El potasio, además de ser uno de los iones mayoritarios de la piel de las bayas, participa en funciones relevantes relacionadas con el control de la turgencia celular, como agente osmótico, o en mecanismos vinculados con el crecimiento y desarrollo de la uva (Clarkson & Hanson, 1980; Pratelli *et al*., 2002; Salt, 2004; Davies *et al*., 2006; Hanana *et al*., 2007). Además, se ha propuesto que este catión podría contribuir al equilibrio de carga que estaría implicado en el transporte de azúcares (Lang, 1983). En el presente trabajo, hemos cuantificado el contenido relativo de potasio y su distribución en las células de la piel de uva Cardinal mediante la técnica de microscopía electrónica de barrido a bajas temperaturas y microanálisis por dispersión de energía de rayos-X (SEM-EDX). Esta técnica no destructiva permite, paralelamente al estudio de superficies de altas resolución mediante imagen, determinar los elementos químicos de una muestra, cuantificarlos y plasmar en imagen su distribución mediante el microanálisis de rayos-X. Asimismo, el contenido de potasio y el de otros iones mayoritarios (Na^+, Ca^{2+}, Mg^{2+}, P) se cuantificaron mediante espectroscopía de emisión óptica de plasma de acoplamiento inductivo (ICP-OES). En esta técnica, los iones o átomos de la muestra son excitados o ionizados mediante una corriente inducida de alta frecuencia y cuando vuelven a su estado fundamental emiten radiaciones de una longitud de onda que es característica de cada elemento. De manera que la intensidad de cada una de las radiaciones está relacionada con la concentración de cada elemento en la muestra. Los resultados de ambos ensayos indicaron que el potasio es el ión más

abundante detectado en las células de la piel. La caracterización de estas células mediante SEM-EDX, en base a la distribución del potasio, mostró una acumulación irregular de este ión en las distintas capas celulares analizadas. Mientras que la capa epidérmica y las células más externas de la hipodermis contenían menos potasio, su concentración fue mayor en las capas hipodérmicas contiguas, al menos hasta la sexta capa de células a partir de la cual empezó a disminuir. Estos resultados son consistentes con los obtenidos por Storey (1987) en el pericarpio de uva. Aunque las razones de esta distribución no uniforme del potasio en las diferentes células no está claro, su localización específica en las células epidérmicas e hipodérmicas de la piel podría posiblemente indicar una diferente funcionalidad de estas células. Aunque no hemos cuantificado las variaciones en el contenido de potasio citoplasmático o vacuolar, nuestros resultados mostraron que los cambios en el contenido de potasio estaban relacionados con la dinámica del agua en las diferentes capas celulares y con las variaciones en el contenido de agua ligada, cuantificada mediante DSC. Cuando se comparó mediante LT-SEM la ultraestructura de las células criofijadas de la capa epidérmica y la primera hipodérmica de la piel de las uvas almacenadas a bajas temperaturas en aire con las tratadas con 20% de CO_2 a 0ºC, así como con la de los frutos recién recolectados, la característica más relevante de las muestras conservadas en aire fue la compresión de estas células. En ellas, también fue evidente la pérdida de volumen y la contracción, posiblemente por la pérdida y liberación de agua libre. Sin embargo, el tratamiento con altos niveles de CO_2 durante 3 días tuvo un efecto beneficioso sobre el mantenimiento del volumen celular, así como de la integridad de las membranas plasmáticas y de las paredes celulares, de manera que la morfología de las células parecía similar a la descrita en los frutos recién recolectados. Por el contrario, la pérdida de volumen de las células de las capas externas de la piel en respuesta a las bajas temperaturas y las alteraciones en el equilibrio hídrico refuerzan y evidencian su daño. Este daño estaría asociado a una pérdida de su integridad, con posibles cambios en la bicapa lipídica y en los sistemas enzimáticos de membrana, como ATPasas, bombas de protones, transportadores, etc., lo que repercutiría en el flujo de iones, y principalmente en el del potasio. En uva, se han caracterizado diferentes canales de potasio y otros transportadores (Pratelli *et al*., 2002; Davies *et al*., 2006), como es el caso de los antiportadores K^+/H^+ de tipo NHX (Hanana *et al*., 2007). En el caso concreto de los NHX, se ha revisado recientemente que podrían participar en

importantes funciones intracelulares en las plantas, tales como la adaptación a estrés y el ajuste osmótico (Bassil & Blumwald, 2014). El mayor contenido de potasio en la piel de uva mesa tras 3 días a 0ºC, junto con la pérdida de volumen, y el descenso de agua no congelable parecen evidenciar un daño celular y tisular durante fase inicial de conservación a 0ºC, lo que estaría en concordancia con trabajos anteriores (Sanchez-Ballesta *et al*., 2007) donde se puso en evidencia que a pesar de que la uva se ha catalogado como un fruto tolerante a las bajas temperaturas, parece ser sensible a los cambios de temperatura a 0ºC. No obstante, la contribución del potasio como osmolito que compense la salida de agua producida en estas condiciones requiere de posteriores análisis. Por el contrario, en las células de la piel de frutos tratados con altas concentraciones de CO_2, no se visualizaron modificaciones en su volumen celular, ni se detectaron variaciones significativas en la liberación de potasio, ni en las poblaciones de agua. Estos resultados indican una limitación en la movilidad del agua en las células de la piel, lo que a su vez podría repercutir en una reducción de su pérdida.

En base a la importancia de mantener los niveles de agua no congelable durante la conservación del fruto, resulta de gran utilidad conocer si el efecto del binomio altos niveles de CO_2-bajas temperaturas en el estado del agua de la uva de mesa va a depender de su grado de madurez. Hasta el momento, se ha estudiado que el grado de madurez influye en diferentes acontecimientos que contribuyen a la calidad de distintos frutos (Shin *et al*., 2008; Lijavetzky *et al*., 2012). Sin embargo, no se había analizado la posible correlación entre grado de madurez y el estado de agua de los tejidos en la fase crítica de conservación a bajas temperaturas. Para ello, se analizaron uvas de mesa con dos grados de madurez distintos, procedentes de dos campañas (G1 y G2), que mostraban diferencias significativas en parámetros de maduración tradicionales como el contenido en sólidos solubles y el color. En cuanto a las características cromáticas, estas diferencias se reflejaron en los valores de luminosidad (L^*), croma (C^*) y ángulo hue (^oh) entre G1 y G2 de las muestras recién recolectadas. En particular, los valores ^oh, uno de los parámetros más representativos de la apariencia visual de la piel de las uvas de esta variedad, corroboraron una menor intensidad del color rojo en la piel de G1 (38,83 ± 0,05) que en el de G2 (12,37 ± 0,15). Igualmente, los resultados sobre las variaciones en las poblaciones de agua ligada mostraron de manera interesante que, independientemente del grado de madurez del fruto, la fase inicial de conservación a 0ºC iba asociada a un descenso en el contenido de agua ligada, no observándose dicho

desajuste en los frutos tratados con alto CO_2. Además, se observó una ligera disminución en la temperatura de inicio de congelación (T_{onset}) en los frutos tratados con altos niveles de CO_2. En conjunto, nuestros resultados evidenciaron que el desequilibrio metabólico provocado por la conservación a 0ºC, independientemente del grado de madurez y dentro de una fase relativamente temprana, se manifestó en un descenso en contenido de agua ligada, pérdida de la integridad de membrana y, con ello, la liberación de potasio y agua libre al espacio extracelular. La aplicación de altas concentraciones de CO_2, posiblemente evitando los desajustes metabólicos del fruto, impidió la manifestación de estos marcadores de daño relacionados con el estado del agua.

2.- Análisis e identificación de marcadores relacionados con el estado oxidativo inducido por las bajas temperaturas en los diferentes tejidos del racimo.

Junto a las alteraciones metabólicas atribuibles a las bajas temperaturas en la fase inicial de conservación, que afectan al contenido de agua ligada, volumen celular, pérdida de la compartimentalización celular y salida de potasio, hay que incluir las relacionadas con los daños oxidativos. Es bien conocido que las bajas temperaturas inducen la producción de ROS (revisado por Sevillano *et al*., 2009) ya que, junto a otros estreses abióticos, pueden ocasionar alteraciones en la homeostasis celular que resultan en un aumento de sus niveles (Jaspers & Kangasjärvi, 2010). Aunque no hemos cuantificado los cambios en el contenido de potasio citoplasmático o vacuolar, los cambios en el pH de los diferentes compartimientos celulares, como resultado del flujo de potasio, podrían haber participado en la producción de ROS. Se sabe que la acidificación citoplasmática es fundamental en la transducción de la señal de la respuesta defensiva, ya que es un paso previo a la generación de ROS y metabolitos secundarios (Shakano, 2001; Zhao *et al*., 2005). No obstante, no está demostrado si los cambios en la generación de ROS a bajas temperaturas están directamente implicados en la activación de las respuestas de defensa de las plantas o son una mera consecuencia del estrés oxidativo que ocurre en las células atacadas. Por tanto, el balance entre la formación y detoxificación de ROS es crítico para la supervivencia de la célula (Mittler, 2002; Blokhina *et al*., 2003; Jaspers & Kangasjärvi, 2010). Asimismo, la producción de ROS en condiciones de estrés se ha asociado con procesos de pardeamiento y

senescencia en frutos (Ruenroengklin *et al*., 2009). La acumulación de estas especies moleculares puede producir la oxidación de los ácidos grasos poliinsaturados de las membranas celulares, originándose productos tóxicos como el malondialdehído (MDA). Por ello, el nivel de peroxidación lipídica, medido mediante el contenido de MDA en frutos (Xu *et al*., 2012), ha sido ampliamente analizado como indicador de estrés oxidativo. En consecuencia, hemos cuantificado los cambios en el contenido de MDA en los tejidos de piel, pulpa y raquis de uva de mesa en respuesta a las bajas temperaturas y altos niveles de CO_2. Nuestros resultados indican que aunque el incremento en el contenido de MDA en piel no fue significativo, sí lo fue en pulpa y raquis de los racimos no tratados como respuesta a su conservación a bajas temperaturas, siendo especialmente evidente en el caso del raquis a lo largo de todo el período de conservación. En contraste, los niveles de MDA fueron, en general, menores en los tejidos de los racimos tratados con altos niveles de CO_2 en comparación con los no tratados, lo que parece indicar que el tratamiento gaseoso reduce el estrés oxidativo asociado a las bajas temperaturas y apoya trabajos previos en los que observamos diferencias en la percepción del frío entre uvas no tratadas y tratadas con CO_2 (Sanchez-Ballesta *et al*., 2006; Romero *et al*., 2008b).

Las células vegetales han desarrollado una gran variedad de sistemas, tanto enzimáticos como no enzimáticos (Zhang *et al*., 1995; Prasad, 1996; Moller, 2001; Mittler, 2002; Kuk *et al*., 2003), que se coordinan cooperativamente y que les protege de los riesgos que conlleva el estrés oxidativo. En nuestro trabajo, hemos analizado el efecto de las bajas temperaturas y el binomio altos niveles de CO_2-bajas temperaturas en el contenido de uno de los principales antioxidantes no enzimáticos, los compuestos fenólicos. Concretamente, hemos cuantificado los antocianos y fenoles totales presentes en la piel de las bayas. Asimismo, también hemos estudiado el efecto del tratamiento con altas concentraciones de CO_2 en la expresión de genes que codifican enzimas relacionadas con la síntesis y oxidación de los compuestos fenólicos (*VcPAL y GPO1*) y con el sistema antioxidante (*GCAT y VcAPX*) en distintos tejidos del racimo durante su conservación a 0ºC.

La uva constituye una de las mayores fuentes de compuestos fenólicos dentro de las distintas especies de frutos (Macheix *et al*., 1990), los cuales abarcan un amplio grupo de metabolitos secundarios implicados en su calidad (Tomás-Barberán & Spin, 2001; Kalt, 2005; Jaakola, 2013), cuya capacidad antioxidante va a depender de su

estructura individual y del número de hidroxilos sustituyentes, así como del peso molecular. La producción de los compuestos fenólicos son consecuencia de importantes cambios en la expresión génica, actividades enzimáticas y metabolismo que caracteriza la maduración del fruto (Deluc *et al*., 2006). Por otro lado, se ha observado la acumulación de compuestos fenólicos en respuesta a un amplio rango de estreses bióticos y abióticos (Dixon & Paiva, 1995). En estudios previos de nuestro grupo, comprobamos que en la fase crítica de conservación a 0ºC (3 días) había un incremento transitorio en el contenido total de antocianos medido mediante el método de pH diferencial, así como en el contenido de antocianos determinado por la suma de los antocianos individuales cuantificados por cromatografía líquida acoplada a espectroscopía de masas (HPLC-MS) (Sanchez-Ballesta *et al*., 2007; Romero *et al*., 2008a). A dicho incremento, contribuía principalmente la peonidina-3-glucósido, que es el antociano mayoritario en la piel de uva Cardinal. Además, los resultados de capacidad antioxidante total (TAC), obtenidos por el método ABTS, al igual que la calculada a partir de la contribución de cada uno de los antocianos identificados en esta variedad, teniendo en cuenta su concentración y capacidad antioxidante medida como valor TEAC (pendiente de antocianos/pendiente Trolox), indicaron un incremento transitorio en la actividad antioxidante en la piel de las bayas durante la conservación a 0ºC, mientras que en los frutos tratados con CO_2 no se observó dicho incremento. A la vista de estos resultados, consideramos que el incremento transitorio observado en lo frutos no tratados podría ser un indicador del daño oxidativo en esta fase inicial de conservación. Puesto que se ha descrito que las bajas temperaturas y el grado de madurez afecta a la calidad y a la capacidad antioxidante de frutos conservados (Shin *et al*., 2008), investigamos si el grado de madurez tenía alguna influencia en el efecto de la exposición durante 3 días con altas concentraciones de CO_2 a lo largo de su conservación a 0ºC en la acumulación de antocianos y fenoles totales, así como en la actividad antioxidante en la piel de la uva. Además, analizamos la expresión del gen que codifica la enzima L-fenilalanina amonio-liasa, PAL, clave en la ruta biosintética de distintos compuestos fenólicos (Hahlbroock & Scheel, 1989; Dixon & Paiva, 1995), que cataliza de desaminación de la fenilalanina procedente de la ruta del ácido siquímico en ácido *trans*-cinámico.

Nuestros resultados mostraron que en la piel de frutos con menor grado de madurez conservados a 0ºC, no se producía el incremento inicial en la capacidad

antioxidante, resultado del mantenimiento de los niveles de antocianos y de fenoles totales. Igualmente, no se observaron cambios en los niveles de los transcritos de *VcPAL*. Únicamente, se detectó un incremento significativo tanto en la acumulación de *VcPAL* como en el contenido de fenoles totales después de 27 días conservación a 0ºC. En consecuencia, al depender el incremento en la capacidad antioxidante del grado de madurez del fruto, no puede generalizarse como posible marcador de daño en la fase inicial de conservación a 0ºC. Asimismo, la aplicación de altas concentraciones de CO_2 no afectó a ninguno de los parámetros analizados. Por otro lado, a pesar de que los frutos con menor grado de madurez presentan un menor contenido de antocianos totales que los frutos con mayor grado, nuestros datos indican que su mayor actividad antioxidante deriva de la acumulación de compuestos fenólicos distintos a los antocianos. Aunque la acumulación de antocianos en la uva se inicia en el envero y va aumentando durante su maduración (Roubelakis-Angelakis & Kliever, 1986; Mateus *et al*., 2002), se ha descrito que su elevada capacidad antioxidante no siempre está relacionada con la acumulación de antocianos en distintos cultivares de uva tinta (Orak *et al*., 2007). Comparativamente, en los ensayos realizados con las uvas mayor grado de madurez, sí se observaba un incremento en la capacidad antioxidante tras 3 días de almacenamiento a bajas temperaturas, que se correlacionó con la acumulación de antocianos (r^2 = 0,892), así como con un aumento de la expresión de *VcPAL*, lo que parece indicar una rápida activación del metabolismo fenólico. A pesar de que algunos autores, como Kalt *et al*. (1999) y Wang & Lin (2000), también encontraron una correlación significativa entre la capacidad antioxidante, los fenoles totales y los antocianos en distintas variedades de frutos, se han observado variaciones en esta respuesta en función del cultivar y el estado de madurez del fruto (Wang & Lin, 2000). En contraste, mientras que la aplicación de un 20% de CO_2 durante 3 días limitó la acumulación de *VcPAL*, antocianos y fenoles totales detectados en los frutos no tratados, la actividad antioxidante de estas uvas tratadas incrementó significativamente. De hecho, no se observó correlación entre la capacidad antioxidante y el contenido en antocianos en estos frutos (r^2 = -0,049). Además, nuestros resultados sugieren que la activación en el metabolismo de los fenilpropanoides en la fase inicial de conservación a 0ºC, posiblemente como consecuencia de la percepción del fruto de una temperatura inferior a su óptima de conservación (Sanchez-Ballesta *et al*., 2007), depende del grado de madurez del fruto en el momento de su recolección. Igualmente, dicha activación se

ve limitada por el tratamiento con CO_2 en los frutos más maduros, lo que indicaría una mejora en su tolerancia a las bajas temperaturas. A este respecto, distintos trabajos han demostrado la existencia de adaptación cruzada en las plantas, por lo que la exposición a un estrés moderado no sólo induce resistencia a dicho estrés, sino que también puede mejorar la tolerancia a otros (Bowler & Fluhr, 2000; Wang *et al*., 2003).

Puesto que el grado de madurez parece jugar un papel importante en las respuestas descritas de la piel de la uva a las bajas temperaturas y altas concentraciones de CO_2, y teniendo en cuenta que la influencia de elevados niveles de CO_2 en el metabolismo general de los frutos puede estar afectado por su concentración y duración del tratamiento (Becatti *et al*., 2010), hemos analizado también el efecto de la exposición a un 20% de CO_2 durante 6 días en la piel de uva de mesa a ambos estados de madurez. En el caso de las uvas con menor grado de madurez, el tratamiento gaseoso durante 6 días tampoco afectó ni a la expresión de *VcPAL* ni a la acumulación de antocianos, aunque incrementó el contenido de fenoles y descendió la actividad antioxidante. No obstante, al final del período de conservación la actividad antioxidante aumentó alcanzando valores semejantes a la de los frutos no tratados. Sin embargo, en la piel de las uvas con mayor grado de madurez se observó una acumulación de los transcritos de *VcPAL* tras 6 días de tratamiento con CO_2, lo que fue acompañado de un incremento en la capacidad antioxidante, fenoles totales y antocianos. Concretamente, los mejores valores de correlación fueron observados con el contenido en antocianos (r^2 = 0,993), lo que probablemente refleje su contribución en la capacidad antioxidante total de este fruto. Además, esta prolongación en el tiempo de exposición con altos niveles de CO_2 mantuvo al final de almacenamiento de los frutos mayores niveles de acumulación de fenoles totales y antocianos, así como de expresión de *VcPAL* que los observados en las uvas no tratadas. Distintos estudios, han indicado que la inducción del metabolismo de los fenilpropanoides en respuesta a diferentes tipos de estreses (Wang *et al*., 2007; Rinaldo *et al*., 2010). Por tanto, al igual que las uvas recolectadas tardíamente no tratadas, el tratamiento con 20% de CO_2 durante 6 días probablemente active el metabolismo fenólico asociado a daño por excesivo tiempo de aplicación del tratamiento gaseoso. Además, dicha inducción no solo es dependiente del tiempo de exposición al tratamiento gaseoso, sino también del grado de madurez al que se recolectan los frutos.

Además de la importancia de los compuestos fenólicos en la apariencia visual de los frutos, su degradación mediante la PPO juega un papel relevante en términos de calidad al participar en el desarrollo de pardeamientos. La PPO cataliza la oxidación de monofenoles y *O*-difenoles a *O*-quinonas, originándose pigmentos de color marrón, negro y rojo (Tomás-Barberán & Espin, 2001; Lei *et al*., 2004; Lichter *et al*., 2011). Concretamente, se ha descrito que la capacidad de oscurecimiento de diferentes variedades de uva de mesa durante su conservación está asociada a la naturaleza y cantidad de la enzima PPO presente en el raquis, así como al contenido de substrato disponible (Carvajal-Millán *et al*., 2001), como son las cantidades significativas de compuestos polifenólicos presentes en estos frutos (Souquet *et al*., 2000). Por ello, junto a la expresión de *VcPAL*, analizamos los cambios de expresión de un gen (*GPO1*) que codifica esta enzima en los distintos tejidos del racimo en respuesta a altas concentraciones de CO_2 durante su conservación a bajas temperaturas. En el caso de la piel, aunque el pardeamiento no fue evidente, se estudió el comportamiento de *GPO1* con el fin de evaluar su participación en las respuestas de defensa de este tejido a las bajas temperaturas. Así, los resultados obtenidos nos indicaron que ni la conservación a 0ºC ni la aplicación de CO_2 indujeron su expresión. Es más, al final del periodo de conservación había un descenso significativo tanto en la piel de frutos tratados como en los mantenidos en aire.

En la pulpa, aunque no se observó un incremento en la expresión de *VcPAL* en la primera fase de conservación a 0ºC, sí tuvo lugar tras 15 días, siendo significativamente menor en los tejidos tratados con CO_2. Esta inducción en el metabolismo fenólico por bajas temperaturas parece corroborar los resultados anteriormente descritos en piel, así como el papel del tratamiento gaseoso evitando y/o modificando estos cambios (Sanchez-Ballesta *et al*., 2007). Sin embargo, en la pulpa, no se encontraron diferencias significativas en la acumulación de *VcPAL* entre frutos tratados y los mantenidos en aire al final del periodo de conservación. Por otro lado, la expresión de *GPO1* se indujo por las bajas temperaturas, alcanzando niveles máximos a los 15 días de conservación. Al igual que la piel, no se observó pardeamiento visual de la pulpa, por lo que esta inducción podría estar asociada con la activación de respuestas de defensa a su exposición a bajas temperaturas. Por su parte, el CO_2 retrasó o redujo dicha inducción, incrementando únicamente de manera transitoria a los 15 días.

El pardeamiento del raquis, junto a la podredumbre de las bayas producida por *B. cinerea* y la pérdida de agua, es una de las disfunciones fisiológicas que conducen a la pérdida de la calidad postcosecha de los racimos de uva de mesa durante su conservación a bajas temperaturas. En concreto, el oscurecimiento y marchitamiento del raquis pueden a ayudar a determinar su período máximo de conservación a estas temperaturas (Sanchez-Ballesta *et al*., 2006), al influir en la apariencia general de los racimos. Nuestros resultados mostraron un incremento transitorio de los niveles de *GPO1* en el raquis en la fase inicial (3 días) de conservación de los racimos mantenidos en aire, sin observarse cambios en la acumulación de *VcPAL*. Este incremento de *GPO1*, al que va unido un aumento en el índice de pardeamiento del raquis, podría constituir un marcador de la fase inicial del daño antes de que la manifestación visual del pardeamiento del mismo fuera más evidente. Por el contrario, la aplicación de un 20% de CO_2 durante 3 días a 0ºC redujo tanto el pardeamiento como el incremento en la expresión de *GPO1* que se detectaron en los raquis no tratados, mientras que la acumulación de los transcritos de *VcPAL* no se vio afectada por el tratamiento gaseoso. Sin embargo, el hecho de que el índice de pardeamiento del raquis y la expresión de *VcPAL* incrementaran a lo largo del almacenamiento a 0ºC en los racimos no tratados, siendo menor en las muestras tratadas con CO_2, mientras que los niveles de los transcritos de *GPO1* disminuyeron tanto en el raquis de los racimos tratados como no tratados hasta alcanzar valores menores a los de los frutos recién recolectados, parecen indicar que otros factores diferentes a la acción de la PPO están implicados en el desarrollo del pardeamiento. Estos resultados corroboran los mencionados anteriormente, donde el tratamiento gaseoso mantiene constante o restringe el incremento por bajas temperaturas de los niveles de compuestos fenólicos observados en la piel de uva en dos estados de madurez diferente. Este hecho podría conducir a una reducción del pardeamiento del raquis debido a una menor disponibilidad del sustrato. En este sentido, Murr & Morris (1974) mostraron que el CO_2 es un inhibidor competitivo de la PPO, ya que altas concentraciones de CO_2 inhibían irreversiblemente la oxidación de fenoles por la PPO. Asimismo, Siriphanich & Kader (1985) argumentaron que altos niveles de CO_2 prevenían el pardeamiento de tejidos de plantas heridas mediante el bloqueo de la producción de nuevos compuestos fenólicos, así como por la inhibición de la actividad de la PPO.

Se sabe que los productos vegetales modulan sus defensas enzimáticas antioxidantes cuando se exponen a bajas temperaturas de conservación (Campos-Vargas *et al*., 2012; Sánchez-Bel *et al*., 2012; Pavez *et al*., 2013; Yuan *et al*., 2014) y a distintos tratamientos gaseosos postcosecha (Wang *et al*., 2005b; Ponce-Valadez *et al*., 2009). En el caso de uva de mesa Cardinal, se ha sugerido que la APX podría participar en la eliminación de H_2O_2 inducido por las bajas temperaturas, y que el pretratamiento gaseoso de 3 días con altas concentraciones de CO_2 podría mitigarlo (Romero *et al*., 2008b). Por ello, hemos estudiado el efecto de altos niveles de CO_2 en la expresión de genes que codifican enzimas relacionadas con el sistema antioxidante (*GCAT y VcAPX*) en distintos tejidos del racimo durante su conservación a 0ºC.

En líneas generales, nuestros resultados parecen indicar que el efecto beneficioso del tratamiento con altas concentraciones de CO_2 en el control del estrés oxidativo mediante la inducción de enzimas antioxidantes depende del tipo de tejido, y podría estar más bien relacionado con la reducción del pardeamiento del raquis que con una respuesta general de la uva de mesa. Así, aunque el tratamiento con CO_2 mantuvo o restringió el contenido de MDA en pulpa y piel, no se observaron diferencias significativas en la expresión de *VcAPX* en pulpa ni de *GCAT* en ambos tejidos. En cambio, a pesar de que no se observó una buena correlación entre el contenido de MDA y el pardeamiento del raquis (r = 0,35), nuestros resultados mostraron ciertas tendencias en el comportamiento de ambos parámetros ya que incrementaron durante su almacenamiento en frío, siendo mayores en comparación con los frutos recién recolectados y los tratados con CO_2. Asimismo, el incremento observado en los niveles de los transcritos de *VcAPX* y *GCAT* en raquis de racimos no tratados parece que, o bien fue tardío, o insuficiente para reducir el contenido de MDA. Hasta el momento, no existe mucha información al respecto, pero los ensayos realizados por Balic *et al*. (2012) aportaron evidencias de la correlación entre el oscurecimiento del raquis de uva Red Globe y cambios en la transcripción de determinados genes implicados en procesos relacionados con el estrés oxidativo. Comparativamente, los raquis de uva Cardinal tratados con altos niveles de CO_2 presentaron un menor nivel de estrés oxidativo que los no tratados, que parecía estar regulada por la inducción de la expresión génica de *VcAPX y GCAT*, especialmente evidente al final del período de conservación donde el contenido de MDA fue bajo y el pardeamiento moderado. En este sentido, aunque solo se observó una moderada correlación negativa entre el contenido en MDA y la

acumulación de los transcritos de *GCAT* (r = -0,53), fue muy buena entre la expresión de este gen y el pardeamiento del tejido (r = -0,93), sugiriendo su implicación en la reducción del desarrollo del deterioro. Wang *et al*. (2005b) indicaron que el empleo de atmosferas modificadas (5% O_2 + 5% CO_2) en melocotones almacenados a 0ºC, redujo los daños por frío y retrasó la disminución de las actividades enzimáticas de la SOD y CAT observados en los frutos control.

3.- Cambios en los patrones de expresión de genes regulados por las bajas temperaturas y altas concentraciones de CO_2.

Las hormonas vegetales son esenciales en la capacidad de las plantas para adaptarse a distintos estreses abióticos (Santner & Estelle, 2009). Concretamente en frutos, se ha descrito que la producción de etileno y la expresión de genes de su biosíntesis (Fonseca *et al*., 2005; Villalobos-Acuña *et al*., 2010; Megías *et al.*, 2014), así como de ABA (Yoshikawa *et al*., 2007; Maul *et al*., 2008; Xia *et al*., 2014), aumentan en frutos en respuesta a las bajas temperaturas. Teniendo en cuenta que se ha estudiado también la implicación de estas hormonas en la coordinación y regulación de respuestas frente al estrés producido por frío (Wang *et al*., 1990; Sevillano *et al*., 2009; Theocharis *et al*., 2012), se presenta interesante analizar su papel durante la fase inicial de conservación a 0ºC, y así entender si el propio estrés es el que induce los genes biosintéticos de ABA o etileno. Por otra parte, tanto el etileno como el ABA participan en los procesos de maduración y senescencia de los frutos, pudiendo producir cambios en su calidad durante su conservación postcosecha, como la aparición de pardeamientos y síntomas de oscurecimiento. En el caso de *Vitis*, se ha propuesto la interconexión entre ABA y etileno para iniciar el proceso de maduración de la baya (Sun *et al*., 2010). No obstante, no hay mucha información sobre la regulación de la síntesis de estas hormonas durante la conservación de uva de mesa a bajas temperaturas, y su participación en los síntomas visibles de envejecimiento. Así, mientras que Palou *et al*. (2003), observaron que la exposición continua a etileno durante la conservación de uva de mesa a bajas temperaturas no afectaba al pardeamiento del raquis, Balic *et al*. (2012) detectaron, en un estudio molecular y fisiológico del pardeamiento postcosecha del raquis de uva de mesa Red Globe, una disminución en la transcripción de un gen que codifica a un miembro de factores de transcripción de respuesta a etileno (*ERF*), cuando

es almacenada a 0º C durante 90 días. Igualmente, en el caso del ABA se ha observado que el tratamiento de racimos de uva Crimson Seedless con esta hormona mejoraba la calidad del raquis durante su conservación (Cantin *et al*., 2007). Por otro lado, tal y como hemos comentado en el apartado anterior, la aparición de pardeamiento en el raquis de uva de mesa parece estar asociada con un incremento en la acumulación de transcritos de *GPO1* en la primera fase de su conservación a 0ºC. Además, se ha publicado que las actividades PAL y PPO inducidas por etileno están relacionadas con el deterioro en la coloración de partes de distintos productos vegetales (Hyodo *et al*., 1978; Pesis *et al*., 2002). Por todo ello, hemos analizamos la expresión de genes implicados en biosíntesis de etileno (*ACO1 y ACS1*) y de ABA (*VvNECD1 y VvNECD2*) en los diferentes tejidos del fruto, tanto en la fase inicial como a lo largo del periodo de conservación, así como su posible correlación con los cambios observados en *PPO* y *PAL*.

Los ensayos realizados en este estudio han mostrado que el pardeamiento del raquis de los racimos no tratados mantenidos en aire estaba relacionado con la inducción de la expresión de genes de la biosíntesis del etileno. Concretamente, existe una correlación positiva con la expresión de *ACS1* (r = 0,70). Asimismo, hemos observado una buena correlación entre los niveles de expresión de *ACO1* y *GPO1* (r = 0,94), siendo moderada entre *ACS1* y *PAL* (r = 0,57). Por el contrario, el tratamiento gaseoso, que redujo el pardeamiento del tejido, también evitó la acumulación de los transcritos de *ACS1* y *ACO1*. Por tanto, consideramos que el efecto beneficioso del tratamiento con altas concentraciones de CO_2 en la reducción del pardeamiento del raquis podría haberse ejercido a través de una modulación de genes de la biosíntesis de etileno evitando su expresión. Sin embargo, esta regulación parece ser específica del raquis, puesto que los niveles de *ACS1* aumentaron en pulpa y los de *ACS1* y *ACO1* en piel de frutos tratados con CO_2. El diferente comportamiento exhibido en los niveles de expresión entre los tejidos del racimo está en consonancia con los resultados obtenidos por otros autores que atribuyen al CO_2 un papel tanto inductor (Mathooko, 1996) como supresor (Mathooko, 1996; de Wild *et al*., 2003) de la ruta de biosíntesis de etileno, dependiendo del producto, del tejido, de la concentración de CO_2 y del tiempo de exposición. En uva de vino, altos niveles de CO_2 indujeron la acumulación de los transcritos de *ACO* y *ACS* en piel y pulpa (Becatti *et al*., 2010). De acuerdo con estos autores, nuestros resultados mostraron una acumulación transitoria en

los niveles de transcritos de *ACS1* y *ACO1* en la fase inicial de conservación a 0ºC en los tejidos de piel tratados con CO_2. Este incremento permite sugerir que la respuesta de estos tejidos al tratamiento gaseoso es dependiente de la señalización de etileno. En consecuencia, estas observaciones también sugirieron una regulación diferente de la biosíntesis de etileno en los distintos tejidos, y reflejó diferencias en sus estructuras génicas y elementos de regulación.

En cuanto a los resultados sobre el ABA, los ensayos de expresión génica revelaron que no existía relación entre el deterioro del raquis y los niveles de expresión de *VvNECD1*, ya que se produjo una regulación negativa tanto en los raquis no tratados como en los tratados. Igualmente, la acumulación de los transcritos de *VvNECD2* no se alteró a lo largo de su conservación en comparación con la de las muestras recién recolectadas. Con respecto al resto de tejidos analizados, observamos que la piel presentaba patrones similares de expresión que los del raquis. En piel de uva de vino tratada con CO_2 se ha detectado también una disminución en la expresión de *NECD* (Becatti *et al*., 2010). Sin embargo, *VvNECD1* sí se indujo específicamente por bajas temperaturas en pulpa, lo que está consonancia con los resultados obtenidos en diferentes investigaciones con frutos (Maul *et al*., 2008; Xia *et al*., 2014). Por el contrario, el tratamiento gaseoso restringió claramente esta inducción en la pulpa. Estos resultados, junto con la hipótesis de la reducción de la síntesis de ABA como respuesta a los altos niveles de CO_2 en la pulpa de uva de mesa, fueron también consistentes con resultados anteriores que indican que el tratamiento gaseoso se correlacionó positivamente con una alta tolerancia a los cambios de temperatura a 0ºC (Sanchez-Ballesta *et al*., 2007).

3.1.- Aislamiento y caracterización de genes que codifican los factores de transcripción *CBF1* y *CBF4*, así como la dehidrina *DHN1a*.

A pesar de los estudios realizados hasta el momento, se conoce poco de los mecanismos moleculares implicados en la respuesta de uva de mesa a las bajas temperaturas, al igual que en repuesta al tratamiento gaseoso coadyuvante. Junto a genes relacionados con la defensa de las plantas frente al estrés oxidativo (Sung *et al*., 2003), se sabe que las bajas temperaturas inducen en las plantas la expresión de genes que forman parte de la superfamilia *COR*, que codifican, entre otras, un conjunto de

proteínas hidrofílicas denominadas LEA (Close *et al*., 1997), familia a la que pertenecen las dehidrinas, así como proteínas con función crioprotectora y/o anticongelante (Hon *et al*., 1994). Se han identificado los activadores transcripcionales CBF/DREB, que se unen a los elementos *cis* CRT/DRE presentes en los promotores de los genes *COR*, que están altamente conservados en plantas y muestran normalmente una muy rápida inducción a nivel transcripcional después de la exposición a bajas temperaturas. Sin embargo, en hojas de *Viti*s, la acumulación de *CBF3* y *CBF4* se observó después de 1-2 días a bajas temperaturas (Xiao *et al*., 2006, 2008). A pesar de que los *CBFs* pertenecen a una de las rutas de señalización mejor caracterizadas implicadas en la tolerancia de las plantas a las bajas temperaturas (revisado por Miura & Furumoto, 2013), y de que se ha observado su inducción durante la conservación frigorífica de productos vegetales (Zhao *et al*., 2009a; Liang *et al*., 2013; Ma *et al.*, 2014), su papel en la respuesta de los frutos a los tratamientos gaseosos durante la conservación a bajas temperaturas es desconocido. En nuestro trabajo de investigación, hemos caracterizado y analizado los patrones de expresión de genes que codifican dos activadores transcripcionales CBF, *VvcCBF1* y *VvcCBF4,* en tejidos del fruto (piel, pulpa y semilla) de racimos no tratados y tratados con CO_2 durante su conservación a 0ºC, así como en el raquis con el fin de comprobar si dicha respuesta era específica para cada uno de ellos. Tal y como hemos citado anteriormente en hojas (Xiao *et al*., 2006, 2008), se ha descrito que la exposición a bajas temperaturas aumenta también la expresión de genes *CBF* en tallos y flores de *Vitis* (Takuhara *et al*., 2011). Sin embargo, nuestros resultados mostraron que, con excepción de las semillas, la exposición a 0ºC por sí sola no fue suficiente para activar *VvcCBF1* y *VvcCBF4* en la piel, pulpa y raquis de los racimos de uva Cardinal. En cambio, el binomio altas concentraciones de CO_2-bajas temperaturas indujo la expresión de ambos *CBFs* en la pulpa, y en el caso de *CBF4* también en el raquis. Además, es importante destacar que la expresión de ambos factores de transcripción fue dependiente del tejido, ya que ninguno de los dos *CBFs* mostró cambios en su expresión en la piel y *CBF1* no se acumuló en el raquis. Otra característica de ambos *CBFs* es que debido a la combinación de altas concentraciones de CO_2 y bajas temperaturas la expresión permaneció estable después de un periodo de tiempo relativamente largo (3 días) en comparación con las pocas horas indicadas en la mayoría de los *CBF*s estudiados. Aunque se desconoce el papel de estos factores de transcripción en la respuesta de uva a los cambios en las condiciones de conservación, es interesante señalar que nuestros

resultados son pioneros ya que se ha observado, por primera vez, que el tratamiento con altos niveles CO_2 a bajas temperaturas induce la expresión de *CBF1* y *CBF4* en diferentes tejidos del racimo. Además de las diferencias en la regulación de la expresión de ambos *CBFs* mencionadas, mediante el análisis de grupos hidrofóbicos (HCA) de la secuencias de amino ácidos completas también identificamos diferencias en la estructura secundaria y terciaria entre los CBF1 y CBF4 de *Vitis*. En *Arabidopsis*, Wang *et al*. (2005c) mostraron que la presencia de distintos grupos hidrofóbicos (HC2-HC6) en el dominio C-terminal ácido de *AtCBF1* lo convierte en un fuerte candidato para poseer propiedades de trans-activación. En nuestro estudio, identificamos en los CBF4 de *Vitis* cinco grupos (HC2-HC6) al igual que ocurre en AtCBF1 (Wang *et al*., 2005c), en comparación con los cuatro grupos (HC2–HC5) identificados previamente por Xiao *et al*. (2008). Asimismo, nuestro análisis mostró por primera vez que en los CBF1 de *Vitis* sólo se identificaban cuatro grupos hidrofóbicos como se había observado en los CBFs de monocotiledóneas. Esto fue debido a que HC3 y HC4 forman un único grupo, ya que un residuo de glutamina reemplaza a la prolina, que es considerado un interruptor de grupos y está presente entre HC3 y HC4 en la mayoría de los CBFs de dicotiledóneas. El efecto que este cambio pueda tener en las propiedades de trans-activación de los CBFs no está claro. Sin embargo, el hecho de que Xiao *et al*. (2008) observaran que CBF4 de *V. riparia* era un activador más efectivo que CBF1 podría estar relacionado con estas diferencias. Además, nuestro análisis mostró que los CBF4 de *Vitis* no pudieron generar un grupo HC5 fiable ya que carecían del residuo de triptófano presente en AtCBF1 y CBF1s de *Vitis*. Curiosamente, es otra de las características que los CBFs de *Vitis* comparten con los de monocotiledóneas (Wang *et al*., 2005c). Aunque es necesario llevar a cabo análisis que ayuden a determinar cómo las diferencias observadas en la región de activación de los CBFs de *Vitis* puede afectar a la respuesta a las bajas temperaturas, en base a nuestros resultados podríamos hipotetizar que la activación de la expresión de *CBF1* y *CBF4* en pulpa por la combinación de altos niveles de CO_2 y bajas temperaturas podría ayudar a los frutos a superar los cambios de temperatura a 0ºC. Por otro lado, los elevados niveles de expresión de *CBF4* observados en el raquis de los racimos tratados destaca el hecho de que el tratamiento con elevados niveles de CO_2 modula las respuestas moleculares de uva de mesa a bajas temperaturas, no sólo en el los tejidos de la baya, sino que también en el raquis como es evidente por

la reducción del pardeamiento del mismo y el incremento en el agua no congelable, descrito en apartados anteriores.

Como hemos comentado previamente, los factores de transcripción CBF reconocen el elemento *cis* CRT/DRE en los promotores de genes *COR*, entre los que se encuentran los que codifican dehidrinas. Por ello, hemos estudiado también los cambios en la expresión de una dehidrina, *VvcDHN1a*, en los distintos tejidos del racimo de uva de mesa en respuesta a las bajas temperaturas y al tratamiento gaseoso. A pesar de que el análisis del ADN genómico reveló la presencia de dos genes *DHN1*, *VvcDHN1a* y *VvcDHN1b*, solamente aislamos el gen *VvcDHN1a* que codifica una dehidrina de tipo YSK_2, cuya secuencia de aminoácidos es idéntica a VvDHN1a de *V. vinifera* descrita previamente por Xiao *et al*. (2006). Además, *VvcDHN1a* presentaba dos variantes de splicing, la forma "spliced" y "unspliced", cuya expresión se indujo por bajas temperaturas de conservación y por altos niveles de CO_2, pero con diferentes patrones de acumulación en los distintos tejidos analizados. Aunque en distintas especies de *Vitis* se ha observado un aumento en la acumulación de transcritos "spliced" y "unspliced" en respuesta a bajas temperaturas (Xiao & Nassuth, 2006), en este trabajo es la primera vez que se ha descrito retención de intrones en los transcritos por un tratamiento gaseoso. Por otro lado, en el caso concreto del raquis, se observó que la expresión de la variante "spliced" era constitutiva en ambas condiciones de almacenamiento, mientras que los transcritos "unspliced" se indujeron en raquis tratados y no tratados tras 15 días a 0ºC. Recientemente nuestro grupo ha llevado a cabo un análisis *in vitro* comparativo de la funcionalidad de las dos variantes de DHN1a, sugiriendo que los dominios K y Φ presentes en la forma "spliced" juegan un papel crucial en la respuesta de las dehidrinas a estreses abióticos, protegiendo frente a la congelación y la deshidratación a enzimas lábiles, así como inhibiendo el crecimiento de *B. cinérea* (Rosales *et al*., 2014). Los resultados apoyan la idea de que DHN1a actúa como un escudo molecular de las proteínas parcialmente desnaturalizadas en lugar de como una chaperona clásica interactuando directamente con proteínas desnaturalizadas. Además, se ha observado que el tratamiento gaseoso inducía los niveles de expresión de las proteínas DHN22 y 27 que presentan homología con DHN4 y DHN2 de *Vitis*, respectivamente, y que este hecho parece estar fuertemente regulado a nivel transcripcional ya que se encontró una buena correlación con los niveles de mRNAs (Navarro *et al*., 2015).

En resumen, el hecho de que no se observe acumulación de *CBF1* en la mayoría de los tejidos analizados, salvo en pulpa, en respuesta a las bajas temperaturas y/o altos niveles de CO_2, y que la inducción de *CBF4* se produzca principalmente en respuesta al tratamiento gaseoso, sugiere que la expresión de *DHN1a* es regulada por otras rutas activadas por frío independientes de estos factores de transcripción CBF. Los resultados específicos del raquis refuerzan esta hipótesis ya que, mientras la expresión de *CBF4* se produjo exclusivamente por acción del tratamiento gaseoso a bajas temperaturas, la de *DHN1a* fue constitutiva en este tejido independientemente de la condición de conservación. Sin embargo, es necesario llevar a cabo análisis de unión *cis*/trans que nos ayuden a corroborar esta hipótesis así como el posible papel de los CBFs en la regulación de la DHN2 y DHN4 identificadas recientemente en nuestro grupo.

4.- Análisis transcriptómico de la respuesta de uva de mesa Cardinal a las bajas temperaturas y altos niveles de CO_2 en dos estados de madurez diferentes.

La mayoría de los trabajos realizados hasta el momento en el campo de la biología molecular relacionados con la respuesta de los frutos a los tratamientos con altas concentraciones de CO_2 se han limitado al nivel de genes individuales o pequeños grupos de genes. Sin embargo, el creciente desarrollo de las técnicas de análisis global o a gran escala, denominadas 'ómicas', ha permitido abordar el análisis del transcriptoma desde un punto de vista más amplio, analizando simultáneamente la expresión de un elevado número de genes. Como hemos indicado anteriormente, el grado de madurez parece jugar un papel importante en las respuestas descritas en la piel de uva conservada a bajas temperaturas y altas concentraciones de CO_2. Con el fin de estudiar las respuestas transcripcionales de la piel de uva Cardinal a las bajas temperaturas y altas concentraciones de CO_2 en la fase crítica de conservación a 0ºC (3 días) y cómo el estado de madurez afecta a estos cambios, hemos realizado un análisis comparativo a gran escala de los cambios en el transcriptoma utilizando el GrapeGen GeneChip®. Dada la complejidad del conjunto de datos de expresión génica, se realizó una primera aproximación mediante un análisis de componentes principales (PCA) y de conglomerados jerárquicos (HCA) sobre los datos de expresión de las 18 muestras analizadas, que permitió agruparlas de acuerdo con su perfil de expresión génica global. Tanto el PCA como el HCA mostraron que en la fase crítica de conservación, las bajas

temperaturas dan lugar a un cambio intenso en el transcriptoma de la piel independientemente del estado de madurez de los frutos, aunque con diferencias en cada uno de ellos. Sin embargo, en los frutos tratados con altas concentraciones de CO_2 sólo se observaron ligeras diferencias en las respuestas transcripcionales en comparación con los frutos recién traídos de campo, independientemente del grado de madurez. Estos resultados están en concordancia con trabajos previos de nuestro grupo, donde analizando genes individuales se observó que la activación de procesos de respuesta a las bajas temperaturas en la primera etapa de conservación parece estar relacionada con la percepción de los cambios de temperatura a 0ºC que tuvo lugar en los frutos no tratados, que fue menos evidente en los tratados con altas concentraciones de CO_2 (Sanchez-Ballesta *et al*., 2007).

Los análisis de expresión diferencial (SAM, FDR 0,001, ratio de cambio de expresión ≥ 1,5 ó ≤ -1,5) revelaron la elevada capacidad de los frutos no tratados para modificar el transcriptoma durante la conservación a 0ºC. Si bien, los cambios más importantes en el número de genes que se expresan diferencialmente tuvo lugar en los frutos de menor grado de madurez. Como ya apuntaban los resultados obtenidos por el PCA y HCA, el número de genes que modificaron su expresión en respuesta al tratamiento de 3 días con altos niveles de CO_2 fueron menos notables en ambos estados de madurez respecto a los frutos recién recolectados. Asimismo, los mayores cambios se observaron en los frutos tratados con menor grado de madurez.

Para entender el significado biológico de los cambios moleculares observados, se llevó a cabo un análisis funcional utilizando DAVID (Database for Annotation, Visualization and Integrated Discovery), que mostró que las respuestas transcripcionales a las bajas temperaturas en la piel de uva en ambos estados de madurez coinciden con procesos biológicos de respuesta a distintos estreses bióticos y abióticos o estímulos. Concretamente, se observó la inducción de la expresión de genes relacionados con los términos de 'respuesta a las bajas temperaturas', 'calor', 'iones metálicos', 'bacteria' y 'privación de agua' en los frutos con menor grado de madurez, y con 'respuesta a la luz', 'estímulos de temperatura' y 'falta de nutrientes', en uva recogida tardíamente. Sin embargo, los términos más representados durante la conservación a 0ºC de los frutos con menor madurez estaban relacionados con la 'gluconeogénesis', con 'procesos catabólicos de quitina' y 'fotosíntesis'. Cabe destacar la inducción en la expresión de fosfoenolpiruvato carboxiquinasa 1 (*PCK1*), dos gliceraldehido-3-fosfato

deshidrogenasas (*GAPC1* y *GAPC2*) y cuatro quitinasas, entre las que se encuentra *chit1b*. Es importante señalar, que la inducción en la expresión de los transcritos de las cuatro quitinasas precedió a la aparición visual de la podredumbre, que tuvo lugar a partir de los 12 días a 0ºC (datos no mostrados). En este sentido, en un trabajo previo observamos un incremento en los niveles de expresión de *chit1b* en la piel de uva no tratada después de 3 días a 0ºC. La proteína recombinante CHIT1b obtenida en *E. coli* mostró actividad crioprotectora *in vitro* y retuvo la actividad catalítica a temperaturas por debajo de 0ºC (Fernandez-Caballero *et al*., 2009), indicando su posible papel protector durante la conservación de la uva de mesa a 0ºC. Por otro lado, es conocido el papel clave que juega PCK en la gluconeogénesis (Benedict & Beevers, 1962; Theodoulou & Eastmond, 2012), proceso metabólico que permite a los organismos obtener azúcares a partir de precursores no carbohidratados, como son los lípidos y proteínas. Bae *et al*. (2013) observaron en *A. thaliana* que GAPC se localizaba en el núcleo en respuesta a las bajas temperaturas y Holtgrefe *et al*. (2008) mostraron su capacidad para unirse al DNA, en particular a la secuencia codificante del gen de la malato deshidrogenasa dependiente de NADP (Hameister *et al*., 2007). Estos hechos parecen indicar que, además de su papel en la gluconeogénesis y glucólisis, GAPC podría estar implicada en la mediación de las señales de estrés y en la transducción de las mismas al núcleo.

En los frutos con menor grado de madurez, las bajas temperaturas también indujeron la expresión de 6 genes que codifican proteínas de transferencia de lípidos no específicas (nsLTPs) y 9 factores de iniciación de eucariotas (eIFs) relacionados con los términos de 'transporte de lípidos' e 'iniciación de la traducción', respectivamente. Las LTPs son capaces de unir ácidos grasos y realizar *in vitro* el intercambio de fosfolípidos entre membranas (Kader, 1996), pudiendo participar en la estabilización de membranas y en la organización de la pared celular como respuesta de los frutos a los cambios de temperatura a 0ºC. Por otro lado, la síntesis proteínas es un paso importante en la expresión génica y está especialmente regulada en la fase de iniciación, donde IF3 juega un importante papel en la elongación de la cadena de polipéptidos, interaccionando con otros factores de iniciación de la traducción, siendo regulado por distintos estreses ambientales (Kawaguchi & Bailey-Serres, 2002). En células de *E. coli* sometidas a un choque frío, IF3 se consideró el factor más importante en términos de traducción selectiva de mRNAs a bajas temperaturas (Giuliodori *et al*., 2004). Nuestros resultados

mostraron un incremento en los niveles de expresión de *IF3* en la piel de uva Cardinal recogida tempranamente y conservada 3 días a 0ºC.

Entre las respuestas transcripcionales específicas a la conservación a 0ºC de los frutos con mayor grado de madurez, se observó la inducción en los niveles de expresión de genes relacionados con el término 'plegamiento de proteínas', cuyo mantenimiento es uno de los retos más importantes de los organismos sometidos a distintas condiciones de estrés. Además de cambios en la expresión de genes que codifican proteínas de choque térmico (HSPs) se observó la inducción de 7 genes que codifican peptidil-prolil cis-trans isomerasas (PPIases), enzimas que catalizan un paso clave del plegamiento de proteínas como es la conversión reversible del enlace peptidil prolil de *cis* a *trans* (Fischer & Schmid, 1999). Otro término GO sobre-representado corresponde a 'transducción de la señal mediada por pequeñas GTPases', donde se incluyeron 8 genes que codifican Rab-GTPases, poniendo en evidencia que el tráfico intracelular de la membrana donde estas proteínas realizan su función (Zerial & McBride, 2001) puede verse afectado por las bajas temperaturas de conservación.

Aunque como ya hemos indicado el número de genes inducidos durante la conservación a bajas temperaturas fue mayor que los reprimidos, dentro de estos últimos cabe destacar en la piel de uva con menor grado de madurez, los cambios en los niveles de 6 ATPasas translocadoras de protones vacuolares (V-ATPasas). Uno de los primeros eventos de los daños por frío que tienen lugar en las plantas parece ser la inhibición de la actividad V-ATPasa, que da lugar a una acidificación del citoplasma (Yoshida *et al*., 1999). Dietz *et al*. (2001), indicaron que las bajas temperaturas conducían a una disminución de la actividad V-ATPasa y en la fuerza motriz de protones que podría afectar a la compartimentación de solutos y en última instancia a la tolerancia de las plantas a las bajas temperaturas. Estos resultados podrían estar en concordancia con resultados previos comentados anteriormente en este trabajo que indicaron que la conservación a bajas temperaturas aumentaba significativamente los niveles de potasio soluble en la piel, mientras que el tratamiento gaseoso los mantuvo. Asimismo, 'la desacetilación de histonas' es otro término enriquecido significativamente en los frutos no tratados de menor grado de madurez. Dentro de este término se incluyeron 5 genes que codifican histonas desacetilasas, entre los que se encuentra *HDA6*, que regula la expresión de genes de respuesta a las bajas temperaturas y participa en la adquisición tolerancia a la congelación en plantas. Así, mutantes *hda6*

de *Arabidopsis* aclimatados al frío mostraron un fenotipo sensible a la congelación en comparación con las plantas silvestres (To *et al*., 2001). Por el contrario, la conservación a 0ºC de uva con mayor grado de madurez parece afectar en la piel a la síntesis de proteínas debido a la represión de genes asociados con 'la elongación de la cadena polipeptídica'.

El efecto del tratamiento con altos niveles de CO_2 en la piel de uva Cardinal parece ser un proceso activo que requiere de factores de transcripción en los frutos recolectados tempranamente y del mantenimiento de energía en los tardíos. En la piel de uva con menor grado de madurez, la aplicación del tratamiento gaseoso dio lugar a la activación de la expresión de 31 genes que codifican distintos factores de transcripción pertenecientes a la familias ERF, una subfamilia de AP2/ERF, así como a WRKY, MYB, bZIP, factores de transcripción de choque térmico y de dedos de zinc. Como ya hemos indicado previamente en este trabajo, los niveles de expresión de los transcritos de *CBF1* y *CBF4* se indujeron en la pulpa y los de *CBF4* también en el raquis, en respuesta al tratamiento gaseoso. Estos resultados parecen indicar que la familia AP2/ERF, a la que también pertenecen los CBFs, podría jugar un importante papel en el efecto del tratamiento gaseoso en los distintos tejidos del racimo. Asimismo, hay abundantes datos que ilustran que la vía señalización que resulta de la regulación de CBFs juega un papel significativo en la tolerancia de las plantas a las bajas temperaturas (Singh *et al*., 2002). Sin embargo, nuestros resultados revelaron que además otros factores de transcripción podrían participar en la menor susceptibilidad de la uva de mesa tratada con altos niveles de CO_2 a los cambios de temperatura a 0ºC. Asimismo, en el efecto del tratamiento gaseoso, los genes relacionados con la fosforilación de proteínas tales como quinasas, parecen también jugar un papel importante. En este sentido, en un trabajo reciente de nuestro grupo se observó que el tratamiento gaseoso inducía la acumulación de la dehidrina DHN44 en la piel de uva Cardinal y los ensayos *in vitro* mostraron que esta isoforma se fosforila (Navarro *et al*., 2015), por lo que este mecanismo podría ser importante en la transducción de las señales mediadas por el tratamiento gaseoso.

CONCLUSIONES

1. La aplicación de un pretratamiento gaseoso (3 días) con un 20% CO_2 durante la conservación a 0ºC aumentó significativamente el contenido de agua ligada en los tejidos del racimo tanto al finalizar el tratamiento como durante su posterior transferencia al aire.

2. El metabolismo de la piel de uva durante la fase crítica de conservación quedó reflejado en el contenido de agua ligada o no congelable, de tal manera que su descenso se podría utilizar como índice de daño.

3. Los efectos asociados a un mayor contenido de agua ligada se manifestaron en un mejor estado de la estructura, volumen celular y apariencia externa de los tejidos de la baya y del raquis de racimos tratados con CO_2.

4. Nuestros resultados sobre el estado del agua justifican su estudio durante la conservación en fresco de frutos como posible indicador de su capacidad potencial de conservación.

5. El incremento de antocianos, fenoles totales y expresión de la *PAL* durante la fase crítica de conservación, posiblemente asociado a un estrés oxidativo, dependió del grado de madurez del fruto. Se observó una mayor predisposición en los frutos en un estado más avanzado de madurez.

6. La aplicación del pretratamiento gaseoso controló también el pardeamiento del raquis a través de la modulación del estado oxidativo y de la biosíntesis de etileno.

7. La activación de la expresión de los factores de transcripción *CBF1* y *CBF4* en la pulpa y raquis de uva de mesa tratada con CO_2 participa en la adaptación a las bajas temperaturas, si bien estos factores no parecen regular la expresión de la *DHN1a*.

8. El estudio transcriptómico mostró que la activación por CO_2 de otros factores de transcripción de las familias ERF, WRKY, MYB, bZIP, HSFs y dedos de zinc es dependiente del grado de madurez del fruto, observándose únicamente en la piel de los frutos con menor grado de madurez. En los frutos en un estado más avanzado de madurez la activación se concretó en genes relacionados con el mantenimiento de su metabolismo energético.

9. Las bajas temperaturas modificaron intensamente el transcriptoma de la piel uva de mesa en ambos estados de madurez, modulando la expresión de genes relacionados con respuestas a estreses abióticos y bióticos. Sin embargo, también se observaron respuestas específicas relacionadas con la gluconeogénesis, fotosíntesis, traducción del mRNA y el transporte de lípidos, en frutos recolectados tempranamente, mientras que el mantenimiento de la estabilidad de plegamiento de proteínas y el tráfico intracelular de la membrana parecen jugar un papel importante en las uvas recolectadas tardíamente.

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Water status and quality improvement in high-CO_2 treated table grapes

Oscar Goñi [1], Carlos Fernandez-Caballero [1], María T. Sanchez-Ballesta, María I. Escribano, Carmen Merodio *

Departamento de Caracterización, Calidad y Seguridad ICTAN-CSIC, José Antonio Novais 10, Ciudad Universitaria, E-28040 Madrid, Spain

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ABSTRACT

Unfreezable water (UFW) content in berry tissues (pulp, skin, seed) and rachis of table grape clusters stored at 0 °C has been studied using differential scanning calorimetry. The effect of short exposure to high CO_2 (20% CO_2 for 3 days) and the transfer to air were also studied. Water status of pulp tissues was related to the thawing behaviour and the structural characteristics, using low-temperature scanning electron microscopy (LT-SEM). The UFW content in all tissues increased rapidly in response to high CO_2 while it remained stable or decreased in untreated clusters. The strong potential of this beneficial gaseous treatment for increasing the UFW content was also evident after transfer to air. The metabolic adjustment caused by exposure to high CO_2, which reduced the amount of water available to be frozen, improved stored fruit quality, thus minimising structural damage and reducing water leakage associated with the freezing–thawing process.

1. Introduction

The quality of table grapes (*Vitis vinifera* L.) is affected by their high sensitivity to water loss and fungal attack. Low temperatures, close to 0 °C and 90–95% relative humidity are used to extend table grape postharvest life (Ginsburg, Combrink, & Truter, 1978) but even under these storage conditions there is a detrimental effect on the quality and appearance of the bunches. Previous studies have indicated that Cardinal table grapes are sensitive when the temperature is lowered to 0 °C (Romero, Sanchez-Ballesta, Escribano & Merodio, 2008). Owing to the fact that severe low temperatures can cause structural damage and fruit quality loss, many efforts have been made to develop effective non-damaging gaseous treatments that could maintain the quality of table grapes (Guillen et al., 2007; Thompson, 2001).

Water is a very important component in fruit, affecting quality, adaptation to environmental storage, shelf-life and processing (Alferez, Alquezar, Burns, & Zacarias, 2010; Hills & Remigereau, 1997; Moraga, Martinez-Navarrete, & Chiralt, 2006). Thus, moisture content has been a parameter widely determined in fruits. However, water in biological materials exists in different states that have strong impact on different processes (Ruan & Chen, 1998). Therefore it is of great interest to quantify the amount of free or freezable water and of the unfreezable water fractions in table grapes that exhibit a large quantity of water (Glidewell, Williamson, Goodman, Chudek, & Hunter, 1997). Our working hypothesis is that variations in water properties could affect table grape quality associated with cellular structural damage and deterioration during storage at severe low temperatures. There are several traditional methods for determining the water status of fresh produce. An approach to the study of water characterisation in plant tissues is the application of magnetic resonance imaging (MRI) (Clark, Hockings, Joyce, & Mazzuco, 1997) or using differential scanning calorimetry (DSC) (Biliaderis, 1983). We previously reported the suitability of DSC to monitor the changes in water status in fruits (Goñi, Muñoz, Ruiz-Cabello, Escribano, & Merodio, 2007) and we found a good correlation between a drop in the T_1 values of whole fruit, as measured by MRI, and the increase in the UFW content determined by DSC. Moreover, it has been reported that T_1 is a better parameter for describing plant water status than the traditional water relation indices (Nagarajan, Chahal, Gambhir, & Tiwari, 1993).

With respect to structural dysfunctions caused by non-freezing and freezing temperatures most of the studies have been focused on rigidification of membrane, loss of membrane integrity and changes in cell wall properties (Kratsch & Wise, 2000; Yamada et al., 2002). Bauchot, Hallett, Redgwell, and Lallu (1999) noted modifications of cell wall composition and properties associated with storage at low temperature in fruits. Rajashekar and Lafta (1996) reported the impact of both the strength and pore size of cell-wall on freezing behaviour of leaves and cell cultures of grapes and apples.

The aim of this work is to determine whether the freezable and unfreezable water content of pulp, skin, seed and rachis of table

* Corresponding author. Tel.: +34 91 5492300; fax: +34 91 5493627.
E-mail address: merodio@ictan.csic.es (C. Merodio).
[1] These authors contributed equally to this work.

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Accumulation and distribution of potassium and its association with water balance in the skin of Cardinal table grapes during storage

Maria Blanch, Carlos Fernandez-Caballero, María T. Sanchez-Ballesta, María I. Escribano, Carmen Merodio*

Department of Characterization, Quality and Security, Institute of Food Science, Technology and Nutrition (ICTAN-CSIC), Jose Antonio Novais 10, Madrid 28040, Spain

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ABSTRACT

Although potassium participates in distinct mechanisms that influence grape growth and development, including osmoregulation, little is known about the association between water and potassium in grape during storage at low temperature. We analyzed the relationship between potassium and the bound water fraction in the skin of early-harvested Cardinal table grapes (*Vitis vinifera* L.) from two different harvest years, both of which were stored at 0 °C for 3 days in air (20% O_2 + 0.03% CO_2) or in air + CO_2 (20% O_2 + 20% CO_2). The relative K^+ content and distribution in the skin cells was determined by energy dispersive X-ray microanalysis, revealing a non-uniform accumulation of K^+ in grape skin cells. Storage at 0 °C in air causes a significant decrease in bound water levels and greater soluble-water K^+ accumulation, irrespective of the harvest year. Furthermore, low temperature-scanning electron microscopy images revealed that the epidermal and the first hypodermal layers of the cells were compressed in the skin of fruit stored in air. However, when exposed to air plus 20% CO_2, there was no decrease in the bound water content or in the associated K^+ accumulation, nor were the outer skin cells compressed.

1. Introduction

Table grapes are very sensitive to fungal decay and water loss, both causing substantial postharvest losses. Susceptibility to disease and water loss is particularly dependent on the cuticular barrier and the condition of the underlying epidermal cells in the skin (Boyer et al., 1997; Comménil et al., 1997). The central vacuole of grape berry cells plays an important role in maintaining their volume, and in controlling the gradients of vacuolar ion concentrations that are essential for acid and sugar balance in the berry. Potassium is perhaps the most important ion in grapes, playing an important role in controlling the vacuolar ion concentration.

The post-harvest quality of table grapes can be enhanced by short-term high CO_2 treatments during low temperature storage (Retamales et al., 2003; Sanchez-Ballesta et al., 2006). The effectiveness of high CO_2 treatment is influenced by the stage of ripeness (Romero et al., 2009) and it also varies according to the temperature at which the commodity can be stored without producing damage (Ahumada et al., 1996; Prange and Lidster, 1992). In grapes, sugar accumulation in the flesh and anthocyanin accumulation in the skin have traditionally been studied to discriminate the harvest maturity. Berry development and ripening also affect the concentration of potassium and its transport by channels (Pratelli et al., 2002), transporters (Davies et al., 2006) and cation/proton antiporters (Hanana et al., 2007). In tomato fruit, changes in pH and K^+ levels have been described in the apoplastic fluid during ripening (Almeida and Huber, 1999, 2007). However, little is known about the relationship between potassium and water fractions during post-harvest storage of table grapes at low temperature.

Different stresses are known to alter a plant's water status (Karen et al., 1992; Ashraf and Foolad, 2007), and bound water is the fraction that probably plays the most important role in tolerance to abiotic stress given that it is responsible for maintaining the structural integrity and cell wall extensibility of living tissues (Singh et al., 2006). In harvested fruits, the ability to cope with changes in internal water content by varying the water fractions seems to be fundamental to maintain quality during storage. We previously demonstrated the benefits of short-term exposure to

* Corresponding author. Tel.: +34 91 5492300; fax: +34 91 5493627.
E-mail address: merodio@ictan.csic.es (C. Merodio).

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Research Note

Influence of the stage of ripeness on phenolic metabolism and antioxidant activity in table grapes exposed to different CO_2 treatments

Irene Romero, Carlos Fernandez Caballero, María T. Sanchez-Ballesta, María I. Escribano, Carmen Merodio*

Departamento de Ciencia y Tecnología de Productos Vegetales, ICTAN-Instituto del Frío, IF-CSIC, José Antonio Novais 10, Ciudad Universitaria, E-28040 Madrid, Spain

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ABSTRACT

We have analyzed the influence of the stage of ripeness on L-phenylalanine ammonia-lyase (PAL) gene expression, accumulation of anthocyanins and total phenolics, and on antioxidant activity in the skin of table grapes treated with 20% CO_2 + 20% O_2 + 60 % N_2 for 3 or 6 d at low temperature (0 °C). The residual effect of high CO_2 treatment after transfer to air was also studied. In early harvested grapes, neither the anthocyanin content nor the accumulation of *VcPAL* mRNA was affected by any of the CO_2 treatments applied. However, in late harvested grapes, the duration of high CO_2 treatment determined its effect and a 6 d treatment with CO_2 sustained higher levels of total phenolics and anthocyanin accumulation, and *VcPAL* expression than observed in untreated late harvested grapes. The increased antioxidant capacity was correlated with the total amount of phenolics and anthocyanins. Conversely, in grapes treated for 3 d with CO_2 the phenylpropanoid pathway did not appear to be induced and a relationship between antioxidant activity and anthocyanins was not observed. Thus, further studies are needed to identify the most important antioxidants in these treated fruit.

1. Introduction

Table grapes are highly perishable and their quality deteriorates rapidly due mainly to water loss and sensitivity to fungal decay. Hence, they should be cooled as soon as possible after harvesting. Postharvest storage at low temperature affects anthocyanin accumulation in grapes and many other fruit (Awad and de Jager, 2002; Romero et al., 2008), and agronomic and environmental factors also have a strong influence on the accumulation of anthocyanins and polyphenols in grapes. Induction of phenylalanine ammonia-lyase (PAL) gene expression, as well as that of other genes involved in anthocyanin biosynthesis, has been reported in response to low temperature stress (Christie et al., 1994). Furthermore, accumulation of anthocyanins and phenolic compounds is developmentally regulated in grapes (Romeyer et al., 1983; Mateus et al., 2002); phenolic compounds determine the characteristics of color and taste in fruit, such as bitterness and astringency. Total phenolics and anthocyanin contents have positively been correlated with antioxidant capacity, and these compounds are considered to contribute significantly to the health benefits of consuming fruit and vegetables. The degree of maturity and harvest date also affect the quality and antioxidant contents of stored fruit (Shin et al., 2008). However, there is little information about the influence of the stage of ripeness of table grapes on PAL gene expression, and on the amount of anthocyanins and total phenolics, as well as their relationship with the overall antioxidant capacity of table grapes exposed to different postharvest treatments. Short-term exposure to high CO_2 concentrations is an effective treatment to maintain quality and to control fungal decay in grapes (Retamales et al., 2003; Sanchez-Ballesta et al., 2007). However, the mode of action of such treatments on health-related compounds is still not understood. The aim of this work was to investigate whether the degree of ripening had any influence on the effect of exposure to high CO_2 during low temperature storage at 0 °C on (1) the expression of the gene encoding the PAL enzyme, (2) accumulation of anthocyanins and total phenolics, and (3) antioxidant activity in the berry skins. Moreover, since gas treatment is affected by concentration and exposure time, we also analyzed the effect of exposing both early and late harvested table grapes to 20% CO_2 for 3 or 6 d.

2. Materials and methods

Table grapes (*Vitis vinifera* L. cv. 'Cardinal') were harvested at a vineyard in Southern Spain (Sevilla) twice over 3 weeks, from 10 vines. The first harvest began on the 13th June 2005 (early harvested, maturity index of 12.45 ± 0.01 and the second on the 5th

* Corresponding author. Tel.: +34 91 5492300; fax: +34 91 5493627.
E-mail address: merodio@if.csic.es (C. Merodio).

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Molecular analysis of the improvement in rachis quality by high CO_2 levels in table grapes stored at low temperature

Raquel Rosales, Carlos Fernandez-Caballero, Irene Romero, Mª Isabel Escribano, Carmen Merodio, Mª Teresa Sanchez-Ballesta*

Departamento de Caracterización, Calidad y Seguridad, Instituto de Ciencia y Tecnología de Alimentos y Nutrición, ICTAN-CSIC, Ciudad Universitaria, E-28040 Madrid, Spain

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Gene expression

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 °C, 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 *GPO1* mRNA 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. This regulation was specific to rachis, showing a different pattern in pulp and skin. Regarding phytohormones, the effect of high CO_2 levels reducing rachis browning seems to be linked to the modulation of ethylene biosynthesis genes. 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.

1. 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 °C, 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 represents about 4% of cluster fresh weight (Carvajal-Millán et al., 2001), such a disadvantage reduces the market where the condition of the 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 work, we have shown the efficacy of a 3-day pretreatment with high CO_2 levels in maintaining the quality of table grapes and reducing rachis browning during storage at 0 °C (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 beneficial 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

* Corresponding author. Tel.: +34 91 5492300; fax: +34 91 5493627.
E-mail address: mballesta@ictan.csic.es (M.T. Sanchez-Ballesta).

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Short communication

Unraveling the roles of *CBF1*, *CBF4* and dehydrin 1 genes in the response of table grapes to high CO_2 levels and low temperature

Carlos Fernandez-Caballero [1], Raquel Rosales [1], Irene Romero, M[a] Isabel Escribano, Carmen Merodio, M[a] Teresa Sanchez-Ballesta *

Departamento de Caracterización, Calidad y Seguridad, Instituto de Ciencia y Tecnología de Alimentos y Nutrición, ICTAN-CSIC, Ciudad Universitaria, E-28040 Madrid, Spain

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ABSTRACT

CBFs (C-repeat binding factors) are transcription factors that are rapidly induced by low temperature and that recognize the CRT/DRE element in the promoter of a set of cold regulated genes, the CBF regulon. Dehydrins are proteins that accumulate in plants under stress conditions, such as low temperature, and some form part of the CBF regulon. To investigate their role in the response of table grape clusters to 0 °C long storage as well as to 3-day high CO_2 postharvest treatment, we isolated two partial *CBF* genes (*VvcCBF1* and *VvcCBF4*) and a full-length dehydrin (*VvcDHN1a*) from *Vitis vinifera* cv. Cardinal. Hydrophobic cluster analysis (HCA) identified differences in the secondary and tertiary structure between *Vitis* CBF4s and CBF1s. Overall, our results showed that, in table grapes, the expression of *CBF* genes is induced mainly in response to CO_2 treatment, suggesting that the response of *DHN1a* in this fruit could be attributed to a cold-inducible CBF-independent pathway.

Introduction

In recent years, transcriptome analyses of cold response have shown that plants induce the expression of genes that encode proteins such as COR (cold-regulated) and dehydrins, which are thought to participate in stress tolerance (Yamaguchi-Shinozaki and Shinozaki, 2006). Dehydrins are induced during periods of water deficit imposed by drought, salinity, and low temperature (Close, 1997). Cold-induced dehydrins have been shown to cryoprotect freezing-labile macromolecules *in vitro* (Hughes and Graether, 2011). In *Vitis*, spliced (*DHN1a* and *DHN1b*) genes encoding YSK_2-type dehydrins as well as unspliced transcripts were induced during exposure to 4 °C for up to 48 h (Xiao and Nassuth, 2006).

Several dehydrin genes contain in their promoter a DNA regulatory element, the C-repeat (CRT) dehydration responsive element (DRE), that imparts responsiveness to low temperature and dehydration (Gilmour et al., 2004). Transcriptional activators that bind to the CRT/DRE, designated as CBF/DREB, are highly conserved in plants and normally show very quick induction at the transcriptional level after exposure to cold. However, in leaves of *Vitis*, the accumulation of *CBF3* and *CBF4* transcripts was observed after 1–2 days (Xiao et al., 2006, 2008). Constitutive overexpression of *CBF* in *Arabidopsis* activated the expression of CRT/DRE-containing target genes, including dehydrins under normal growing conditions and enhanced freezing tolerance in non-acclimated plants (Gilmour et al., 2004). Similar results were obtained recently in *Arabidopsis* transformed with *V. riparia CBF1* and *CBF4* (Siddiqua and Nassuth, 2011).

Low temperature is the most important mechanism for maintaining postharvest fruit quality. Table grapes are classified as chilling tolerant, but their storage life at low temperature is limited by their high susceptibility to fungal decay and sensitivity to serious water loss after harvest. In previous work, we have shown the efficacy of a 3-day CO_2 pretreatment in maintaining table grape quality (Romero et al., 2006; Sanchez-Ballesta et al., 2006). Likewise, the activation of cold-defense responses in the first stage of grape storage at 0 °C seems to be related to the perception of temperature shifts by the fruit, which was less noticeable in CO_2-treated grapes (Sanchez-Ballesta et al., 2007). Different studies have shown the importance of C-repeat binding factors (CBFs) and dehydrins in the response of *Vitis* plants to cold stress. However, their role in fruit under postharvest cold long storage as well as their relationship with cold tolerance induced by high CO_2 levels is still unknown. To investigate whether CBFs and DHNs would also be involved in these processes, we present the characterization and expression pattern

Abbreviations: CBF, CRT binding factor; COR, cold-regulated; CRT/DRE, C-repeat dehydration responsive element; DHN, dehydrin; HCA, hydrophobic cluster analysis.

* Corresponding author. Tel.: +34 91 5492300; fax: +34 91 5493627.
E-mail address: mballesta@ictan.csic.es (M.T. Sanchez-Ballesta).

[1] These authors contributed equally to this work.

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