Centro de Investigación en Alimentación y Desarrollo, A.C.

ANÁLISIS TRANSCRIPTÓMICO DE YEMAS DE VID (*Vitis vinifera* L.) DURANTE LA LIBERACIÓN DE LA LATENCIA INDUCIDA POR CIANAMIDA DE HIDRÓGENO Y COMPUESTOS AZUFRADOS

Por:

CLAUDIA VANESSA GARCÍA BALDENEGRO

TESIS APROBADA POR LA

COORDINACIÓN DE TECNOLOGÍA DE ALIMENTOS DE ORIGEN VEGETAL

Como requisito parcial para obtener el grado de

DOCTOR EN CIENCIAS

Hermosillo, Sonora Agosto de 2016

APROBACIÓN

Los miembros del comité designado para la revisión de la tesis de Claudia Vanessa García Baldenegro, la han encontrado satisfactoria y recomiendan que sea aceptada como requisito parcial para obtener el grado de Doctor en Ciencias.

Dr. Miguel Ángel Martínez Téllez
Director de Tesis

Dra. Irasema del Carmen Vargas Arspuro
Co-directora de Tesis

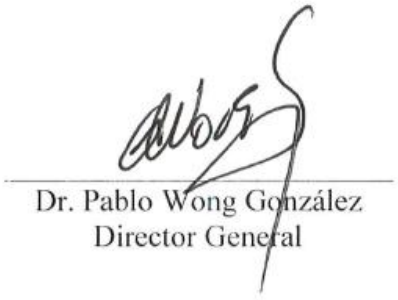
Dra. María Auxiliadora Islas Osuna
Asesora

Dra. Marisela Rivera Domínguez
Asesora

DECLARACIÓN INSTITUCIONAL

La información generada en esta tesis es propiedad intelectual del Centro de Investigación en Alimentación y Desarrollo, A.C. (CIAD). Se permiten y agradecen las citas breves del material contenido en esta tesis sin permiso especial del autor, siempre y cuando se dé crédito correspondiente. Para la reproducción parcial o total de la tesis con fines académicos, se deberá contar con la autorización escrita del Director General del CIAD.

La publicación en comunicaciones científicas o de divulgación popular de los datos contenidos en esta tesis, deberá dar los créditos al CIAD, previa autorización escrita del manuscrito en cuestión del director de tesis.



Dr. Pablo Wong González
Director General

AGRADECIMIENTOS

Agradezco al Consejo Nacional de Ciencia y Tecnología (CONACyT) por el apoyo económico brindado durante mis estudios de doctorado.

Al Centro de Investigación en Alimentación y Desarrollo, A. C. por permitirme realizar mis estudios doctorales en su programa y en sus instalaciones.

Agradezco al Instituto de la Ciencia de la Vid y el Vino (ICVV) por permitirme realizar mi estancia doctoral en sus instalaciones.

A mis Directores de Tesis, Dra. Irasema Vargas Arispuro, gracias por poner en mis manos este proyecto y por guiarme en el camino, por no dejarme caer ni rendirme, por sus aportaciones a este trabajo. Al Dr. Miguel Ángel Martínez Téllez, por aceptarme como estudiante y darme las herramientas necesarias para llevar a cabo esta tesis. A ambos les agradezco de corazón y les admiro, son un gran ejemplo a seguir.

Agradezco también a mis asesoras, la Dra. María Auxiliadora Islas Osuna, quien me ha guiado en gran parte de mi tesis, aconsejado y animarme. A la Dra. Marisela Rivera Domínguez, quien también me ha apoyado en el transcurso de este proyecto.

También quiero agradecer a los colaboradores de este trabajo, el Dr. José Miguel Martínez Zapater, quien me recibió en España para poder asesorarme en relación a la primer parte y muy fundamental de este trabajo.

Agradezco muy profundamente al Dr. Miguel Ángel Hernández Oñate, su colaboración fue fundamental en este proyecto, quien sin su ayuda y su guía, este trabajo estuviera inconcluso.

A mis compañeros del Laboratorio de Fisiología Vegetal, especialmente por el apoyo técnico de Emmanuel Aispuro Hernández, Marisol Ochoa Villarreal y Francisco Soto Córdova, del Laboratorio de Ecología Química a Consuelo Corrales Maldonado y Socorro Vallejo. Más que compañeros, amigos, familia. Gracias chicos por todo su apoyo laboral y moral, así como espiritual, la constante compañía y amistad que Uds. me brindaron en todos estos años, que son de mucho tiempo atrás de este proyecto.

Por el apoyo técnico brindado durante mi estancia en el Centro Nacional de Biotecnología de España a Virginia Rodríguez y, del Laboratorio de Genética y Genómica de la Vid en el Instituto de la Ciencia de la Vid y el Vino a Pablo Carbonell. A las personas que me ayudaron en España, en todas las situaciones posibles, tanto académicas como personales.

A mis compañeros de laboratorio, los de antes y de ahora, ocho años me ha permitido conocer a muchas personas con los mismos objetivos que yo, haciéndome sentir uno más del grupo. A todos los tengo en mi corazón y me encanta pasar tiempo con Uds., fueron esos momentos juntos los que me ayudaron a sobrellevar el tedio del trabajo.

Agradezco a Dios por darme la oportunidad de estar con mi familia en muchos momentos importantes, tantos felices como tristes. Todos los momentos que paso junto a Uds. los atesoro en mi corazón, no cabe duda que no hubiera querido estar en otra familia.

Agradezco a mis Padres, María Jesús y Jesús Alfredo, quienes me han apoyado todos estos años en cada una de las ocurrencias que me vienen a la cabeza. Por ser mi pilar, mi ejemplo, mi gran apoyo, por su amor y por seguir cuidando de mí a pesar de mi edad. Los amo.

DEDICATORIA

A Dios, por el privilegio de prestarme vida y guiarme en el camino personal y profesional. Sigo en tus manos.

A mis padres, Jesús Alfredo García y María de Jesús Baldenegro, a quienes debo lo que soy y a quienes dedico mis éxitos.

Los amo.

LISTA DE ABREVIATURAS

ABA: Ácido abscícico

ADH: Alcohol deshidrogenasa

ACT: Actina

AGC: Ciclo del ascorbato-glutatión

ANOVA: Análisis de varianza

APX: Ascorbato peroxidasa

AOX: Alternativa oxidasa

ARN: Ácido ribonucleico

ATP: Adenosín trifosfato

atpB: ATP sintasa subunidad beta

$CaCN_2$: Cianamida de calcio

CAT: Catalasa

cDNA: Ácido desoxirribonucleico complementario

CL: Control

CL0/CL24: Comparación de Control a las 0 h versus Control a las 24 h

CNB: Centro Nacional de Biotecnología

CO_2: Dióxido de Carbono

C_T: Umbral de ciclo

CTAB: Bromuro de hexadeciltrimetilamonio

Cy3: Cianina 3

DEG: Genes diferencialmente expresados

DEPC: Dietilpirocarbonato

DHAR: Dehidroascorbato reductasa

DNA: Ácido desoxirribonucleico

DW: Peso seco

ED: Endodormancia

EDTA: Ácido etilendiaminotetraacético

EST: Marcador de secuencia expresada

EtOH: Etanol

FDR: Tasa de falso descubrimiento

G6PDH: Glucosa 6-fosfato deshidrogenasa

GR: Glutatión reductasa

GSH: Glutatión reducido

GSSG: Glutatión oxidado

GST: Glutatión S-transferasa

GDBRPK: Proteína cinasa relacionada con el rompimiento de la latencia de la uva

GO: Gene Ontology

HF: Horas frío

H_2O_2: Peróxido de hidrógeno

H_2S: Ácido sulfhídrico

HC: Cianamida de hidrógeno

HC24/CL0: Comparación de Cianamida de hidrógeno a las 24 h versus Control a las 0 h

HC24/CL24: Comparación de Cianamida de hidrógeno a las 24 h versus Control a las 24 h

HS: Choque térmico

IATA: International Air Transport Association

ICVV: Instituto de la Ciencia de la Vid y el Vino

KNO_3: Nitrato de potasio

LiCl: Cloruro de litio

LIMMA: Modelos lineales para datos de microarreglos

LN_2: Nitrógeno líquido

MADS: Acrónimo de MCM1, AGAMOUS, DEFICIENS y SRF

MAP: Proteínas cinasas activadas por mitógenos

MDHAR: Monodehidroascorbato reductasa

MeV: Multiexperiment viewer

MYB: mieloblastosis

N_2: Nitrógeno

NAC: Acrónimo de NAM (para no meristemo apical), ATAF1 y −2, y CUC2 (para cotiledones en forma de taza)

NaCl: Cloruro de sodio

NADH: Nicotinamida adenina dinucleótido

NADPH: Nicotinamida adenina dinucleótido fosfato

NaOAC: Acetato de sodio

PAL: Fenilalanina amonio liasa

PCA: Análisis de componentes principales

PCR: Reacción en cadena de la polimerasa

PDC: Piruvato decarboxilasa

PFK: Fosfofructocinasa

PFT1: Fitocromo y tiempo de floración 1

PPP: Ciclo de las pentosas fosfato

PSII: Fotosistema II

PVP40: Polivinil pirrolidona (peso molecular 40,000)

qRT-PCR: Reacción en cadena de la polimerasa con transcriptasa inversa de tiempo real

RT-PCR: Reacción en cadena de la polimerasa con transcriptasa inversa

RIN: Número de Integridad del ARN

RMA: Promedio de multi-arreglos robusto

ROS: Especies reactivas al oxígeno

rpm: revoluciones por minuto

rRNA: Ácido ribonucleico ribosomal

Ser: Serina

SD: Derivados de azufre

SO_2: Óxido de azufre

SuSy: Succinato sintasa

SNF: Sacarosa no fermentativa

SD24/CL0: Comparación de Derivados Azufrados a las 24 h versus Control a las 0 h

SD24/CL24: Comparación de Derivados Azufrados a las 24 h versus Control a las 24 h

TCA: Ciclo del ácido tricarboxílico

TE: Buffer de TrisHCl y EDTA

Thr: Treonina

Tris HCL: Hidrocloruro de tris(hidroximetil)aminometano

TRX2: tiorredoxina 2

TRXh: Tiorredoxina H

tsf: factor de traducción de elongación Ts

WRKY: Factor de transcripción formado por un dominio conservado heptapéptido integrado por los aminoácidos triptófano (W), arginina (R), lisina (K), tirosina (Y), glicina (G), glutamina (Q), lisina (K) en el extremo amino terminal.

ycf4: marco de lectura hipotético de cloroplasto número 4

CONTENIDO

RESUMEN

Uno de los mayores obstáculos para la producción comercial de uva de mesa en lugares con inviernos cálidos, es la latencia prolongada, provocada por la ausencia de frío invernal, por lo que la aplicación de medios artificiales para romper la latencia es un factor dominante para mantener una producción económicamente exitosa. El rompedor de latencia por excelencia es la cianamida de hidrógeno (HC). Regionalmente, una mezcla de compuestos azufrados (SD) presenta resultados similares a HC. Poco es conocido de los eventos metabólicos y celulares que desencadena la aplicación de HC y mucho menos se ha estudiado para los SD. Mediante el uso de microarreglos, se analizó el transcriptoma de yemas de vid durante la liberación de la latencia inducida por HC y SD. Los genes expresados diferencialmente por los tratamientos fueron enriquecidos en funciones biológicas y se evaluó la expresión de los genes del sistema antioxidante, *ascorbato peroxidasa* (*APX*), *dehidroascorbato reductasa* (*DHAR*), *glutatión reductasa* (*GR*) y *catalasa* (*CAT*), mediante PCR de tiempo real. Para HC se expresaron diferencialmente genes relacionados a respuestas al estrés, metabolismo secundario, oxidación-reducción y se enriquecieron funciones moleculares de glutatión S-transferasas (GST). Los SD indujeron la expresión de genes involucrados en la biosíntesis de pared celular, al igual que el control. Se encontró inhibido el enriquecimiento del metabolismo secundario y oxidación-reducción por efecto de SD e inducida la función de GST. Utilizando el perfil de MapMan, confirmamos que HC induce genes del metabolismo secundario y de oxidación-reducción, específicamente monodehidroascorbato reductasa (*MDHAR*), tiorredoxina 2 (*TRX2*), glutarredoxina (*GRX*). Para los SD se confirma la inhibición de la expresión del metabolismo secundario y oxido-reducción, similar al control. Por qPCR confirmamos que HC inhibe la expresión de *APX*, *DHAR* y *CAT*, provocando una mayor peroxidación. Por el contrario, los SD presentan un perfil de expresión similar al control, manteniendo la homeostasis redox. Ambos tratamientos presentaron niveles altos de expresión de *GR*, indicándonos una participación del ciclo del glutatión. Conjuntando estos resultados, podemos concluir que el tratamiento HC induce a un estrés abiótico y oxidativo en las yemas de vid, lo que podría ser la causa de su fitotoxicidad. En cambio, los SD inducen a la expresión de genes relacionados al sistema antioxidante como *GR* y *GST*s.

Palabras clave: Sistema antioxidante, microarreglos, ciclo del ascorbato-glutatió, catalasa.

ABSTRACT

One of the biggest obstacles for the commercial production of table grapes in regions with warm winter is the prolonged dormancy caused by the absence of cold winter. Thereby, the application of artificial means to break dormancy is a key factor for maintaining a financially successful production. The breaking dormancy agent per excellence is hydrogen cyanamide (HC). Regionally, a mixture of sulfur compounds (SD) showed similar results than HC. Little is known about the metabolic and cellular events triggered by the application of HC, and much less has been studied for SD. By using microarrays, we analyzed the transcriptome of grapevine buds, during dormancy release induced by HC and SD. The differentially expressed genes by treatments were enriched in biological functions and gene expression of the antioxidant system, ascorbate peroxidase (*APX*) dehydroascorbate reductase (*DHAR*), glutathione reductase (*GR*) and catalase (*CAT*) was evaluated by real time PCR. Genes related to stress responses, secondary metabolism and redox were differentially expressed by HC. Also, molecular functions of glutathione S-transferases (*GST*) were enriched. The SD induced the expression of genes involved in cell wall biosynthesis in a similar way to the control. The enrichment of secondary metabolism and redox was inhibited, while GST function was induced by SD. Using the MapMan tool, we confirmed that HC induced genes of the secondary metabolism and oxidation-reduction, specifically monodehydroascorbate reductase (*MDHAR*), thioredoxin 2 (*TRX2*) and glutarredoxine (*GRX*). The inhibition of the expression of secondary metabolism and redox genes by SD was similar to the control. In the qPCR analysis, HC inhibited the expression of *APX*, *DHAR* and *CAT*, increasing peroxidation processes. By the contrary, the SD showed a similar expression profile to the control, keeping the redox homeostasis. Both treatments showed high expression levels of *GR*, indicating an implication of the glutathione cycle. Taking together these results, we are able to conclude that the HC treatment induces an oxidative and abiotic stress in grapevine buds, which could be the cause of its phytotoxicity. Instead, the SD induced an expression of genes related to the antioxidant system, like *GR* and *GST*s.

Key Words: Antioxidant system, microarrays, Ascorbate-Glutahtione cycle, catalase.

SINOPSIS

La vid es una planta caducifolia cultivada en todos los continentes del mundo, excepto en la Antártica, y es uno de los cultivos más ampliamente distribuidos (Williams et al., 1994). En México, en el 2014, se plantaron alrededor de 29,466 hectáreas de vid, produciendo 335,739 toneladas de frutos. Dejando un total de $4,531,830.26 de miles de pesos por la producción de uva tanto industrial como de mesa (SIAP, 2016).

Particularmente, la producción de uva de mesa en México se concentra en la zona norte de su territorio, principalmente en el estado de Sonora, que es responsable del 69% del total de la uva cultivada en el país. Las condiciones climáticas de la región vitícola del estado de Sonora han favorecido la productividad y calidad de las variedades actuales de uva de mesa, tomando ventaja competitiva de producción con otras regiones viticultoras (SIAP, 2016; Vázquez, 2011). Sin embargo, los cambios climáticos que se han presentado los últimos años han acentuado la variabilidad de las temperaturas entre años y regiones, acrecentándose problemas relacionados con la insuficiencia de frío, el cual es necesario para una adecuada latencia o dormancia de las vides. El incumplimiento en los requerimientos de frío, provoca brotaciones improductivas, afectando el rendimiento de los viñedos (Vázquez, 2011).

La dormancia es un estado característico de los frutales caducifolios que les permite sobrellevar las condiciones desfavorables, se caracteriza por el cese de crecimiento, detención de la división celular, y actividades metabólica y respiratoria reducidas (Lang et al., 1987). La dormancia en plantas, no es un estado uniforme, sino que es un fenómeno de diferentes condiciones fisiológicas, por lo cual se divide en tres etapas: paradormancia (pre-dormancia), endodormancia (dormancia invernal) y ecodormancia (quiescente) (Lang et al., 1987; Horvath et al., 2003). De estos tres períodos, la endodormancia (ED) es la que más implicaciones tiene en la productividad de las

plantas, ya que es un receso profundo del sistema metabólico de la planta, que está regulada por factores endógenos en las yemas, las cuales permanecen latentes por períodos prolongados (Wang et al., 1986). Para que la planta salga del estado de endodormancia, requiere estar expuesta de manera continua a bajas temperaturas, con la finalidad de que ésta, acumule sus requerimientos de horas frío (HF), los cuales dependen de la especie y de la variedad. Cubiertos los requerimientos de frío, las vides inician la brotación de manera sincronizada, al aumentar la temperatura en la época de la primavera (Arora et al., 2003). Comparando con otras especies de frutos caducifolios, para completar el período de frío, las variedades de vid de mesa, requieren una exposición corta al frío, que va entre 50 y 400 horas a temperaturas menores a 7 °C (Vasconcelos et al., 2007; Andrenini et al., 2009).

En lugares con inviernos templados, como los que se presentan en Sonora y en otros países cultivadores de la vid que se encuentran en la misma latitud, la dormancia prolongada se considera uno de los mayores obstáculos para la producción comercial de frutos templados, por lo que son utilizados medios artificiales para compensar la falta de frío natural, convirtiéndolo en un factor dominante para mantener una producción económica (Or et al., 2000). La falta de frío invernal en la vid, afecta procesos de brotación de yema, como el retraso en la brotación, brotación errática o no uniforme, disminución del número de brotes por sarmiento, lo que conlleva a la disminución de racimos por sarmiento, desigualdad en el desarrollo de los racimos y efectos como el retraso en la maduración de las bayas (Pinto et al., 2007). Todos estos efectos realzan la importancia del uso de métodos artificiales para compensar la falta de frío natural y forzar a las vides a romper con la dormancia (Erez et al., 1987). El rompedor de dormancia químico más utilizado a nivel mundial en campo, es la cianamida de hidrógeno (HC) (Blanco y Díaz, 2010). Otros productos químicos que se reportan como liberadores de dormancia son la azida de sodio (Pérez et al., 2009), KNO_3 (Chang y Sung, 2000), thidiazuron (Wang et al., 1991), H_2O_2 (Pérez et al., 2008), giberelinas (Chang y Sung, 2000), así como compuestos azufrados, H_2S (ácido sulfhídrico) y SO_2 (óxido de azufre) (Wang y Faust, 1994), sulfuro de alilo, disulfuro de metilo (Hosoki et al., 1986). También se han reportado inductores físicos como la temperatura con horas

frío acumuladas (Vergara y Pérez, 2010) y temperaturas altas de 50 °C (HS) (Halaly et al., 2008).

La HC es uno de los agentes rompedores de dormancia más efectivos en los países con inviernos templados, ya que la aplicación de HC a un tiempo adecuado en vides que no lograron cumplir con los requerimientos de frío, aumenta y sincroniza la brotación, promoviendo una maduración temprana de frutos (Erez, 1987). La reproducibilidad del proceso de la liberación de la endodormancia de la yema de vid con la aplicación de HC, ha permitido desarrollar un marco de tiempo bien caracterizado, lo cual ayuda a crear un sistema modelo, rastreable y confiable para la identificación de rutas bioquímicas involucradas en el mecanismo de liberación de la ED de la yema (Pérez et al., 2009, Dokoozlian et al., 1995). La elucidación completa del mecanismo por el cuál la cianamida de hidrógeno actúa liberando a la planta de la dormancia, aún es desconocido. Sin embargo, está bien elucidado que la HC disminuye la actividad de la enzima catalasa de manera similar como la afecta las temperaturas frías invernales. La disminución de la actividad de catalasa causa un aumento en el nivel de H_2O_2 en tejidos de yema de vid provocando un estrés oxidativo (Nir et al., 1986; Pérez et al., 2009); sin embargo, la conexión entre el estrés oxidativo inducido por HC, causado por reducción de la actividad de la catalasa, y el efecto de la cianamida en la liberación de la dormancia aún no está claro (Or et al., 2002; Pérez y Burgos, 2004; Halaly et al., 2008). Se ha propuesto que la molécula H_2O_2 tiene una participación en la liberación de la endodormancia como molécula señal (Pérez y Burgos, 2004), donde el aumento en los niveles de H_2O_2 pueden inducir la activación temporal del ciclo de la pentosa fosfato, el cual, activa al ciclo ascorbato-glutatión, mediante la producción de NADPH, restableciendo el ciclo de crecimiento de la planta y por consecuencia la terminación de la dormancia, brotación y crecimiento rápido (Nir et al., 1986; Pérez et al., 2009).

Fortaleciendo estos trabajos, Halaly et al. (2008), reportó que la aplicación de HC a yemas de vid, puede inducir la expresión de genes del ciclo ascorbato-glutatión como ascorbato peroxidasa (*APX*) y glutatión reductasa (*GR*). Además, la elevación transitoria de la relación glutatión reducido: glutatión oxidado (GSH:GSSG) observada por Pacey-

Miller et al. (2003) en yemas latentes de vid aplicadas con HC , confirma la activación del ciclo ascorbato-glutatión y del ciclo de las pentosas fosfato (Pérez et al., 2009).

A pesar de que se ha demostrado la efectividad de la HC para inducir la brotación de la vid, manzano, durazno y ciruelo, demostrando que es el químico más potente como rompedor de dormancia, se ha reportado fitotoxicidad en las yemas florales y brotes jóvenes de duraznos, ciruelos y vid (Erez, 1987; Or et al., 2000). Debido a la toxicidad de la HC para plantas y humanos, la Agencia de Protección Ambiental (Estados Unidos de América) lo ha clasificado como un compuesto de la más alta categoría tóxica (Categoría I), y la Unión Europea ha prohibido su utilización (Vasconcelos-Botehlo y Müller, 2007). Entre las diversas alternativas al uso de la HC, están los compuestos azufrados provenientes de ajo, los cuales han demostrado ser efectivos para romper la dormancia en vides de mesa (Vargas-Arispuro et al., 2008; Corrales-Maldonado et al., 2010). Kubota y Miyamuki (1992), aplicaron pasta de ajo a yemas de diferentes variedades de vid como "Kyoho", "Delaware", "Neo Muscat", concluyendo que fue más eficiente para inducir la brotación que la aplicación de cianamida de calcio ($CaCN_2$), y que la pasta de ajo protegía a las vides de daño por bajas temperaturas. También la aplicación de compuestos volátiles azufrados como H_2S, SO_2, sulfuro de alilo, isotiocianato de alilo indujo el rompimiento de la dormancia en cormos de gladiola, tubérculos y árboles de peonías (Hosoki et al., 1986; Wang et al., 1994). También Vasconcelo-Botehlo et al. (2010), aplicaron un extracto de ajo a yemas latentes de vid cv. 'Niagara Rosada' induciendo la brotación de manera similar a la de HC. La aplicación de compuestos azufrados derivados de ajo, además de inducir la brotación ha demostrado que mejora el rendimiento de las plantas caducifolias aumentando la fructificación (Corrales-Maldonado et al., 2010; Abd El-Razek et al., 2013; Vasconcelos-Botehlo et al, 2007). Razón por la cual surge el interés en la utilización de las mezclas de ajo como agentes inductores de brotación en los cultivos agroecológicos. Sin embargo, la información sobre procesos celulares, moleculares y bioquímicos que regulan la liberación de la latencia en plantas, de manera general, es muy limitada y más escasa con el uso de agentes azufrados.

La terminación de la endodormancia se ha relacionado con varios genes que participan en la fotosíntesis, genes de resistencia a enfermedades y defensa, energía, metabolismo, producción de proteínas y procesamiento, estructura celular y crecimiento de plantas, desconociendo la interacción entre estos procesos (Pacey-Miller et al., 2003). Un mecanismo común en químicos utilizados para romper la dormancia, es que inhiben la actividad de la enzima catalasa, dando a lugar a la activación de ciertas peroxidasas. Otros químicos intervienen en la respiración aeróbica (Erez, 1987). Algunos autores han concluido que la aplicación de cianamida regula la expresión de genes relacionados con la hipoxia, como son piruvato descarboxilasa (*PDC*), alcohol deshidrogenasa (*ADH*) y sacarosa sintasa (*SuSy*) los cuales están relacionados con el ciclo de la pentosa fosfato, así como de genes relacionados con el estrés oxidativo como tioredoxina *h* (*TRXh*), glutatión S-transferasa (*GST*), ascorbato peroxidasa (*APX*), glutatión reductasa (*GR*) (Halaly et al., 2008; Pérez et al., 2008; Ophir et al. 2009; Vergara et al., 2010).

También la proteína relacionada con el rompimiento de dormancia de la uva (GDBRPK, por sus siglas en inglés) la cual es un transcrito para la proteína cinasa tipo SNF para sacarosa no fermentativa (Or et al., 2000). De todos estos hallazgos, se hipotetiza que las distorsiones respiratorias desencadenadas por la cianamida son necesarias para el rompimiento de la endodormancia en vid (Pérez et al., 2008).

El incremento en los procesos oxidativos, activa los sistemas antioxidantes, por lo que durante el rompimiento de la dormancia de las yemas inducido por agentes químicos, se ha encontrado activado el del ciclo del ascorbato-glutatión (AGC, por sus siglas en inglés), el cual puede detoxificar el exceso de H_2O_2 de manera alternativa a la catalasa. Durante la secuestración del H_2O_2 por el AGC se da una subsecuente activación del ciclo de la pentosa fosfato (PPP, por sus siglas en inglés) que podría ser crucial para el rompimiento de la endodormancia de las yemas por la acción de la cianamida. Sin embargo, la aplicación directa de H_2O_2 no activa este ciclo de la pentosa fosfato (Pérez et al., 2008). Estas respuestas nos indican que es posible tener distintos mecanismos para el rompimiento de la dormancia en función del estímulo utilizado, como sería el frío de manera natural, la cianamida como compuesto nitrogenado y uno diferente para los compuestos azufrados.

Siguiendo este razonamiento, en este trabajo se realizó un análisis transcriptómico para evaluar la expresión diferencial de genes en un sistema antioxidante, cuando utilizamos cianamida o compuestos azufrados como estímulos para romper la dormancia en las yemas de vid cv. 'Flame Seedless'. El análisis transcriptómico se realizó en el CNB (Centro Nacional de Biotecnología) en España, por medio del Instituto de las Ciencias de la Vid y el Vino (ICVV) en la Rioja. El impedimento para introducir a España material vegetal proveniente de otros países, para nuestro caso, las yemas endodormantes de vid, nos condujo a evaluar métodos para la obtención de ARN, que al ser transportado en largas distancias, mantuviera la calidad para el análisis de transcripción masiva de yemas endodormantes. En las revisiones de la literatura, no encontramos un método que nos resultara factible, razón por la cual se evaluó la liofilización de las yemas, previo a la extracción del ARN total, así como diferentes tiempos de almacenamiento durante los cuales se obtuviera un ARN de muy buena calidad de las muestras liofilizadas. En el capítulo I de este escrito, se muestra que la liofilización de las yemas y tiempos prolongados de almacenamiento, no afectó a la calidad del ARN. Resolviendo de esta forma el envío a largas distancias del material vegetal y la introducción de éste a otros países, para continuar con los objetivos del trabajo.

En el Capítulo II partimos de la premisa de que la cianamida de hidrógeno estimula la brotación de yemas dormantes de vid, mediante la inducción de un estrés oxidativo por efecto de la inhibición de catalasa, lo que conlleva la activación de la expresión de genes del ciclo ascorbato-glutatión. Para los compuestos azufrados, que también estimulan la brotación de las yemas en una magnitud similar a HC (Vargas et al., 2008), se desconoce la utilización de este mecanismo. En razón de que los compuestos de ajo utilizados en este trabajo, incluye las moléculas azufradas reportadas que inducen la brotación (Vargas et al., 2008), se asume que el azufre podría jugar un papel importante en el rompimiento de la dormancia, actuando como un pool de azufre para la formación de los aminoácidos azufrados, cisteína y metionina. La cisteína es un precursor de la molécula glutatión (GSH), el cual se ha especulado que está involucrada en la liberación de la dormancia debido a que un aumento en los niveles de GSH está asociado con la

activación del ciclo de ascorbato-glutatión y de las pentosas fosfato (Pacey-Miller et al., 2003). En este segundo capítulo, titulado "Dormancy breaking-stimuli cyanamide and sulfur derivatives regulate the expression of ascorbate-glutathione cycle genes in grapevine (*Vitis vinifera* L.) buds", se investigó la expresión de los genes del ciclo del ascorbato-glutatión por la aplicación de los rompedores de dormancia, cianamida de hidrógeno y una mezcla de compuestos azufrados derivados de ajo. Los resultados muestran que CH induce la expresión de *GR* y reprime la expresión de los genes *APX*, *DHAR* y *CAT*. Por el contrario los derivados de ajo, inducen la expresión de *GR*, y mantiene los niveles de expresión de *APX*, *DHAR* y *CAT* en un patrón similar al del control. De esta manera, la aplicación de compuestos azufrados mantiene patrones de expresión similares a los del control, sugiriendo un balance en la redox homeostasis. En ambos tratamientos, se observa el aumento en la expresión de *GR*, sugiriendo un incremento en el glutatión, el cual se conoce participa en la liberación de la dormancia.

En el capítulo III de este escrito, se describen los primeros eventos metabólicos y celulares relacionados al sistema antioxidante Ascorbato-Glutatión que fueron activados por CH y compuestos azufrados utilizados como liberadores de latencia de yemas endodormantes de vid. Este estudio se realizó mediante la aplicación de tecnología genómica como son los microarreglos. Para el análisis se utilizaron las herramientas de enriquecimiento funcional de GO (Gene Ontology) y la visualización de rutas metabólicas (MapMan), que permitieron comprobar la compleja red de procesos bioquímicos y celulares involucrados en la regulación de la liberación de la latencia de las yemas de vid. Los resultados muestran que a las 24 h, después de la aplicación de CH, se encuentran activas las rutas del ciclo del ascorbato-glutatión, apareciendo inducidos los genes *Glutatión S-transferasas* y monodehidroxiascorbato reductasa (*MDHAR*) como respuestas al estrés. En cambio, para los compuestos azufrados a las 24 h después de la aplicación, están expresados diferencialmente los genes *Glutatión S-transferasas*, lo cual pudiera indicar que es el inicio de la activación del ciclo ascorbato-glutatión. Estos resultados, nos indican que los compuestos azufrados tienen una manera muy similar de dar lugar a la brotación a la manera natural de la planta. Una mayor discusión de estos resultados se describen en el tercer manuscrito titulado "Differential

expression in endodormant grapevine buds evoked by sulphur derivatives and cyanamide stimuli".

REFERENCIAS

Abd El-Razek E, El-Migeed MMMA, Abdel-Hamid N (2013) Response of 'Le Conte' pear trees to garlic extract and GA_3 as budbreak dormancy agents. 14(11):1407-1413.

Andrenini L, Viti R, Scalabrelli G (2009) Study on morphological evolution of bud break in *Vitis vinifera* L. Vitis 48:153-158

Arora R, Rowland LJ, Tanino K (2003) Induction and reléase of bud dormancy in Woody perennials: a science comes of age. HortScience, 38:911-921

Blanco JLM, Diaz GM (2010) Manejo de la cianamida de hidrógeno en vid de mesa. Seminario de Viticultura, 9

Chang YS, Sung FH (2000) Effects of gibberellic acid and dormancy-breaking chemicals on flower development of Rhododendron pulchrum Sweet and R. scabrum Don. Scientia Horticulturae 83(3):331-337

Corrales-Maldonado C, Martínez-Téllez MA, Gardea AA, Orozco-Avitia A, Vargas-Arispuro I (2010) organic alternative for breaking dormancy in table grapes grown in hot regions. American Journal of Agricultural and biological Sciences. 5:194-198.

Dookoozlian N, Wiliams E (1995) Chilling exposure and hydrogen cyanamide interact in breaking dormancy of grape buds. HortScience, 30:1244-1247.

Erez A (1987) Chemical control of budbeak. HorScience, 22: 1240-1243.

Halaly T, Pang X, Batikoff T, Crane O, Keren A, Venkateswari J., Ogrodovitch A, Sadka A, Lavee S, Or E (2008) Similar mechanisms might be triggered by alternative external stimuli that induce dormancy release in grape buds. Planta. 228 :79-88.

Horvath DP, Anderson JV, Chao WS, Foley ME (2003) Knowing when to grow: signals regulating bud dormancy. TRENDS in Plant Science. 8 (11):534-540

Hosoki T, Sakai Y, Hamada M, Taketani K (1986) Breaking bud dormancy in corms and trees with sulfide compounds in garlic and horseradish. HortScience 21:114-116.

Kubota N, Miyamuki M, Yamane Y, Kobayashi A, Mizutani F(1999) Breaking bud dormancy in grapevine cuttings with garlic volatiles. J. Japan. Soc. Hort. Sci. 68:927-931

Kubota, N. and M. Miyamuki. 1992. Breaking bud dormancy in grapevines with garlic paste. J. Amer. Soc. Hort. Sci. 117:898–902.

Kubota, N., Matthews, M.A., Takahagi, T. and Kliewer, W.M. 2000. Budbreak with garlic preparations. Effect of garlic preparations and of calcium and hydrogen cyanamides on budbreak of grapevines grown in greenhouses. Am. J. Enol. Vitic. Vol. 51(4):409-414.

Lang GA, Early JD, Darnell RD, Martin JC (1987) Endo-, Para-, and Ecodromancy: Physiological terminology and classification for dormancy research. HortSciene, 22:371-377.

Nir G, Shulman Y, Fanberstein L, Lavee S (1986) Changes in the activity of catalase (E.C. 1.11.1.6.) in relation to the dormancy of grapevines (*Vitis vinifera* L.) buds. Plant Physiology 81:1140-1142.

Or E, Belausov E, Popilevsky I, Ben-Tal Y (2000) Changes in endogenous ABA level in relation to the dormancy cycle in grapevine grown in hot climate. J. Hortic. Sci Biotechnol. 75:190-194.

Or E, Vilozny I, Eyal Y, Ogrodovith A (2000) The transduction of the signal for grape bud dormancy breaking induced by hydrogen cyanamide may involve the SNF-like protein kinase GDBRPK. Plant Molecular Biology 43: 483-494.

Or E, Vilozny I, Fennel A, Eyal Y, Ogradovich A (2002) Dormancy in grape buds: Isolation and Characterization of catalase cDNA and analysis of its expression following chemical induction of bud dormancy release. Plant. Sci. 162:121-130.

Ophir R, Pang X, Halaly T, Venkateswari J, Lavee S, Galbraith D, Or E (2009) Gene-expression profiling of grape bud response to two alternative dormancy-release stimuli expose possible links between impaired mitochondrial activity, hypoxia, ethylene-ABA interplay and cell enlargement. Plant Molecular Biology 71:403-423.

Pacey-Miller T, Scott K, Ablett E, Tingey S, Ching A, Henry R (2003) Genes associated with the end of dormancy in grapes. Funct Integr Genomics. 3:144-152

Perez FJ, Burgos B (2004) Alterations in the pattern of peroxidase isoenzymes and transient increases in its activity and in H2O2 levels take place during the dormancy cycle of grapevines buds: the effect of hydrogen cyanamide. Plant Growth Regul. 43:213-220.

Pérez FJ, Vergara R, Rubio S (2008) H2O2 is involved in the dormancy-breaking effect of hydrogen cyanamide in grapevine buds. Plant Growth Regul. 55:149-155

Perez FJ, Vergara R, Or E (2009) On the mechanism of dormancy release in grapevine buds: a comparative study between hydrogen cyanamide and sodium azide. Plan Grewth regul. 59:145-152.

Pinto M, Lira W, Ugalde H, Pérez F (2006) Fisiología de la latencia de las yemas de vid: hipótesis actuales http://www.gie.uchile.cl/publicaciones/index.html. Consultada en Diciembre 2015.

SIAP (2016) Servicio de Información Agroalimentaria y Pesquera. http://www.siap.gob.mx Consultada en Junio de 2016.

Vargas-Arispuro I, Corrales-Maldonado C, Martinez-Tellez MA (2008) Compounds derived from garlic as bud induction agents in organic farming of table grape. Chilean journal of agricultural research 68:94-101.

Vasconcelos Botehlo R, Pozzobom PA, Paioli PE, Monteiro TM, Lopes MM (2007) Effects of chilling and garlic on bud dormancy release in Cabernet Sauvignon in grapevine cuttings. Am. J. Enol. Vitic. 58 (3):402-405

Vasconcelos Botehlo R, Paioli Pires EJ, Fernandes Moura M, Monteiro Terra M, Tecchio MA (2010) Garlic extract improves budbreak of the 'Niagara Rosada' grapevines on sub-tropicarl regions. Ciencia Rural. 40 (11): 2282-2287.

Vasconcelos Botehlo R, Müller MM (2007) Evaluation of garlic extracto n bud dormancy reléase of 'Royal Gala' Apple trees. Australian Journal of Experimental agriculture. 4: 1-4

Vázquez N (2011) Asociación Agrícola de Productores de Uva de Mesa. Modelo de la Agricultura Moderna en México en el Siglo XXI. L Deschamps, ed., Fundación Produce Sonora, A.C. . http://www.redinnovagro.in Consultada en Marzo, 2012.

Vergara R, Pérez FJ (2010) Similarities between natural and chemically induced bud-endodormancy release in grapevine Vitis vinifera L. Scientia Horticulturae. 125:648-653

Wang SY, Faust M (1994) Changes in the antioxidant system associated with budbreak in Anna Apple (*Malus domestica* Borkh.) buds. Journal of the American Society of Horticultural Science. 119:735-741.

Wang SY, Steffens GL, Faust M, (1986) Breaking bud dormancy with a plant bioregulator, thidiazuron. Phytochemistry. 25:311-317.

Williams LE, Dokoozlian NK, Wample R (1994) Chapter 4: Grape, IN: Handbook of Environmental Physiology of Fruit Crops Volume I: Temperate Crops, B Schaffer y PC Andersen, eds., CRC Press, Boca Raton, Florida, U.S.A. pp. 85-133.

Capítulo 1

Total RNA quality of lyophilized and cryopreserved dormant grapevine buds

---

Manuscrito publicado en el Electronic Journal of Biotechnology

Electronic Journal of Biotechnology 18 (2015) 134–137

HOSTED BY

ELSEVIER

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Short communication

Total RNA quality of lyophilized and cryopreserved dormant grapevine buds

Claudia Vanessa García-Baldenegro, Irasema Vargas-Arispuro, María Islas-Osuna, Marisela Rivera-Domínguez, Emmanuel Aispuro-Hernández, Miguel Ángel Martínez-Téllez *

Centro de Investigación en Alimentación y Desarrollo, A.C. Carretera a la Victoria Km 0.6, Hermosillo, Sonora C.P. 83304, México

ARTICLE INFO

Article history:
Received 31 October 2014
Accepted 12 December 2014
Available online 16 February 2015

Keywords:
Freeze-dried
RNA integrity number
RT-qPCR
Vitis vinifera L
Woody tissue

ABSTRACT

Background: Plant tissues must be preserved in their collection state, especially for genome-wide expression profile studies. Lyophilization is a feasible, affordable tool when fresh tissues cannot be shipped at ultralow temperatures from their origin to the place of analysis. In this study, the total RNA quality of dormant grapevine buds (*Vitis vinifera* L. cv. 'Flame Seedless') of freeze-dried samples stored at room temperature conditions was evaluated and compared to that of cryopreserved (-80°C) grapevine buds.
Results: Good yield and quality of RNA were obtained from freeze-dried dormant buds stored at room temperature for 0, 3 and 6 weeks after they were lyophilized. Further experiments confirmed that the extracted total RNA could be used for actin and β-tubulin PCR gene amplification.
Conclusion: High-quality RNA that is useful for downstream applications was obtained from freeze-dried dormant grapevine bud tissue, similarly to the RNA obtained from cryopreserved dormant grapevine buds.

1. Introduction

The isolation of high-quality RNA and DNA is very important for biological studies, and the ability to obtain this material depends on tissue preservation after collection. Maintaining the integrity of RNA represents an important problem, particularly for the preservation and long-distance shipment of biological samples for international exchange between collaborators or for analysis. Generally, RNA methodologies require fresh tissues, minimum processing, or freezing (liquid nitrogen [LN_2]) at ultralow temperatures. The use of LN_2 and dry ice for long-term tissue preservation has been well established; however, it is not suited for long transportation times or unexpected delays. In order to maintain RNA integrity, samples must be shipped with expedited delivery, resulting in high fees, and in bulky LN_2 or dry ice containers. Additionally, sample transportation in dry ice or LN_2 has to meet several troublesome requirements according to the International Air Transport Association (IATA) [1]. In some countries, samples in dry ice are not approved by transportation companies, especially airlines, because the gases released (CO_2 and N_2) may cause explosion and suffocation and are considered Hazard Class 9 and Class 2, respectively [1,2]. Accordingly, these methods are unsuitable for storage and/or international exchange because of the potential safety, high costs and inconvenience of operation. Over long distances, the sample may thaw, and multiple freeze-thaw cycles and prolonged exposure to increased temperatures must be avoided, as these conditions promote degradation of labile RNA samples [3].

There are new technologies that help preserve RNA at room temperature, such as RNALater® (Ambion, Carlsbad, CA, USA), which helps preserve sample tissues for further RNA extraction, and RNA stable® (Biometrica, San Diego, CA, USA), which keeps isolated RNA in anhydrobiosis at room temperature for weeks [3,4]. However, these methods are costly and need to be in hand at the laboratory at the moment of use. Lyophilization is an alternative appropriate method for processing samples for transportation, as well as for room temperature storage, when an ultralow freezer is a limiting factor [5]. Lyophilization has been widely used for the freeze-drying and storage of various biological samples in the food industry, pharmacy biotechnology and tissue engineering. Despite the advantages that this tool offers, RNA extraction from lyophilized tissues, such as mouse tissues [6], tea leaves [7], tuber and root tissues, such as potato, turnip, sweet potato and radish [5], has not been extensively reported. Saha et al. [8] reported the disadvantage that lyophilized cotton tissue had total RNA low quality. Theoretically, lyophilization should limit or delay cellular component degradation by inactivation of proteolytic enzymes and nucleases [7,8], allowing long-term room temperature storage or long-distance transportation if the seal is maintained. In the present study, we evaluated the effects of the freeze-dried process using dormant grapevine buds on the quality of the obtained RNA that will be used for further cDNA synthesis, amplification and gene expression in transcriptomic contexts.

* Corresponding author.
E-mail address: norawa@ciad.mx (M.Á. Martínez-Téllez).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2015.01.002

2. Materials and methods

2.1. Plant material

Cuttings were collected from ecodormant grapevine plants (*Vitis vinifera* L. cv. 'Flame Seedless'). The plant material came from a commercial vineyard in an agricultural zone in San Miguel de Horcasitas, Sonora, Mexico (29° 20′N, 110° 51′O). Grapevine buds were dissected and immediately frozen in liquid nitrogen and were cryopreserved at -80°C for 6 months. Cryopreserved buds were divided into 4 batches. One batch was kept in an ultralow freezer (-80°C), and 3 batches were lyophilized and packed in Falcon tubes that were closed and sealed with parafilm and stored at room temperature for 0, 3 and 6 weeks. Total RNA extractions of the lyophilized buds were performed after each storage time.

2.2. Total RNA extraction

RNA extraction was performed according to Reid et al. [9], using 8 grapevine buds per sample with 3 replications. The samples were purified using the commercial system Spectrum Plant Total RNA (Sigma-Aldrich, St. Louis, MO, USA) starting from binding columns. Genomic DNA traces were eliminated using DNase I (QIAGEN, Valencia, CA, USA). The integrity and quantity of the total RNA were evaluated by the spectrophotometric absorbance ratios A_{260}/A_{280} and A_{260}/A_{230} using a NanoDrop 2000 (Thermo Scientific NanoDrop, USA). The integrity of RNA was determined by electrophoresis on a denaturing (formaldehyde) 1% agarose gel, as well as by using a Bioanalyzer 2100 RNA LabChip (Agilent Technologies, Palo Alto, CA, USA). The obtained RNA was used for synthesis of cDNA.

2.3. Semi-quantitative and real-time RT-PCR

First-strand cDNA was synthesized with 1 μg of total RNA (DNA free) using the SuperScript™ First-strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Semi-quantitative and real-time PCR amplifications were performed for actin and β-tubulin. The primers used for actin were VvACTFw (5′-GCT GAG AGA TTC CGT TGT CC-3′) and VvACTRv (5′-GCC ACC ACC TTG ATC TTC AT-3′) (GenBank accession no. AF369524), and the primers used for β-tubulin were Vvβ8TUBFw (5′-GCA GTG AAC CTG ATC CCA TTT CC-3′) and VvβTUBRv (5′-GCT CAC TCA CCC TCC TGA ACA-3′) (GenBank accession no. AF196485) (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). To prove the suitability of the cDNA prepared from the RNA from freeze-dried grapevine buds, semi-quantitative PCR was conducted with Go Taq DNA polymerase (Promega, Madison, WI, USA) using the gene-specific primers above. The samples were initially denatured at 95°C for 1 min, followed by 30 cycles of 1 min at 95°C, 1 min at 59°C (β-tubulin) or 63°C (actin) and 2 min at 72°C with a final extension at 72°C for 10 min. The reactions were analyzed by electrophoresis in a 1% agarose gel stained with GelRed™ (Biotium, Hayward, CA, USA).

Real-time PCR was performed in triplicate reactions for each sample using iTaq™ SYBR® Green Supermix kit (BIO-RAD, CA, USA) in a 48-well plate with a StepOne™ Real-time PCR system (Applied Biosystems, CA, USA). Reactions were done in 20 μL volume containing 125 nM of each primer, 5 μL cDNA (corresponding to 4 ng) and 10 μL 2× iTaq™ SYBR® Green Supermix reagent. Aliquots from the same cDNA sample were used with both set of primers. Reactions were run using the manufacturer's recommended cycling parameters of 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. No-template controls were included for each primer pair.

2.4. Statistical analysis

The data were subjected to analysis of variance using the NCSS software (2006, Kaysville, UT, USA). The effect of cryopreservation and lyophilization on the RNA quality was evaluated by the Tukey–Kramer's comparison test. Differences were considered significant at $p < 0.05$.

3. Results and discussion

The RNA extraction results obtained from freeze-dried and cryopreserved grapevine buds are shown in Table 1. The RNA quality and yield from freeze-dried grapevine buds that were stored at room temperature for 0, 3, and 6 weeks after lyophilization were very similar among all the samples and those obtained from cryopreserved samples. Although the RNA yield from lyophilized buds was somewhat lower than those obtained with cryopreserved buds, no significant differences ($p > 0.05$) were observed. High-quality RNA was obtained in all samples (Fig. 1), as two sharp bands corresponding to 18S and 28S rRNA were obtained from each sample using denaturing 1% agarose gel electrophoresis (Fig. 1a), Bioanalyzer electrophoresis (Fig. 1b) and electropherograms (Fig. 1c). Pearson et al. [10] reported similar results in a study where they compared the RNA quality and yield of frozen and lyophilized brown algae and seagrasses, which did not show significant differences in the RNA quality and yield obtained from frozen and lyophilized tissues [10]. Contrary to this, Saha et al. [8] reported a complete degradation of RNA from freeze-drying cotton tissues compared with RNA from non-freeze-dried tissues. However, in this study, the RNA integrity was also very good in all samples evaluated, as is shown in the values of the RNA integrity number (RIN) in Table 1. The RIN is a standardized method for the interpretation of quality RNA control that takes into account the entire electrophoretic trace, generating an automatic ratio of the 18S and 28S ribosomal RNAs that is based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact RNA [11]. The RIN values of the RNA obtained from freeze-dried grapevine buds immediately after lyophilization was 7.03 ± 0.05, which was very good (week 0, samples 1, 2, and 3). The RNA obtained from samples of freeze-dried buds that were stored for 3 (samples 4–6) and 6 (samples 7–9) weeks at room temperature had RIN values of 6.80 ± 0.00 and 6.46 ± 0.05, respectively, which are also good values. These values were slightly lower than the RIN values for RNA from week 0 but were higher compared to the results obtained for the RNA from the cryopreserved buds (samples 10–12) with a RIN value of 6.60 ± 0.17; it is considered good that the value was still greater than 6. RNA samples with RIN values from 3 to 6 are generally considered degraded [12]. The electropherograms generated by the Bioanalyzer (Fig. 1c) showed a similar peaks pattern in RNA obtained from both tissues (lyophilized and cryopreserved). Schroeder et al. [12] take degraded RNA into consideration when there are small peaks in the area prior to the 18S rRNA peak [12]. However, the presence of these bands in the electropherograms in our samples seems to be more related to other smaller rRNA fragments [12] because the electrophoresis (Fig. 1a and Fig. 1b) shows defined bands instead of degraded bands in these areas. The few degraded RNAs were related to the $A_{260/280}$ ratio values of RNA obtained for each sample (Table 1); the cryopreserved buds showed a higher value ratio of 2.11 ± 0.01, followed by the RNA obtained from freeze-dried buds immediately after lyophilization (time 0) and from the buds stored for 3 weeks, with ratio values of 2.09 ± 0.01, and from the tissue stored for 6 weeks, with a ratio of 2.10 ± 0.01. There was no significant difference ($p > 0.05$) among these values. Additionally, the polyphenol and polysaccharide contamination was low in the RNA obtained from buds, with $A_{260/230}$ ratio values that ranged between 2.34 to 2.39, indicating a low presence of contamination [13]; there was no significant difference ($p > 0.05$) among these values. The low values of polyphenol and polysaccharide contamination in lyophilized buds may be related to the lyophilization process because this may inhibit the

Table 1
Quality of total RNA isolated from lyophilized and cryopreserved dormant grapevine buds. Each date is the average of three independent extractions of each sample and the ± SD.

Sample	RNA (ng/μL)	RIN	Absorbance ratio		C_T ratio
			260/280	260/230	actin/tubulin
Lyophilized					
1–3	231.60 ± 51.35[a]	7.03 ± 0.05[a]	2.09 ± 0.01[a]	2.35 ± 0.03[a]	0.617 ± 0.01[a]
4–6	211.90 ± 35.19[a]	6.80 ± 0.00[ab]	2.09 ± 0.01[a]	2.39 ± 0.03[a]	0.619 ± 0.02[a]
7–9	250.10 ± 19.32[a]	6.46 ± 0.05[c]	2.10 ± 0.01[a]	2.36 ± 0.02[a]	0.625 ± 0.01[a]
Cryopreserved					
10–12	252.00 ± 30.49[a]	6.60 ± 0.17[bc]	2.11 ± 0.01[a]	2.34 ± 0.07[a]	0.627 ± 0.01[a]

Eight grapevine buds were used for each RNA extraction. Samples 1–3 correspond to lyophilized grapevine buds at time zero; samples 4–6 were lyophilized and stored at room temperature for 3 weeks; and samples 7–9 were lyophilized and stored at room temperature for 6 weeks. Samples 10–12 were cryopreserved at -80°C. Different letters mean a significant difference ($p < 0.05$) between treatments.

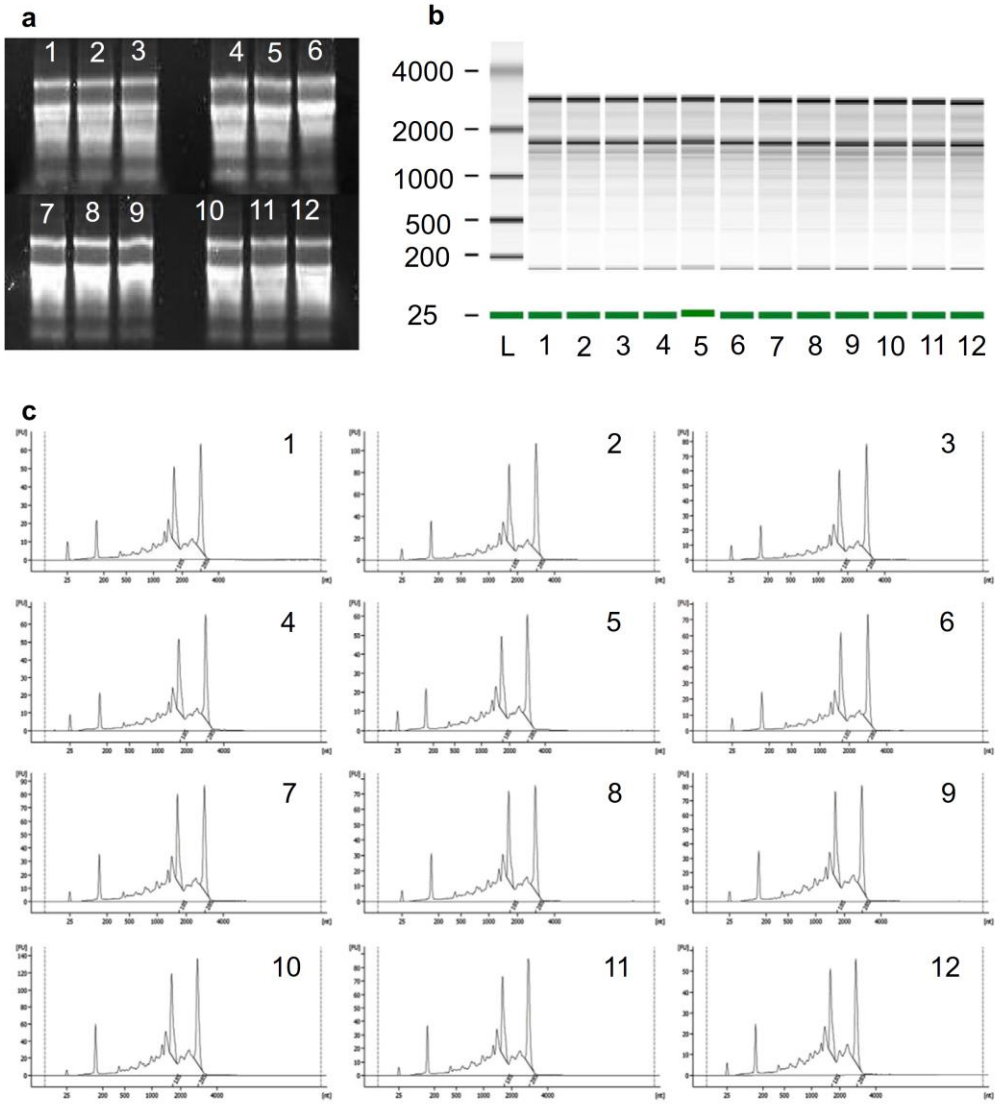


Fig. 1. Quality RNA comparison among samples extracted from dormant grapevine buds. (a) Agarose gel image stained with GelRed™. (b) Bioanalyzer gel image. (c) Electropherograms; note that the scales differ. Samples 1–3, lyophilized buds time zero; 4–6, lyophilized buds stored for 3 weeks at room temperature; 7–9, lyophilized buds stored for 6 weeks at room temperature; 10–12, cryopreserved buds. L: ladder.

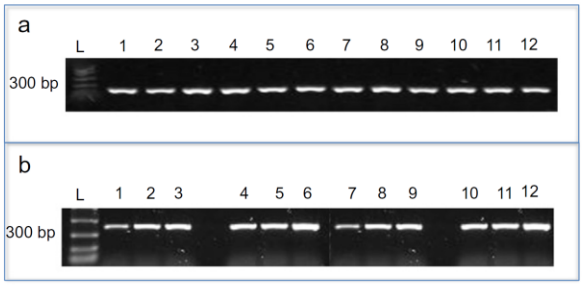


Fig. 2. PCR amplifications performed with the cDNA synthesized from RNA obtained from lyophilized and cryopreserved dormant grapevine buds. (a) Gel bands corresponding to actin (239 bp). (b) Gel bands corresponding to β-tubulin (390 bp). The amplifications were performed using template cDNA synthesized from RNA of lyophilized buds at week zero (lanes 1–3); lyophilized buds stored at room temperature for 3 weeks (lanes 4–6); lyophilized buds stored at room temperature for 6 weeks (lanes 7–9); and buds cryopreserved at -80°C (lanes 10–12). L: ladder (PCR Markers, Promega).

activity of proteolytic enzymes and nucleases, diminishing oxidation and cellular component degradation [7].

According to the RIN values in all the samples evaluated, we report that RNA obtained from these tissues has good integrity [13]. These results demonstrate that the lyophilization process did not affect the quality, yield and integrity of total RNA from grapevine buds.

To assess the suitability of isolated RNA from lyophilized buds for use in further cDNA synthesis, we used reverse transcription coupled to PCR. The cDNA that was synthesized from RNA obtained from the cryopreserved buds and from the lyophilized stored buds at 0, 3, and 6 weeks was used to perform PCR to amplify the housekeeping genes actin (239 bp) (Fig. 2a) and β-tubulin (390 bp) (Fig. 2b). No significant differences were found among the amplified DNA samples using cDNA as the template. Also real-time PCR was performed to assess the amplification efficiency and the C_T ratio was calculated. C_T ratio from lyophilized samples showed no significant differences ($p > 0.05$) from the C_T ratio obtained from cryopreserved samples (Table 1), which may indicate that the transcript abundance ratio of the evaluated genes is similar among samples. Therefore, the RNA stability is not affected by the lyophilization of grapevine buds that are kept for 6 weeks at room temperature prior to RNA extraction. Pearson et al. [10] demonstrated that 3-month-old stored lyophilized sea grasses lead to successful PCR for 16S rRNA, *tsf*, *atpB* and *ycf4* [10]. In this study, the PCR analysis demonstrated that the RNA obtained from lyophilized tissues was well suited for downstream applications, such as gene amplification, which can be further used in studies involving analysis of gene expression.

In conclusion, the freeze-drying process is an adequate method for preserving woody tissues for RNA extraction. In this way, samples can be transported by air or surface mail over short or long distances. It is also good for maintaining RNA quality and integrity for 3 weeks of storage at room temperature. Six weeks after tissue lyophilization, the RNA quality undergoes slight degradation. However, the RNA is still competent for downstream applications such as cDNA and PCR. Freeze-drying is a useful tool for samples with high polyphenol and polysaccharide contents, as it decreases nuclease and protease activities, diminishing the RNA contamination mediated by these compounds.

Conflict of interest

None.

Financial support

This research was sponsored by National Research and Technology Council, Mexico, (CONACyT) grant CB-2010-1000157334. C.V. García-Baldenegro received a scholarship from CONACyT, which funded her doctoral thesis.

References

[1] IATA. (International Air Transport Association): Acceptance checklist for dry ice. [cited January 1, 2015]. Available from Internet at: http://www.iata.org/whatwedo/cargo/dgr/Documents/Acceptance-Checklist-Dry-Ice2014-EN.pdf.
[2] Prendini L, Hanner R, DeSalle R. Obtaining, storing and archiving specimens and tissue samples for use in molecular studies. In: DeSalle R, Giribet G, Wheeler W, editors. Techniques in Molecular Systematics and Evolution. Berlin: Birkhäuser Basel; 2002. p. 176–248.
[3] Hernández GE, Mondala TS, Head SR. Assessing a novel room-temperature RNA storage medium for compatibility in microarray gene expression analysis. BioTechniques 2009;47:667–70. http://dx.doi.org/10.2144/000113209.
[4] Mutter G, Zahrieh D, Liu C, Neuberg D, Finkelstein D, Baker HE, et al. Comparison of frozen and RNALater solid tissue storage methods for use in RNA expression microarrays. BMC Genomics 2004;5:88. http://dx.doi.org/10.1186/1471-2164-5-88.
[5] Kumar GNM, Iyer S, Knowles NR. Extraction of RNA from fresh, frozen, and lyophilized tuber and root tissues. J Agric Food Chem 2007;55:1674–8. http://dx.doi.org/10.1021/jf062941m.
[6] Wu Y, Wu M, Zhang Y, Li W, Gao Y, Li Z, et al. Lyophilization is suitable for storage and shipment of fresh tissue samples without altering RNA and protein levels stored at room temperature. Amino Acids 2012;43:1383–8. http://dx.doi.org/10.1007/s00726-011-1212-8.
[7] Jaiprakash MR, Pillai B, Venkatesh P, Subramanian N, Sinkar VP, Sadhale PP. RNA isolation from high-phenolic freeze-dried tea (*Camellia sinensis*) leaves. Plant Mol Biol Rep 2003;21:465–6. http://dx.doi.org/10.1007/bf02772599.
[8] Saha S, Callahan FE, Dollar DA, Creech JB. Effect of lyophilization of cotton tissue on quality of extractable DNA, RNA, and protein. J Cotton Sci 1997;1:10–4 [cited January 1, 2015. Available from Internet at: http://www.cotton.org/journal/1997-01/1/].
[9] Reid KE, Olsson N, Schlosser J, Peng F, Lund ST. An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. BMC Plant Biol 2006;6:27. http://dx.doi.org/10.1186/1471-2229-6-27.
[10] Pearson G, Lago-Leston A, Valente M, Serrão E. Simple and rapid RNA extraction from freeze-dried tissue of brown algae and seagrasses. Eur J Phycol 2006;41:97–104. http://dx.doi.org/10.1080/09670260500505011.
[11] Mueller O, Lightfoot S, Schroeder A. RNA integrity number (RIN) — standardization of RNA quality control. Agilent Technol 2004:1–7 [cited January 1, 2015. Available from Internet at: http://www.chem.agilent.com/Library/applications/5989-1165EN.pdf].
[12] Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, et al. The RIN: An RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol 2006;7:1–14. http://dx.doi.org/10.1186/1471-2199-7-3.
[13] Tattersal EAR, Ergul A, AlKayal F, DeLuc L, Cushman JC, Cramer GR. Comparison of methods for isolating high-quality RNA from leaves of grapevine. Am J Enol Vitic 2005;56:400–6 [cited January 1, 2015. Available from Internet at: http://www.ajevonline.org/content/56/4/400.abstract].

Capítulo 2

Dormancy breaking-stimuli cyanamide and sulfur derivatives regulate the expression of ascorbate-glutathione cycle genes in grapevine (*Vitis vinifera* L.) buds

---

Manuscrito sometido a la revista Plant Growth Regulation

Dormancy breaking-stimuli cyanamide and sulfur derivatives regulate the expression of ascorbate-glutathione cycle genes in grapevine (*Vitis vinifera* L.) buds.

Claudia Vanessa García-Baldenegro[1], Emmanuel Aispuro-Hernández[1], Miguel Ángel Martínez-Téllez[1], Irasema Vargas-Arispuro[2]*.

[1] Coordinación de Tecnología de Alimentos de Origen Vegetal, Centro de Investigación en Alimentación y Desarrollo, A.C. Carretera a la Victoria Km 0.6, Hermosillo, Sonora, México, C.P. 83304.

[2] Coordinación de Ciencias en Alimentos, Centro de Investigación en Alimentación y Desarrollo, A.C. Carretera a la Victoria Km 0.6, Hermosillo, Sonora, México, C.P. 83304.

*Corresponding autor: iris@ciad.mx

For: Plant Growth Regulation

http://www.springer.com/life+sciences/plant+sciences/journal/10725

Abstract

In temperate winter regions, dormancy-breaking chemicals as hydrogen cyanamide (HC) or sulfur derivatives (SD) are used in grapevine cultivars to overcome dormancy. HC inhibits catalase activity and the concomitant production of H_2O_2 activates the ascorbate-glutathione cycle (AGC). Nevertheless, the mechanism that leads to dormancy release is not well known at molecular levels. This study compares the expression of the genes ascorbate peroxidase (*APX)*, dehydroascorbate peroxidase (*DHAR)*, glutathione reductase (*GR)* and catalase (*CAT)* between HC- or SD-treated grapevine dormant buds to learn about the implication of the AGC on dormancy release. We found that HC treatment down-regulates the expression of *APX*, *DHAR* and *CAT*. Conversely, SD may lead to a plant dormancy release more similar to controls by maintaining comparable patterns, similar expression levels of *APX*, *DHAR* and *CAT*, and augmenting the *GR* expression, which may lead to a more efficient homeostasis recovery due to a reduced glutathione (GSH) increment. Therefore arises the hypothesis that GSH is a key molecule for breaking dormancy.

Keywords
Sulfur derivatives
Glutathione reductase
Hydrogen peroxide
Endodormancy
Catalase
Hydrogen cyanamide

Introduction

Dormant grapevines cultivated in regions with desert climate are forced to budbreak mainly by applying chemical elicitors due to the lack of sufficient chilling accumulation during winter, which is the natural stimulus to overcome dormancy. The increment of hydrogen peroxide (H_2O_2) levels inside the grapevine buds by inhibition of the enzyme

catalase is a necessary event preceding dormancy release; therefore, the catalase inhibitor hydrogen cyanamide (HC) is a very effective agent to stimulate an early and vigorous vegetative growth (Corrales et al., 2010; Halaly et al., 2008; Or et al., 2000), albeit phytotoxic side effects have also been proved (Arora et al., 2003; Siller-Cepeda et al., 1992). In order to restore the cellular homeostasis and avoid damage after oxidative burst signaling, reactive oxygen species (ROS) have to be scavenged by catalase non-dependent alternative mechanisms. HC has been found to increase the transcript levels of *ascorbate peroxidase* (*APX*) and *glutathione reductase* (*GR*), triggering the ascorbate glutathione cycle (AGC) (Halaly et al., 2008; Pérez et al., 2009), which is a metabolic pathway that detoxifies H_2O_2 by the reduction of metabolites such as ascorbate, glutathione and NADPH (Vergara and Pérez, 2010). In the search of less toxic dormancy breakers, extracts and volatiles of garlic successfully broke the dormancy of grapevine cuttings (Kubota et al., 1999; Kubota and Miyamuki, 1992), in the same magnitude than HC (Vargas-Arispuro et al, 2008; Corrales-Maldonado et al., 2010). These authors found that this garlic extract was rich in sulfur derivatives (SD) as allicin, diallyl disulfide, diallyl trisulfide, S-methyl cysteine sulfoxide, dimethyl disulfide, dimethyl trisulfide and dimethyl thiosulphonate (Vargas-Arispuro et al., 2008). A mix of these compounds is in patent process and called Bro-T (MX/A/2008000929). The mechanisms by which SD promote the release of dormancy in grapevine is not yet well understood, but it may be related to the capacity to supply the plant with sulfur precursors of compounds involved in the response to oxidative stress and growth resumption (Pacey-Miller et al., 2003; Mohamed et al., 2012). In order to gain a better understanding of the similarities or differences between the mechanisms of grapevine dormancy release induced by HC and SD, this study compares the expression of the AGC genes *APX*, *dehydroascorbate reductase* (*DHAR*), and *GR*, as well as *catalase* (*CAT*) by effect of both treatments.

Materials and Methods

Vegetal material and treatments

Cuttings with six buds were collected from endodormant grapevine plants (*Vitis vinifera* L. cv. 'Flame Seedless') with ~150 h of naturally accumulated cold (under 7.2 °C) in a commercial vineyard located in San Miguel de Horcasitas, Sonora, Mexico (29° 20′N, 110° 51′O). The experiments were carried out in the "Centro de Investigación en Alimentación y Desarrollo, A.C.". Six bud cuttings were dipped for 30 s in 4 % (v/v) hydrogen cyanamide (Compensor® Arysta LifeScience, Mexico), 10 % sulfur derivatives (patent in progress MX/A/2008000929), or water as control. Buds were collected at 0, 4, 12, 24 and 48 h after application, immediately frozen in liquid nitrogen and stored at -80 ºC until analysis.

Calorimetric measurement

Metabolic heat production by buds was measured by calorimetric activity before treatments application. Six node cuttings of grapevine cv. 'Flame Seedless' recently brought from the vineyard were kept at room temperature with their basal ends immersed in water until measurement. Metabolic heat was measured with a differential scanning calorimeter (DSC) (CSC 4100; Calorimetry Science Corporation, Pleasant Grove, Utah) working in the isothermal mode at 25 °C for 3000 sec. The instrument has a baseline sensitivity ±1 μW and a working range of -30-110°C. Temperature around the DSC chamber was maintained at 15°C with a refrigerated circulating bath (*Polyscience, Niles*, IL). A flux of dry nitrogen at 175 g cm^{-2} was used to prevent moisture condensation inside the instrument. Samples were measured in three 1 cm^3 hastelloy ampoules with removable lids. Metabolic heat (Rq) rate was expressed on a dry-weight basis (Gardea et al., 2000). Rq means were calculated on six replicates.

Total RNA isolation

RNA isolation was performed according to Read et al., (2006), with slights modifications from García-Baldenegro et al. (2015). The extraction buffer contained 300 mM Tris HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 2% CTAB, 2% PVP40, 0.05% spermidine trihydrochloride, and 2% ß-mercaptoethanol. Tissue was ground to a fine

powder in liquid nitrogen using a mortar and pestle. The powder was added to pre-warmed (65ºC) extraction buffer at 20 mL/g of tissue and shaken vigorously. Tubes were incubated in 65 ºC water bath for 12 min and shaken every 3 min. Mixtures were extracted twice with equal volume chloroform: isoamyl alcohol (24:1) then centrifuged at 13,200 rpm for 10 min at 4 ºC each time and the aqueous layer was transferred to a new tube and centrifuged at 13,200 for 15 min at 4 ºC to remove remaining insoluble material. 0.1 vol. 3M NaOAc (pH 5.2) and 0.6 vol. isopropanol were added to the supernatant, mixed and stored at -80 ºC for 10 min. Nucleic acid pellets were collected by centrifugation at 13,200 rpm for 30 min at 4 ºC. The pellet was dissolved in 1 mL TE (pH 7.5). To selectively precipitate RNA, 0.25 vol. of 10 M LiCl was added and the sample was stored overnight at 4 ºC. RNA was pelleted by centrifugation at 13,200 rpm for 30 min at 4 ºC, and then washed with 1mL of ice cold 70% EtOH, air dried, and dissolved in 60 µL DEPC-treated water.

RNA purification and cDNA synthesis

The samples were purified using the commercial system Spectrum Plant Total RNA (Sigma-Aldrich, St. Louis, MO, USA) starting from binding columns. Genomic DNA traces were eliminated using RNase-Free DNase (QIAGEN, Valencia, CA, USA). The integrity and quantity of the total RNA were evaluated by the spectrophotometric absorbance ratios A_{260}/A_{280} and A_{260}/A_{230} using a NanoDrop 2000 (Thermo Scientific NanoDrop, USA). The integrity of RNA was determined by electrophoresis on a denaturing (formaldehyde) 1% agarose gel. cDNAs were synthesized from 1µg of total RNA using the Superscript™ II First-Strand Synthesis System (Invitrogen, CA, USA), according to the manufacturer's instructions.

Real time RT-PCR

The expression of the AGC (ascorbate-glutathione cycle) genes *APX*, *DHAR*, *GR*, and other oxidative stress system gene *CAT* was evaluated by PCR in triplicate reactions including a 5µL cDNA template (4 ng), 10 µL of iTaq Universal SYBR Green Supermix

(Bio-Rad Laboratories, CA, USA), 1 µL of 5µM of each sense and antisense primer and 0.1% DEPC-treated water to a final volume of 20 µL. Specific primers are listed in Table 1. PCR amplification was done in a 48-well Step-One™ Real-time PCR System (Applied Biosystem Inc., CA, USA). Amplification conditions were 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. PCR specificity was confirmed by constructing a melt curve after amplification in a range from 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. Non-template controls were also included. The results were normalized with *actin* (*ACT*) expression levels and analyzed with the $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Statistical analysis was performed using one-way ANOVA, with a significance level of $p<0.05$, using the NCSS (2007) software.

Table 1. List of primers used in real time PCR.

Gene	Accession no.	Forward primer (5'-3')	Reverse primer (5'-3')
APX	NM_001281059	GCCTTCCGCTTCTTCTGAGC	TCAGCGATGAACCCTCTGAGC
DHAR	NM_001281063	TCAGAGCAGGCTTTGCTGGAT	AATGGCCAAGAGCCACCTGA
GR	AF019907	GCACGAGCTCATTGTCGACGC	AGAGAGAGAGCCTGGGGCTAC
CAT	XM_003631877	AGCAAGCAAGGGTGTTCATCAGTC	TCAACCGTAGGATTTGTCTGGCTC
ACT	AF369524	GCTGAGAGATTCCGTTGTCC	GCCACCACCTTGATCTTCAT

Results and Discussion

Endodormancy

In order to know the developmental stage of the grapevine buds, the calorimetric measurements were performed. Our results show that the 'Flame Seedless' buds used in this work had a heat metabolic of Rq= 2.1564 µJ• s-1• mg-1 DW with 150 natural chilling hours accumulated. According to Gardea et al. (1994), this value of Rq indicates that the developmental stage of the buds were still endodormant. Flame Seedless buds

need to accumulate from 200-250 hours of natural chilling for budbreak, measured under 7.2 °C (Siller et al., 1993).

Effects of SD and HC on the expression of *APX* and *DHAR* in endodormant grapevine buds

During dormancy, the low metabolic level of the plant leads to the production and accumulation of ROS such as H_2O_2. Strong evidence suggests H_2O_2 acts as a signaling molecule in plants, triggering the endodormancy release (Or et al., 2000, 2002; Foyer and Noctor, 2005; Pérez et al., 2008). AGC is the major H_2O_2 metabolizing pathway with the primary peroxidation of ascorbate by APX as it uses reduced ascorbate as a donor (Chew et al., 2003; Foyer and Halliwell, 1976). Our work evidences that the stimuli of SD and HC treatments on first instance induced an oxidative burst by the 5-fold reduced transcript accumulation of *APX* at 12 h compared to control ($p<0.05$) (Fig. 1A), which may lead to a raise in H_2O_2 and to the consequent diminish of the ascorbate metabolite. A similar expression pattern for *DHAR* was given along the experiment, suggesting a coordinated regulation in the antioxidant machinery of AGC as evidenced by the close inter-relation between *APX* and *DHAR* (Fig. 1A, 1B). As *DHAR* is down-regulated by both treatments at time point 12 h, ascorbate could not be produced and reduced, with a consequent accumulation of H_2O_2. The increment of H_2O_2 levels could be an evidence of abiotic stress and would trigger the signaling events as described by Pérez et al. (2008).

The expression of *APX* and *DHAR* transcripts at 24 and 48 h are regulated by SD treatment, but not by HC (Fig. 1A, 1B). Contrary to evidences in other works (Halaly et al., 2008; Ophir et al., 2010) where *APX* expression enhances at the first 24 h after HC application, thus permitting its product acting as a detoxifier enzyme. The down-regulation of *APX* and *DHAR* by HC at 24 and 48 h after treatment in this work may mean that there is a further increase in H_2O_2 causing phytotoxicity in the plant (Arora et al., 2003; Siller-Cepeda et al., 1992). For SD treated buds, the regulation of the transcripts of *APX* and *DHAR* was similar to their control at 24 and 48 h. These results

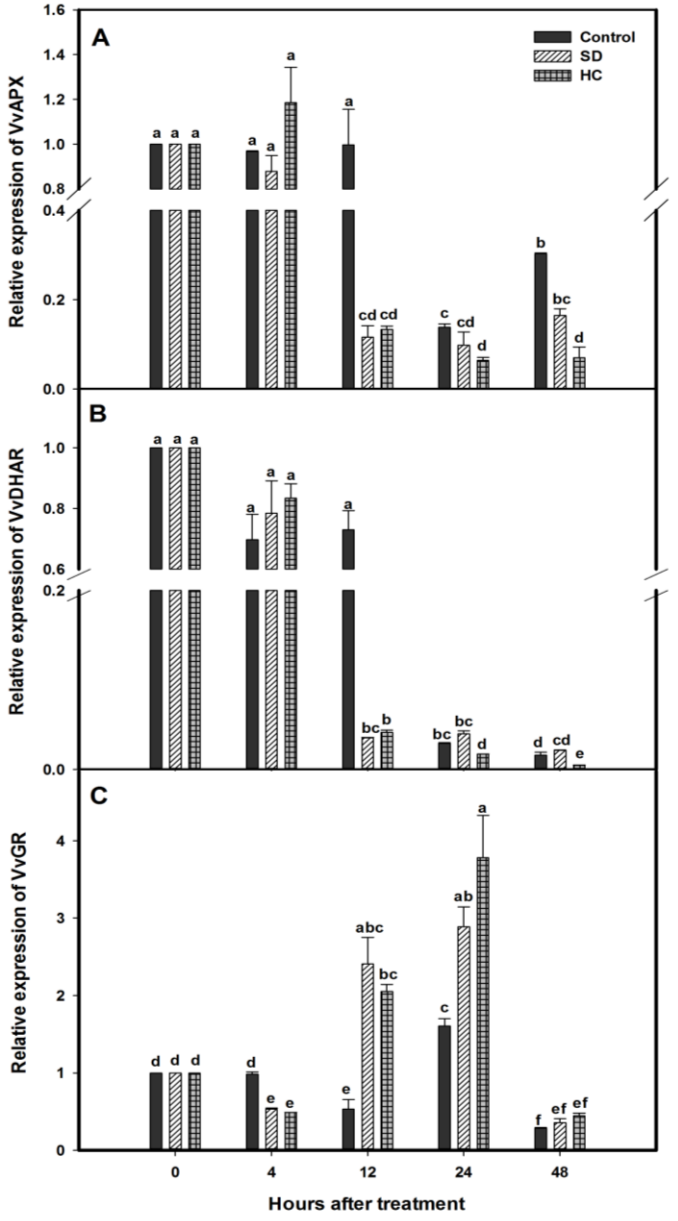


Figure 1. Effects of sulfur derivatives (SD) and hydrogen cyanamide (HC) on the expression of the AGC genes *APX* (A), *DHAR* (B), and *GR* (C). Data normalized against *ACT*. Values correspond to the average of three technical replicates and bars represent standard error. Different letters mean statistical differences ($p<0.05$).

suggest that SD may induce a lower oxidative stress than HC treatment, and its regulatory effect is similar to non-treated buds (control). This would allow a transient regulation in the production of ascorbate and a faster homeostasis recovery. Wang and Faust (1994) reported an increase in ascorbate concentration two days after application of allyl disulfide in 'Anna' apple buds and observed free radical removal, events known to precede budbreak. A study by Orrantia-Araujo (2015), reported an increment in the production of ascorbate in Flame Seedless grapevine dormant buds, after SD application, reaching a higher concentration of 3.56 µM/ g FW at 24 h compared to control and HC ($p<0.05$). The ascorbate molecule is an excellent detoxifier of ROS (Gallie, 2013) such as the H_2O_2, which is a small molecule that can be transported easily through the cell, causing damage at locations far from its site of generation, and oxidizing other cellular structures by peroxidation of lipids, oxidation of proteins, damage to nucleic acids, activation of programed cell death pathway and cell death (Sharma et al., 2012).

Effects of SD and HC on the expression of *GR* in endodormant grapevine buds

Despite the strong evidence of sulfur compounds effectiveness to release dormancy (Vargas et al., 2008; Corrales et al., 2010; Kubota and Miyamuki, 1992; Kubota et al., 2003) the effect of sulfur derivatives on the expression of *GR* was unknown. Applying compounds like HC and sodium azide in grapevine buds to break dormancy induces the expression of *GR* gene and also the activity of its enzyme (Halaly et al., 2008; Pérez et al., 2009; Fukoka and Enomoto, 2007; Vergara and Pérez, 2010). In this study the SD and HC treatments had an abrupt increase in the expression of *GR* at 12 and 24 h after treatments application on grapevine buds (Fig. 1C). The up-regulation of *GR* may lead to an increment of reduced glutathione (GSH). Evidence to support this was provided by Wang and Faust (1994), who demonstrated that the application of allyl disulfide on apple buds increased the GR specific activity and consequently the GSH content. Also Orrantia-Araujo (2015), reported that the application of SD from garlic extract in grapevine buds, lead to an increase in the reduced glutathione: oxidized glutathione (GSH:GSSG) ratio at 48 h after treatment, with a value of 27.40, followed by the application of HC and control with 17.81 and 13, respectively. According to Pacey-

Miller et al. (2003) and Tobhe et al. (1998), a high GSH:GSSG ratio leads to the end of dormancy, which may be related to a high GSH content activating and deactivating redox-dependent enzyme systems (Ziegler, 1985). The increment in GR activity without the decreasing of ascorbate production suggests that the sulfur derivatives of garlic may be acting as a pool of sulfur compounds that nurse the plant and helps it to enhance optimal GSH levels, enabling the synthesis of cysteine and methionine. Which could be the explanation of these effects reported after the application of garlic extracts rich in sulfur compounds that induce the breaking of dormancy as well as increments in the fructification yield in apple trees, grapevines, and pear trees (Botelho et al., 2007; Corrales-Maldonado et al., 2010; El-Razek et al., 2013).

Effects of SD and HC on the expression of *CAT* in endodormant grapevine buds

Another scavenger of hydrogen peroxide is *CAT*, which catalyzes the reduction of $2H_2O_2$ into two water molecules and oxygen. The application of the treatments SD and HC on grapevine buds showed a differential expression on *CAT*. The SD treatment did not induce differences in *CAT* expression, being similar to their control ($p>0.05$) during 48 h after the treatments application (Fig. 2). On the other hand, the application of HC induced *CAT* down-regulation from 12 h to 48 h ($p<0.05$) (Fig. 2). It has been shown that catalase expression and activity are inhibited shortly after HC application leading to an oxidative stress (Nir et al., 1986; Or et al., 2002; Halaly et al., 2008). This might indicate that other authors have reported that a certain level of H_2O_2 needs to be maintained in order to trigger a signaling cascade to activate other pathways (Pérez et al., 2008; Halaly et al., 2008). The results of *CAT* expression showed here are coincidental with those described by Halaly et al. (2008), where *CAT* transcripts were completely null by applying HC in dormant grapevine buds at 24 h and 48 h (Halaly et al., 2008; Or et al., 2002). This could lead to a transitory low enzyme production and consequently to a low activity, as reported by Pacey-Miller et al. (2003) and others (Siller-Cepeda et al., 1992; Nir et al., 1986).

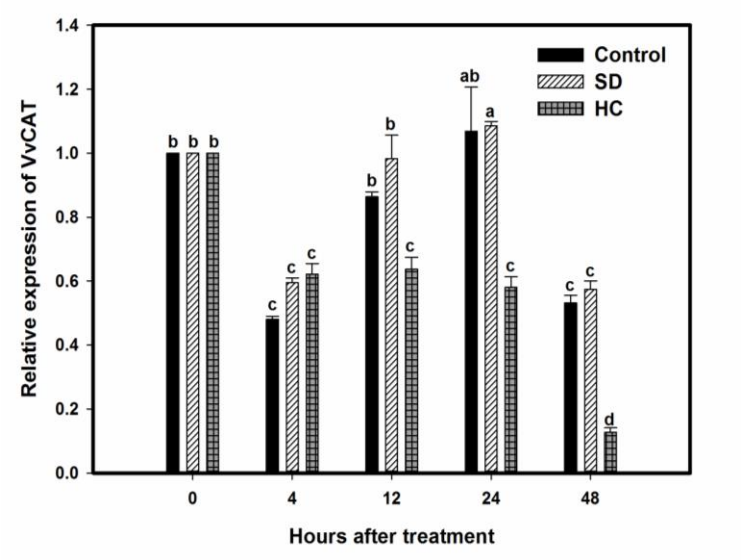


Fig. 2. Relative expression of *CAT* by effect of sulfur derivatives (SD) and hydrogen cyanamide (HC) application. Data normalized against *ACT*. Values correspond to the average of three technical replicates and bars represent standard error. Different letters mean statistical differences ($p<0.05$).

Overall, our results show both dormancy-breaking treatments influence the expression of AGC genes in grapevine buds, on the one hand by regulating the *APX* and *DHAR*, and on the other hand, up-regulating *GR*. Increased levels of GSH may be favoring and adequate redox balance by allowing the activity of other enzymatic redox systems like glutathione-S-transferases, glutaredoxins and peroxiredoxins (Foyer and Noctor, 2005). While the transient *APX* and *DHAR* down-regulation would trigger the oxidative signaling for dormancy release, prolonged oxidative conditions may jeopardize the process viability. Therefore SD, which promote a prompt recovery of *APX* and *DHAR* transcript levels, along with the fact that the expression of *CAT* is not affected as in HC-treated plants, may lead to a more efficient homeostasis recovery.

Conclusions

These results showed that sulphur derivatives treatment maintains a large sulphur pool for redox homeostasis leading to budbreak release in a natural way without affecting negatively the plant. Sulfur derivatives (SD) and hydrogen cyanamide (HC) treated grapevine buds showed similarities in the expression of the ascorbate-glutathione cycle genes (AGC). Nevertheless, HC induce a downregulation of *APX*, *DHAR* and *CAT* expression that might lead to producing stress in the plant. On the other hand, sulfur derivatives maintain the *APX*, *DHAR* and *CAT* expression levels and might promote the activation of AGC that is an important H_2O_2 scavenging. In both treatments, *GR* expression level was higher than in control, suggesting a glutathione increment that has been hypothesized to be responsible for budbreak in grapevines.

Acknowledgements

This research was sponsored by National Research and Technology Council, Mexico, (CONACyT) grant CB-2010-1000157334. C.V. García-Baldenegro received a scholarship from CONACyT, which funded her doctoral thesis. We would like to thank to Alfonso A. Gardea for the calorimetric analysis.

References

Arora R, Rowland LJ, Tanino K (2003) Induction and release of bud dormancy in woody perennials: a science comes of age. HortScience 38: 911–921.

Botehlo RV, Müller MML (2007) Evaluation of garlic extract on bud dormancy release of 'Royal Gala' apple trees. Aust J Exp Agric 47:1-4. doi: 10.1071/EA06135

Chew O, Whelan J, Millar AH (2003) Molecular definition of the ascorbate-glutathione cycle in *Arabidopsis* mitochondria reveals dual targeting of antioxidant defenses in plants. J Biol
Chem, 278: 46869–46877. doi: 10.1074/jbc.M307525200

Corrales-Maldonado C, Martinez-Tellez MA, Gardea AA, Orozco-Avitia A, Vargas-Arispuro I (2010) Organic alternative for breaking dormancy in table grapes grown in hot regions. Am J Agri & Biol Sci 5: 194-198. doi: 10.3844/ajabssp

El-Razek EA, El-Migeed MMM, Abdel-Hamid N (2013) Response of 'Le Conte' Pear Trees to Garlic Extract and GA3 as Budbreak Dormancy Agents. Middle East J Sci Res 14: 1407-1413. doi: 10.5829/idosi.mejsr.2013.14.11.3573

Foyer CH, Halliwell B (1976) The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133:21-25. doi: 10.1007/bf00386001

Foyer CH, Noctor G (2005) Redox Homeostasis and Antioxidant signaling: A Metabolic Interface between Stress Perception and Physiological Responses. The Plant Cell 17: 1866–1875. doi: 10.1105/tpc.105.033589

Fukoka N, Enomoto T (2007) Effects of sulfur application on enzyme activities in relation to ascorbate-glutathione cycle and the occurrence of internal browning in *Raphanus* roots. J Japan Soc Hort Sci 76:305-309. doi: 10.1080/14620316.2002.11511521

Gallie DR (2013) L-Ascorbic Acid: A Multifunctional Molecule Supporting Plant Growth and Development. Scientifica. doi:10.1155/2013/795964

García-Baldenegro CV, Vargas-Arispuro I, Islas-Osuna M, Rivera-Domínguez M, Aispuro-Hernáandez E, Martínez-Téllez MA (2015) Total RNA quality of lyophilized and cryopreserved dormant grapevine buds. Electron J Biotechnol. doi:10.1016/j.ejbt.2015.01.002

Gardea AA, Carvajal-Millan E, Orozco JA, Guerrero VM, Llamas J (2000) Effect of chilling on calorimetric responses of dormant vegetative apple buds. Thermochimica Acta 349: 89-94. doi:10.1016/S0040-6031(99)00500-6

Gardea AA, Moreno YM, Azarenko AN, Lombard PB, Daley LS (1994) Changes in metabolic properties of grape buds during development. J Amer Soc Hort Sci 119: 756–760

Halaly T, Pang X, BatikoT, Crane O, Keren A, Venkateswari J, Ogrodovitch A, Sadka A, Lavee S, Or E (2008) Similar mechanisms might be triggered by alternative external stimuli that induce dormancy release in grape buds. Planta 228:79–88. doi: 10.1007/s00425-008-0720-6

Kubota N, Miyamuki M (1992) Breaking bud dormancy in grapevines with garlic paste. J Amer Soc Hort Sci 117: 898-901

Kubota N , Miyamuki M, Yamane y, Kobayashi A, Mitzutani F (1999) Breaking bud dormancy in grapevine cuttings with garlic volatiles. J Japan Soc Hort Sci 68:927-931. doi: 10.2503/jjshs.68.927

Kubota N, Toriu K, Yamane Y, Kawazu K, Higushi T, Nishimura S (2003) Identification of active substances in Chinese chive and rakkyo plants responsible for breaking bud dormancy in grape cuttings. J Japan Soc Hort Sci 72:268-274. doi: 10.2503/jjshs.72.268

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25: 402–408. doi:10.1006/meth.2001.1262

Mohamed HB, Vadel AM, Geuns JMC, Khemira H (2012) Effects of hydrogen cyanamide on antioxidant enzymes' activity, proline and polyamine contents during bud dormancy release in Superior Seedless grapevine buds. Acta Physiol Plant 34:429-437. doi: 10.1007/s11738-011-0839-0

Nir G, Shulman Y, Fanberstein L, Lavee S (1986) Changes in the activity of catalase (EC 1.11.1.6) in relation to dormancy of grapevine (*Vitis vinifera* L.) buds. Plant Physiol 81: 1140. doi: 0032-0889/86/81/1 140/00/$0 1.00/0

Orrantia-Araujo MA (2015) Cuantificación de metabolitos del ciclo ascorbato-glutatión en yemas latentes de vid de mesa, después de aplicar inductores de brotación. Dissertation, Centro de Investigación en Alimentación y Desarrollo, A.C.

Or E, Vilozny I, Eyal Y, Ogrodovitch A (2000) The transduction of the signal for grape bud dormancy breaking induced by hydrogen cyanamide may involve the SNF-like protein kinase GDBRPK. Plant Mol Biol 43: 483–494. doi: 10.1023/a:1006450516982

Or E, Vilozny I, Fennell A, Eyal Y, Ogrodovitch A (2002) Dormancy in grape buds: isolation and characterization of catalase cDNA and analysis of its expression following chemical induction of bud dormancy release. Plant Science 162:121–130. doi: 10.1016/S0168-9452(01)00542-8

Ophir R, Pang X, Halaly T, Venkateswari J, Lavee S, Galbraith D, Or E (2009) Gene-expression profiling of grape bud response to two alternative dormancy-release stimuli expose possible links between impaired mitochondrial activity, hypoxia, ethylene-ABA interplay and cell enlargement. Plant Mol Biol 71:403–423. doi: 10.1007/s11103-009-9531-9

Pacey-Miller T, Scott K, Ablett E, Tingey S, Ching A, Henry R (2003) Genes associated with the end of dormancy in grapes. Funct Integr Genomics 3:144-152. doi: 10.1007/s10142-003-0094-6

Pérez FJ, Vergara R, Or E (2009) On the mechanism of dormancy release in grapevine buds: a comparative study between hydrogen cyanamide and sodium azide. Plant Growth Regul 59:145-152. doi: 10.1007/s10725-009-9397-5

Pérez FJ, Vergara R, Rubio S (2008) H_2O_2 is involved in the dormancy-breaking effect of hydrogen cyanamide in grapevine buds. Plant Growth Regul 55:149–155. doi: 10.1007/s10725-008-9269-4

Reid KE, Olsson N, Schlosser J, Peng F, Lund ST (2006) An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. BMC Plant Biology 6:27. doi: 10.1186/1471-2229-6-27

Sharma P, Jha AB, Dubey RS, Pessarakli M (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Journal of Botany 1-26 doi:10.1155/2012/217037

Siller-Cepeda JH, Fuchigami LH, Chen THH (1992) Hydrogen cyanamide induced budbreak and phytotoxicity in 'Redhaven' peach buds. Hort Science 27:874-876.

Siller CJ, Osorio G, Baez R, Gardea A (1993) Requerimientos de frio y rompimiento del reposo en vid (Vitis vinifera L.) Memorias. II ciclo internacional de conferencias sobre viticultura. Hermosillo, Sonora, México. p. 120-132

Tobhe M, Mochioka R, Horiuchi S, Ogata T, Shiozaki S, Kurooka H (1998) The role of glutathione on the onset of endodormancy of grape buds. J Japan Soc Hort Sci 67: 912-916

Vargas-Arispuro I, Corrales-Maldonado C, Martínez-Téllez MA (2008) Compounds derived from garlic as bud induction agents in organic farming of table grape. Chil J Agr Res 68:94-101. doi: 10.4067/S0718-58392008000100009

Vergara R, Pérez FJ (2010) Similarities between natural and chemically induced bud-endodormancy release in grapevine *Vitis vinifera* L. Sci Hort 125: 648–653. doi:10.1016/j.scienta.2010.05.020

Wang SY, Faust M (1994) Changes in the antioxidant system associated with budbreak in `Anna´ apple (*Malus domestica* Borkh.) buds. J Amer Soc Hort Sci 119: 735-741

Ziegler D (1985) Role of reversible oxidation-reduction of enzyme thiols-disulfides in metabolic regulation. Annu Rev Biochem 54:305-329. doi: 10.1146/annurev.bi.54.070185.001513

Capítulo 3

Differential gene expression in endodormant grapevine buds evoked by sulphur derivatives and cyanamide stimuli

---

Manuscrito preparado para enviarse a la revista Plant Molecular Biology Reporter

Differential gene expression in endodormant grapevine buds evoked by sulphur derivatives and cyanamide stimuli

Claudia Vanessa García-Baldenegro[1], Irasema Vargas-Arispuro[2], Miguel Ángel Hernández-Oñate[1], María Auxiliadora Islas-Osuna[1], Marisela Rivera-Domínguez[2], Miguel Ángel Martínez-Téllez[1]*.

[1]Coordinación de Tecnología de Alimentos de Origen Vegetal, Centro de Investigación en Alimentación y Desarrollo, A.C. Carretera a la Victoria Km 0.6, Hermosillo, Sonora, México, C.P. 83304.

[2]Coordinación de Ciencias de los Alimentos, Centro de Investigación en Alimentación y Desarrollo, A.C. Carretera a la Victoria Km 0.6, Hermosillo, Sonora, México, C.P. 83304.

*Corresponding autor: norawa@ciad.mx

For: Plant Molecular Biology Reporter
http://www.springer.com/life+sciences/plant+sciences/journal/11105

Abstract

Viticulture in warm winter regions is restricted due to the lack of enough chilling hours for breaking dormancy and must be chemically stimulated, mainly by the application of hydrogen cyanamide (HC), which inhibits catalase activity leading to an H_2O_2-mediated oxidative stress and inducing plant toxicity. Therefore, less toxic alternatives to release bud dormancy need to be addressed. An organic alternative to break dormancy is the use of sulphur derivatives (SD) from garlic, which have shown the ability to lead the early grapevine budbreak and good cluster yield, but under less stressful conditions for plant in comparison to the conventional HC. The sulphur derivatives are very likely a great supplier of sulphur to the plant. Sulphur is a precursor of cysteine and methionine, metabolites used to form glutathione, molecule related to stress and dormancy release. Nevertheless, the mechanism that leads to dormancy resumption is not well known at molecular levels. In this context, here we show a transcriptional analysis using microarrays to evaluate the differential gene expression of the grapevine buds in response to the treatments with sulfur derivatives (SD) and hydrogen cyanamide (HC) 24 hours after application. Our analysis shows that HC-treatment provokes the induction of transport functions, signaling, secondary metabolism, hormone metabolism and response to stress, primarily oxidative stress. The SD-treated buds up-regulates genes transcripts involved in cell wall organization, and glutathione S-transferases (GST), it also presented down-regulation of the genes associated to secondary metabolism and oxidation-reduction. These results demonstrate that SD leads to budbreak resumption in a natural way without affecting negatively the plant.

Keywords: sulphur compounds, Nimblegen array, glutathione S-transferases, secondary metabolism, budbreak, dormancy

Introduction

In warm winter regions table grape productivity is limited for the lack of natural chilling to release dormancy and becomes a major obstacle to commercial production of

temperate fruits, including grapevine (Erez, 1995; Or et al., 1999). Therefore, in these regions, dormancy release needs to be controlled by the use of artificial dormancy-breaking compounds to compensate the lack of natural chilling and enable the economic productivity of table grapes. Transcriptional studies have been performed to provide an insight of the biochemical and cellular processes involved in the dormancy release mechanisms during development of grapevine (Díaz-Riquelme et al., 2012), leafy spurge (Horvath et al., 2008), and kiwifruit (Walton et al., 2009). Other works have studied the transcriptional expression of dormancy release in grapevine buds by chilling application (Mathiason et al., 2009) and by hydrogen cyanamide (HC) application (Keilin et al., 2007, Halaly et al., 2008, Ophir et al., 2009). Also, the use of HC as a powerful tool to control the dormancy release in grapevines, provide us a controlled system for the analysis of dormancy release in grapevines. Keilin et al., (2007), studied the global transcription of dormancy release in grapevines buds cv. 'Perlette' using a digital expression profiling from an EST collection controlling the timing of the induction of dormancy release using HC as a budbreak agent. Their work gave an approach of the biochemical processes occurring during dormancy release, suggesting that HC regulates oxidative stress, calcium signaling, and anaerobic carbohydrate metabolism (Keilin et al., 2007; Halaly et al., 2008; Ophir et al., 2009). Notwithstanding HC is an excellent agent to induce bud break, it is well known that it causes phytotoxicity to the plant (Arora et al., 2003; Siller-Cepeda et al., 1992). Thus in the search for less toxic dormancy breakers, garlic extracts and volatiles are known to be highly effective for breaking dormancy of grapevine cuttings (Kubota et al., 1999; Kubota and Miyamuki, 1992). Vargas-Arispuro et al., (2008) and Corrales-Maldonado et al. (2010) obtained an extract with several sulfur compounds derived from garlic (SD) that promoted dormancy release in grapevine. However, the cellular mechanisms by which SD promote the release of dormancy in grapevine is not yet well understood, but it may be related to the capacity to supply the plant with sulfur precursors of compounds involved in the response to oxidative stress and growth resumption (Pacey-Miller et al., 2003; Rady and Seif El-Yazal, 2014). These reports point to a number of potential mode of actions for SD action in budbreak and in the present work, an analysis of differential gene expression was made to identify early transcriptional events following the application of

SD and HC to understand the similarities or differences between the mechanisms of grapevine dormancy breaking agents.

Materials and Methods

Vegetal material and treatments

Cuttings with six buds were collected from endodormant grapevine plants (*Vitis vinifera* L. cv. 'Flame Seedless') with 156 h of naturally accumulated cold (under 7.2 °C). The vegetal material came from a commercial vineyard in an agricultural zone in San Miguel de Horcasitas, Sonora, Mexico (29° 20′N, 110° 51′O). The experiments were carried out in the Centro de Investigación en Alimentación y Desarrollo, A.C. Canes were placed in vases with their bases immersed in water. The following day, six bud cuttings were dipped for 30 s in a 4 % (v/v) HC (Compensor® Arysta LifeScience, Mexico), 10 % sulfur derivatives (SD) (patent in progress MX/A/2008000929), and water as control (CL). Buds were collected at 0 h and 24 h after application, immediately frozen in liquid nitrogen upon collection and then stored at -80 °C until analysis. The canes bases were maintained in water along the experiment.

Calorimetric measurement

Metabolic heat production by buds was measured by calorimetric activity before treatments application. Six node cuttings of grapevine cv. 'Flame Seedless' recently brought from the vineyard with their basal ends were kept immersed in water until measurement was done at room temperature. Six replications were analyzed. Metabolic heat was measured with a differential scanning calorimeter (CSC 4100; Calorimetry Science Corporation, Pleasant Grove, Utah) working in the isothermal mode at 25°C for 3000 sec. The instrument has a baseline sensitivity ±1 μW and a working range of -30 to -110°C. Temperature around the DSC chamber was maintained at 15°C with a refrigerated circulating bath (Polyscience, Niles, IL). A flux of dry nitrogen at 175 g cm−2 was used to prevent moisture condensation inside the instrument. Samples were

measured in three hastelloy of 1 cm^3 ampoules with removable lids. Metabolic heat (Rq) rate was expressed on a dry-weight basis (Gardea et al., 2000). Rq means were calculated on six replicates on each sampling date.

Total RNA isolation

RNA isolation was performed according to Reid et al. (2006) with a slight modification using lyophilized endodormant grape buds as in García-Baldenegro et al. (2015). The extraction buffer contained 300 mM Tris HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 2% CTAB, 2% PVP40, 0.05% spermidine trihydrochloride, and 2% ß-mercaptoethanol. Lyophilized bud tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle. Pre-warmed (65 °C) extraction buffer was added to powder at 2 mL/mg of ground tissue, and 2% ß-mercaptoethanol was added and shaken vigorously. Tubes were incubated in a 65 °C water bath for 12 min and shaken every 3 min. Mixtures were extracted twice with equal volume of chloroform: isoamyl alcohol (24:1), then centrifuged at 13,200 rpm for 10 min at 4 °C each time and the aqueous layer was transferred to a new tube and centrifuged at 13,200 rpm for 15 min at 4 °C to remove remaining insoluble material. 0.1 vol. 3M NaOAc (pH 5.2) and 0.6 vol isopropanol were added to the supernatant, mixed and stored at -80 °C for 10 min. Nucleic acid pellets were collected by centrifugation at 13,200 rpm for 30 min at 4 °C. The pellet was dissolved in 1 mL TE (pH 7.5). To selectively precipitate RNA, 0.25 vol. of 10 M LiCl was added and the sample was stored overnight at 4 °C. RNA was pelleted by centrifugation at 13,200 rpm for 30 min at 4 °C, and then washed with 1mL of ice cold 70% EtOH, air dried, and dissolved in 60 µL DEPC-treated water. The samples were purified using the commercial system Spectrum Plant Total RNA (Sigma-Aldrich, St. Louis, MO, USA) starting from binding columns. Genomic DNA traces were eliminated using RNase-Free DNase (QIAGEN, Valencia, CA, USA). The integrity and quantity of the total RNA were evaluated by the spectrophotometric absorbance ratios A_{260}/A_{280} and A_{260}/A_{230} using a NanoDrop 2000 (Thermo Scientific NanoDrop, USA). Before microarray hybridization, the RNA integrity for each RNA preparation was tested using an Agilent 2100 Bioanalyzer (Agilent Technologies).

Microarray Hybridization and Data Extraction

Microarray hybridizations were performed at the Genomics Unit of the Centro Nacional de Biotecnología (CNB-CSIC, Spain). The cDNA was synthesized from 10 μg of total RNA using the cDNA Synthesis System Kit (NimbleGen-Roche). The cDNA preparation (1 μg) was amplified and labelled with Cy3-random nonamers using the One-Color Labelling Kit (NimbleGen-Roche). If the bioanalyzer quality control was correct, then 4 μg of labelled cDNA were hybridized on a NimbleGen microarray 090818 Vitis exp HX12 (NimbleGen-Roche). Hybridization solution (NimbleGen Hybridization kit) was added to each labelled cDNA and hybridization was performed for 16 h at 42°C in a HS 4 Hybridization station (NimbleGen-Roche). Hybridized microarrays were washed with the Wash buffer kit (NimbleGen-Roche) and scanned at 532 nm and 2 μm resolution in a DNA Microarray Scanner with the Surescan High-Resolution Technology (Agilent Technologies). After evaluation of hybridization quality by the experimental metrics report implemented in the NimbleScan Software version 2.6 (NimbleGen-Roche), probeset signal values from all microarray hybridizations were background corrected and normalized together using the robust microarray average (RMA) with the Nimble- Scan Software as well, which produces calls file for each sample with normalized expression data condensed for each gene. A dataset for the 12 samples was generated from normalized data including the expression of all 29,549 genes represented in the microarray with $\log_2$ formation representing 98.6% of the genes predicted from the V1 annotation of the 12x grapevine genome (http://genomes.cribi.unipd.it/grape/) and 19,091 random probes as negative controls. Differential expression analyses were performed in Multi Experiment Viewer (Saeed et al., 2003) using LIMMA with experimental design of the multi class and significance level of 0.05, and later applying a Benjamini-Hochberg correction with an adjusted p-value < 0.05 (Benjamini and Hochberg, 1995). The genes with a $\log_2$ of fold change ≥ 1, ≤ -1 and an adjust p-value<0.05 were considered as differentially expressed in each of the treatment comparisons. Principal components analysis was conducted using MeV (Saeed et al., 2003) to perform data normalization and corroborated with a Pearson

correlation analysis to assess similarities between biological replicates of each sample using RStudio (version 0.98.1103, RStudio Inc., 2014).

Functional Annotation and GO Analyses

The gene annotation were obtained from the V1 version of the 12x draft annotation of the grapevine genome (http://genomes.cribi.unipd.it/DATA/), genes were grouped into most represented functional categories based on functional annotation. The grape GO terms were obtained from the data of V1 coding sequences in CRIBI (http://genomes.cribi.unipd.it/grape/), AgriGO (http://bioinfo.cau.edu.cn/agriGO/download/item2term_3) and completed with the data proportionated by Grimplet et al. (2012). GO annotations, classification, and enrichment analysis were performed using Blast2GO software (http://www.blast2go.org). The statistical significance of GO terms enrichment was measured by Fisher's exact test. For each ontology category, the p-value was calculated as the probability under which we would observe at least k genes to have a given GO term, t, if we randomly selected m genes from the given M genes in the genome, where n genes were associated with term t. Given a term and a list of genes, k follows the hyper-geometric distribution. We also reported the False Discovery Rate (FDR) based on Benjamini-Hochberg multiple testing corrections (Benjamini and Hochberg, 1995).

MapMan visualization

In order to obtain a pictorical representation of the of changes in transcript abundance for SD- and HC- treated grapevine buds compared with the control at 0 h and 24 h, the MapMan software was used (http:// gabi.rzpd.de/projects/MapMan/) (Thimm *et al*. 2004), with *Vitis vinifera* mapping found in resources in MapMan page (http://mapman.gabipd.org/), and annotation files as described in Rotter *et al*. (2009). From the 29,549 grapevine sequences, 16,255 were sorted in 36 BINs and several subBINs, resulting in the pictorical pathways of metabolism overview and regulation.

Only transcripts with significant changes after FDR correction where displayed in MapMan images.

Microarray validation

To validate the reliability of the transcriptional response results, 4 genes were selected for qRT-PCR evaluation. Validation of microarray data was carried out in biological triplicates on the same RNA samples subjected to microarray analysis. qRT-PCR were performed in triplicate reactions including a 5µL of cDNA template (4 ng), 10 µL of iTaq Universal SYBR Green Supermix, 1 µL of 5µM of each sense and antisense primer and 0.1% DEPC-treated water to a final volume of 20 µL. Gene-specific primers were designed using a free online primer design tool (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) listed in Supplemental electronic data Table 1. PCR amplification was done in a 48-well Step-One™ Real-time PCR System (Applied Biosystem Inc., CA, USA). Amplification conditions were 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. PCR specificity was confirmed by constructing a melt curve after amplification in a range from 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. Non-template controls were also included. The results were normalized with actin (ACT) expression levels and analyzed with the $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Gene expression was determined as the mean, and standard errors were calculated over all biological and technical replicates. Statistical analysis was performed using one-way ANOVA, with a significance level of $p<0.05$, using the NCSS (2007) software.

Results

Endodormancy stage of latent grapevine buds

To study the development stage of the grapevine buds along transcriptomic analysis, we collected grapevine buds attached to their canes from the vineyard and brought them to the laboratory and analyzed by calorimetric activity. The metabolic heat of the buds was

Rq= 2.1564 μJ• s^{-1}• mg^{-1} DW, which confirms that the buds were endodormant since the low rate of chilling hours was about 154 h.

Transcriptional modulation induced by budbreak stimulation

To investigate the molecular changes that take place in response to treatments, we carried out a comparative microarray analysis of grapevine endodormant buds treated with sulphur derivatives (SD) at 24 h, cyanamide (HC) at 24 h and control (CL) at 0 and 24 h after treatment. The Nimblegen 090918_Vitus_exp_HX12 array (>98% whole genome) was used to compare global gene expression among the treatments and control was used. A global analysis of expression levels using Principal Components Analysis (PCA) revealed enough uniformity among the three biological replicates to defined associations between treatments (Fig. 1), where the two principal components, explaining about 61% of the overall variance, allowed us to separate HC from SD and CL, whereas the separation between SD and CL was less clear. In addition, we generated a Pearson's distance correlation matrix to compare the transcriptomes from each sample (Figure Supplemental 1), which showed a strong correlation between SD-treated and control samples and a clear distinction from HC treated samples ($P<0.01$).

Sulfur derivatives and hydrogen cyanamide effects on the transcriptome of endodormant grapevine buds

We carry out comparisons between treatments at time points 0 h for control, and at 24 h for control and treatments SD and HC. Our analysis showed a total of 5,773 differentially expressed genes between all comparisons, representing ~ 19.54 % of all genes in the array (Fig. 2). A hierarchical clustering of the expression pattern of the differentially expressed genes (DEG) within treatment comparisons is presented in figure 2A. Red, white, and blue elements in the matrix indicate up-, no change, and down-regulated expression of differential genes, respectively. In this analysis, we compared the expression of the control buds at 0 h and 24 h as a natural transition of the

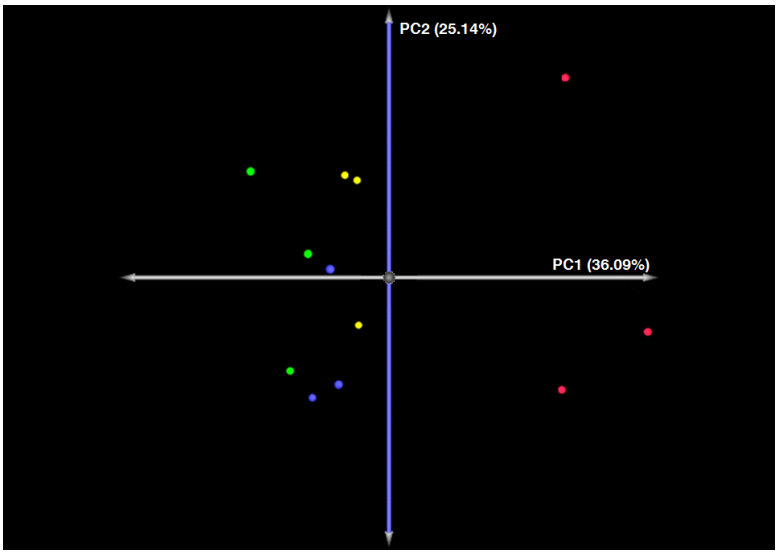


Fig. 1 PCA plot of ‘Flame seedless’ grapevine endodormant buds according to their expression data. PCA plot of grapevine endodormant buds treated with water as control at 0 h (yellow), control at 24 h (blue), 10% sulfur compounds at 24 h (green), and 4% hydrogen cyanamide (red).

grapevine bud during dormancy (CL24/CL0). We found that there were 939 DEGs, among them 236 were up-regulated and 703 were down-regulated representing a 16.27 % of the transcripts affected (Fig. 2B). To learn about the effect of the treatments during the transition of time and specific treatment effect, we normalized the HC- and SD-treated buds against the control at 0h and at 24 h, respectively. The SD application after 24 h compared to control at 0 h (SD24/CL0) resulted in 231 up-regulated and 480 down-regulated genes. Interestingly, the SD treatment at time point 24 h (SD24/CL24) did not showed any DEG. In contrast, the HC treatment at 24 h compared with control at 0 h (HC24/CL0) indicates the effect of HC after 24 h of treatment application, and it induced a large number of significant changes in transcript abundance, 1317 up-regulated and 2199 down-regulated representing nearly 60.90 % the transcripts affected. We found that HC treatment (HC24/CL24) specifically induced the higher number of transcripts abundance with 2049 up-regulated genes and 1757 down-regulated genes, about the 65.88 % of the total DEG (Fig. 2B).

A greater proportion of the comparisons performed for the treatments SD-, HC- and the Control at 24 h versus the Control time 0 h, generated a larger amount of down-regulated transcripts, whereas HC-treated buds compared to Control at 24 h, known as a specific differential expression by treatment, had an up-regulated differential expression. The specific SD-treatment effect (SD24/CL24) had non- significant effect on effect on expressed genes. A four-way Venn diagram showed the common DEG among the treatments, and specific DEG for each treatment comparison (Fig. S2, Supplemental electronic data Table 2). All treatments comparisons of the modulated genes (except SD24/CL24) shared 35 differentially expressed genes. The CL24/CL0 specifically had 145 genes expressed differentially (4.94%).

The application of SD affected to a 12.32% (711) of the transcripts differentially expressed, there were specific for SD application 110 genes (1.90%). About 94% of the differentially expressed transcripts were affected by HC application (5424 DEG) and HC application affected specifically 48.36% (2792 DEG) of all the DEG. In response to treatments, the modulation of gene expression was variable between treatments comparisons, and no modulated transcripts were shared among the five treatments comparisons (Fig. S2). A Venn diagram is given for common and specific up-regulated

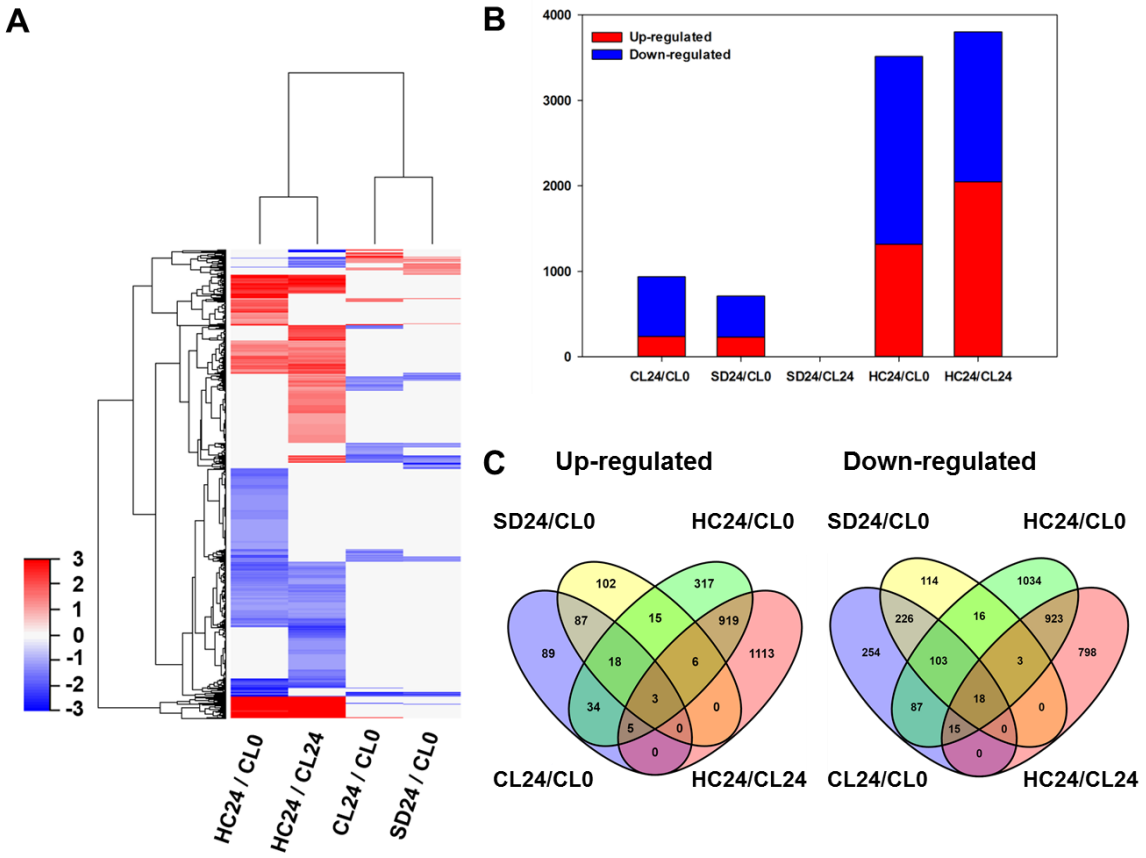


Fig. 2 Summary of significant changes in transcript abundances in dormant grapevine buds at 0 h and 24 h after treatment with sulfur derivatives 24 (SD24), hydrogen cyanamide 24 h (HC24) and Control at 0 h (CL0) and 24 h (CL24). **(A)** Expression heat-map of differentially expressed genes (P<0.05 and |Fold change|≥ 2 at least) **(B)** Figure showing the number of transcripts up-regulated (red) and down-regulated (blue) for each treatment compared with controls. **(C)** Four-way Venn diagrams showing overlapping transcripts that are significantly changing (up- and down-regulated) across multiple treatments, as distinct from transcripts that are uniquely altered by single treatments.

DEG (2708; Fig. 2C, Supplemental data Table 3), where there were no up-regulated genes in common between all treatments comparison. Moreover, the distribution of the common and specific down-regulated genes is shown where neither was displayed common down-regulated genes between all the comparisons (3591; Fig. 2C; Supplemental data Table 4). Specifically for control, there were 89 up-regulated genes and 254 down-regulated genes. And specifically for each treatment, there were 102 up- and 114 down-regulated genes for SD treated buds, and for HC- treated buds we found 1430 up- and 1832 down-regulated genes. Thus, there were more down-regulated genes for treatment specific than there were during the natural transition of endodormancy in grapevine buds.

Functional categories analysis in the SD and HC treatment comparisons

Differentially expressed transcripts were grouped into 16 functional classes, on the basis of their first functional classification in which they were involved. The distribution of functional categories is represented in bar graphics showing the number of DEG in each comparison (Fig. 3). In response to treatments, the response was highly variable to the application of SD and HC. However, a similar expression patter was found for the transcripts expressed in grapevine buds during the natural transition of endodormancy (CL24/CL0) and the SD-treated buds, being the 38% of the total of shared genes and a 64% of the transcripts expressed by SD-treated buds. In the 'cellular process' category cellulose synthases and polygalacturonase genes are up-regulated for treatments SD and natural transition control (CL24/CL0). In the 'development' category, the regulators of transcription, *Constans-like,* are down-regulated. For 'carbohydrate metabolism', the starch and sucrose metabolism genes are down-regulated, like *trehalose synthase*. In 'nucleic acids' category, there is a higher number of DEG for CL24/CL0 than for SD24/CL0. However, there are more down-regulated DEGs for CL24/CL0. The genes for DNA replication in 'nucleic acids metabolism', *Replication factor A 1* (*rfa 1*) is up-regulated in the CL24/CL0 comparison, but it is not significant for the SD treatment and there are two DEG heat shock proteins up-regulated in SD24/CL0. For 'secondary metabolism' category, a large family of stilbene synthase genes is down-regulated for

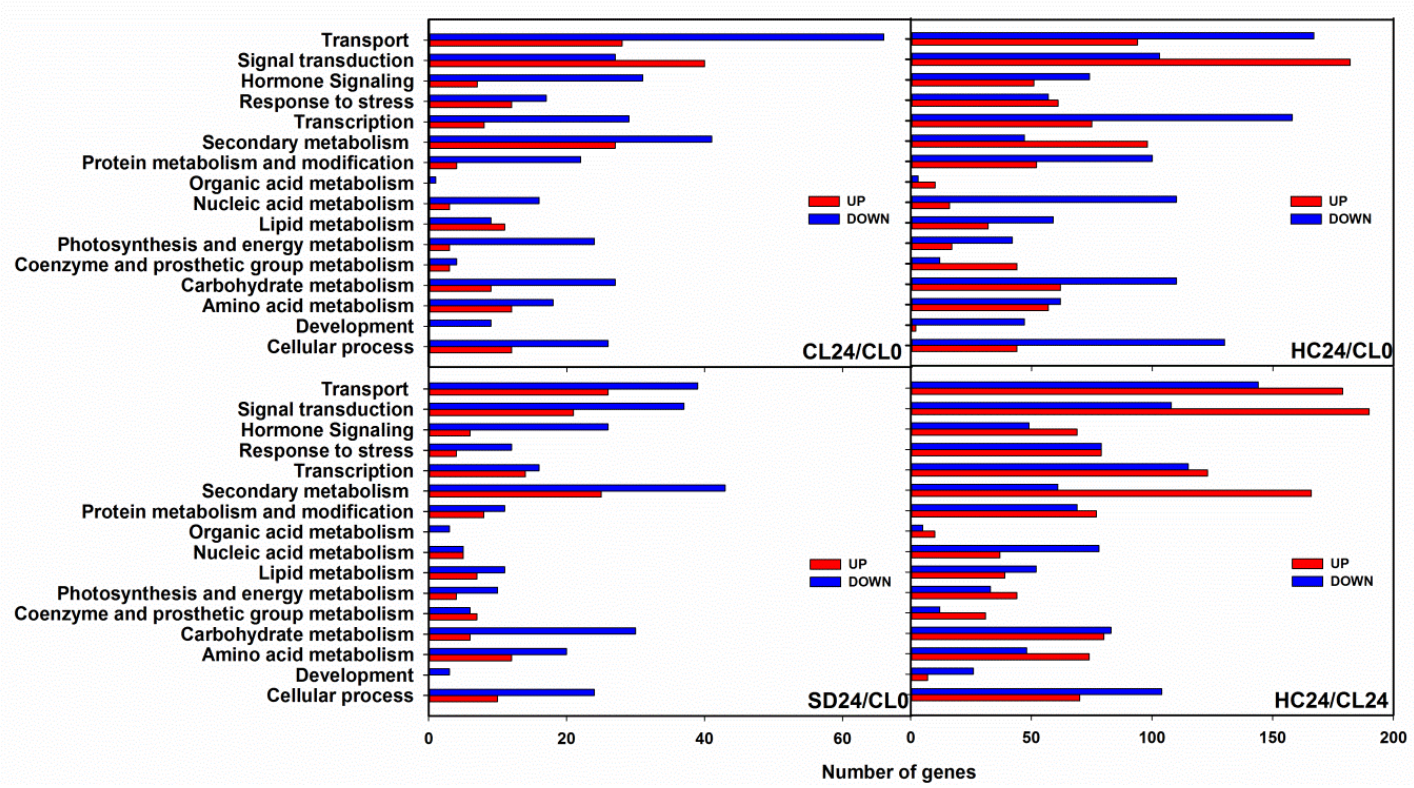


Fig. 3 Functional distribution of transcripts significantly induced and repressed in treated grapevine buds ($p<0.05$). Bars represent the number of transcripts up-regulated (red) and down-regulated (blue) within each functional category.

SD24/CL0, and these are not induced in natural transition. Another difference between SD-treated buds and natural transition transcripts is in the category of 'coenzyme and prosthetic group metabolism', where there is a high transcription of *glutathione S-transferases* that are used in the glutathione metabolism. In 'amino acid metabolism', seven *PAL* genes are down-regulated in SD while there are no *PAL* genes expressed in CL24/CL0. In the 'response to stress' category, during natural transition there are pathogenesis genes up-regulated, but in the SD-treated buds these transcripts are not differentiated. As for 'hormones', most of DEGs are down-regulated for CL24/CL0 and SD24/CL0, there are three auxins induced in SD treatment, and in CL24/CL0 there is an ABA glycosidase gene induced. In 'transcription' category, SD-treated buds had a larger number of up-regulated genes, including two MYB, Zinc finger and others. In 'signaling' the *protein kinase* genes are up-regulated and the calcium and calmodulin sensors are down regulated for both CL24/CL0 and SD24/CL0. For transport category, CL24/CL0 there were up-regulated one *Aquaporin PIP2B*, 2 sulfate transporter genes, and 7 ABC-transporters including one *Glutathione S-conjugate ABC transporter*, whereas sugar transport proteins were inhibited. For SD-treated buds, transcripts of ABC-transporters were induced, including the *Glutathione S-conjugated ABC transporter*.

As for the HC treated buds, in both comparisons (HC24/CL0 and HC24/CL24), there is a higher plethora of DEG (Fig. 3). As cyclins, involved in the regulation of cell cycle, induced the up-regulation of genes of pectinesterase family for pectin modification, and down-regulated polygalacturonase genes family. Also, in cellular process, HC down-regulated the genes involved in cellular redox homeostasis, except for *Thioredoxin 2* (*TRX2*). In development, all the genes were down-regulated for HC24/CL0, and the HC affected the transition down-regulating genes like *sepallata1*, constans-like genes, vernalization genes, embryo defective genes. For the specific HC treatment effect (HC24/CL24) it was seen a gene *vernalization 3*, induced. HC did not differentiate genes constans-like, and there was a *PFT1* down-regulated. In the 'amino acid metabolism' HC exert the induction of several peroxidases and PAL involved in the phenylalanine biosynthesis and tyrosine biosynthesis, respectively. Also a glutamate biosynthesis gene

was up-regulated by HC, *gamma-glutamylcysteine synthetase*, which is a precursor of glutathione. For the carbohydrate metabolism, it was found that there are induced genes like *G6PDH, ADH, PFK, β-1,3-glucanase*, among others by HC treatment. For the coenzyme category, it was found a high stimulus of up-regulated genes in comparison of SD-treated buds and control at 24 h. It induced to 35 and 22 *glutathione S-transferases* for HC24/CL0 and HC24/CL24, respectively. Another category that was down-regulated by HC it was the 'generation of energy' where it depleted a larger number of transcripts. The citric acid was induced by HC application; *phosphoenolpyruvate carboxykinase* and *ATP-citrate synthase* were up-regulated. It was also induced the electron transport respiratory chain phosphorylation with *NADH dehydrogenase* and several *quinone synthase* up-regulated. But HC-treatment inhibited the photosynthesis genes contrary to SD treated buds. In the production of organic acids, it was found that HC exerts the up-regulation of ascorbate metabolism like *ascorbate oxidase* genes and one *MDHAR* found in HC24/CL0. The L-ascorbate peroxidase was reduced by the application of HC. For proteins metabolism and modification, HC up-regulates a high number of heat shock proteins and heat shock transcription factors, also up-regulates genes of the F-box family protein. The secondary metabolism in HC- treated buds had a significant up-regulation of DEG inducing the production of flavonoids like *chalcone synthase*, and phenylpropanoids inducing a large quantity of genes like *stilbene synthase*. For 'transcription' category, it was a higher number of transcripts for HC-treated buds. There were as much induced as inhibited genes that codified for NAC domain containing protein, WRKY DNA binding protein, zinc finger family, MYB family, MADS-box agamous-like among others. For 'response to stress' there was found induced pathogenesis defense genes for HC treated buds, also in response to oxidative stress, there are several *glutaredoxin* genes induced. For hormone signaling, there are genes inducing ABA, auxins, cytokinins, brassinosteroids, gibberellins, and highly for ethylene biosynthesis. In signal transduction there was a large number of induced DEG stimulated by HC application, like calmodulin binding proteins and protein kinases, like MAP kinases. In transport category, HC induced the up-regulation of sugar, nitrate and sulfate transporters, as well as a large amount of multidrug ABC transporters.

GO enriched categories

Gene ontology enrichment analysis was conducted to explore possible functions of the DEGs detected in the different treatments comparisons. The most frequent and significant GO terms of biological processes associated with DEGs of CL24/CL0, SD24/CL0, HC24/CL0 and HC24/CL24 are represented in Figure 4. According to the GO analysis, the comparison CL24/CL0 was enriched in three biological process: protein phosphorylation, cellulose biosynthetic, and oxidation-reduction. In SD24/CL0 comparison, 4 biological processes were enriched. For the enrichment in the repressed DEGs, L-phenylalanine catabolic related to the secondary metabolic, oxidation-reduction process and for trehalose biosynthetic was also a repressed category for SD compounds. Interestingly, SD compounds was enriched for up-regulated DEGs in biological process related to cellulose biosynthetic and, which are sharing with to the natural transition of the grapevine bud during dormancy (CL24/CL0), suggesting that SD compounds not drastically affect the essential biological processes of grapevine bud during dormancy. On the other hand, the application of HC during transition induced to a high number of biological functions, those that were up-regulated were involved with transport, amino acid biosynthesis, cell wall biogenesis, signaling and response to stimulus. The down-regulated categories for HC application involved in transition (HC24/CL0) were related to photosynthesis and the regulation of the cell cycle, as well as lipid metabolism. Those biological processes that were affected by HC specifically (HC24/CL24) were involved in toxin catabolism, cell wall organization, signaling, secondary metabolism, and fatty acid biosynthesis was down-regulated. Oxidation-reduction category was found down regulated in the CL24/CL0 and SD24/CL0 comparisons and up-regulated for HC24/CL0. It has been demonstrated that the application of HC in dormant grapevine buds induced to the expression of genes involved in defense, like secondary metabolism, antioxidant machinery, oxidative stress, signaling and ethylene metabolism in dormant grapevine buds (Ophir et al., 2009).

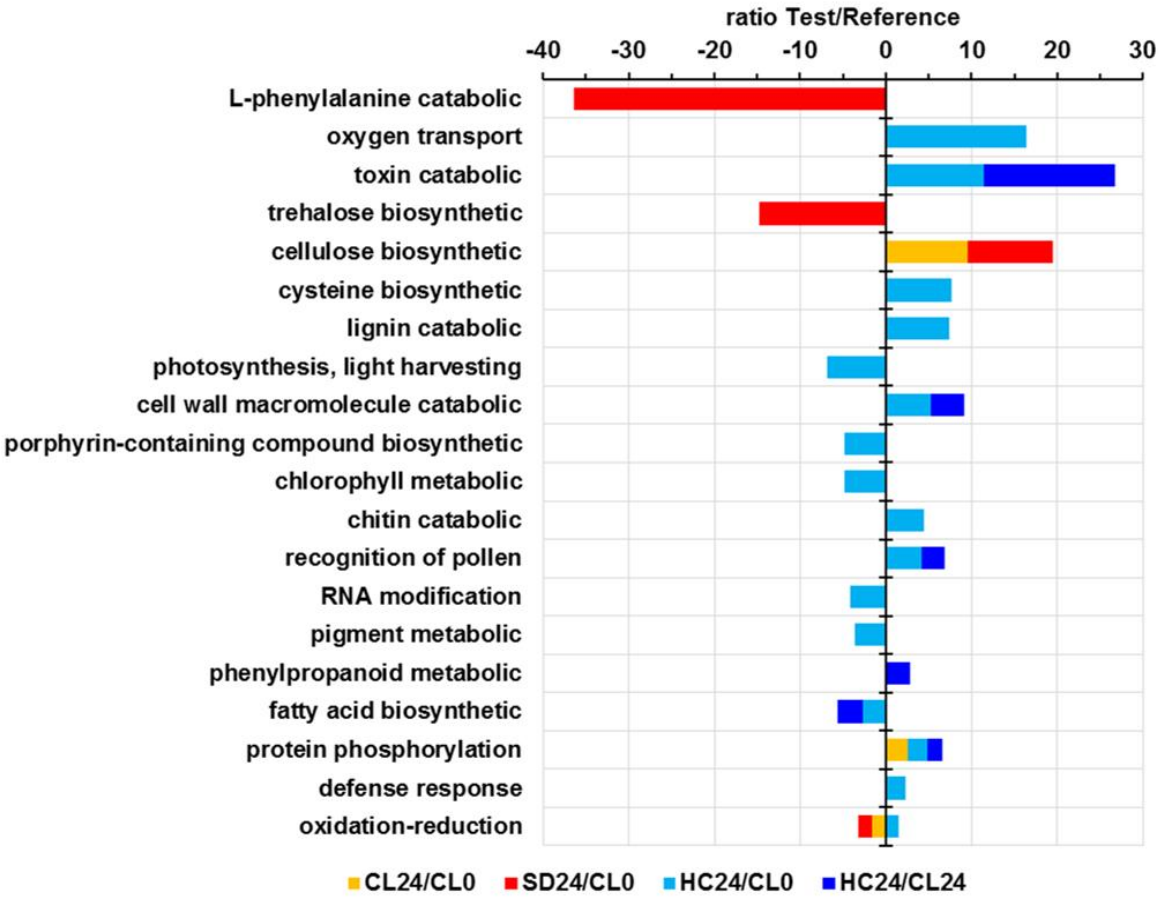


Fig. 4 Functional categorization of DEGs induced by SD- and HC-treatments and compared versus Control at 0 and 24 h based on the biological process of Gene Ontology (GO).

According to the GO enrichment analysis, the DEGs were enriched in several molecular functions (Fig. 5). For the natural transition of dormant buds (CL24/CL0), the up-regulated genes were enriched in molecular functions related to auxin transport, alkaloid metabolism, organization of the cell wall, and it was found down-regulated for nutrient storage. Interestingly, for SD24/CL0 comparison the up-regulated genes were enriched in molecular functions related to glutathione metabolism and transport, as well as those DEGs involved in cellulose synthase. However, there were down-regulated functions related to the phenylpropanoids and flavonoid synthesis, and carbohydrate metabolism. On the other hand, the HC-treated buds evoked a larger number of up-regulated molecular functions related to nitrogen assimilation, jasmonate biosynthesis, phenylpropanoid and flavonoid biosynthesis, and a large induction of protein kinases for signaling, and glutathione metabolism and transport. There were down-regulated DEGs related to activities involved in cell wall biosynthesis by HC application. These results suggest that HC induce to a higher signaling expression promoting antioxidant defense genes. It has been reported that the environmental, chemical and cold conditions, known to break dormancy, induce a sublethal stress, affecting respiration creating anaerobic conditions leading to budbreak in grapevines (Or et al., 2000).

Overview of metabolism changes in grapevine buds in response to breaking dormancy agents

To enable the metabolic processes visualization of the DEGs of complex data sets and to provide a rapid interpretation, we used the MapMan algorithm to investigate the responses of the DEGs in the metabolism of the endodormant grapevine buds. We were able to map 4,337 genes of the 5,773 significantly DEG. In Figure 6, we show the response of the genes assigned to metabolism. The mapping of significant DEGs of the comparison of CL24/CL0 and SD24/CL0 are depicted in Figure 6A and 6B, respectively. In Fig. 6A, corresponding to the genes expressed during a natural transition, we can observe that carbohydrates participating in cell wall metabolism, cell wall degradation, and lipids genes expression up-regulated, the carbohydrates corresponding to trehalose are down-regulated. In the redox Bin, we observed a

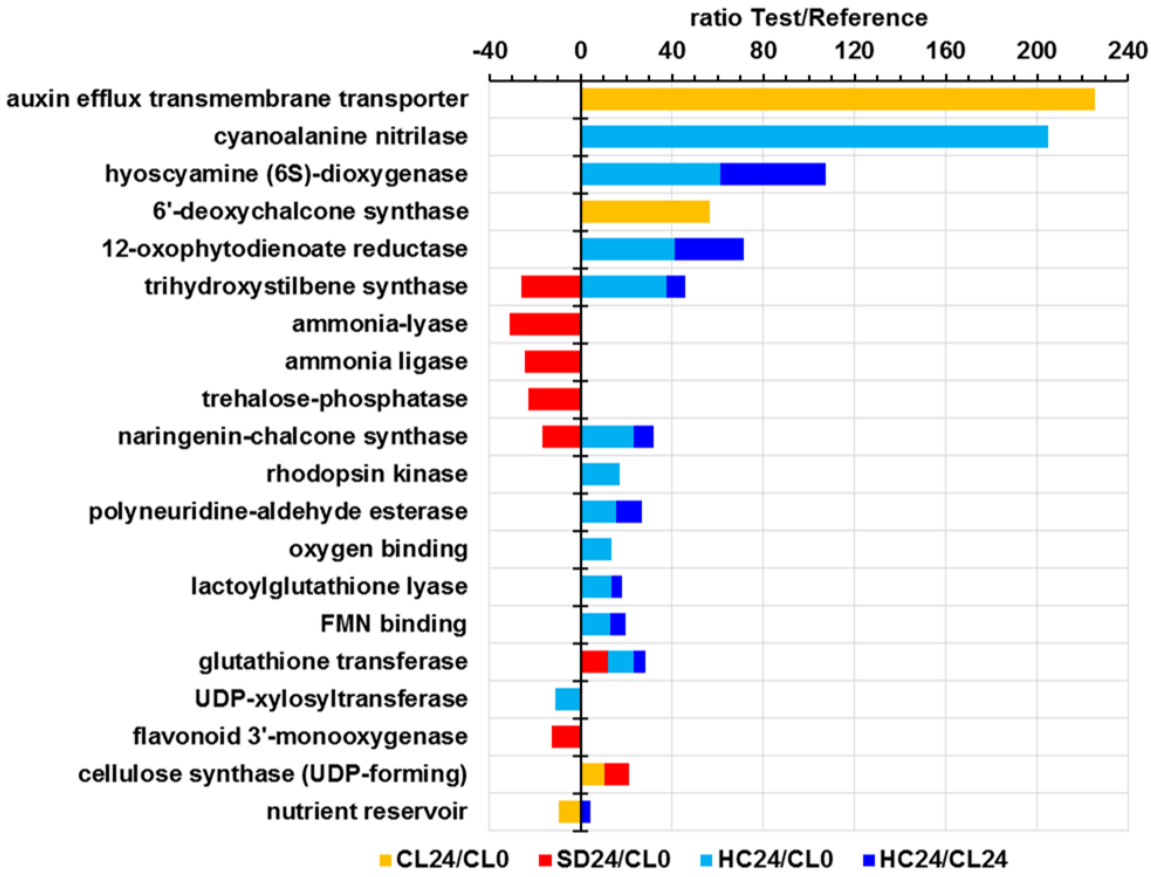


Fig. 5 Functional categorization of DEGs induced by SD- and HC-treatments and compared versus Control at 0 and 24 h based on the molecular function of Gene Ontology (GO).

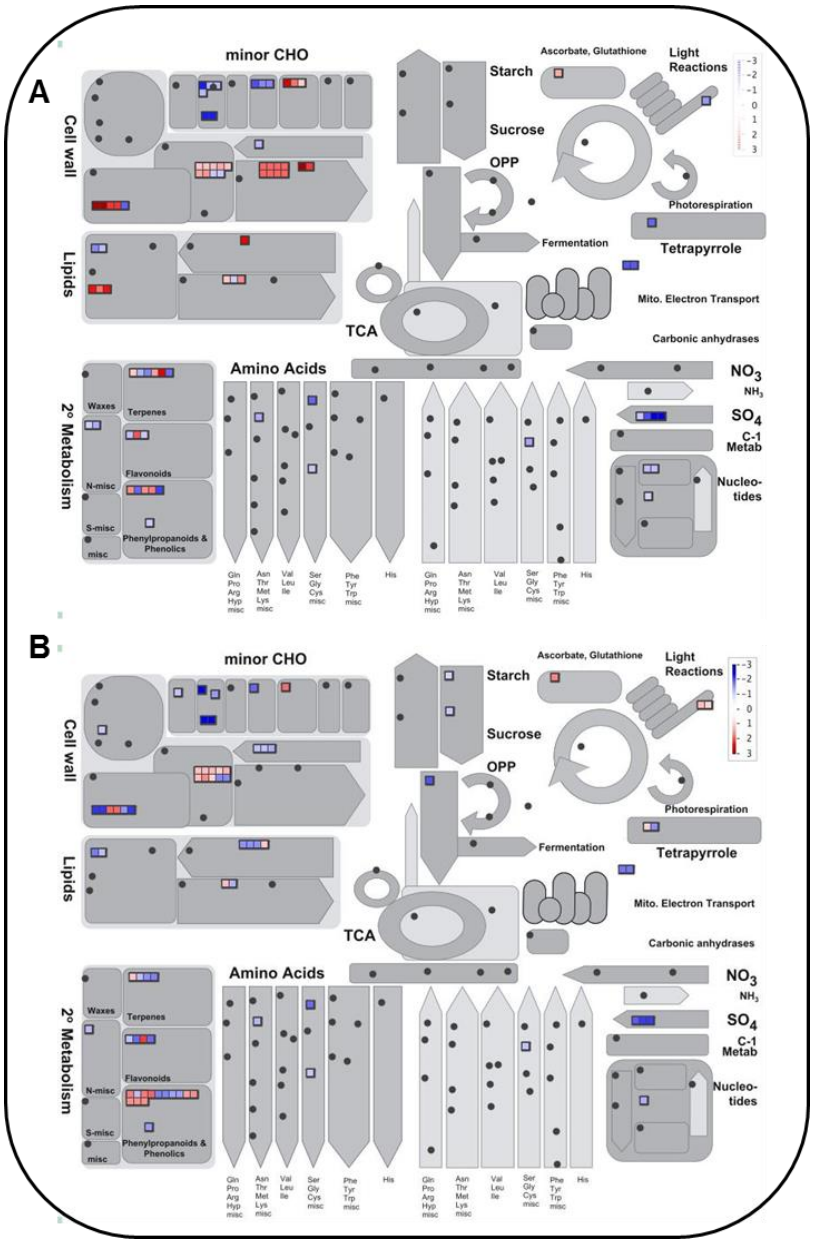


Fig. 6 General overview display of DEG ($p<0.05$) involved in metabolism. The image represents the genes (A) Change in the transcripts during endodormancy transition in 24 h lapse comparing Control at 24 h versus Control at 0 h. (B) Direct comparison of the SD-treatment on grapevine buds 24 h after treatment compared to Control at 0 h. Log_2 ratios for average transcript abundance were based on three replicates of NimbleGen microarray. The resulting file was loaded into the MapMan Image Annotator module to generate the metabolism overview map. On the logarithmic color scale, blue represents down-regulated transcripts, and red represents up-regulated transcripts.

Cytochrome B561 up-regulated, light reaction, tetrapyrrole and mitochondrial electron transport encoding genes are down-regulated during natural transition. Most of the genes of secondary metabolism are also down-regulated, as well as for amino acid synthesis and degradation. The sulfur assimilation and nucleotide metabolism process are also down-regulated. As for SD-treated buds (SD24/CL0), the DEGs were depleted in a small number and intensity of expression (Fig. 6B), but very similar behavior to the control (CL24/CL0). We can only highlight a few mechanisms differentiated for SD-treated buds, like there were no cell wall degradation genes induced, lipids metabolism was down-regulated. The SD-treatment induced the down-regulation of genes involved in carbohydrate sucrose and starch degradation and glycolysis. Also, two genes of the PSII were induced in the SD24/CL0 comparison.

The metabolic response of grapevine buds to HC-treatment application is shown in figure 7. As shown in figure 7, the application of HC causes drastic effects in general metabolic processes in comparison with normal transition (CL24/CL0) and SD compounds (SD24/CL0). In general, the HC treatment repress genes related to cell wall organization and carbohydrate metabolism, except for trehalose which is highly up-regulated. Also for lipids synthesis, we observed mostly down-regulated genes. For the major carbohydrates, we observed the starch and sucrose degradation down-regulated, except for an up-regulated *hexokinase* in the specific HC-treatment comparison. The secondary metabolism was up-regulated by the HC application. In the HC24/CL24 comparison, we found differential genes for the secondary metabolism, like a wax gene and alkaloids are induced. Flavonoids up-regulate their expression while during transition of HC application are down-regulated (HC24/CL0). There are *G6PDH* induced in the pentose phosphate pathway (PPP), *pyruvate kinase* from glycolysis is up-regulated, the photorespiration and Calvin cycle genes are down-regulated and we observed some TCA cycle up-regulated. The photosystem II encoding genes were down-regulated by the HC application. And for the ascorbate-glutathione cycle, *APX* was down-regulated and *gamma-glutamylcysteine synthetase* upregulated for both HC comparisons, and we found a *MDHAR* and *glutathione synthetase* up-regulated at

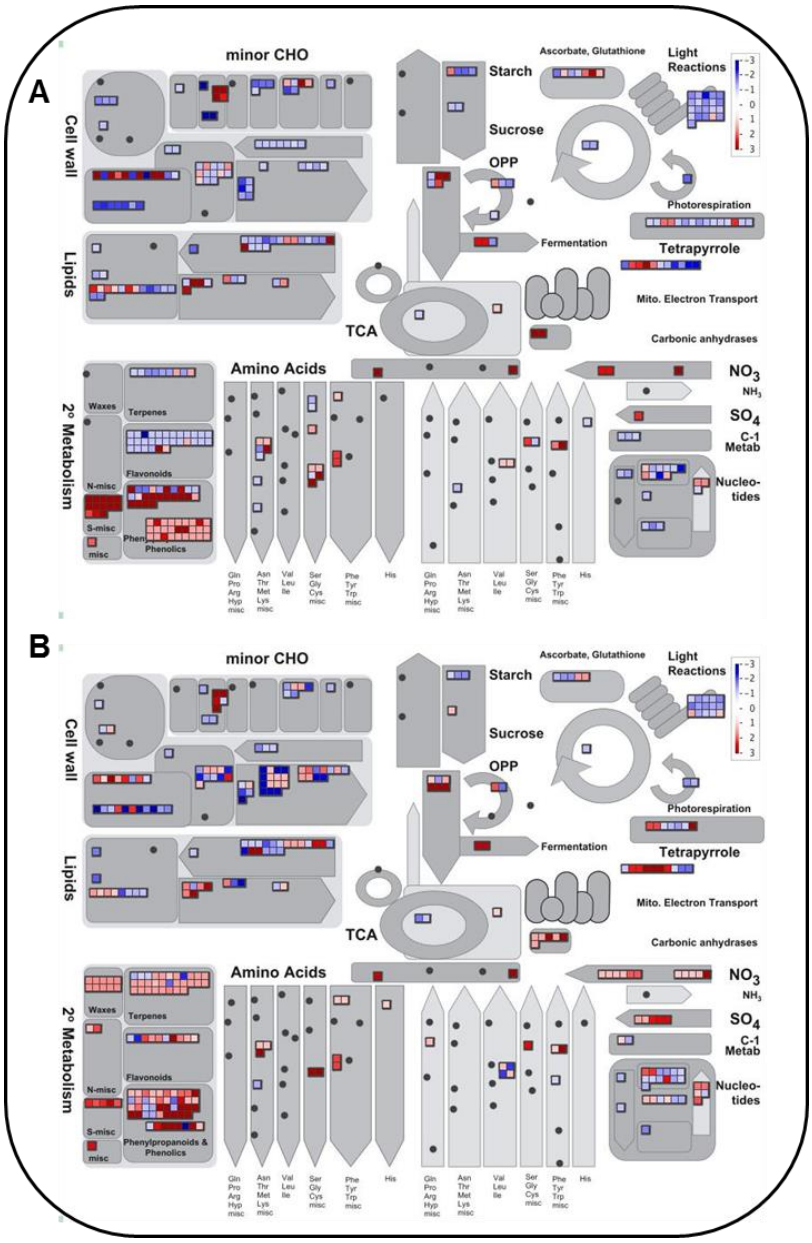


Fig. 7 MapMan metabolism overview maps showing differences in transcript levels of HC application versus (A) Control at 0 h showing the effect of HC during the transition and (B) the specific effect of HC application on DEG in endodormant grapevine buds. Log_2 ratios for average transcript abundance were based on three replicates of NimbleGen microarray. The resulting file was loaded into the MapMan Image Annotator module to generate the metabolism overview map. On the logarithmic color scale, blue represents down-regulated transcripts, and red represents up-regulated transcripts.

HC24/CL0, but not differential for the specific HC treatment (HC24/CL24). HC application induced the differential expression of N- and S- assimilation, and *glutamate decarboxylase*. The specific HC-treatment augmented the DEGs and intensity of the expression of those mechanisms.

The representation of bud dormancy regulation provided by MapMan highlights the genes involved in dormancy developmental control, including genes involved in transcription regulation hormonal metabolism and signaling and protein modification for the comparisons CL24/CL0 and SD24/CL0 (Figure 8) and for HC application at 0 h and 24 h after application (Figure 9). In a glance, we observed in Fig. 8 that most of the genes involved in regulation of dormancy were down-regulated, where transcription factors, protein modification and protein modification are down-regulated, the hormone metabolism, and signaling is also down-regulated (CL24/CL0). For the SD application, the number of the DEGs is lower than in natural transition and we observed that the intensity in expression is also diminished. For the HC application, the number of DEGs involved in regulation is larger than for SD-treated and control grapevine buds. In figure 9, we observe a high number of DEGs in the transcription regulation, protein modification and protein degradation, where we can found WRKY, MYB, NAC, BZIP transcription factors, as ethylene response factors; receptor kinases DUF26 type, and calcium signaling and receptor kinases. In the hormones, we observe induced the ethylene synthesis like *ACC synthase* and ethylene responsive transcription factors. In the redox, there were found up-regulated genes involved in the glutathione synthesis, and *MDHAR,* a *glutaredoxin* and *Thioredoxin 2* are also up-regulated by HC24/CL0. In the display of HC24/CL24 (Fig. 9B), we observed a similar number of DEGs than for the effect of HC during dormancy transition. The transcription factors increased in up-regulated gene. Some DEG involved in ethylene synthesis, auxins and jasmonates were upregulated. For signaling, the calcium regulation, receptor kinases and MAP kinases were up-regulated. And for the redox response, the glutathione synthesis genes were up-regulated, but not the ascorbate related genes, *glutaredoxins* and *thioredoxin 2* kept up-regulated in a higher intensity.

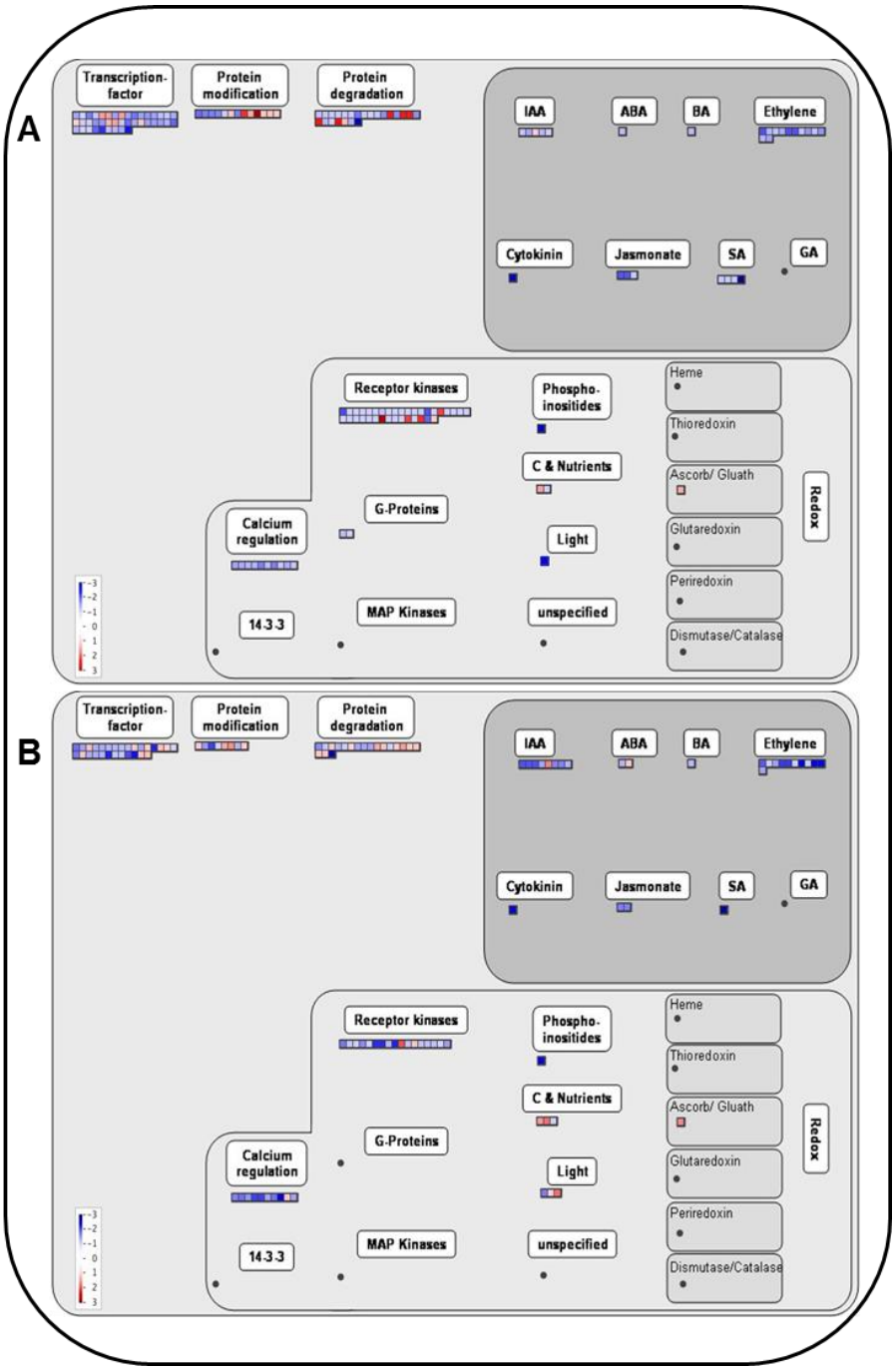


Fig. 8 Endodormancy gene regulation. MapMan Overview of Flame Seedless grapevine buds gene regulation during dormancy. The image represents the DEG ($p<0.05$) during (A) dormancy transition in 24 h comparing Control at 24 h versus Control at 0 h, and (B) the genes differentiated by the SD application after 24 h versus Control at 0 h.

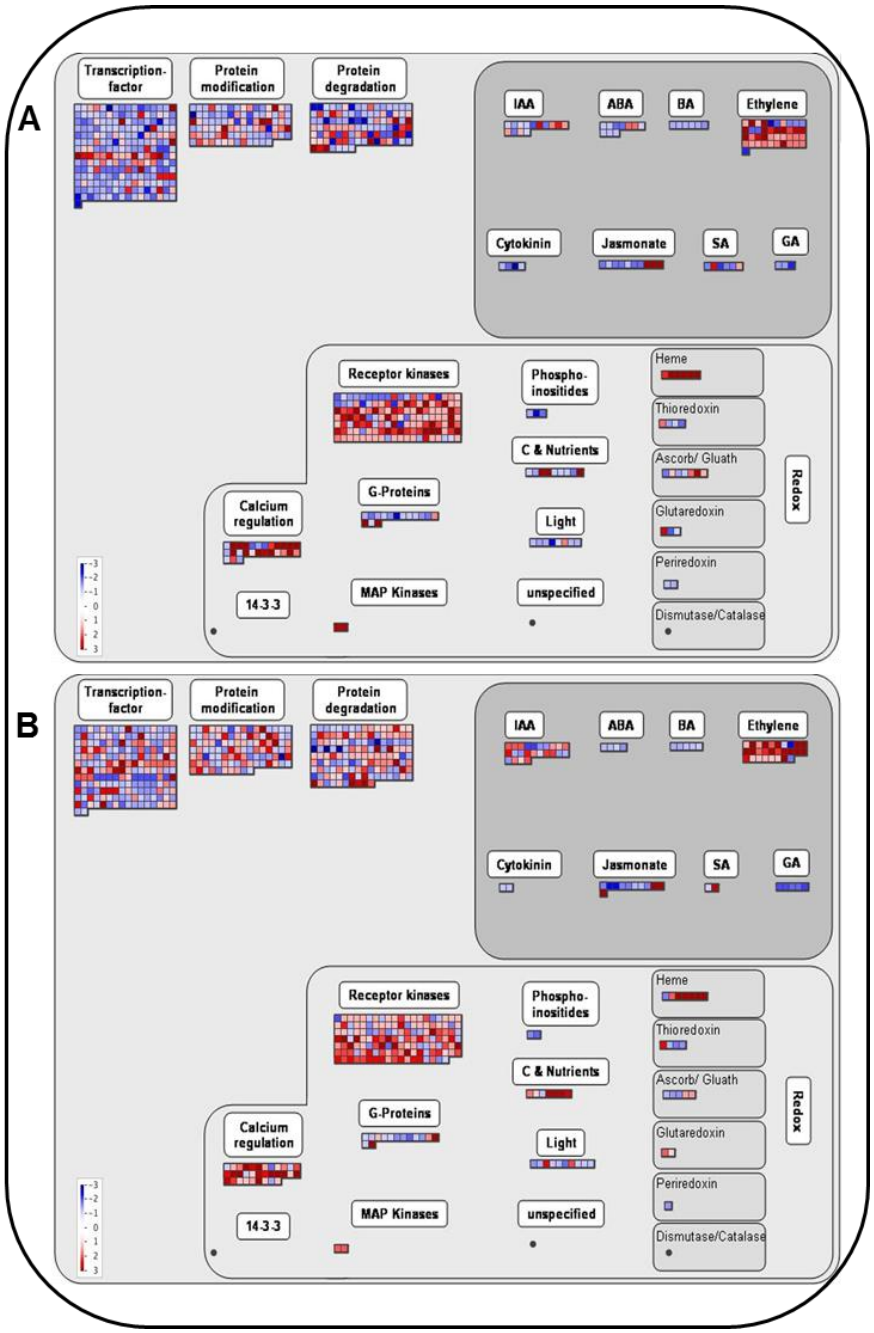


Fig. 9 MapMan visualization of gene regulation by HC treatment application. The differences in the transcript levels of HC application against (A) Control at time point 0 h during the transition and (B) the specific effect of HC 24 h after application on DEG in endodormant grapevine buds (HC24 vs CL24). Log_2 ratios for average transcript abundance were based on three replicates of NimbleGen microarray. The resulting file was loaded into the MapMan Image Annotator module to generate the metabolism overview map. On the logarithmic color scale, blue represents down-regulated transcripts, and red represents up-regulated transcripts.

Discussion

The effect of SD- and HC- treatments is directly related to the endodormant stage. The metabolic activity pattern of grapevines decreases during endodormy. Gardea et al. (1994), reported that the pattern of bud heat metabolism (Rq) along endodormancy observed during autumn when around 132 chilling hours are beginning to be accumulated in mature grapevines cv. 'Pinot Noir' with an Rq <2 $\mu J \bullet s^{-1} \bullet mg^{-1}$ DW. Also Corrales-Maldonado et al. (2010) found endodormant 'Flame Seedless' buds with a low Rq 1.5 $\mu J \bullet s^{-1} \bullet mg^{-1}$ DW. In accordance with these reports, the metabolic heat of the buds analyzed here was Rq= 2.1564 $\mu J \bullet s^{-1} \bullet mg^{-1}$ DW, which confirms that the buds are endodormant since the low rate of chilling hours was about 154 h. Therefore, the metabolic heat of buds was used as an indicator of the dormancy status of grapevine buds to guide the application of treatments on an adequate timing.

The PCA and Pearson's correlation matrix (not shown) previously ran to the data onto the 12X Grape Genome (Fig.1) confirmed that HC-treated buds showed a different transcriptomic behavior since it had a larger amount of differentially expressed genes. However, the SD-treated buds had a similar behavior to the control and were in the same component with a close Pearson's correlation, suggesting that the SD treatment has a low biological activity similar to the plant in natural conditions to achieve budbreak, since it is known that SD induce earlier budbreak initiation (Corrales-Maldonado et al., 2010). In this context, our analysis of the comparison between Control at 24 h and Control at 0 h (CL24/CL0) reveals the expression of genes in a moment during natural transition of deep dormancy. According to our results, we found that in this period, there were expression of genes involved in the up-regulation of the cell wall biosynthesis, like *cellulose synthase*, pathogenesis and signaling are up-regulated during dormancy. In this sense, Fasoli et al. (2012) studied the dormancy transcription during development of grapevine and found up-regulation in genes involved in defense/stress response and cell wall assembly. In CL24/CL0, signaling was also up-regulated in the GO process protein phosphorylation, with a large amount of protein kinases like Ser/Thr protein kinases. The expression of genes related to hormones might be due to the

presence of sulphur derivatives from the garlic extract. SD-treated buds induced to a high number of *glutathione s-transferases* (*GST*), which are related to stress and signaling. The GSTs catalyzes the conjugation of glutathione to a variety of hydrophobic, electrophilic and cytotoxic substances that have the ability to detoxify herbicides (Walton et al., 2009). GSTs are important in response to a range of abiotic and biotic stress catalyzing the conjugation of GSH to other molecules like nitrous compounds controlling the damage effects of nitrates exposure (Dixon et al., 2002). GSTs act by catalyzing the conjugation of reduced glutathione (GSH) with electrophilic, often hydrophobic toxic compounds to form derivatives that can be secreted from the cell, sequestered in the vacuole, or catabolized, and its importance is in response to a range of abiotic and biotic stresses (Dalton et al., 2009).

HC stimuli in budbreak of dormant buds have been studied earlier by Ophir et al. (2009). They found that the application of cyanamide and also heat shock to dormant buds were linked to sub lethal stress, mitochondrial activity, hypoxic conditions, interplay between ethylene and ABA metabolism, and cell enlargement during bud dormancy release by HC and heat shock application has been reported (Ophir et al., 2009). In accordance to our results, the greatest fluctuations were seen in buds treated with HC (Fig. 2B). In contrast, SD presented the lowest fluctuations but in a very similar patron to the control, sharing 64% of the genes regulated by SD24/CL0 (Fig. 2B). Shared expression and treatment-specific transcripts are detailed in Fig. S2. Although both treatment, HC and SD, induced to early budbreak, SD evokes a lower number of DEG, and most of the DEGs are same to the natural transition (CL24/CL0).

HC application induced to the expression of a wide diversity of functions, those that were enriched by the GO algorithm, were related to nitrogen assimilation, since HC is a molecule with nitrogen in it. The plant starts to assimilate and catalyzes it to form ammonium to be used by the plant. Also, we could found that HC-treated buds had transmembranal movements and antioxidant activity. This indicates us that the plant is processing and transporting metabolites and energy. We found up-regulated genes from oxidative stress like *peroxidases*, *glutathione S-transferases*, *monodehydroascorbate*

reductase (*MDHAR*), and from redox homeostasis like *alternative oxidase* (*AOX*) and *thioredoxin 2* (*TRX2*). In the study realized by Ophir et al., (2009), it was found an induction of an *AOX* gene after heat shock and hydrogen cyanamide application. This also up-regulated thioredoxin genes, which products serve as hydrogen donors involved in AOX activation (Buchanan et al., 2000).

Conclusions

The analysis clearly shows differences in the transcriptional response between treatment comparisons with HC and SD, nevertheless, both treatments had the same effect on dormancy release. Therefore, further analysis is needed between the common DEG between HC and SD treatments on bud response, which could be responsible for early budbreak. The broad plethora of HC-induced and HC-reduced genes, we can observe that cyanamide induce to the plant to a high level of oxidative stress, activating different redox metabolic pathways like alternative oxidase and thioredoxin. The plant recognizes HC as a biotic stress by the activation of pathogenesis genes, stilbenes production, therefore, recognizes that HC cause phytotoxicity by activating defense functions during dormancy. On the other hand, the small amount of the SD-stimulated differentially expressed genes and as for those genes shared to control, as well as for the activations of biological functions and metabolic pathways, SD induce to budbreak in a very similar manner to that from the natural way. The down-regulation of genes involved in stilbenes, aminoacids related to methionine, molecules related to stress and defense of the plant is presented by SD- treatment. The down-regulation of these molecules and the up-regulation of the *GST*s is an indicative that SD is not nocive to the plant and caused no toxicity to the plant, and the GSTs are acting as molecules of stress response. There is little known about the mechanisms of budbreak stimulation with sulphur derivatives. Further analysis is recommended to unveil the metabolic and cellular events release by SD stimuli during dormancy.

References

Arora R, Rowland LJ, Tanino K (2003) Induction and release of bud dormancy in woody perennials: a science comes of age. HortScience 38: 911–921.

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B, 57:289–300

Buchanan BB, Gruissem W, Jones RL (2000) Biochemistry and molecular biology of plants. American Society of Plant Physiologists, Rockville.

Corrales-Maldonado C, Martinez-Tellez MA, Gardea AA, Orozco-Avitia A, Vargas-Arispuro I (2010) Organic alternative for breaking dormancy in table grapes grown in hot regions. Am. J. Agri. & Biol. Sci., 5: 194-198. doi: 10.3844/ajabssp

Dalton DA, Boniface C, Turner Z, Lindahl A, Kim HJ, Jelinek L, Govindarajulu M, Finger RE, Taylor CG (2009) Physiological roles of glutathione S-transferases in soybean root nodules. Plant Physiol. 1:521-530. doi: 10.1104/pp.109.136630

Díaz-Riquelme J, Grimplet J, Martínez-Zapater JM, Carmona MJ (2012) Transcriptome variation along bud development in grapevine (*Vitis vinifera* L.). BMC Plant Biol. 12:181 doi: 10.1186/1471-2229-12-181

Dixon DP, Lapthorn A, Edwards R (2002) Plant glutathione transferases. Genome Biol, 3: reviews3004.1-reviews3004.10. http://genomebiology.com/2002/3/3/reviews/3004

Erez A (1995) Means to compensate for insufficient chilling to improve bloom and leafing. Acta Hort. 395: 81–95.

Fasoli M, Dal Sanro S, Zenoni S, Battista Tornielli G, Farina L, Zamboni A, Porceddu A, Venturini L, Bicego M, Murino V, Ferranini A, Delledonne M, Pezzotti M (2012)

The grapevine expression atlas reveals a deep transcriptome shift driving the entire plant into a maturation program. The Plant Cell. 24: 3489-3505. doi:10.1105/tpc.112.100230

García-Baldenegro CV, Vargas-Arispuro I, Islas-Osuna M, Rivera-Domínguez M, Aispuro-Hernández E, Martínez-Téllez MA (2015) Total RNA quality of lyophilized and cryopreserved dormant grapevine buds. Electronic Journal of Biotechnology; 18:134-137. doi: 10.1016/j.ejbt.2015.01.002

Gardea AA, Moreno YM, Azarenko AN, Lombard PB, Daley LS (1994) Changes in metabolic properties of grape buds during development. J Amer Soc Hort Sci. 119:756–760

Gardea, AA, Carvajal-Millán E, Llamas J, Orozco JA (2002). Effect of chilling on calorimetric responses of dormant vegetative apple buds. Thermochimica Acta, 349:89-94

Giraud E, Ivanova A, Gordon CS, Whelan J, Considine MJ (2012) Sulphur dioxide evokes a large scale reprogramming of the grape berry transcriptome associated with oxidative signalling and biotic defence responses. Plant, Cell and Environment, 35:405–417. doi: 10.1111/j.1365-3040.2011.02379.x

Grimplet J, John Van Hemert JV, Pablo Carbonell-Bejerano P, José Díaz-Riquelme J, Dickerson J, Fennell A, Pezzotti M, Martínez-Zapater JM (2012) Comparative analysis of grapevine whole-genome gene predictions, functional annotation, categorization and integration of the predicted gene sequences. BMC Research Notes, 5:213 http://www.biomedcentral.com/1756-0500/5/213

Halaly T, Pang X, BatikoT, Crane O, Keren A, Venkateswari J, Ogrodovitch A, Sadka A, Lavee S, Or E (2008) Similar mechanisms might be triggered by alternative external stimuli that induce dormancy release in grape buds. Planta 228:79–88. doi: 10.1007/s00425-008-0720-6

Horvath DP, Chao WS, SuttleJC, Thimmapuram J, Anderson JV (2008) Transcriptome analysis identifies novel responses and potential regulatory genes involved in seasonal dormancy transitions of leafy spurge (Euphorbia esula L.) BMC Genomics, 9:536. doi:10.1186/1471-2164-9-536

Keilin T, Pang X, Venkateswari J, Halaly T, Crane O, Keren A, Ogrodovitch A, Ophir R, Volpin H, Galbraith D, Or E (2007) Digital expression profiling of a grape-bud EST collection leads to new insight into molecular events during grape-bud dormancy release. Plant Science, 173: 446-457. doi: 10.1016/j.plantsci.2007.07.004

Kubota N, Miyamuki M (1992) Breaking bud dormancy in grapevines with garlic paste. J Amer Soc Hort Sci 117:898-901

Kubota N, Miyamuki M, Yamane y, Kobayashi A, Mitzutani F (1999) Breaking bud dormancy in grapevine cuttings with garlic volatiles. J Japan Soc Hort Sci 68:927-931

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25: 402–408. doi:10.1006/meth.2001.1262

Mohamed HB,• Vadel AM, Geuns JMC, Khemira H (2012) Effects of hydrogen cyanamide on antioxidant enzymes' activity, proline and polyamine contents during bud dormancy release in Superior Seedless rapevine buds. Acta Physiol Plant 34:429-437. doi: 10.1007/s11738-011-0839-0

Ophir R, Pang X, Halaly T, Venkateswari J, Lavee S, Galbraith D, Or E (2009) Gene-expression profiling of grape bud response to two alternative dormancy-release stimuli expose possible links between impaired mitochondrial activity, hypoxia, ethylene-ABA interplay and cell enlargement. Plant Mol Biol 71:403–423. doi: 10.1007/s11103-009-9531-9

Or E, Nir G, Vilozny I (1999) Timing of hydrogen cyanamide application to grapevine buds. Vitis 38:1–6.

Or E, Vilozny I, Eyal Y, Ogrodovitch A (2000) The transduction of the signal for grape bud dormancy breaking induced by hydrogen cyanamide may involve the SNF-like protein kinase GDBRPK. Plant Mol Biol 43: 483–494. doi: 10.1023/a:1006450516982

Pacey-Miller T, Scott K, Ablett E, Tingey S, Ching A, Henry R (2003) Genes associated with the end of dormancy in grapes. Funct Integr Genomics 3:144-152. doi: 10.1007/s10142-003-0094-6

Pérez FJ, Vergara R, Or E (2009) On the mechanism of dormancy release in grapevine buds:a comparative study between hydrogen cyanamide and sodium azide. Plant Growth Regul 59:145-152. doi: 10.1007/s10725-009-9397-5

Rady MM, Seif El-Yazal MA (2014) Garlic extract as a novel strategy to hasten dormancy release in buds of 'Anna' apple trees. South African Journal of Botany, 92: 105-111. doi: 10.1016/j.sajb.2014.02.012

Reid KE, Olsson N, Schlosser J, Peng F, Lund ST (2006) An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. BMC Plant Biol, 6:27. doi: 10. 1186/1471-2229-6-27.

Rotter A, Camps C, Lohse M, Kappel C, Pilati S, Hren M, Stitt M, Coutos-Thévenot P, Moser C, Usadel B, Delrot S, Gruden K (2009). Gene expression profiling in susceptible interaction of grapevine with its fungal pathogen Eutypa lata: Extending MapMan ontology for grapevine. BMC Plant Biol, 9:104. doi:10.1186/1471-2229-9-104.

Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, Sturn A, Snuffin M, Rezantsev A, Popov D, Ryltsov A, Kostukovich

E, Borisovsky I, Liu Z, Vinsavich A, Trush V, Quackenbush J (2003) TM4: a free, open-source system for microarray data management and analysis. Biotechniques 34(2):374-8.

Siller-Cepeda JH, Fuchigami LH, Chen THH (1992) Hydrogen cyanamide induced budbreak and phytotoxicity in 'Redhaven' peach buds. Hort Science 27:874-876.

Susuki M, Suzuki, Ryo Nakabayashi R, Ogata, Y, Sakurai N, Tokimatsu T, Goto S, Suzuki M, Jasinski M, Martinoia E, Otagaki S, Matsumoto S, Saito K, Shiratake K (2015) Multiomics in Grape Berry Skin Revealed Specific Induction of the Stilbene Synthetic Pathway by Ultraviolet-C Irradiation. Plant Physiology, 168:47–59. doi: 10.1104/pp.114.254375

Thimm O, Blasing O, Gibon Y, et al. (2004) MapMan: a userdriven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. The Plant Journal 37, 914–939. doi: 10.1111/j.1365-313X.2004.02016.x

Vargas-Arispuro I, Corrales-Maldonado C, Martínez-Téllez MA (2008) Compounds derived from garlic as bud induction agents in organic farming of table grape. Chilean Journal Of Agricultural Research 68:94-101. http://www.bionline.org.br/cj

Vergara R, Pérez FJ (2010) Similarities between natural and chemically induced bud-endodormancy release in grapevine *Vitis vinifera* L. Scientia Horticulturae 125: 648–653. doi:10.1016/j.scienta.2010.05.020

Walton EF, Wu RM, Richardson AC, Davy M, Hellens RP, Thodey K, Janssen BJ, Gleave AP, Rae GM, Wood M, Schaffer RJ (2009) A rapid transcriptional activation is induced by the dormancy-breaking chemical hydrogen cyanamide in kiwifruit (*Actinidia deliciosa*) buds. Journal of Experimental Botany, 60: 3835-3848. doi:10.1093/jxb/erp231

Additional files captions.

Supplementary electronic Table 1. List of primers used in real time PCR for Nimblegen array validation.

Supplementary electronic Table 2. Transcripts expression modulated throughout 24 h in Flame Seedless grapevine endodormant buds. Differentially expressed genes according to 0.05 Benjamini-Hochberg adjusted p-value in a multi-class LIMMA comparison and ≥2-fold change between SD-, HC-treatment and Controls at 24 h. Log_2 expression is normalized to the controls at 0 h and 24 h (CL0 and CL24, respectively). The DEG are grouped as common and specific for each treatment comparison generated by Venn diagram analysis. Transcript annotations are shown**.**

Supplementary electronic Table 3. Transcripts expression up-regulated throughout 24 h in Flame Seedless grapevine endodormant buds. Up-regulated differentially expressed genes according to 0.05 Benjamini-Hochberg adjusted *p*-value in a multi-class LIMMA comparison and ≥2-fold change between SD-, HC-treatment and Controls at 24 h. Log_2 expression is normalized to the controls at 0 h and 24 h (CL0 and CL24, respectively). The up-regulated DEG are grouped as common and specific for each treatment comparison generated by Venn diagram analysis. Transcript annotations are shown**.**

Supplementary electronic Table 4. Transcripts expression down-regulated throughout 24 h in Flame Seedless grapevine endodormant buds. Downregulated differentially expressed genes according to 0.05 Benjamini-Hochberg adjusted *p*-value in a multi-class LIMMA comparison and ≥2-fold change between SD-, HC-treatment and Controls at 24 h. Log_2 expression is normalized to the controls at 0 h and 24 h (CL0 and CL24, respectively). The down-regulated DEG are grouped as common and specific for each treatment comparison generated by Venn diagram analysis. Transcript annotations are shown**.**

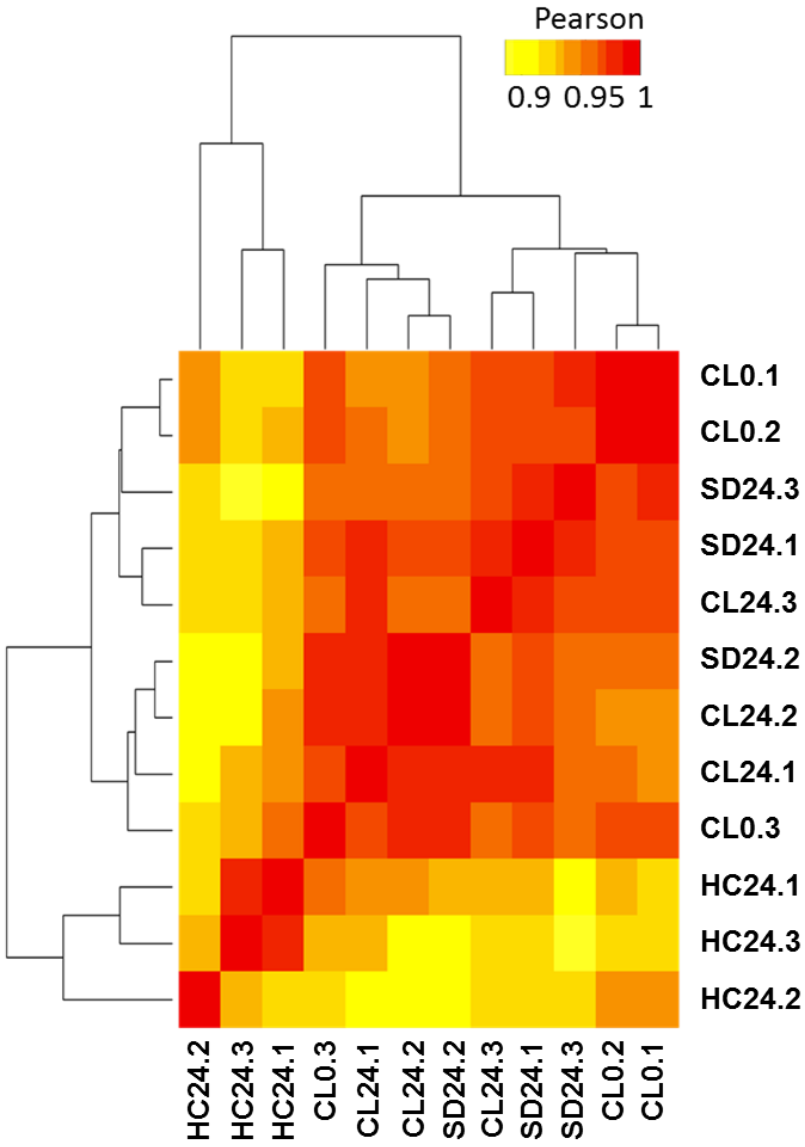
Supplemental Fig. 1. Pearson's correlation matrix among different biological replicates of grapevine buds treated at 24 h with SD, HC, and a control at 0 h and 24 h.

Supplemental Fig. 2 Venn diagram of differentially expressed genes compared between treatments Control 0 h (CL0), Control 24 h (CL24), sulfur derivatives 24 (SD24), and

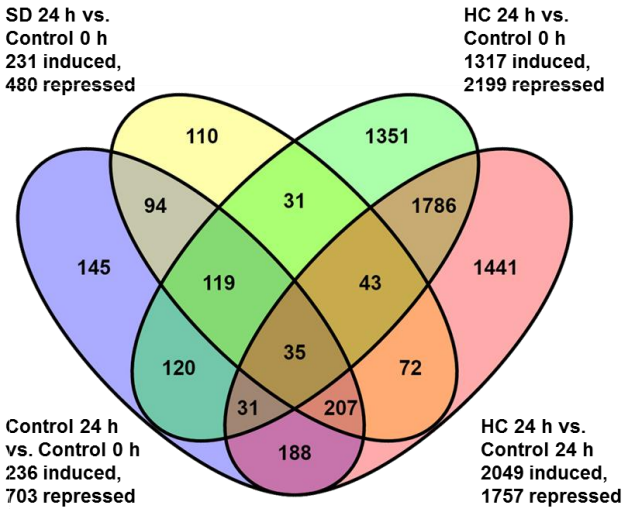
hydrogen cyanamide 24 h (HC24). The number of up-regulated and down-regulated genes for each comparison is given in the diagram.

Supplementary Table 1. List of primers used in real time PCR for Nimblegen array validation.

Gene	Accession no.	Forward primer (5'-3')	Reverse primer (5'-3')
APX	NM_001281059	GCCTTCCGCTTCTTCTGAGC	TCAGCGATGAACCCTCTGAGC
DHAR	NM_001281063	TCAGAGCAGGCTTTGCTGGAT	AATGGCCAAGAGCCACCTGA
GR	AF019907	GCACGAGCTCATTGTCGACGC	AGAGAGAGAGCCTGGGGCTAC
CAT	XM_003631877.2	AGCAAGCAAGGGTGTTCATCAGTC	TCAACCGTAGGATTTGTCTGGCTC
ACT	AF369524	GCTGAGAGATTCCGTTGTCC	GCCACCACCTTGATCTTCAT
ADH	XM_002271314.3	TCCATTTGGCAAAGCGGGGTTG	TGCCCACTGCCTCATCAAAAACC
AOX	XM_010667166.1	AACTTGAGCAGCACGGTGTCG	GGTCAGCTTCACAGGAGCCAC
GA20ox	XM_002263088.3	CGGGCGTCCAAATCCACACA	CTTCCGTTGCTCCACACCGT
ABCGs	XM_002281034.2	TTCAATGCCACTGTACGGGGAAAC	TGCCCACCGCTGATATTCACC
MDHAR	XP_002278648.2	ACAGGTTGATGGTCAGTTCCGAAC	AATGCTGTGCTGAGCGACGAG
TRX2	XM_002282290.3	CGGGTCTTGCTCCCACTCAA	TCCCACCATTTTGGCTGCTCA
TRXH	XM_002280911.3	AGCCCATTTCTGGCAGAGCT	GCACCCACAACCTTGTCCACTA
PDC	AF195868	GTCGTCACCTTCACGGTCGG	ACCGATGGTGTGGTGGAGGA
SAMDC	XM_010658339	CCCAGTTCTCAGTGGCGGTC	CACGTACACCACGCACCCTT
ACCO	XM_002273394.3	CTACCCTCCATGCCCACAGC	ATGGAGTGTCGCATCGGTGG
ABA1	AY337615.1	TAATGTGGGGAGGCGGCAAC	AACCGCCTGGAAGAGCTTGC
GRX	XM_002278616	ACGACGAGGAGATCGGAGCC	ACCAACTTCGACGAGGCGAG
GSTU	XM_002264401	GCCCCTTGCTCCTTGAGATGA	GGGTCACTAGGCAACAATGGGG
SULTR	XM_002281212	TCGTTCCCAGGCGTCCTAGT	GCACATGCTCGACATTGGTCC
SOD	NM_001281138.1	AGCCCCAACCCTAGTCCTCAG	GGCTTTCTTGGTGACGGCGA
G6PDH	XM_002266894.3	AGGAGGTGTTACATACCCAGCCT	TGGAACCCCATCCCATCTGG
PFK1	XM_002277982.2	AGCAATGCACGCTTCGCTTTC	CCTGCTCCTTCAGCCACACA



Supplemental Fig. 1 Pearson's correlation matrix among different biological replicates of grapevine buds treated at 24 h with SD, HC, and a control at 0 h and 24 h.



Supplemental Fig. 2. Venn diagram of differentially expressed genes compared between treatments Control 0 h (CL0), Control 24 h (CL24), sulfur derivatives 24 (SD24), and hydrogen cyanamide 24 h (HC24). The number of up-regulated and down-regulated genes for each comparison is given in the diagram.