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Biochemistry

The dormancy-breaking stimuli "chilling, hypoxia and cyanamide exposure" up-regulate the expression of α-amylase genes in grapevine buds

Sebastián Rubio, Amanda Donoso, Francisco J. Pérez *

Universidad de Chile, Facultad de Ciencias, Laboratorio de Bioquímica Vegetal, Casilla 653, Santiago, Chile

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ABSTRACT

It has been suggested that respiratory stress is involved in the mechanism underlying the dormancy-breaking effect of hydrogen cyanamide (H_2CN_2) and sodium azide in grapevine buds; indeed, reductions in oxygen levels (hypoxia) and inhibitors of respiration promote bud-break in grapevines. In this study, we showed that, hypoxia increased starch hydrolysis soluble sugar consumption and up-regulated the expression of α-amylase genes (*Vvα-AMYs*) in grapevine buds, suggesting that these biochemical changes induced by hypoxia, may play a relevant role in the release of buds from endodormancy (ED). Three of the four *Vvα-AMY* genes that are expressed in grapevine buds were up-regulated by hypoxia and a correlation between changes in sugar content and level of *Vvα-AMY* gene expression during the hypoxia treatment was found, suggesting that soluble sugars mediate the effect of hypoxia on *Vvα-AMY* gene expression. Exogenous applications of soluble sugars and sugar analogs confirmed this finding and revealed that osmotic stress induces the expression of *Vvα-AMY1* and *Vvα-AMY3* and that soluble sugars induces *Vvα-AMY2* and *Vvα-AMY4* gene expression. Interestingly, the plant hormone gibberellic acid (GA_3) induced the expression of *Vvα-AMY3* and *Vvα-AMY4* genes, while dormancy breaking stimuli, chilling and cyanamide exposure, mainly induced the expression of *Vvα-AMY1* and *Vvα-AMY2* genes, suggesting that these two α-amylase genes might be involved in the release of grapevine buds from the ED.

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Introduction

The ability to degrade starch reserves is a crucial biochemical event for the survival of plant tissues under oxygen-limited conditions and for the germination of cereal seeds (Perata et al., 1997, 1998; Loreti et al., 2003). In the first case, sugars, such as sucrose (Suc) and glucose (Glc), are rapidly channeled into the glycolytic and fermentative pathway as soon as oxygen availability decreases below a threshold value to alleviate the limited production of ATP and recycling of NADH to NAD (Dennis et al., 2000). However, because the amount of hexoses and disaccharides stored in plant cells is usually low, the ability to degrade starch reserves becomes crucial for survival under oxygen-limited conditions (Loreti et al., 2003). Therefore, it has been suggested that sugar starvation in plant tissues might function as a signal that triggers starch breakdown (Hajirezei et al., 2003). During the germination of cereal seeds, the transition from a quiescent tissue to one with high metabolic activity requires starch mobilization to satisfy the energetic demands of growth (Yu, 1999; Lee et al., 2009). After seed imbibition, sugars are rapidly consumed in the embryo, leading to a transient sugar depletion that acts as a signal for starch breakdown (Yu et al., 1996). A set of enzymes is necessary for starch degradation, namely α, β and iso-amylase. However, only α-amylase is considered to play a major role in starch degradation (Sun and Henson, 1991). In cereals, the expression of α-amylase genes during seed germination and seedling growth is negatively regulated by sugars in the embryo and positively regulated by gibberellins in endosperm through the sugar-response complex (SRC) and GA-response complex (GARC), respectively (Lu et al., 2002; Chen et al., 2006; Hong et al., 2012). Indeed, the expression of α-amylase genes in the embryos of germinating rice (Yu et al., 1996), in barley seeds (Perata et al., 1997) and in cultured rice suspension cells (Yu et al., 1991) is activated by sugar starvation and is repressed by sugar provision, and sugar repression of α-amylase gene expression involves the control of both transcription rate and mRNA stability (Chan and Yu, 1998). Interestingly, hypoxia

Abbreviations: BR_{50}, time required to reach 50% bud-break under forcing conditions; 2DOG, 2 deoxi-glucose; 6DOG, 6 deoxi-glucose; ED, endodormancy; GA_3, gibberellic acid; Glc, glucose; Fru, fructose; 3MG, 3 methyl glucose; Suc, sucrose; *Vvα-AMYs*, *Vitis vinifera* α-amylase genes.

* Corresponding author. Tel.: +56 2 9787249; fax: +56 22727363.
E-mail address: frperez@uchile.cl (F.J. Pérez).

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has a negative impact on germination and seedling emergence in cereals (Guglielminetti et al., 1995). However, rice (*Oryza sativa*) is the only cereal that has the ability to germinate and grow under oxygen-limited conditions (Perata et al., 1997). One crucial feature that distinguishes rice from other cereals and that explains its ability to germinate and grow post-germinatively under oxygen-limited conditions is its ability to mobilize endosperm starch under reduced oxygen levels (Perata et al., 1997; Park et al., 2010).

In grapevines, respiratory stress is involved in the release mechanism of buds from ED (Ophir et al., 2009; Pérez et al., 2009), hypoxia induces budburst (Vergara et al., 2012), and H_2CN_2, a well-known dormancy-breaking compound, increases starch hydrolysis (Ben-Mohamed et al., 2012). Therefore, in this study, we hypothesized that the transcriptional activation of α-amylase genes (*Vvα-AMYs*) via respiratory stress would be part of the mechanism by which buds are released from ED.

Material and methods

Plant material

Plant material was collected from 8-year-old (*Vitis vinifera* L, cv. Thompson seedless) vines growing at the experimental field station of the Agronomic and Forestry Sciences faculty, University of Chile, located in Santiago (33°34′S). The canes were randomly collected on 26 April (fully endodormancy $BR_{50} > 40$), 18 June (ED release $BR_{50} = 22$) and 3 July of 2012 (earlier ecodormancy $BR_{50} < 20$). BR_{50} is a parameter developed to estimate the mean time (days) required for reaching 50% of bud-break under forcing conditions (Pérez et al., 2007; Vergara and Pérez, 2010). Although, the buds used in the experiments were all dormant, some differences in the depth or intensity of ED exist between them; however, to our knowledge, this fact should not affect their response. The canes were cut off at both ends, leaving the central section with 10–12 buds for further analysis.

Hypoxia treatments

Canes collected on 3 July were separated into six groups of 30 single-bud cuttings each. The first group was maintained at room temperature in the dark (control), and the other five groups were subjected to hypoxia for 3, 6, 12, 18 and 24 h. After the treatment, each group was divided into two subgroups of 15 buds each (biological replicates) for the analysis. To obtain a low oxygen concentration (hypoxia), the single-bud cuttings were placed in a glass chamber with water in the bottom, and N_2 was flushed continuously at a rate of 100 ml min^{-1}. The oxygen concentration in the bulk solution of the measuring flask was recorded polarographically using a Clark-type O_2 electrode; the O_2 concentrations in the control varied between 250 and 260 nmol ml^{-1} and after 3, 6, 12, 18 and 24 h of N_2 bubbling, were 162, 112, 91, 91 and 91 nmol ml^{-1}, respectively. Three biological replicates were performed for each treatment.

Exogenous applications of soluble sugars and sugar analogs

Canes collected on 26 April 2012 were separated into eight groups of 30 single-bud cuttings each. Each group was divided into two subgroups of 15 buds each (biological replicates) and sprayed to runoff with solutions of 2.5% (w/v) soluble sugars sucrose (Suc), glucose (Glc), fructose (Fru) and the sugar analogs 3-o-methyl glucose (3MG) (Sigma–Aldrich, USA), 6-deoxyglucose (6DOG) (Sigma–Aldrich, USA) 2-deoxyglucose (2DOG) (Sigma–Aldrich, USA) and mannitol (Sigma–Aldrich, USA); water was used as the control. After the treatments, the buds were maintained for 24 h in a growth chamber set at 23 ± 2 °C with 16 h photoperiod.

Exogenous application of hydrogen cyanamide (H_2CN_2) and gibberellic acid (GA_3)

Canes collected on 18 June 2012 were separated into three groups of 30 single-bud cuttings each. Each group was divided into two subgroups of 15 buds each (biological replicates) and sprayed to runoff with solutions of 100 ppm GA_3 or 2.5% (w/v) H_2CN_2; water was used as the control. After the GA_3 treatment buds were maintained for 24 h in a growth chamber set at 23 ± 2 °C with 16 h photoperiod. After H_2CN_2 treatment buds were maintained for 4, 24 and 48 h in the growth-chamber before analysis.

Cold treatments

Canes collected on 26 April 2012 were prepared and mounted on a polypropylene sheet and floated in tap water in a plastic vessel Immediately after mounting, the samples were separated into two groups of 60 cuttings each: one group was placed in a refrigerator set at 5 ±1 °C and the other in a growth chamber set at 14 ± 1 °C, both treatments were carried-out in the darkness. Samples of 10 cuttings were collected and separated into two subgroups of 5 cuttings (biological replicates) for gene expression analyses after 0, 138, 336, 504 and 1008 h of treatment.

Determination of starch and sugar concentrations

Grapevine buds (0.2 g approximately) were ground with a mortar and pestle in liquid nitrogen and extracted 3× with 3 ml of cold acetone and 1× with a mixture of chloroform and isoamyl alcohol (24:1). The suspension was centrifuged at 13,000 × *g* for 3 min, and the pellet was dried and extracted with 2 ml 80% (v/v) ethanol for 30 min in a water bath heated to 60 °C. This extraction was repeated 3 times, and the supernatants were collected, pooled and dried. The starch content of the pellet was determined after ethanol extraction of the soluble sugars by acid extraction using the anthrone reagent (Hansen and Moller, 1975). The dried supernatant obtained from ethanol extraction was dissolved in 15 μl of pyridine, and 5 μL BSTFA was added (Sigma–Aldrich, USA); the mixture was then heated at 90 °C for 30 min. The chromatographic analyses of the derivatized samples were performed using a Shimadzu GC 2014 gas chromatograph equipped with a CBP1 capillary column and an FID detector. The operating conditions were as follows: injector and detector temperature were 180 °C and 300 °C, respectively; carrier gas flow (helium) at 1.0 ml/min; injection volume of 1 μL with a flow splitter at a ratio of 50:5. The oven was programmed to temperatures of 60 °C to 200 °C at a rate of 30 °C min^{-1} and from 200 °C to 280 °C at a rate of 5 °C min^{-1}. Standard curves were constructed for the determination of the sucrose, glucose, fructose and starch concentrations.

RNA purification and cDNA synthesis

Total RNA was isolated and purified from grapevine buds (0.5–0.7 g^{-1} fr.wt) using a modification of the method of Chang et al. (1993), as described in Noriega et al. (2007). DNA was removed by treatment with RNAase-free DNAase (1 U/μg) (Invitrogen, CA, USA) at 37 °C for 30 min. First-strand cDNA was synthesized from 5 μg of purified RNA with 1 μL oligo$(dT)_{12-18}$ (0.5 μg $μL^{-1}$) as primer, 1 μL dNTP mix (10 mM) and Superscript® II RT (Invitrogen, USA).

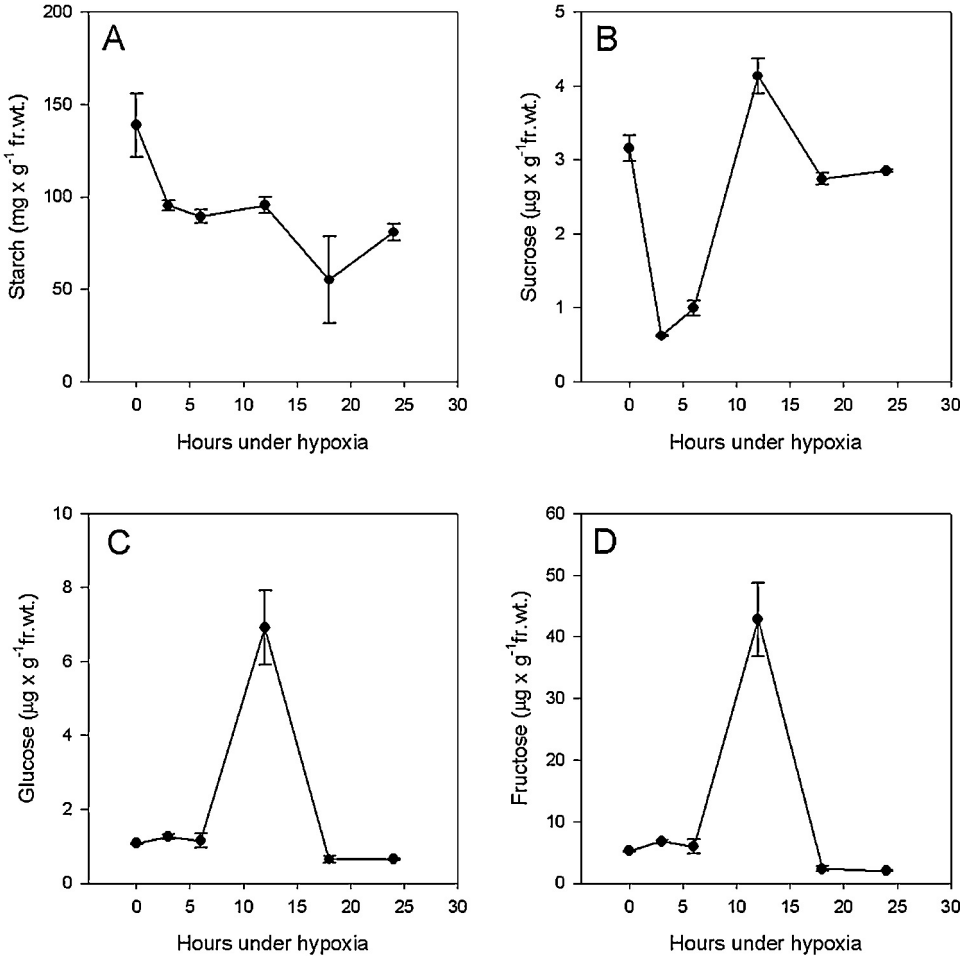

Fig. 1. Effect of hypoxia on starch and soluble sugar content in grapevine buds. Values are the average of three biological replicates and bars represent the standard deviation.

Quantitative real-time PCR

Quantitative real-time PCR was performed using an Eco Real-Time PCR system (Illumina, Inc. SD, USA) with the intercalation dye SYBRGreen I as a fluorescent reporter and Platinum Taq DNA Polymerase (Invitrogen, USA). Primers suitable for the amplification of 100–150 bp products for each gene under study were designed using the PRIMER3 software (Rozen and Skaletsky, 2000) (Table 1). The amplification of cDNA was performed under the following conditions: denaturation at 94 °C for 2 min and 40 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s. Two biological replicates with three technical repetitions were performed for each treatment. Melting curves for each PCR were determined by measuring the decrease in fluorescence with increasing temperature (from 55 to 95 °C). The induction or repression of transcription was calculated by the ΔΔCq method (Livak and Schmittgen, 2001) using *VvUBIQUITIN* as the reference gene. *VvUBIQUITIN* was selected as a reference gene because the transcript level was stable across the treatments.

Phylogenetic tree

Full-length amylase protein sequences retrieved from the public GenBank database were used to construct a phylogenetic tree. Multiple alignments were generated using Muscle, and the tree was calculated with the Phylogeny.fr program (Dereeper et al., 2008).

Sequence analysis of promoter regions

The sequences comprising the 1000 bp upstream from the transcription start site of the selected genes were downloaded from the *Vitis vinifera* genomic database GENOSCOPE (http://www.genoscope.cns.fr). The identification of putative

Table 1
Primers used for real-time quantitative RT-qPCR experiments.

Gene	Locus (GENOSCOPE)	Forward primer	Reverse primer
Vvα-AMY1	GSVIVT01031746001	5′ACTCTGCAACACTGGCCTTT 3′	5′CCTCTTTCAGACCCCACTCA 3′
Vvα-AMY2	GSVIVT01020069001	5′TGAAGCGAACTGAAGTGGTG 3′	5′AGAAACACCCCAATGCAGAA 3′
Vvα-AMY3	GSVIVT01032922001	5′GCCATTTCCACGAGATAAGC 3′	5′CGGAGGCCAAAATCATAGAA 3′
Vvα-AMY4	GSVIVT01008714001	5′TGGCACAGGACAACTTTCAG 3′	5′TGAATGTGACAGCCCTTGAA 3′

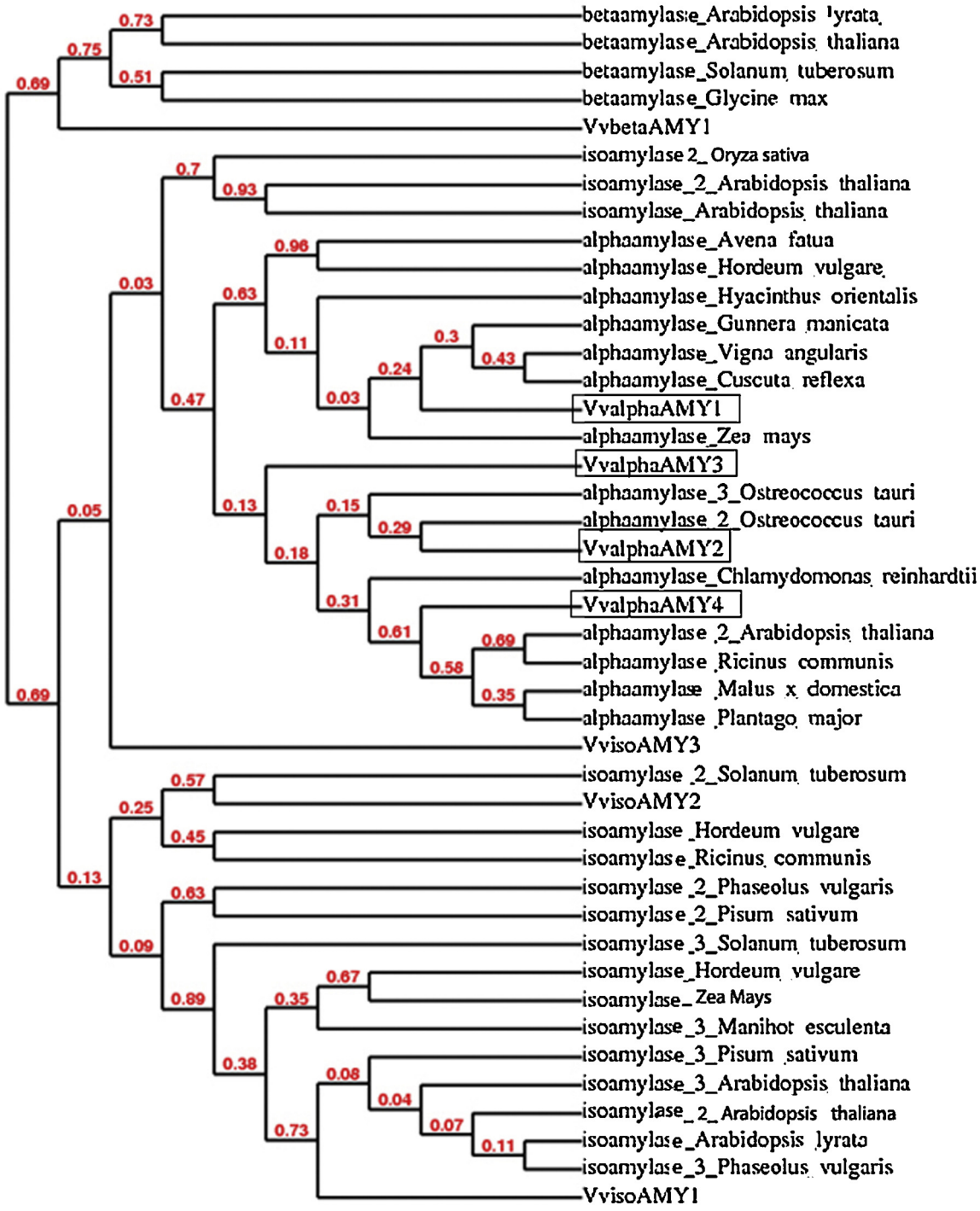

Fig. 2. Comparison of amino acid sequences of plant amylases. Dendrogram was generated by comparing the complete sequences of plant amylase from a public database (GenBank). The *V. vinifera* amylases are highlighted.

cis-acting regulatory elements (CARE) within the promoter dataset was achieved using the web-based analysis tool PlantCare (Rombauts et al., 1999).

Statistic

Data were analyzed by ANOVA using a repeated measure design for variables that were measured more than once, and Duncan's new multiple range tests was used for comparisons of treatment means using Statistica (StatSoft, Tulsa, OK, USA).

Results

Temporal effect of hypoxia on the content of starch and soluble sugars in grapevine buds

Hypoxia rapidly reduced the Suc and starch content in the grapevine buds (Fig. 1A and B). During the first 3 h of the hypoxia treatment, starch and Suc levels decreased by 1.5 and 6 times, while Glc and Fru content increased 1.2 and 1.3 fold, respectively. After 12 h of treatment, Glc and Fru peaked and their content increased

6.4 and 8.2 fold, while Suc recovered to above its initial value (Fig. 1B–D). In addition, starch content decreased steadily throughout treatment (Fig. 1A).

Temporal effect of hypoxia on the expression of Vvα-AMY genes in grapevine buds

A survey of amylase genes in GENOSCOPE showed the presence of 23 putative amylase genes: 6 α, 12 β and 5 iso. A Blast n analysis of these sequences against the EST database of grapevine buds (Ophir et al., 2009) indicated that four *Vvα-AMYs*, three *Vviso-AMYs* and one *Vvβ-AMY* are expressed in buds. However, *Vvβ-AMY* was not induced by hypoxia or H_2CN_2 (results not shown), suggesting that this gene is not involved in dormancy-release events. The phylogenetic tree analysis showed that the sequences of α, β and iso-amylases expressed in grapevine buds match with the amylase genes of other species, confirming that their assignment is correct (Fig. 2). Hypoxia rapidly induced *Vvα-AMY2*, *Vvα-AMY3* and *Vvα-AMY4* and slightly repressed *Vvα-AMY1* gene expression (Fig. 3). After 12 h of hypoxia treatment, the transcript levels of *Vvα-AMY2* (Fig. 3) and the content of soluble sugars (Fig. 1) peaked simultaneously. In contrast, *Vvα-AMY3* peaked after 6 and 18 h of hypoxia treatment (Fig. 3), coinciding with a reduced content of soluble sugars (Fig. 1).

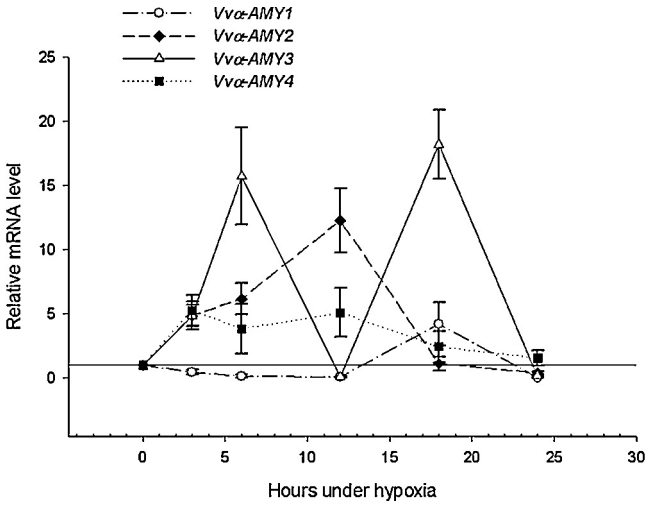

Fig. 3. Expression profile of *Vvα-AMY* genes in grapevine buds under hypoxia. Transcript levels were determined by RT-qPCR and are expressed relative to the control and normalized to *VvUBIQUITIN*. Values are the average of two biological replicates, each with three technical repetitions and bars represent the standard deviation.

Effect of soluble sugars and sugar analogs on the expression of Vvα-AMY genes in grapevine buds

Because hypoxia rapidly enhanced starch and Suc consumption in the grapevine buds, we tested whether exogenous applications of the soluble sugars (2.5%, w/v) Glc, Fru and Suc regulates the expression of *Vvα-AMY* genes. To remove osmotic effects, grapevine buds were also treated with mannitol (2.5%, w/v). Sugars can act as signaling molecules and/or as regulator of gene expression, genetic analyses have revealed a central role for hexokinase (HXK) as a conserved Glc sensor. Therefore, to determine whether the effect of the soluble sugars is mediated by the HXK signaling pathway (Moore et al., 2003) or requires HXK catalytic activity (Xiao et al., 2000), the Glc analogs 3-o-methyl-Glc (3MG), which is transported into plant cells but is metabolized very slowly (Cortés et al., 2003), 6-deoxy-Glc (6DOG), which can also be transported into plant cells

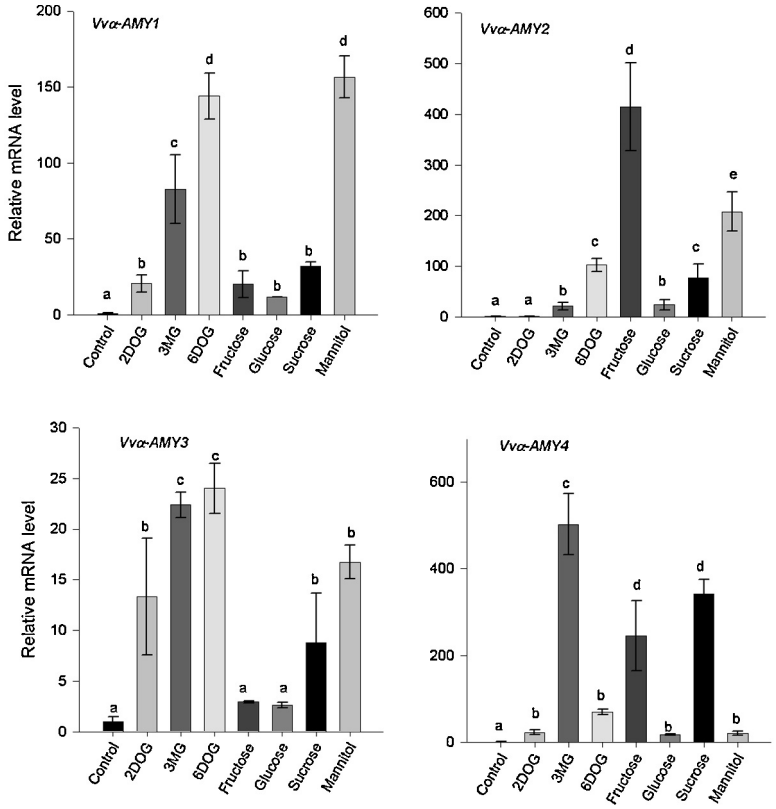

Fig. 4. Effect of soluble sugars Suc, Glc and Fru and sugar analogs 2DOG, 6DOG and 3MG on *Vvα-AMY* gene expression. Transcript levels were determined by RT-qPCR and expressed relative to the control and normalized to *VvUBIQUITIN*. Values are the average of two biological replicates each with three technical repetitions, bars marked with different letters are judged to be significantly different from each other (ANOVA, $P < 0.05$).

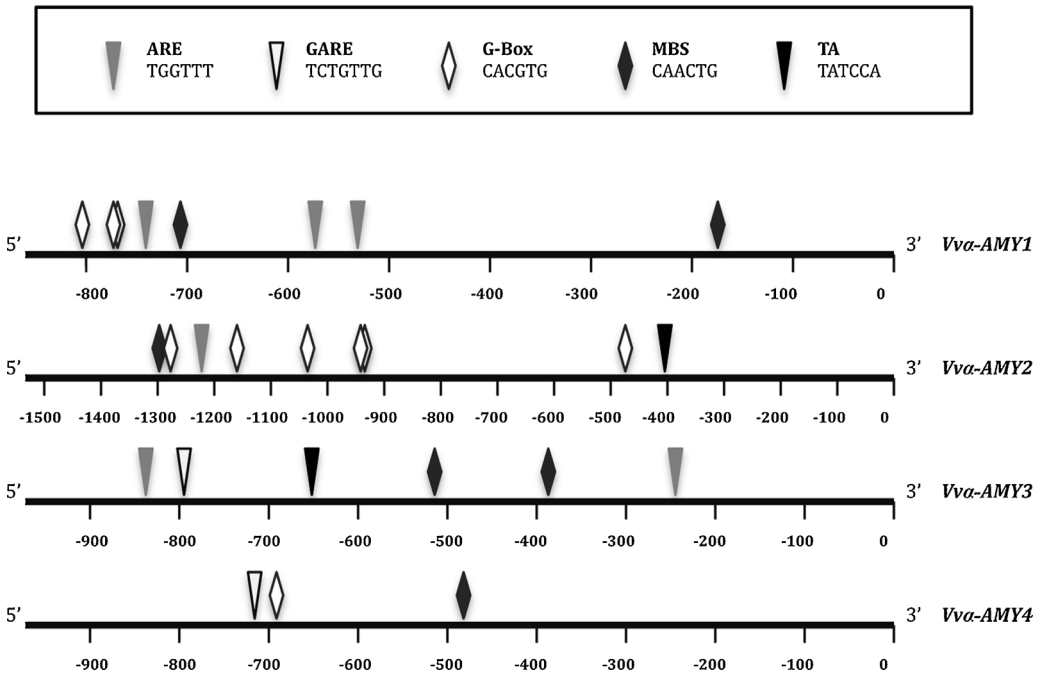

Fig. 5. Positions of the anaerobic-response element (ARE), gibberellin-response element (GARE), G-box, MBS and TA-box, putative regulator *cis*-elements within the 1 kb promoter region of *Vvα-AMY* genes.

but cannot be phosphorylated by HXK (Gibson, 2000), and 2-deoxy-Glc (2DOG), which is phosphorylated by HXK but not metabolized further by the cells (Jan and Sheen, 1994), were tested. A soluble sugar response similar to that of 2DOG would be indicative of the HXK signaling pathway, whereas a response similar to that of 6DOG would be indicative of an HXK-independent pathway. Fig. 4 shows that *Vvα-AMY1* and *Vvα-AMY3* gene expressions are strongly up-regulated by mannitol 157 ± 14 and 17 ± 2 by 6DOG 144 ± 15 and 24 ± 3 and by 3MG 82 ± 22 and 22 ± 2 fold respectively. Conversely, *Vvα-AMY2* and *Vvα-AMY4* were strongly induced by Suc 76 ± 27 and 342 ± 33 and Fru 415 ± 87 and 245 ± 80 respectively.

GA_3 up-regulates the expression of Vvα-AMYs that contains GARE elements in their promoter

Because gibberellin response elements (GAREs) were found in the promoter of the *Vvα-AMY3* and *Vvα-AMY4* genes (Fig. 5), we tested whether the application of gibberellic acid (GA_3) induces the expression of these genes in grapevine buds. Fig. 6A shows that GA_3 up-regulated *Vvα-AMY3* and *Vvα-AMY4* gene expression though did not affect the expression of the *Vvα-AMY1* and *Vvα-AMY2* genes.

The dormancy-breaking stimuli chilling and H_2CN_2 mainly induced Vvα-AMY1 and Vvα-AMY2 gene expression in grapevine buds

Hydrogen cyanamide (H_2CN_2) is used worldwide as an artificial dormancy-breaking stimulus in vineyards, and it has been reported that inhibits mitochondrial respiration (Pérez et al., 2009) and stimulates starch degradation in grapevine buds (Ben-Mohamed et al., 2012). Due to its temperate origin, grapevine requires chilling to overcome bud dormancy. However, this chilling requirement is not fulfilled in warm-winter regions, resulting in a poor an uneven bud-break (Saure, 1985). Therefore, the effects of both artificial and natural dormancy-breaking stimuli were studied in single-bud cuttings with regard to *Vvα-AMY* gene expression. Transient increases in the expression of *Vvα-AMY2* were observed soon after the H_2CN_2 application (>300-fold at 4 h) (Fig. 6B) and after prolonged exposure of endodormant buds to chilling (>1500-fold at 336 h) (Fig. 7). In contrast, the exposure of buds to room temperature (14 °C) for the same period of time did not affect the transcript accumulation of this gene (Fig. 7). Additionally, cyanamide and chilling treatments up-regulated to a lesser extent the expression of *Vvα-AMY1* (Figs. 6B and 7), and the expression of the gibberellin-responsive genes, *Vvα-AMY3* and *Vvα-AMY4*, were not as greatly affected by the dormancy-breaking stimuli (Fig. 6A).

Discussion

Hypoxia increases sugar consumption and up-regulates Vvα-AMYs gene expression in grapevine buds

The absence of oxidative-phosphorylation under hypoxia means that in order to produce adequate amounts of ATP via the less efficient fermentation pathway, greater sugar consumption is required. This is supported by the hydrolysis of starch (Dennis et al., 2000; Perata et al., 1998; Vergara et al., 2012). Therefore, the transcriptional activation of *Vvα-AMYs* genes becomes crucial for the survival of grapevine buds under oxygen-limited conditions. The ability of rice to germinate and grow under oxygen-limited conditions can be explained by the presence of a subfamily of α-amylase genes (*Amy3*) that are expressed under anaerobic conditions, with expression under sugar control and not hormonal control (Perata et al., 1997; Park et al., 2010). In grapevines, three (*Vvα-AMY2*, *Vvα-AMY3* and *Vvα-AMY4*) of the four *Vvα-AMY* genes that are expressed in the buds are up-regulated by hypoxia, and two of these (*Vvα-AMY1* and *Vvα-AMY2*) did not respond to gibberellic acid (GA_3). Thus, it appears reasonable that sugars may regulate *Vvα-AMY* genes expressions and that their expression pattern under hypoxia is a consequence of an alteration in the soluble sugar levels induced by hypoxic stress (Loreti et al., 2003). The fact

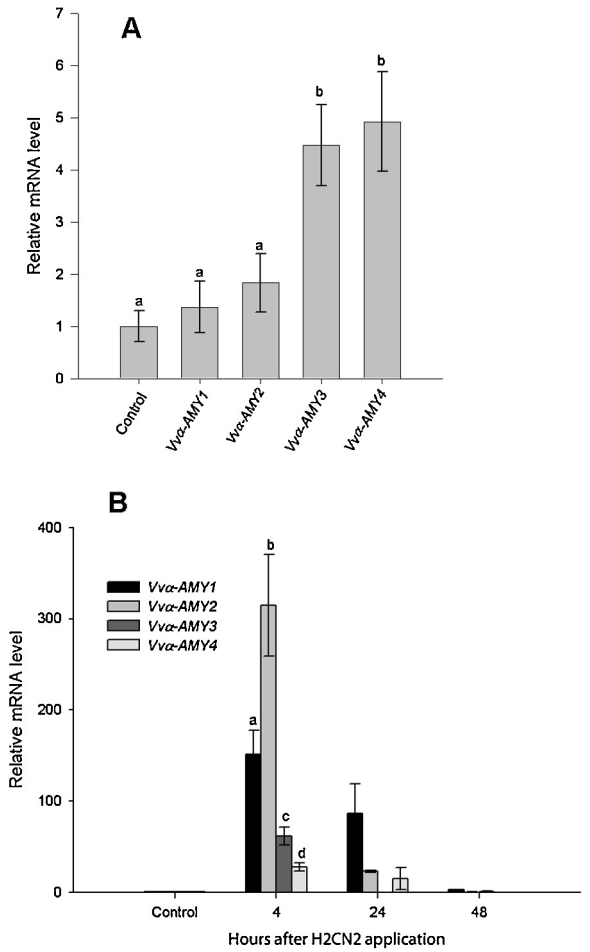

Fig. 6. Effect of (A) gibberellic acid (GA_3) and (B) hydrogen cyanamide (H_2CN_2) on the expression of *Vvα-AMY* genes. Transcripts levels were determined by RT-qPCR at 24 h after GA_3 treatment and at 4, 24 and 48 h after H_2CN_2 treatment and are expressed relative to the control and normalized to *VvUBIQUITIN*. Values are the average of two biological replicates each with three technical repetitions, bars marked with different letters are judged to be significantly different from each other (ANOVA, $P < 0.05$).

that transcript level of *Vvα-AMY2* and the content of soluble sugars simultaneously peaked after 12 h of hypoxia treatment, suggests that soluble sugars up-regulates the expression of this gene. Moreover, the exogenous application of soluble sugars and sugar analogs confirmed the above results, demonstrating that *Vvα-AMY2* gene expression is up-regulated by Fru, whereas *Vvα-AMY4* is induced by Suc and Fru. Furthermore, the same experiments showed that *Vvα-AMY3* is down-regulated by soluble sugars, as the transcript level of this gene peaked after 6 and 18 h of hypoxia treatment, coinciding with a reduction in the content of soluble sugars. Additionally, the fact that *Vvα-AMY1* gene expression was induced by mannitol and by the sugar analogs 3MG and 6DOG, whereas Glc, Fru and the sugar analog 2DOG applied at the same concentration had a slight effect, indicates that osmotic stress induces the expression of this gene and that a sugar repressive effect overrides the osmotic stress effect. This sugar repressive effect most likely occurs through the HXK signaling pathway because 2DOG, a Glc analog that is readily phosphorylated by HXK but not further metabolized by the cells (Jan and Sheen, 1994), behaved in a similar fashion as the soluble sugars Glc and Fru. Indeed, biochemical and genetic evidence suggests that HXK acts as a sugar sensor, triggering the repression of genes regulated by sugars in many higher plants (Moore et al., 2003; Cho et al., 2007, 2009).

Promoter analysis of Vvα-AMY genes

In rice and barley, α-amylase gene expression is negatively regulated by sugars in the embryo (Perata et al., 1997) and positively by gibberellins (GA) in the endosperm (Yu et al., 1996; Perata et al., 1997; Loreti et al., 2000). The α-amylase promoter is activated by sugar starvation via the sugar–response complex (SRC) of which the TA/Amy-box is a key element (Lu et al., 1998). The α-amylase promoter is also activated by GA through the gibberellin-response complex (GARC) of which the adjacent gibberellin-response element (GARE) and TA/Amy-box are key elements and act synergistically (Hong et al., 2012). In fact, the TA/Amy-box and GARE element have been identified in the promoter of a variety of GA-inducible hydrolase genes (Sun and Gubler, 2004). A promoter analysis of the *Vvα-AMY* genes showed that the GARE element is present in the promoters of the *Vvα-AMY3* and *Vvα-AMY4* genes; consistent with this finding, the expression of both genes was induced by gibberellic acid (GA_3). The presence of the TA/Amy box was found in the promoters of the *Vvα-AMY2* and *Vvα-AMY3* genes, indicating that *Vvα-AMY3* 1 Kb upstream fragment is the only *Vvα-AMY* gene that contain both a GARE element and TA/Amy-box and that is up-regulated by sugar starvation and GAs, resembling the α-amylase genes of rice and barley (Hong et al., 2012). This particular characteristic of the *Vvα-AMY3* promoter could be the reason why small decreases in the content of soluble sugars induced by hypoxia positively regulate the expression of this gene. In addition, the lack of a GARE element in the promoter of *Vvα-AMY1* could make this gene less sensitive to the small changes in the soluble sugar content induced by hypoxia, which would explain why *Vvα-AMY1* is not induced by hypoxia and is repressed by high concentrations of soluble sugars.

Possible role of Vvα-AMYs during bud-ED release in grapevines

Starch hydrolysis in bud sprouting as in seed germination provides essential soluble sugars for the emerging seedling prior to the commencement of photosynthesis. Of the starch-degrading enzymes, α-amylase is considered to be the most important in seeds, as it is capable of hydrolyzing intact starch grains (Beck and Ziegler, 1989). Interestingly, selection for low dormancy in annual rye grass results in the high constitutive expression of a specific α-amylase isoform (Goggin and Powles, 2012). A relationship between carbon metabolism and outgrowth potential of buds has been reported in peach (Maurel et al., 2004). In grapevine buds, the dormancy breaking stimuli chilling and cyanamide exposure mainly induced the expression of *Vvα-AMY1* and *Vvα-AMY2*, suggesting that both genes might be involved in the release of buds from ED. *Vvα-AMY1* was induced by mannitol and by the non-phosphorytable sugar analog 6DOG, suggesting that osmotic stress regulated positively its expression. Fru, on the other hand, regulates positively *Vvα-AMY2* gene expression. Under respiratory stress, the replacement of invertase by susy in the breakdown of Suc, favors Fru accumulation in plant tissues (Geigenberger, 2003), and since in grapevine buds *VvSUSY* is induced by hypoxia and by H_2CN_2 treatments (Vergara et al., 2012), a potential accumulation of Fru within the bud could explain the stronger induction of *Vvα-AMY2* by H_2CN_2. *Vvα-AMY3* resembles the behavior of cereal α-amylases genes, since its expression was down-regulated by Fru and up-regulated by GA_3. *Vvα-AMY4* seems to be developmentally regulated, since its expression pattern over time evolves similarly in cooled and non-cooled buds. Because the *Vvα-AMY* genes up-regulated by GA_3 are not induced by the dormancy breaking stimuli, we concluded that gibberellins (GAs) are not involved in the release of grapevine buds from the ED.

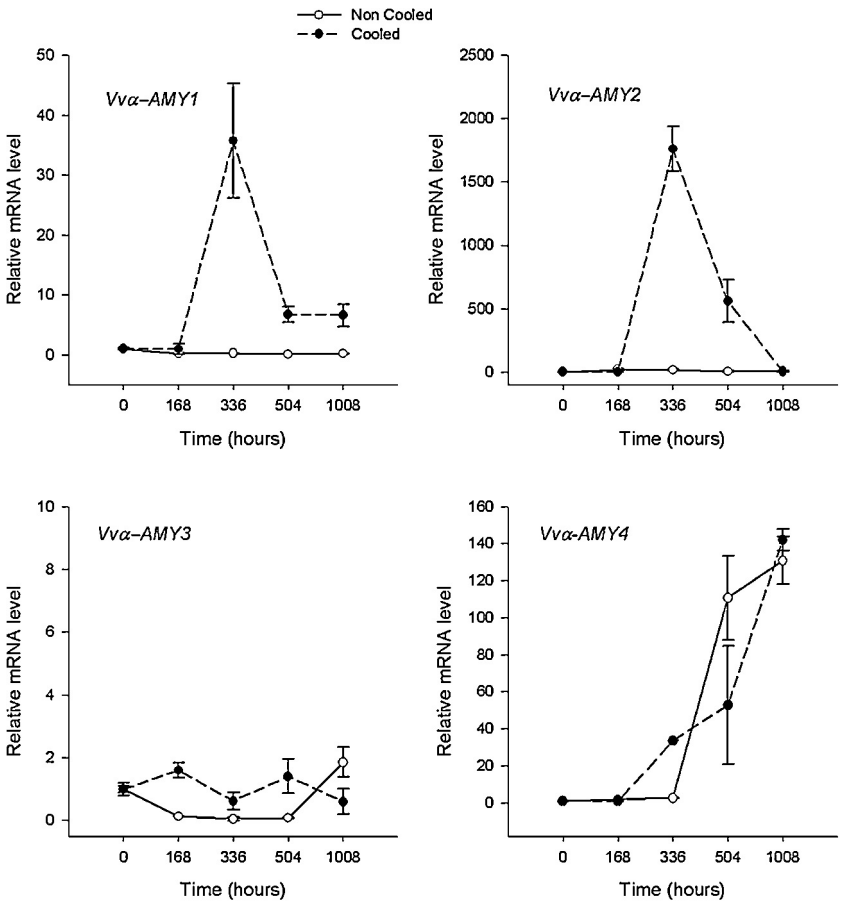

Fig. 7. The effect of chilling temperature on the expression of *Vvα-AMY* genes in endodormant grapevine buds. Transcript levels were determined by RT-qPCR and are expressed relative to the control and normalized to *VvUBIQUITIN*. Values are the average of two biological replicates each with three technical repetitions, bars represent the standard deviation.

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