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RESEARCH PAPER

Hypoxia induces H_2O_2 production and activates antioxidant defence system in grapevine buds through mediation of H_2O_2 and ethylene

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Abstract

Paradoxically, in eukaryotic cells, hydrogen peroxide (H_2O_2) accumulates in response to oxygen deprivation (hypoxia). The source of H_2O_2 under hypoxia varies according to the species, organs, and tissue. In non-photosynthetic tissues, H_2O_2 is mainly produced by activation of NAD(P)H-oxidases or by disruption of the mitochondrial electron transport chain (m-ETC). This study showed that hypoxia, and inhibitors of respiration like potassium cyanide (KCN) and sodium nitroprusside (SNP), trigger the production of H_2O_2 in grapevine buds. However, diphenyleneiodonium, an inhibitor of NAD(P)H-oxidase, did not reduce the H_2O_2 levels induced by KCN, suggesting that, under respiratory stress, H_2O_2 is mainly produced by disruption of the m-ETC. On the other hand, γ-aminobutyric acid (GABA), a metabolite that in plants alleviates oxidative stress by activating antioxidant enzymes, reduced significantly the levels of H_2O_2 induced by KCN and, surprisingly, repressed the expression of genes encoding antioxidant enzymes such as *ASCORBATE PEROXIDASE* (*VvAPX*), *GLUTATHIONE PEROXIDASE* (*VvGLPX*), *SUPEROXIDE DISMUTASE* (*VvSOD*), and one of the *CATALASE* isoforms (*VvCAT1*), while *VvCAT2* was upregulated. In contrast to GABA, hypoxia, H_2O_2, and ethylene increased dramatically the expression of genes encoding antioxidant enzymes and enzymes of the alternative respiratory pathway such as *ALTERNATIVE NADH-DEHYDROGENASES* (*VvaNDs*) and *ALTERNATIVE OXIDASES* (*VvAOXs*). Hence, it is concluded that H_2O_2 production is stimulated by respiratory stress in grapevine buds, that H_2O_2 and ethylene act as signalling molecules and activate genes related to the antioxidant defence system, and finally that GABA reduces H_2O_2 levels by up-regulating the expression of *VvCAT2*.

Key words: antioxidant system, buds, ethylene, GABA, hypoxia, hydrogen peroxide, *Vitis vinifera*.

Introduction

A general response of plants to oxygen deprivation (hypoxia) involves a reconfiguration of carbon metabolism to overcome limited ATP production and a regeneration of NAD^+, by enhancing the glycolysis and fermentative pathways respectively (Fukao and Bailey-Serres, 2004; Bailey-Serres and Chang, 2005). It has also been reported that, under hypoxia, plants stimulate H_2O_2 production (Blokhina *et al.*, 2001). However, the source of H_2O_2 produced during hypoxia should be determined (Fukao and Bailey-Serres, 2004). In *Arabidopsis*, hypoxia stimulates a Rop-signalling transduction pathway that activates a diphenyleneiodonium (DPI)-sensitive NADPH oxidase that results in increased H_2O_2 production (Baxter-Burrell *et al.*, 2002). H_2O_2 could also be produced as a consequence of the disruption of the mitochondria electron transport chain (m-ETC). In yeast and mammalian cells, hypoxia stimulates the production of the superoxide anion at the complex III of mitochondria by increasing the abundance of the partially reduced ubisemiquinone anion (Moller, 2001). The superoxide anion is rapidly converted to H_2O_2 by spontaneous dismutation or by a reaction catalysed by the mitochondrial enzyme superoxide dismutase (SOD) (Moller, 2001).

In grapevines, several lines of evidence indicate that a respiratory stress is involved in the release of buds from dormancy and that perturbations of mitochondrial functions caused by dormancy-breaking compounds, hydrogen cyanamide (HC) and sodium azide, leads to the development of oxidative-stress, induction of glycolysis and fermentation, and increased ethylene production (Ophir *et al.*, 2009; Pérez *et al.*, 2009). Recently, the current study group found that hypoxia promotes the bud-break response in grapevines and induces the expression of genes encoding for enzymes of the fermentative pathway, *PYRUVATE DECARBOXYLASE* (*VvPDC*) and *ALCOHOL DEHYDROGENASE* (*VvADH2*) (Vergara *et al.*, 2012), giving thus further support to the hypothesis that a respiratory stress is involved in the release of buds from dormancy.

This study reports that hypoxia and inhibitors of respiration such as potassium cyanide (KCN) and sodium nitroprusside (SNP) trigger the production of H_2O_2 in grapevine buds. Hypoxia also induced the expression of reactive oxygen species (ROS) scavenging genes (*VvAPX*, *VvSOD*, *VvGLPX*, and *VvCAT*) and genes related to the alternative respiratory pathway [*ALTERNATIVE OXIDASES* (*VvAOXs*) and *ALTERNATIVE NAD(P)H-DEHYDROGENASES* (*VvaNDs*)]. This study also determined whether H_2O_2 and ethylene mediate the antioxidant response of buds to hypoxia. It has been reported that GABA inhibits the accumulation of H_2O_2 brought about by salt stress and that it also alleviates the oxidative damage caused by aluminium toxicities through the activation of the antioxidant defence system (Shi *et al.*, 2010; Song *et al.*, 2010). Therefore, the current study also determined the effect of γ-aminobutyric acid (GABA) on the accumulation of H_2O_2 triggered by respiratory stresses and on the expression of antioxidative genes and genes related to the alternative respiratory pathway.

Materials and methods

Plant material

Eight-year-old *Vitis vinifera* L cv. Thompson seedless grown in the experimental field of the faculty of Agronomic and Forestry Sciences, University of Chile located in Santiago (33° 34′ S) were used as plant material. Canes were collected between 20 June and 28 July 2010 at the stage of endodormancy release, according to previous assessments of bud-dormancy status (Pérez *et al.*, 2007; Vergara and Pérez, 2010). Canes were cut off at both ends, leaving the central section with 10–12 buds for further experiments

Chemical and hypoxia treatments

Canes collected on 20 June 2010 were separated in four groups of six single bud cuttings each. The first group was maintained under ambient conditions (control), while the other three groups were submitted to hypoxia for 24 h: two of these groups were pretreated with 1 and 10 μM DPI before being submitted to hypoxia. To obtain low oxygen concentrations (hypoxia), cuttings were placed in a glass chamber with water in the bottom and N_2 flushed continuously at a rate of 100 ml min^{-1}. The oxygen concentration in the bulk solution of the measuring chamber was recorded polarographically using a Clark type electrode and after 24 h of bubbling, the O_2 concentration was 105 nmol ml^{-1} (8.5%). Analysis was performed immediately after the hypoxia treatment. Three biological replicates were performed for each treatment.

Canes collected on 12 July 2010 were separated in three groups of 12 single bud cuttings each. The first group was sprayed with 0.1% (w/v) KCN, the second with 0.1% (w/v) SNP, and the third with water (control). Analysis was carried out 24 and 48 h after treatment. Three biological replicates were performed for each treatment.

Canes collected on 28 July 2010 were separated in three groups of six single bud cuttings each. The first group was pretreated with 2% (w/v) GABA (Sigma-Aldrich) and afterwards sprayed with 0.1% KCN, the second was sprayed with 0.1% KCN, and the third with water (control). Analysis was carried out 24 h after treatment. Three biological replicates were performed for each treatment.

Determination of H_2O_2 content

H_2O_2 concentration was measured by chemiluminescence in a Cobalt-catalysed oxidation of luminol (5-amino-2,3-dihydro-1,4-phtalazinedione) (Pérez and Rubio, 2006).

Exogenous applications of GABA, H_2O_2, and ethylene

Canes collected on 8 July 2010 were separated in four treatment groups of 30 single bud cuttings each. Each group was divided into two subgroups of 15 buds each and the subgroups were treated separately. Treatments were spraying with (i) 2% GABA, (ii) 1% H_2O_2 (Merck), (iii) 10 mg ethephon (Sigma-Aldrich) dissolved in 10 ml 100 mM phosphate buffer (pH 5.0), or (iv) water (as control). One day post treatment, total RNA was extracted from each subgroup, obtaining thus two biological replicates for each treatment. Gene expression was analysed by quantitative real-time (qRT)-PCR.

RNA purification and cDNA synthesis

Total RNA was isolated and purified from grapevine buds (0.5–0.7 g freshweight) using a modification of the method of Chang *et al.* (1993), described in Noriega *et al.* (2007). DNA was removed by treatment with RNAase-free DNAase (1 U/μg) (Invitrogen) at 37 °C for 30 min. First-strand cDNA was synthesized from 5 μg 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).

Quantitative real-time PCR

Quantitative real-time PCR was carried out in an Eco Real-Time PCR system (Illumina, SD, USA) using the intercalation dye SYBRGreen I as a fluorescent reporter and Platinum Taq DNA Polymerase (Invitrogen). Primers suitable for amplification of 100–150 bp products for each gene under study were designed using the PRIMER3 software (Rozen and Skaletsky, 2000) (Table 1). Amplification of cDNA was carried out under the following conditions: denaturation at 94 °C for 2 min and 40 cycles of 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). PCR products were run on in 1.5% (w/v) agarose gel to confirm the size and presence of a unique PCR product. Induction or repression of the transcription level was calculated by the ΔΔCq method (Livak and Schmittgen, 2001) using VvACTIN as reference gene. VvACTIN was selected as a reference because the transcript level was stable across the treatments. The efficiency for reference and studied genes were determined by standard curves and was 95%. The expression of the reference gene did not varied between samples and gave a Cq value between 10 and 11.

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

Genes	Locus (GENOSCOPE)	Forward primer (5′–3′)	Reverse primers (5′–3′)
VvASPX1	GSVIVT00024455001	AAATGGGTCTCAGCGACAAG	CAGGGTCCTTCAAATCCAGA
VvASPX2	GSVIVT00037064001	TAGCCGTTAGGCTCTTGGAA	AGCAACAACTCCAGCCAACT
VvASPX3	GSVIVT00015409001	GAAATTTGTGGCAGCCAAGT	GCTTCCACCAACTGCTTCAT
VvCAT1	GSVIVT00004081001	TGCGGAGACATTCCCTATTC	TGTTGATGAAACGCTCTTGC
VvCAT2	GSVIVT00002880001	GCTGAGAGGTACCCCATTC	TCTCCTGGCTGCTTGAAGTT
VvGLPX1	GSVIVT00032791001	CCCGAGATTAAGCAGTTTGC	GTGGTTGGCCCATTAACATC
VvGLPX2	GSVIVT00000409001	GCACAGGAACCTGGGAGTAA	CAGCACTATCGCCATTCACA
VvGLPX3	GSVIVT00000410001	AGAGGAGGAACCGGGAAGTA	GCAGCATTTTCGCCATTTAC
VvSOD1	GSVIVT00029451001	GGCGATTCATCTACGGTTGT	CAACCCAGTGAACCTTTTGG
VvSOD2	GSVIVT00014163001	CATGTTCAAGGAAGGGCAAT	CCCAATGGTATTCCAACGTC
VvSOD3	GSVIVT00008877001	AATGAGGGTGCTTGTGGAAC	AGGCCAGAAAGACTCCCAGT
VvAOX23	GSVIVT00003173001	ACGTTGTGGAGGTGATCAGG	GCGGGAACATCCTTGAACT
VvAOX53	GSVIVT00000267001	AACTGTGATCCGTGCTGATG	GGTAACCAATAGGGGCFTGGT
VvaND1	GSVIVT00024082001	GCATCCCTTGGCAGATACA	ATAACCGGTTCCTCCAGCTT
VvaND2	GSVIVT00029180001	TTCAATCGCATGGAAGAGTG	GCCCAAGATGCTTGTACCTG

Sequence analysis of promoter regions

Sequences of 1000 bp upstream from the transcription start site of the selected genes were download from the *V. vinifera* genomic database GENOSCOPE (http://www.genoscope.cns.fr). Identification of putative *cis*-acting regulatory elements within the promoter dataset was carried out using the web-based analysis tool PlantCare (Rombauts *et al.*, 1999).

Results

Hypoxia triggers H_2O_2 production in grapevine buds

Hypoxia (8% O_2 for 24 h) doubled the level of H_2O_2 in grapevine buds (Fig. 1A). To test whether the increase in H_2O_2 was due to the activation of NAD(P)H-oxidase, single bud cuttings were pretreated with DPI, an inhibitor of NAD(P)H-oxidase, before being submitted to hypoxic treatments (Riganti *et al.*, 2004). Fig. 1A shows that DPI at the two concentrations tested increased instead of reduced the level of H_2O_2, suggesting that increases in H_2O_2 levels may be due to disruption in m-ETC rather than activation of NAD(P)H-oxidase.

KCN and SNP trigger H_2O_2 production in grapevine buds

KCN and SNP, which decompose to nitric oxide (NO) and cyanide (Bethke *et al.*, 2006), increased the level of H_2O_2 in grapevine buds (Fig. 1B). The effect of KCN was higher than that of SNP, but in both cases, the increase in H_2O_2 level was transient, since 24 h after treatment the level of H_2O_2 was significantly higher in treated that in control buds, while 48 h after treatment there were no major differences between control and treated samples (Fig. 1C).

GABA reduces H_2O_2 levels in grapevine buds

GABA reduced significantly the levels of H_2O_2 in grape cuttings sprayed with KCN (Fig. 1D). The levels of H_2O_2 in buds pretreated with GABA was even lower than in control buds (not sprayed with KCN). Analysis was performed 24 h after treatment and showed clearly that GABA neutralizes the effect of KCN on H_2O_2 accumulation in grapevine buds.

GABA represses the expression of genes encoding antioxidant enzymes and enzymes of the alternative respiratory pathway

To test whether exogenous GABA application reduced H_2O_2 levels because of increased transcription of genes related to the antioxidant defence system, this study analysed by qRT-PCR the expression of genes coding for the antioxidant enzymes ascorbate peroxidase (*APX*), catalase (*CAT*), glutathion-peroxidase (*GLPX*), and superoxide dismutase (*SOD*), in control and in GABA treated buds. The three genes encoding ascorbate peroxidase in GENOSCOPE, *VvAPX1*, *VvAPX2*, and *VvAPX3*, were markedly repressed by the GABA application. *VvAPX2* was the most repressed isogene, followed by *VvAPX3* and *VvAPX1* respectively (Fig. 2A). The same occurred with the three glutathione peroxidases (*VvGLPX1*, *VvGLPX2*, and *VvGLPX3*) and superoxide dismutase genes (*VvSOD1*, *VvSOD2*, and *VvSOD3*) (Fig. 2A). Although GABA repressed *VvCAT1*, one of the genes encoding catalase, it up-regulated the other catalase isogene *VvCAT2* (Fig. 2A). Since GABA can be transformed into succinate to feed the TCA cycle (Bouché and Fromm, 2004), the current study analysed its effect on the expression of genes encoding enzymes of the alternative respiratory pathway, *VvaNDs* and *VvAOXs*. GABA regulated differently the expression of the two NAD(P)H-dehydrogenase genes that are expressed in grape buds (*VvaND1* and *VvaND2*): the first was repressed and the second was slightly induced (Fig. 2B). Similarly, the two alternative-oxidase genes that are expressed in grape buds, *VvAOX23* and *VvAOX53*, were regulated differently by GABA: *VvAOX23* was repressed while *VvAOX53* was up-regulated (Fig. 2B).

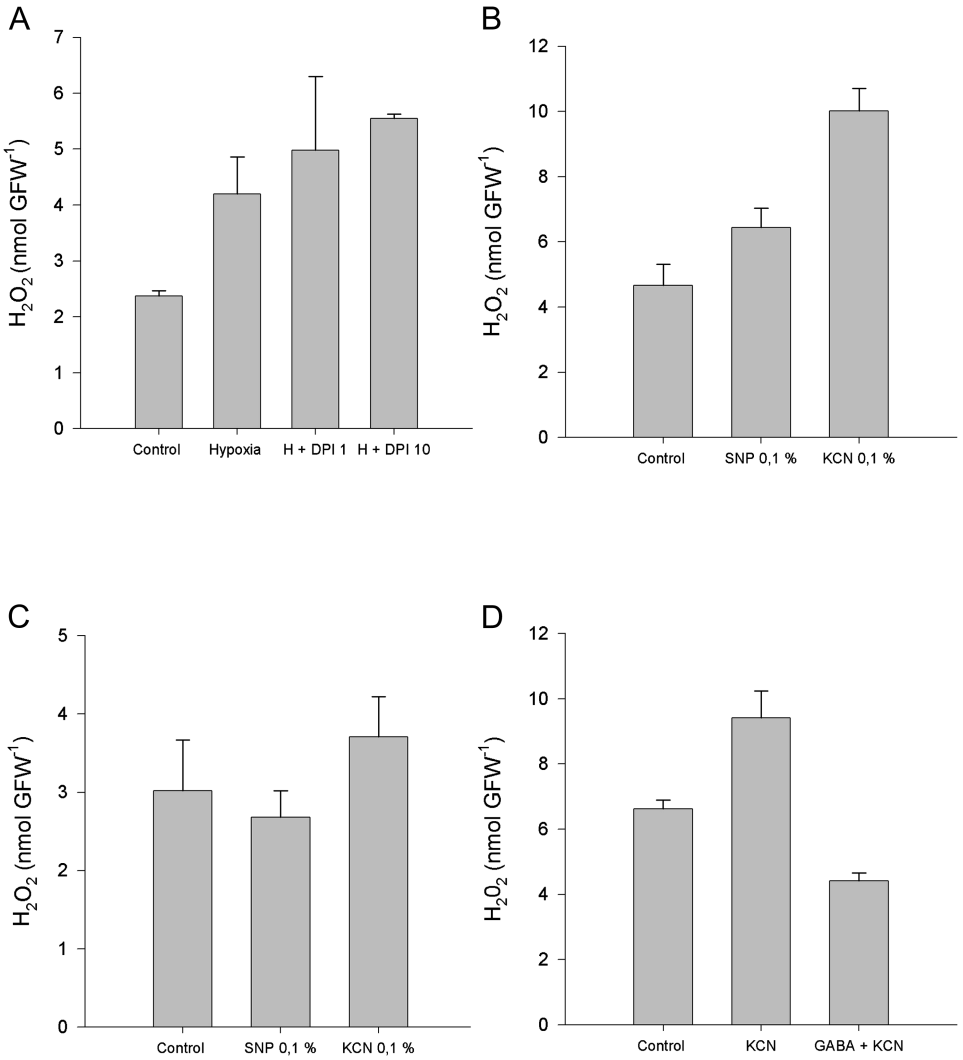

Fig. 1. (a) Hypoxia increases H_2O_2 level in grapevine buds. Groups of buds were pretreated with 1 and 10 μM diphenyleneiodonium (DPI) before hypoxia treatment and H_2O_2 was determined 24 h after hypoxia treatment. (b, c) Sodium nitroprusside (SNP) and potassium cyanide (KCN) increase temporarily the level of H_2O_2 in grapevine buds. SNP and KCN were applied at a concentration of 0.1% (w/v) before hypoxia treatment and H_2O_2 was determined 24 h (b) and 48 h (c) after hypoxia treatment. (d) γ-Aminobutyric acid (GABA) reduced the content of H_2O_2 induced by KCN in grapevine buds. Bud cuttings were pretreated with a solution 2% (w/v) GABA before treatment with 0.1% KCN. H_2O_2 was determined 24 h after hypoxia treatment. All values are mean ± standard deviation ($n = 3$).

Hypoxia, H_2O_2, and ethylene increase the expression of genes encoding antioxidant enzymes and enzymes of the alternative respiratory pathway

To test whether hypoxia stimulates the antioxidant defence system in grapevine buds and whether this response is mediated by H_2O_2, ethylene or both, this study analysed by qRT-PCR the effect of hypoxia and exogenous applications of H_2O_2 and ethylene on the expression of genes encoding antioxidant enzymes and enzymes of the alternative respiratory pathway. Genes belonging to the alternative respiratory pathway *VvaND1* and *VvAOX23* were dramatically induced by the three stimuli, but hypoxic induction was larger than that with H_2O_2 and ethylene (Figs. 3, 4, and 5). The other genes of this pathway, *VvaND2* and *VvAOX53*, were slightly induced by the three stimuli and again hypoxic induction was stronger than that with H_2O_2 and ethylene (Figs. 3, 4, and 5). The three stimuli also up-regulated significantly the expression of antioxidant genes; however, differences in the expression pattern of these genes were observed between the three stimuli. Thus, while the genes most induced by hypoxia were *VvCAT1*, *VvGLPX3*, and *VvSOD3*, the most induced by H_2O_2 were *VvAPX2*, *VvCAT1*, and *VvGLPX2*, and by ethylene were *VvGLPX3*, *VvGLPX2*, and *VvCAT1* (Figs. 3, 4, and 5).

Promoters of VvAOX23 *and* VvaND1 *share common putative regulatory elements*

Similarities observed among the expression patterns of *VvAOX23* and *VvaND1* in response to the three stimuli investigated prompted this study to look for common

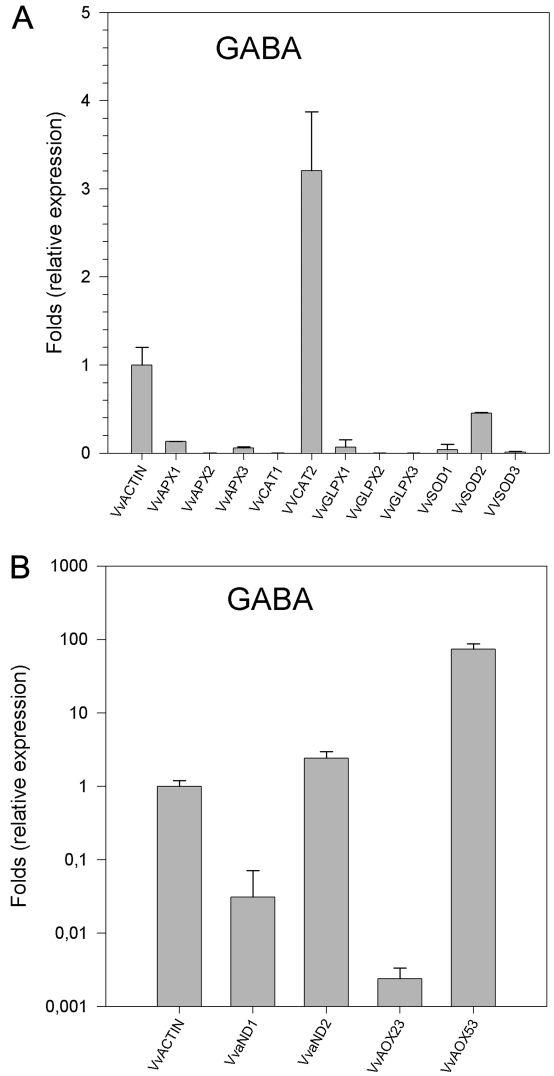

Fig. 2. Effect of γ-aminobutyric acid (GABA) on the expression of genes encoding (A) antioxidant enzymes and (B) enzymes of the alternative respiratory pathway in grapevine buds. GABA was applied at a concentration of 2% (w/v) and gene expression analysis was performed by quantitative real-time PCR 24 h after treatment. Expression of genes encoding for antioxidant enzymes [ascorbate peroxidase (*VvAPX1*, *VvAPX2*, and *VvAPX3*), glutathione peroxidase (*VvGLPX1*, *VvGLPX2*, and *VvGLPX3*), superoxide dismutase (*VvSOD1*, *VvSOD2* and *VvSOD3*), and catalase (*VvCAT1* and *VvCAT2*)] and for enzymes of the alternative respiratory pathway [oxidase alternative (*VvAOX23* and *VvAOX53*) and alternative dehydrogenases (*VvaND1* and *VvaND2*)] were analysed. Transcript levels were normalized to *VvACTIN* using the ΔΔCT method (Livak and Schmittgen, 2001). Values are means of two biological replicates and bars represent the range of variation of technical replicates.

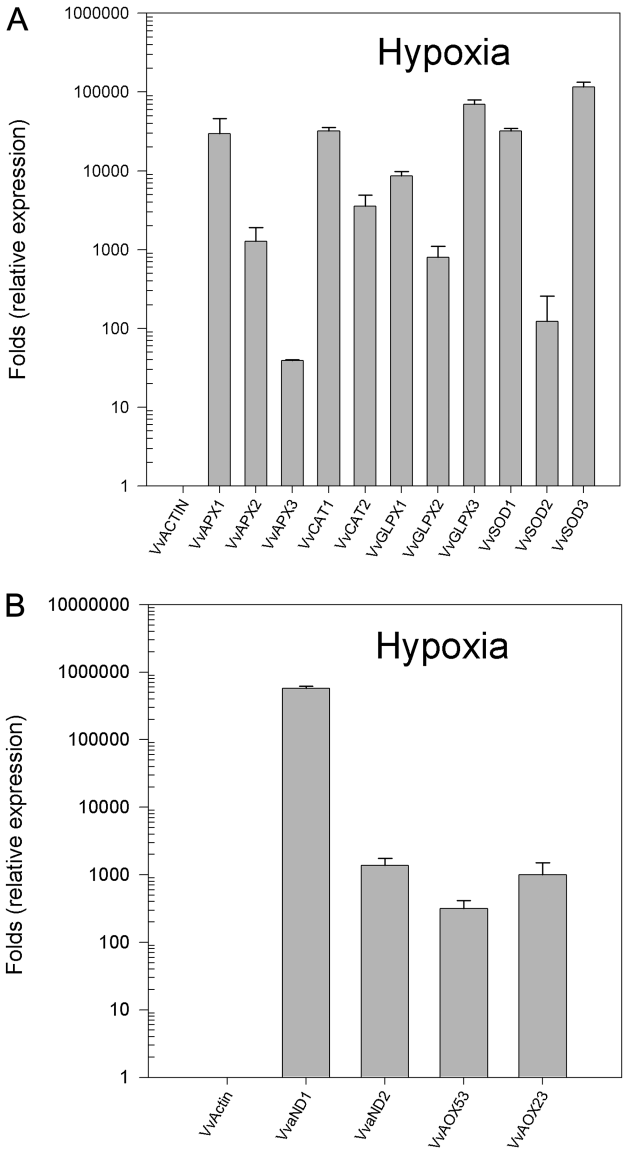

Fig. 3. Effect of hypoxia on the expression of genes encoding (A) antioxidant enzymes and (B) enzymes of the alternative respiratory pathway in grapevine buds. Hypoxia (8% O_2) was applied for 24 h and immediately after treatment gene expression analysis was performed through quantitative real-time PCR. Expression of genes encoding for antioxidant enzymes [ascorbate peroxidase (*VvAPX1*, *VvAPX2*, and *VvAPX3*), glutathione peroxidase (*VvGLPX1*, *VvGLPX2*, and *VvGLPX3*), superoxide dismutase (*VvSOD1*, *VvSOD2* and *VvSOD3*), and catalase (*VvCAT1* and *VvCAT2*)] and for enzymes of the alternative respiratory pathway [oxidase alternative (*VvAOX23* and *VvAOX53*) and alternative dehydrogenases (*VvaND1* and *VvaND2*)] were analysed. Transcript levels were normalized to *VvACTIN* using the ΔΔCT method (Livak and Schmittgen, 2001). Values are means of two biological replicates and bars represent the range of variation of technical replicates.

cis-acting regulatory elements in their promoters and to investigate whether these elements are also present in the promoter of the orthologue of these genes in *Arabidopsis* and in poplar (*Populus trichocarpa*). Fig. 6 shows that common *cis*-acting regulatory elements, anaerobic response element and heat shock element, are present in the promoters of *VvAOX23* and *VvaND1* and in the promoters of their orthologues in *Arabidopsis* and in poplar.

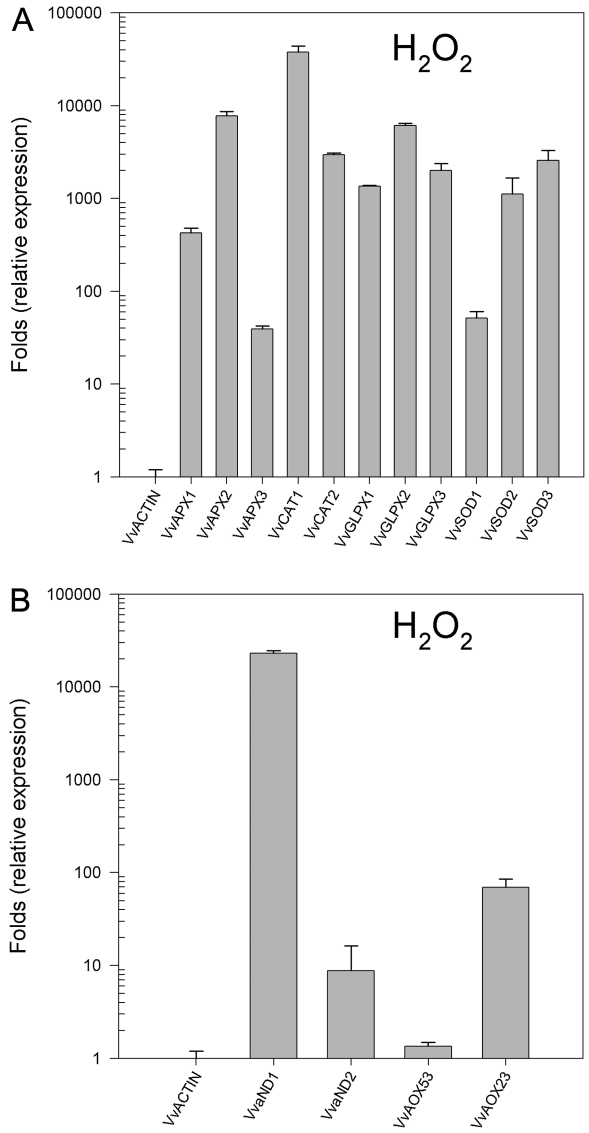

Fig. 4. Effect of H_2O_2 on the expression of genes encoding (A) antioxidant enzymes and (B) enzymes of the alternative respiratory pathway in grapevine buds. H_2O_2 was applied at a concentration of 1% (v/v) and gene expression analysis was performed through quantitative real-time PCR analysis 24 h after treatment. Expression of genes encoding for antioxidant enzymes [ascorbate peroxidase (*VvAPX1*, *VvAPX2*, and *VvAPX3*), glutathione peroxidase (*VvGLPX1*, *VvGLPX2*, and *VvGLPX3*), superoxide dismutase (*VvSOD1*, *VvSOD2* and *VvSOD3*), and catalase (*VvCAT1* and *VvCAT2*)] and for enzymes of the alternative respiratory pathway [oxidase alternative (*VvAOX23* and *VvAOX53*) and alternative dehydrogenases (*VvaND1* and *VvaND2*)] were analysed. Transcript levels were normalized to *VvACTIN* using the ΔΔCT method (Livak and Schmittgen, 2001). Values are means of two biological replicates and bars represent the range of variation of technical replicates.

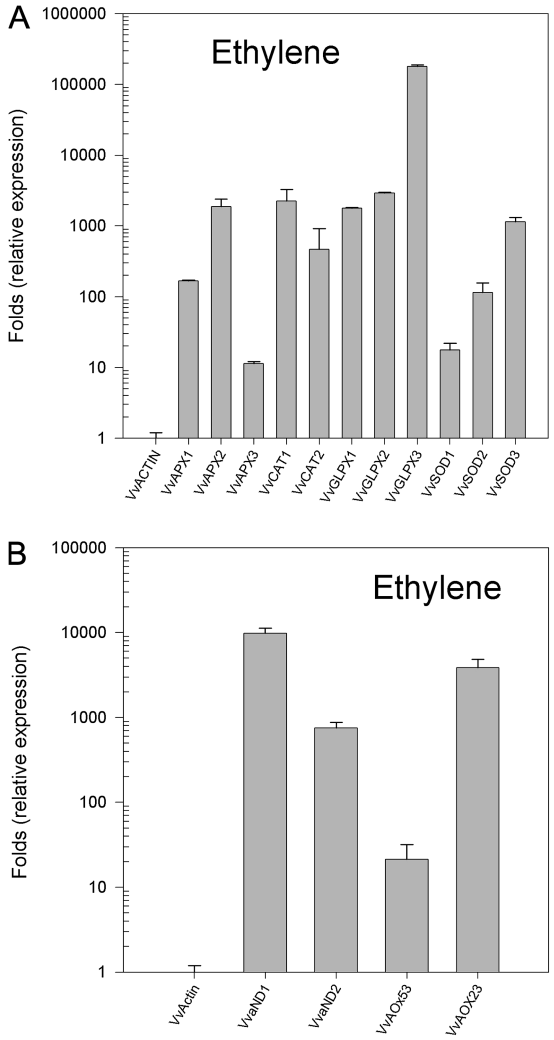

Fig. 5. Effect of ethylene on the expression of genes encoding (A) antioxidant enzymes and (B) enzymes of the alternative respiratory pathway in grapevine buds. Ethylene was applied at a concentration of 1000 ppm and gene expression analysis was performed by quantitative real-time PCR 24 h after treatment. Expression of genes encoding for antioxidant enzymes [ascorbate peroxidase (*VvAPX1*, *VvAPX2*, and *VvAPX3*), glutathione peroxidase (*VvGLPX1*, *VvGLPX2*, and *VvGLPX3*), superoxide dismutase (*VvSOD1*, *VvSOD2* and *VvSOD3*), and catalase (*VvCAT1* and *VvCAT2*)] and for enzymes of the alternative respiratory pathway [oxidase alternative (*VvAOX23* and *VvAOX53*) and alternative dehydrogenases (*VvaND1* and *VvaND2*)] were analysed. Transcript levels were normalized to *VvACTIN* using the ΔΔCT method (Livak and Schmittgen, 2001). Values are means of two biological replicates and bars represent the range of variation of technical replicates.

Discussion

Hypoxia and inhibitors of respiration trigger the production of H_2O_2 in grapevine buds

Hypoxia (8% O_2, 24 h) and inhibitors of respiration (KCN and SNP) triggered H_2O_2 production in grapevine buds. In plants, increases in H_2O_2 levels under hypoxic conditions have been detected in the roots and leaves of *Hordeum vulgare* (Kalashnikov *et al.*, 1994), in wheat roots (Biemelt *et al.*, 2000; Blokhina *et al.*, 2001), and more recently in *Arabidopsis* (Baxter-Burrell *et al.*, 2002). In all cases, the source of H_2O_2 has been assigned to the activation of a NAD(P)H-oxidase sensitive to inhibition by DPI (Riganti *et al.*, 2004). In the current study, DPI did not reduce

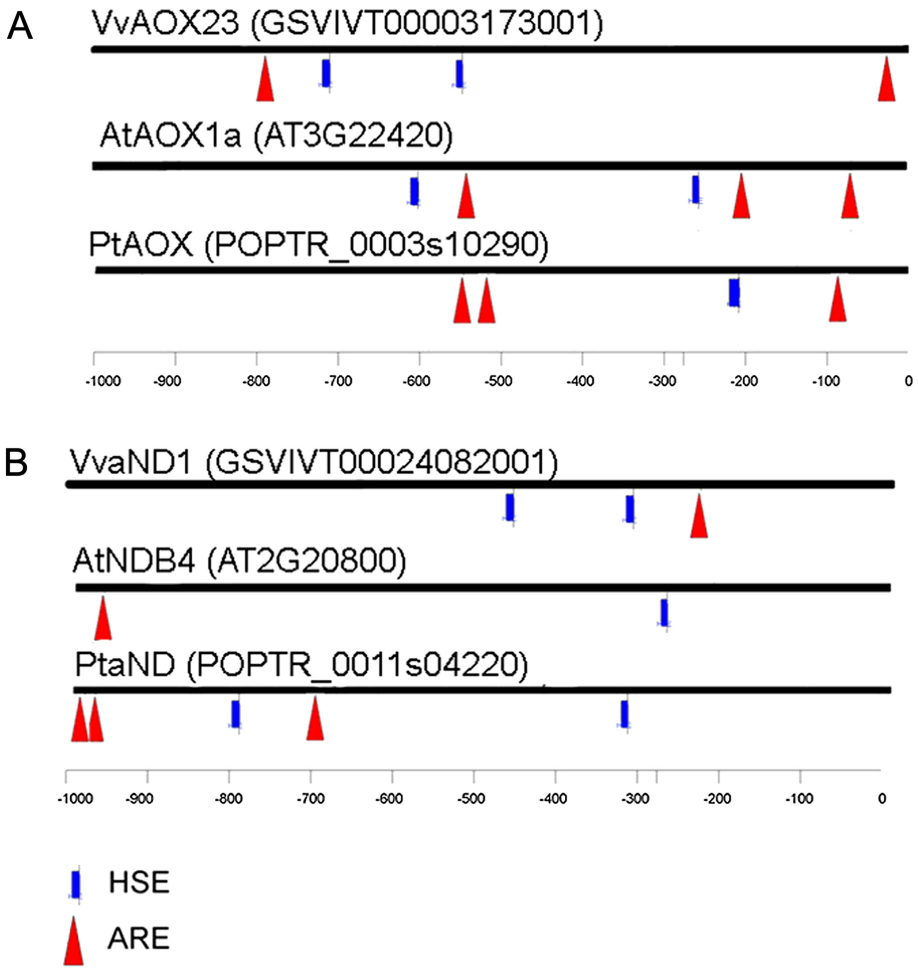

Fig. 6. Position of anaerobic-responsive elements (ARE) and heat shock elements (HSE) putative *cis*-regulators within the 1-kb promoter region of (a) *VvAOX23* and (b) *VvaND1* and their orthologues in *Arabidopsis* and poplar.

the levels of H_2O_2 induced by KCN, suggesting that the disruption of the m-ETC could be potentially the source of H_2O_2 under hypoxic conditions. In cultured mammalian cells, mitochondrial production of ROS increases under conditions of hypoxia, and H_2O_2 plays a crucial role in cell response to hypoxia by regulating the hypoxic responsive factor (HIFα), a transcription factor that regulates the transcription of a number of genes in response to hypoxia (Schofield and Ractliffe, 2004). In plant mitochondria, complex I (Hirst *et al.*, 2008) and complex III (Turrens *et al.*, 1985) are the main sites of ROS production. Nevertheless, in plants there are no reports indicating that low oxygen concentrations trigger increases in mitochondrial ROS production. Interestingly, in grapevine buds among the three stimuli tested, hypoxia was the only one that induced significantly the expression of *VvSOD1*, a gene that encodes a mitochondrial SOD isoenzyme. The increased expression of *VvSOD1* under low oxygen environments could be the result of increased production of the superoxide anion by complex I and III in the mitochondria and supports the hypothesis that increased H_2O_2 production under hypoxia is mainly due to m-ETC disruption.

GABA reduces H_2O_2 levels in grapevine buds by up-regulating VvCAT2

In plants, GABA is produced rapidly and extensively in response to biotic and abiotic stresses and has been linked to the regulation of cytosolic pH, carbon flux in the Krebs cycle, nitrogen metabolism, and protection against oxidative stress (Bouché and Fromm, 2004). Recently, it has been reported that exogenous applications of GABA to seedlings of barley relieves stress caused by oxidative damage of Al^{3+}, increasing the activity of antioxidant enzymes and decreasing the levels of ROS (Song *et al.*, 2010). In intermediate *Caragana*, a leguminous shrub, exogenous application of GABA inhibits the production of H_2O_2 during NaCl stress, which suggests that the reduction in the levels of H_2O_2 may be the result of induced expression of genes related to ROS scavenging (Shi *et al.*, 2010). Contrasting with these results, the current study found that exogenous applications of GABA to grapevine buds repressed the expression of genes encoding antioxidant enzymes (*VvAPX*, *VvGLPX*, *VvSOD*, and *VvCAT1*), except *VvCAT2*, whose expression increased. Furthermore, GABA repressed the expression of genes related to the alternative respiratory pathway, *VvaND1* and

VvAOX23, while increased slightly *VvaND2* and strongly *VvAOX53* expression. GABA can function as a metabolite or as a signalling molecule. As a metabolite, it is converted into succinate, through the action of enzymes γ-aminotransferase and succinyl semilaldehyde dehydrogenase (SSADH) (Bouché and Fromm, 2004), which donates electrons to the ubiquinone pool of the m-ETC, activating the aerobic respiratory pathway. As a signalling molecule, it has been reported that GABA stimulates ethylene production by up-regulating the expression of genes coding for two enzymes of ethylene biosynthesis, 1-aminocyclopropane-1-carboxylic acid synthase and aminocyclopropane-1-carboxylic acid oxidase (Kathiresan *et al.*, 1997). In the current case, GABA did not stimulate ethylene production, since it did not up-regulate the expression of antioxidant genes and genes related to the alternative respiratory pathway, like ethylene. On the contrary, GABA repressed the expression of those genes, suggesting that GABA was acting as a metabolite rather than as a signalling molecule. Furthermore, GABA can reduce the level of H_2O_2 induced by KCN by up-regulating the expression of *VvCAT2* or by stimulating the TCA cycle and simultaneously up-regulating the expression of *VvAOX53*, reducing in this way the redox charge of the ubiquinone pool.

Hypoxia, H_2O_2, and ethylene up-regulate genes encoding antioxidant enzymes and enzymes of the alternative respiratory pathway

H_2O_2 and ethylene production is triggered by hypoxia or inhibitors of respiration and exogenous applications of these molecules induce the expression of antioxidant genes (*VvAPX*, *VvGLPX*, *VvCAT*, and *VvSOD*) and genes related to the alternative respiratory pathway (*VvaNDs* and *VvAOX*) in the same way as hypoxia. Thus, it seems likely that H_2O_2 and ethylene act as signalling molecules in the response of grapevine buds to hypoxia. However, differences in the expression patterns of antioxidant genes induced by the three stimuli suggested that other signalling molecules could also be involved in the response of grapevine buds to hypoxia. Thus, while hypoxia enhanced significantly the expression of *VvAPX1* and *VvSOD1*, the other stimuli, H_2O_2 and ethylene, did not induce their expression. On the other hand, genes related to the alternative respiratory pathway, *VvaND1* and *VvAOX23*, showed a similar expression pattern towards the three stimuli, suggesting that they are co-expressed. Furthermore, sequence analysis of promoter regions of these genes and their orthologues in *Arabidopsis* and poplar showed that they share the regulatory elements anaerobic response element and heat shock element in their promoters. In *Arabidopsis*, the orthologues *AOX1a* and *NDB4*, are up-regulated by H_2O_2 and rotenone and are co-regulated (Clifton *et al.*, 2005; Ho *et al.*, 2008).

The positive regulation of antioxidant genes by H_2O_2 might be the reason for the transient increase in its concentration in grape buds treated with KCN. Compared with other ROS, H_2O_2 is a relatively long-lived molecule that is able to cross cell membranes (Bienert *et al.*, 2006), and this feature is compatible with its role as a signalling molecule (Neill *et al.*, 2002). Gene expression studies clearly show that increased cellular H_2O_2 levels have a significant impact on the transcriptome of all species, changing the expression of hundreds of genes (Vandenbroucke *et al.*, 2008). A meta-analysis of genes regulated by H_2O_2 indicates that induction of genes coding for antioxidant enzymes like APX, GLPX, CAT, and SOD is limited to unicellular organisms, and in *Arabidopsis* none of the antioxidant genes was induced by H_2O_2 (Vandenbroucke *et al.*, 2008). In another study, Vanderauwera *et al.* (2005), using an *Arabidopsis* mutant deficient in catalase, also found that expression of genes encoding APX, GLPX, and SOD enzymes are not up-regulated by H_2O_2. Although, H_2O_2 did not induce the expression of antioxidant genes in *Arabidopsis*, manila grass (*Zoysia matrella*), or mascarene grass (*Zoysia tenuifolia*), exogenous application of H_2O_2 significantly increased the activity of antioxidant enzymes APX, GLPX, CAT, and SOD and improved tolerance to cold stress (Wang *et al.*, 2010), suggesting that among plant species, H_2O_2 regulates the expression of antioxidant genes differently. In grapevines, it will be interesting to investigate if the up-regulation of antioxidant genes by H_2O_2 is restricted to the bud or is a general phenomenon affecting all organs of the plant.

In addition to the general response to oxygen deprivation, consisting in the reconfiguration of central carbon metabolism (Mustroph *et al.*, 2010), the grape bud also increases its antioxidant defence system. These two responses of the bud to hypoxia highlight the importance of respiratory stress as a signal that, on one hand, triggers the release of buds from endodormancy, and, on the other, prepares it to face a stage of high metabolic activity such as bud sprouting.

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