Hypoxia induces $\text{H}_2\text{O}_2$ production and activates antioxidant defence system in grapevine buds through mediation of $\text{H}_2\text{O}_2$ and ethylene

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Abstract

Paradoxically, in eukaryotic cells, hydrogen peroxide ($\text{H}_2\text{O}_2$) accumulates in response to oxygen deprivation (hypoxia). The source of $\text{H}_2\text{O}_2$ under hypoxia varies according to the species, organs, and tissue. In non-photosynthetic tissues, $\text{H}_2\text{O}_2$ 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 $\text{H}_2\text{O}_2$ in grapevine buds. However, diphenyleneiodonium, an inhibitor of NAD(P)H-oxidase, did not reduce the $\text{H}_2\text{O}_2$ levels induced by KCN, suggesting that, under respiratory stress, $\text{H}_2\text{O}_2$ 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 $\text{H}_2\text{O}_2$ induced by KCN and, surprisingly, repressed the expression of genes encoding antioxidant enzymes such as ASCORBATE PEROXIDASE ($\text{VvAPX}$), GLUTATHIONE PEROXIDASE ($\text{VvGLPX}$), SUPEROXIDE DISMUTASE ($\text{VvSOD}$), and one of the CATALASE isoforms ($\text{VvCAT1}$), while $\text{VvCAT2}$ was upregulated. In contrast to GABA, hypoxia, $\text{H}_2\text{O}_2$, and ethylene increased dramatically the expression of genes encoding antioxidant enzymes and enzymes of the alternative respiratory pathway such as ALTERNATIVE NADH-DEHYDROGENASES ($\text{VvNDs}$) and ALTERNATIVE OXIDASES ($\text{VvAOXs}$). Hence, it is concluded that $\text{H}_2\text{O}_2$ production is stimulated by respiratory stress in grapevine buds, that $\text{H}_2\text{O}_2$ and ethylene act as signalling molecules and activate genes related to the antioxidant defence system, and finally that GABA reduces $\text{H}_2\text{O}_2$ levels by up-regulating the expression of $\text{VvCAT2}$.

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 NADP⁺, 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 $\text{H}_2\text{O}_2$ production (Blokchina et al., 2001). However, the source of $\text{H}_2\text{O}_2$ 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 $\text{H}_2\text{O}_2$ production (Baxter-Burrell et al., 2002). $\text{H}_2\text{O}_2$ 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 ubiquinone anion (Moller, 2001). The superoxide anion is rapidly converted to $\text{H}_2\text{O}_2$ 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$_2$O$_2$ 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 (VvNADs)]. This study also determined whether H$_2$O$_2$ and ethylene mediate the antioxidant response of buds to hypoxia. It has been reported that GABA inhibits the accumulation of H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ content

H$_2$O$_2$ concentration was measured by chemiluminescence in a Cobalt-catalysed oxidation of luminol (5-amino-2,3-dihydro-1,4-phthlazinedione) (Pérez and Rubio, 2006).

#### Exogenous applications of GABA, H$_2$O$_2$, 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$_2$O$_2$ (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 Skalitsky, 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 ΔΔCt 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.
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**Table 1. Primers used for real-time quantitative RT-PCR experiments**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Locus (GENOSCOPE)</th>
<th>Forward primer (5’ → 3’)</th>
<th>Reverse primers (5’ → 3’)</th>
</tr>
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<td>VvASPX1</td>
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<td>AAATGGGTCTCTAGGGCAGAAG</td>
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<td>GCCGATCCACAGCTTCTCAT</td>
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</tr>
<tr>
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<tr>
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<td>GCCCAAGATGCTTGTACCTT</td>
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Sequence analysis of promoter regions

Sequences of 1000 bp upstream from the transcription start site of the selected genes were downloaded 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 \( \text{H}_2\text{O}_2 \) production in grapevine buds**

Hypoxia (8% \( \text{O}_2 \) for 24 h) doubled the level of \( \text{H}_2\text{O}_2 \) in grapevine buds (Fig. 1A). To test whether the increase in \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \), suggesting that increases in \( \text{H}_2\text{O}_2 \) levels may be due to disruption in m-ETC rather than activation of NAD(P)H-oxidase.

**KCN and SNP trigger \( \text{H}_2\text{O}_2 \) production in grapevine buds**

KCN and SNP, which decompose to nitric oxide (NO) and cyanide (Bethke et al., 2006), increased the level of \( \text{H}_2\text{O}_2 \) in grapevine buds (Fig. 1B). The effect of KCN was higher than that of SNP, but in both cases, the increase in \( \text{H}_2\text{O}_2 \) level was transient, since 24 h after treatment the level of \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) levels in grapevine buds**

GABA reduced significantly the levels of \( \text{H}_2\text{O}_2 \) in grape cuttings sprayed with KCN (Fig. 1D). The levels of \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) 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), glutathione-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, \( VvNDs \) and \( VvAOXs \). GABA regulated differently the expression of the two NAD(P)H-dehydrogenase genes that are expressed in grape buds (\( VvND1 \) and \( VvND2 \)); 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).
Hypoxia, H$_2$O$_2$, 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$_2$O$_2$, ethylene or both, this study analysed by qRT-PCR the effect of hypoxia and exogenous applications of H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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
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.
Hypoxia and inhibitors of respiration trigger the production of H$_2$O$_2$ in grapevine buds

Hypoxia (8% O$_2$, 24 h) and inhibitors of respiration (KCN and SNP) triggered H$_2$O$_2$ production in grapevine buds. In plants, increases in H$_2$O$_2$ 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$_2$O$_2$ has been assigned to the activation of a NAD(P)H-oxidase sensitive to inhibition by DPI (Riganti et al., 2004).

**Discussion**

Hypoxia and inhibitors of respiration trigger the production of H$_2$O$_2$ in grapevine buds

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the levels of H$_2$O$_2$ induced by KCN, suggesting that the disruption of the m-ETC could be potentially the source of H$_2$O$_2$ under hypoxic conditions. In cultured mammalian cells, mitochondrial production of ROS increases under conditions of hypoxia, and H$_2$O$_2$ plays a crucial role in cell response to hypoxia by regulating the hypoxic responsive factor (HIF), 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 iso-enzyme. 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$_2$O$_2$ production under hypoxia is mainly due to m-ETC disruption.

GABA reduces H$_2$O$_2$ 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$_2$O$_2$ during NaCl stress, which suggests that the reduction in the levels of H$_2$O$_2$ 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

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**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.

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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 semialdehyde 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-aminoacyclopropane-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 H2O2 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, H2O2, and ethylene up-regulate genes encoding antioxidant enzymes and enzymes of the alternative respiratory pathway

H2O2 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 (VvaND1 and VvAOX) in the same way as hypoxia. Thus, it seems likely that H2O2 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, H2O2 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 H2O2 and rotenone and are co-regulated (Clifton et al., 2005; Ho et al., 2008).

The positive regulation of antioxidant genes by H2O2 might be the reason for the transient increase in its concentration in grape buds treated with KCN. Compared with other ROS, H2O2 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 H2O2 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 H2O2 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 H2O2 (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 H2O2. Although, H2O2 did not induce the expression of antioxidant genes in Arabidopsis, manila grass (Zoysia matrella), or mascarene grass (Zoysia tenuifolia), exogenous application of H2O2 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, H2O2 regulates the expression of antioxidant genes differently. In grapevines, it will be interesting to investigate if the up-regulation of antioxidant genes by H2O2 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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References


