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ORIGINAL PAPER

Is GABA-shunt functional in endodormant grapevine buds under respiratory stress?

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Abstract It has been suggested that a respiratory stress is part of the mechanism through which the dormancy-breaking compounds, hydrogen cyanamide (HC) and sodium azide, induce the release of buds from the endodormancy (ED) in grapevines. The accumulation of metabolites like succinate, alanine (Ala) and γ-amino butyric acid (GABA), together with the activation of the GABA-shunt pathway, is a general feature of plants in response to oxygen deprivation and to respiratory stress. Unexpectedly, in a previous study, we found that GABA applied exogenously to grapevine buds, down-regulated the expression of most genes encoding for antioxidant enzymes, suggesting that its accumulation under respiratory stress conditions could be deleterious for the bud. In order to analyze whether GABA accumulates under respiratory stress conditions in grapevine buds, we analysed in this study, the effect of hypoxia, the respiration inhibitor KCN and the dormancy breaker compound HC, on the level of GABA, and on the expression levels of the GABA-shunt genes (*VvGAD*, *VvGABA-T*, *VvSSADH*). Additionally, genes from the Ala fermentative pathway (*VvAlaAT*, V*vAspAT*) were also analysed. The results revealed that although the three treatments mentioned above, up-regulated the expression of *VvGAD1*, the content of GABA remained constant, while Ala content increased. The lack of GABA accumulation under respiratory stress is an important physiological fact in grapevine buds, since it avoids the down-regulation of antioxidant genes, and promotes the incorporation of succinate into the TCA cycle, a fact that would be important in the release of buds from the ED.

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Introduction

In response to oxygen deprivation, plants accumulate alanine (Ala), succinate and γ-amino butyric acid (GABA) (Miyashita and Good 2008). This phenomenon is currently explained by the activation of the GABA-shunt or the Ala fermentative pathway (Fait et al. 2007; Rocha et al. 2010). The GABA-shunt is a metabolic pathway that incorporates glutamate (Glut) in the TCA cycle, bypassing two enzymes of the cycle, specifically α-ketoglutarate dehydrogenase and Succinyl-CoA ligase (Sweetlove et al. 2010). The GABA-shunt, in turn is composed of three enzymes; the cytosolic enzyme glutamate decarboxylase (GAD) that transforms glutamate (Glut) into GABA, the mitochondrial enzymes GABA transaminase (GABA-T) that converts GABA into succinic semialdehyde (SSA), and succinic semialdehyde dehydrogenase (SSADH) that converts SSA into succinate (Bouché and Fromm 2004). SSA an intermediate of the GABA-shunt could either be oxidized to succinate (Bouché et al. 2003) or reduced to γ-hydroxybutyrate, by the enzyme γ-hydroxy butyrate reductase (GHBDH) (Breitkreuz et al. 2003). In plants, disruption of the gene encoding SSADH leads to high levels of reactive oxygen species (ROS) associated with dwarfism, chlorotic leaves and extensive necrotic lesions (Bouché et al. 2003). This phenotype is exacerbated upon exposure to environmental stresses such as high light intensity, UV-B and heat. Treatment with γ-vinyl-γ-aminobutyrate (VGB), a specific GABA-T inhibitor, prevents the accumulation of ROS in *ssadh* Arabidopsis mutants (Fait et al. 2005). The double mutant *pop2* and *ssadh* also suppress the severe phenotype

of *ssadh* mutant indicating that *pop2* is epistatic to *ssadh* (Ludewig et al. 2008). Both results, suggest that SSA or γ-hydroxybutyrate, but not GABA could be responsible of ROS accumulation. In a recent study, we found that GABA applied exogenously to grapevine buds, down-regulated the expression of genes encoding for antioxidant enzymes such ascorbate peroxidase (*VvAPX*), glutathione peroxidase (*VvGLPX*), superoxide dismutase (*VvSOD*) and one of the catalase isoforms (*VvCAT1*), while the expression of *VvCAT2* was up-regulated (Vergara et al. 2012a). Since in plants, GABA accumulates in response to respiratory stress and hypoxia (Fait et al. 2007; Miyashita and Good 2008), and since respiratory stress is involved in the induction of bud-ED release in grapevines (Ophir et al. 2009; Pérez et al. 2009), it would be interesting to analyze whether GABA accumulates during respiratory stress in grapevine buds. In this study, the effects of hypoxia, of the respiration inhibitor (KCN) and of the dormancy breaker compound HC on the levels of GABA and on the expression of the GABA-shunt genes were studied.

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 on the 26 April of 2012, at the endodormant stage, 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 the 26 April were separated in four groups of 30 single bud cuttings each. Group one and two were sprayed with 2.5 % (w/v) HC, (Dormex, SKW, Trotsberg, Germany) and 2 % (w/v) potassium cyanide KCN (Merck, Germany) respectively, group three was exposed to hypoxia for 24 h and group four was sprayed with water and used as control. 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 carried-out immediately (0), 3, 6 and 24 h post-treatment. For chemical treatments, the analysis was performed 4, 24 and 48 h post-treatment, during the post-treatment period, buds were placed under forcing conditions in a growth chamber set at 23 ± 2 °C, 14 h light (150 μmol m^{-2} s^{-1}). Three biological replicates ($n = 10$) were performed for each treatment.

GABA and Ala determinations

γ-amino butyric acid was determined using the GABase assay as described by Zhang and Bown (1997). Buds were ground in liquid N_2 using a mortar and pestle. To about 0.1 g of the frozen powder 1 ml of 70 mM lanthanum chloride (Sigma–Aldrich, St Louis, USA) was added and the sample shaken for 15 min, centrifuged at 13,000*g* for 15 min, and 0.8 ml of the supernatant transferred to a second tube. To this was added 200 μl of 1 M KOH followed by shaking for 5 min and centrifuging as before. The resulting supernatant was used for the spectrophotometric determination of GABA and Ala. The 1 ml assay contained 550 μl of the sample, 150 μl 4 mM $NADP^+$, 200 μl 0.5 M buffer pyrophosphate K^+ (pH 8.6), 50 μl of 2 units GABASE from *pseudomonas fluorescens* (Sigma–Aldrich, St Louis, USA) and 50 μl of 20 mM α-ketoglutarate. The difference in Absorbance at 340 nm between samples with and without GABASE was determined after 1 h and a calibration curve in the range of 10–100 nmol of GABA was constructed. Ala was determined by the AlaDH assay (Miyashita and Good 2008) in a mix containing 150 mM Tris–HCl (pH 9.0), 1 mM NAD^+ and 0.02 U of *Bacillus subtilis* AlaDH (Sigma–Aldrich, St Louis, USA). The difference in absorbance at 340 nm between samples with and without AlaDH was determined after 1 h at 30 °C and a calibration curve in the range 10–500 nmol ml^{-1} of Ala was constructed.

RNA purification and cDNA synthesis

Total RNA was isolated and purified from grapevine buds (0.5–0.7 g. fr. wt) using a modification of Chang et al. (2000) method, described in Noriega et al. (2007). DNA was removed by treatment with RNAase-free DNAase (1 U μg^{-1}) (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, CA, USA).

Quantitative real-time PCR

Quantitative real-time PCR was carried-out in an Eco Real-Time PCR system (Illumina, Inc. SD, USA) using the intercalation dye SYBRGreen I as a fluorescent reporter and Platinum Taq DNA Polymerase (Invitrogen, CA, USA). Primers suitable for amplification of 100–150 bp

products for each gene under study were designed using the PRIMER3 software (Rozen and Skaletsky 2000) and are presented in Table 1. For *V. vinifera* glutamate decarboxylase (*VvGAD*) three genes were identified by Blat search in GENOSCOPE (http://www.genoscope.cns.fr) using the sequences of the five *Arabidopsis* GAD_{1-5} as query (Bouché and Fromm 2004). Unique genes for *VvGABA-T*, *VvSSADH*, *VvGHBDH* and *VvAlaAT* were identified in GENOSCOPE using *Arabidopsis* sequences as query and their sequences were used for primers design. Amplification of cDNA was carried-out under the following conditions: 2 min denaturation 94 °C; 40 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s. Three 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). Induction or repression of gene expression was calculated by the ΔΔCq method (Livak and Schmittgen 2001) using *VvUBIQUITIN* as reference gene. *VvUBIQUITIN* was selected as a reference gene because the transcript level was stable across the treatments.

Results

Hypoxia, respiratory inhibitors KCN and the dormancy breaker HC up-regulated *VvGAD1* gene expression in grapevine buds

Three glutamate decarboxylase genes were identified in the public *V. vinifera* genomic database GENOSCOPE; *VvGAD1*, *VvGAD2* and *VvGAD3* (Table 1). Figure 1a, shows that hypoxia (24 h treatment), up-regulated transiently the level of *VvGAD1* without affecting the levels of *VvGAD2* and *VvGAD3*, in grapevine buds. However, 3 h post-treatment, while the level of expression *VvGAD1* fell to its initial value, the levels of expression of *VvGAD2* and *VvGAD3* increased, peaking 6 h post-treatment. The expression level of *VvGAD1* responded rapidly to the current level of oxygen in the grapevine buds, since after 3 h under hypoxia, reached its highest level of expression (Fig. 1b), while 3 h post-treatment (24 h treatment), its expression level fell drastically (Fig. 1a). Similarly to the hypoxia effects, KCN up-regulated the level of *VvGAD1* without affecting the levels of *VvGAD2* and *VvGAD3* (Fig. 1c), while HC in addition to up-regulated *VvGAD1*, down-regulated *VvGAD2* in the short time, while at longer times, strongly induced the expression of *VvGAD3*. Interestingly, in control buds, transcript levels of *VvGAD1* and *VvGAD3* were significantly lower than those of *VvGAD2* (Fig. 2).

Hypoxia, KCN and HC regulated differently *VvGABA-T*, *VvSSADH* and *VvGHBDH* gene expression in grapevine-buds

Single genes of *VvGABA-T*, *VvSSADH* and *VvGHBDH* were identified in the public *V. vinifera* genomic database GENOSCOPE (Table 1), they all showed high identity with the corresponding genes of Arabidopsis. The three treatments did not significantly alter the expression level of *VvGABA-T* in relation to the control. However, they affect differently the level of expression of *VvSSADH* because, while KCN and HC up-regulated, hypoxia down-regulated its expression level. Furthermore, *VvGHBDH* expression level was significantly down-regulated by hypoxia and KCN, but not by HC (Fig. 3b, c).

Hypoxia, KCN and HC regulated differently *VvAlaAT* and *VvAspAT1* gene expression in grapevine-buds

A single gene of *VvAlaAT* was identified in the *V. vinifera* genomic database, GENOSCOPE (Table 1). In grapevine buds, gene expression analysis revealed that *VvAlaAT* was up-regulated by hypoxia (24 h treatment), transcript levels increased further during 3–6 h post-treatment (re-oxygenation period), but later 24 h post-treatment fell significantly (Fig. 4a). In contrast KCN and HC down-regulated the expression of *VvAlaT* (Figs. 4b, c, 5). Aspartate

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

Genes	Locus (GENOSCOPE)	Forward primer	Reverse primer
VvGAD1	GSVIVT00030677001	5′ CCAAGGTGCTCCATGAACCTT 3′	5′ CATCGGTGATTTCCCTCTGT 3′
VvGAD2	GSVIVT00006041001	5′ CGTGAGGAGATTGCCATGTA 3′	5′ AGAAGACGCATTTGGAGGTG 3′
VvGAD3	GSVIVT00023484001	5′ AGACGGCATCAGAGTCCAAT 3′	5′ CTTGGGAGCGAAACTCTAACA 3′
VvGABA-T	GSVIVT00037592001	5′ GTTGCGGCTGCTAGAAGTTT 3′	5′ CATCTTCCCTTTGCATGGAT 3′
VvSSADH	GSVIVT00002101001	5′ TTGTGGCTCGGCTGAATAG 3′	5′ CGGTATGGTCTTCCCATCAT 3′
VvGHBDH	GSVIVT00013336001	5′ GACCTGGGTGGAATTGCTAA 3′	5′ CAAGAGCAAGAGCCAACCTC 3′
VvAlaAT	GSVIVT00033997001	5′ GGTGCACAATACTGCCTCAA 3′	5′ CGAAACTCCTCCATGAATCC 3′
VvAspAT1	GSVIVT00032723001	5′ TTGACAGCCTCTCTGCAAAA 3′	5′ CAGCCCTGTGAATGAGAA 3′

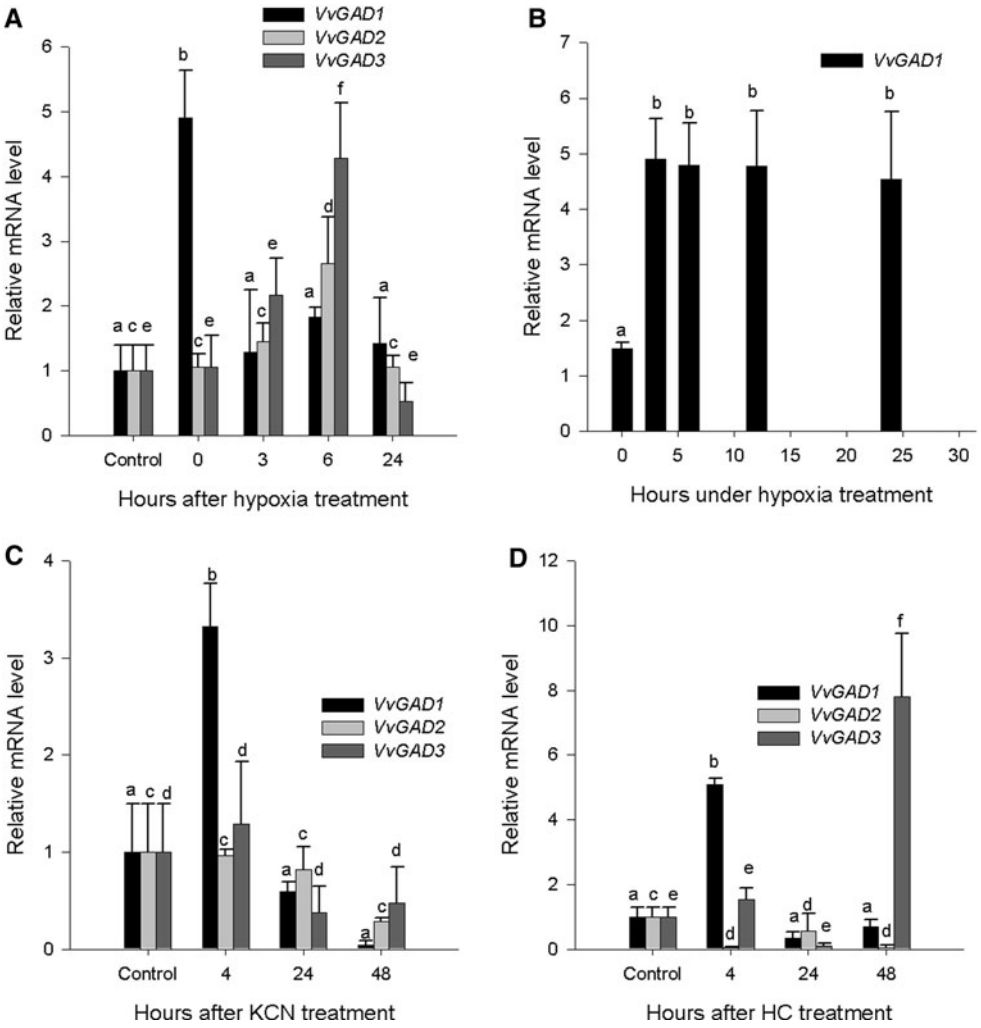

Fig. 1 Effect of hypoxia, KCN and HC on the expression of glutamate decarboxylase genes *VvGAD1*, *VvGAD2* and *VvGAD3* in endodormant grapevine-buds. **a** Hypoxia treated buds (24 h of hypoxia) were analyzed immediately and, 3, 6 and 24 h post-treatment. **b** Hypoxia effect on grapevine buds was also analyzed after 3, 6, 12 and 24 h of treatment. Effect of KCN and HC was analyzed 4, 24 and 48 h post-treatment (**c**, **d**). Gene expression analysis was performed by RT-qPCR using *VvUBIQUITIN* as control gene. Values are means of three biological replicates, *bars* marked with *different letters* are judged to be significantly different from each other (one way ANOVA, $P < 0.05$)

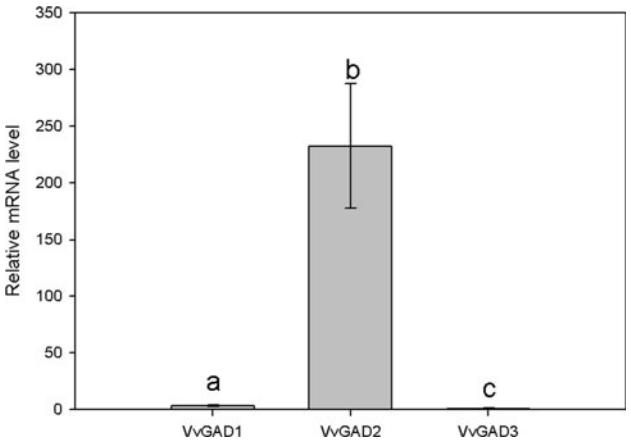

Fig. 2 Level of expression of *VvGAD1* and *VvGAD2* relative to *VvGAD3* in control grapevine buds. Gene expression analysis was performed by RT-qPCR using *VvUBIQUITIN* as reference gene. Values are means of three biological replicates, *bars* marked with *different letters* are judged to be significantly different from each other (one way ANOVA, $P < 0.05$)

aminotransferase (AspAT) catalyses the reversible transfer of an amino group from aspartate to 2-oxoglutarate to form Glut and oxalacetate (Fig. 6). In grapevines, *VvAspAT1* corresponds to one of the three aspartate aminotransferase genes that is expressed in the bud, its expression level was induced by hypoxia and KCN, but not by HC (Fig. 4a, b, c).

Hypoxia, KCN and HC increased Ala without affecting GABA concentration in grapevine-buds

GABA and Ala concentrations were determined in grapevine buds collected on April 26, and exposed to hypoxia or treated with KCN and HC. Buds exposed to hypoxia for 24 h, were analyzed immediately after treatment, whereas buds treated with KCN or HC, were analyzed 48 h after treatment. Results showed that none of the treatments significantly affected the concentration of GABA (Fig. 5a), but they all increased by approximately 20 % the concentration of Ala (Fig. 5b).

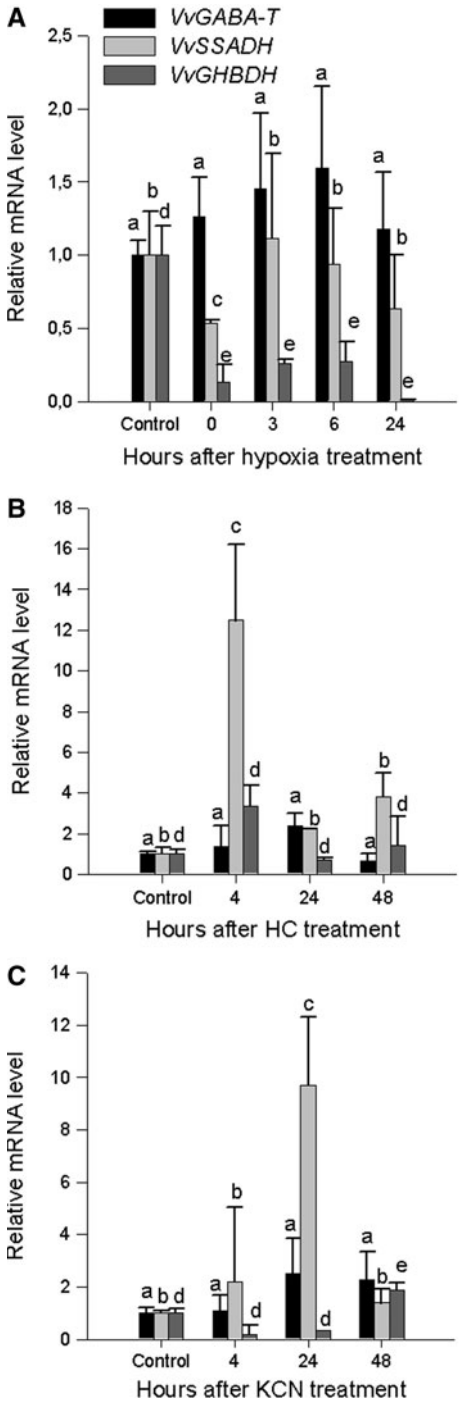

Fig. 3 Effect of hypoxia, KCN and HC on the expression of the GABA-shunt genes *VvGABA-T*, *VvSSADH* and *VvGHBDH* in endodormant grapevine-buds. **a** Hypoxia treated buds (24 h of hypoxia) were analyzed immediately 0, 3, 6 and 24 h post-treatment. **b**, **c** Effect of KCN and HC was analyzed 4, 24 and 48 h after the respective treatments. Gene expression analysis was carried-out by RT-qPCR using *VvUBIQUITIN* as reference gene. Values are means of three biological replicates, *bars* marked with *different letters* are judged to be significantly different from each other (one way ANOVA, $P < 0.05$)

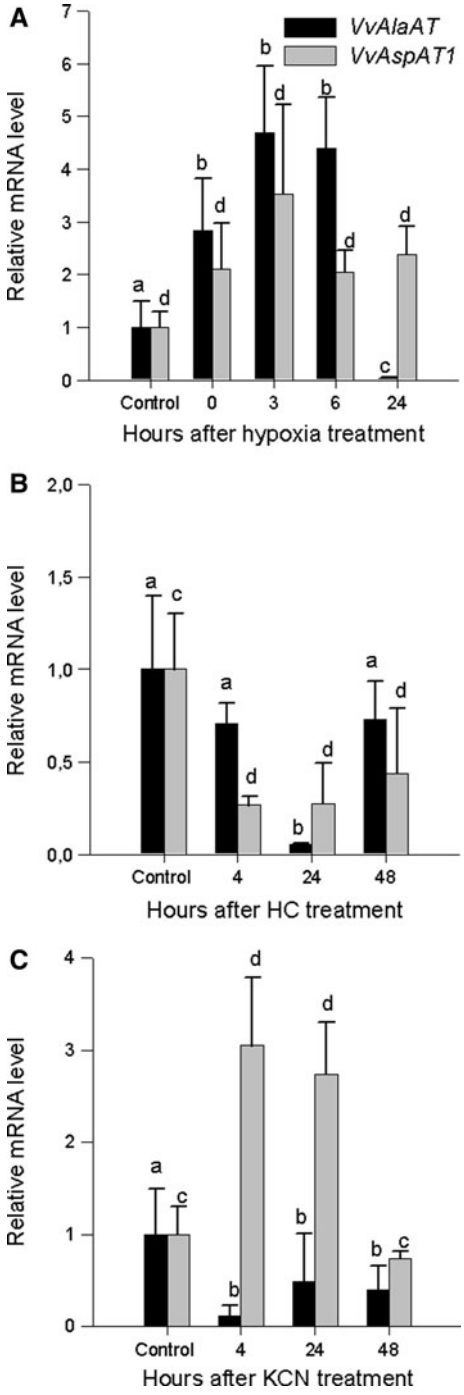

Fig. 4 Effect of hypoxia, KCN and HC on the expression of *VvAlaAT* and *AspAT1* in endodormant grapevine-buds. **a** Hypoxia treated buds (24 h of hypoxia) were analyzed immediately treatment 0, 3, 6 and 24 h post-treatment. **b**, **c** Effect of KCN and HC was analyzed 4, 24 and 48 h after the respective treatments. Gene expression analysis was carried-out by RT-qPCR using *VvUBIQUITIN* as reference gene. Values are means of three biological replicates, *bars* marked with *different letters* are judged to be significantly different from each other (one way ANOVA, $P < 0.05$)

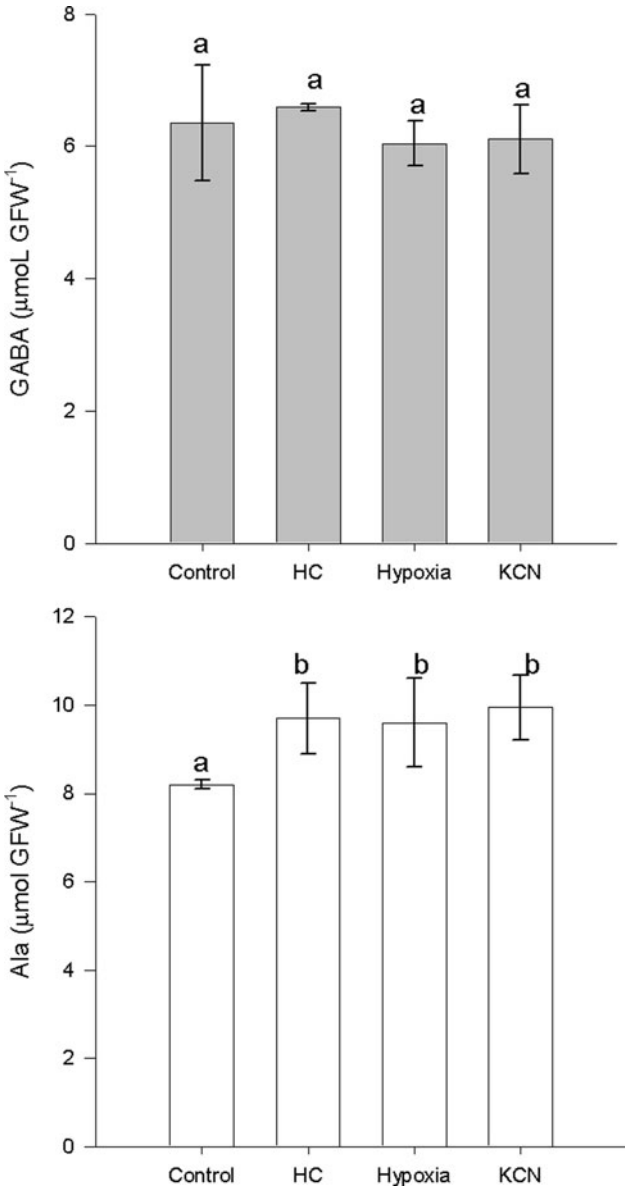

Fig. 5 Effect of hypoxia, KCN and HC on the concentration of GABA and Ala in endodormant grapevine-buds. Hypoxia treated buds (24 h of hypoxia) were analyzed immediately after the treatment, while KCN and HC treated buds were analyzed 48 h after the treatments. Values are means of three biological replicates, *bars* marked with *different letters* are judged to be significantly different from each other (one way ANOVA, $P < 0.05$)

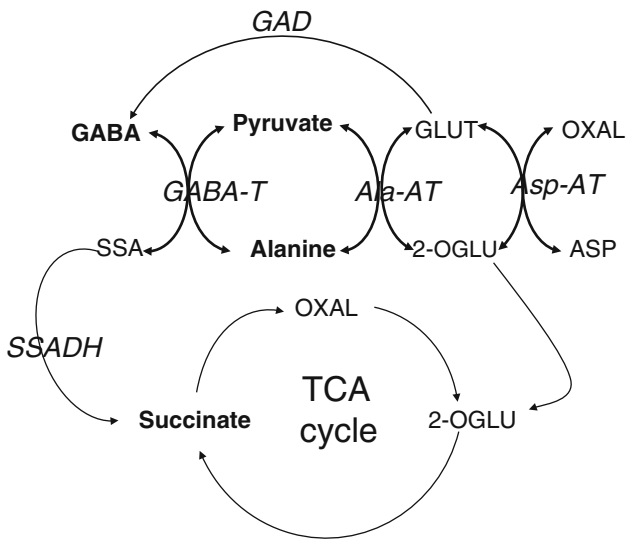

Fig. 6 Metabolic model of primary metabolism in grapevine-buds under respiratory stress. A model is described that agree with transcriptomic changes observed in grapevine-buds during respiratory stress. Pyruvate production is enhanced due to the activation of glycolysis (Pasteur effects). Glut is mainly transformed to Ala by the Ala fermentative pathway (*VvAlaAT*) during hypoxia, while in KCN and HC treated buds, Glut is mainly transformed to GABA and further to succinate by the GABA-shunt pathway (*VvGABA-T* and *VvSSADH*)

Discussion

Hypoxia, KCN and HC up-regulate *VvGAD1* gene expression without affecting the content of GABA in grapevine buds

In plants, GABA is largely and rapidly accumulated in response to stressful conditions (hypoxia, salt stress, heat or cold shock, drought and mechanical injury) (Bouché et al. 2003; Youn et al. 2011), and activation of the GABA-shunt has been associated with regulation of the cytosolic pH, carbon fluxes in the TCA cycle, nitrogen metabolism, protection against oxidative stress, osmoregulation and signalling (Bouché and Fromm 2004; Fait et al. 2007). Glutamate decarboxylase (GAD) and diamine-oxidase (DAO) are the rate limiting enzymes for GABA synthesis (Wakte et al. 2011). Among the three glutamate-decarboxylase genes that expressed in grapevine buds, only *VvGAD1* was up-regulated by the three treatments; hypoxia, KCN and HC, suggesting that a respiratory stress produced either by oxygen deprivation or by chemical inhibition of the cytochrome oxidase (COX), with the subsequent inhibition of the oxidative phosphorylation, might be a signal that regulates the transcriptional activation of *VvGAD1*. It has been reported that HC inhibits mitochondrial respiration (Pérez et al. 2009). Although, the three treatments up-regulated the expression level of *VvGAD1*, the content of GABA remained constant. The fact that under respiratory stress, GABA content remains constant, despite the increase in the level of *VvGAD1* could be interpreted as if the highest level of expression of *VvGAD1* had no effect on GABA synthesis, or if the expression of genes related to GABA degradation (*VvGABA-T*, *VvSSADH*) increases proportionally to *VvGAD1*. The level of *VvGABA-T* remained relatively stable under the three treatments, while the level of *VvSSADH* was strongly induced by KCN and HC, suggesting that these

two chemicals favour the degradation of GABA into succinate. Furthermore, the fact that the Ala content increased in parallel with a reduction in the level of *VvAlaAT* in KCN and HC-treated buds, indicates that Ala accumulation comes from the reaction catalyzed by the *VvGABA-T* enzyme (Fig. 6), and that the up-regulation of *VvGAD1* by the respiratory stress, increases the synthesis of GABA. In Arabidopsis, five GAD genes have been identified in its genome and only one of them (*GAD4*) is up-regulated by hypoxia (Miyashita and Good 2008). Sequence homology studies revealed that *AtGAD4* has the closest identity match (82 %) with *VvGAD3* which is not induced by hypoxia, while *VvGAD1* has the closest identity match (83 %) with *AtGAD1* which express only in roots (Miyashita and Good 2008).

Respiratory stress induces Ala accumulation in grapevine buds

Alanine aminotransferase (AlaAT) catalyses the reversible transfer of an amino group from glutamate (Glut) to pyruvate to form 2-oxoglutarate and alanine (Ala) (Fig. 6). The regulation of the *AlaAT* gene has been studied in response to low oxygen stress and in different plant species, it has been reported that Ala is accumulated under respiratory stress caused either by inhibitors of respiration or by oxygen limitations (Garnier et al. 2008; Miyashita et al. 2007). Several metabolic pathways have been proposed to explain Ala accumulation under oxygen deficiency. A rapid induction of *AlaAT*, a gene encoding for the enzyme Ala aminotransferase, as well as an increase in its activity (Muench and Good 1994) could potentially explain the increased production of Ala. However, Ala production does not depend solely on the catalytic activity of AlaAT, an alternative reaction, that produces Ala is catalysed by GABA-T using pyruvate as co-substrate (Fig. 6) (Miyashita and Good 2008). In grapevines, the three treatments mentioned above, increased the Ala content in the bud-tissue. However, while hypoxia induced *VvAlaAT* without affecting *VvSSADH*, KCN and HC repressed *VvAlaAT*, inducing the expression of *VvSSADH*. These results, suggest that the accumulation of Ala has distinct origins depending on the treatment. In hypoxia treatment, Ala comes mainly from the reaction of Glut and pyruvate catalyzed by the *VvAlaAT*, while in KCN and HC treatments Ala comes from the reaction of GABA with pyruvate catalyzed by the *VvGABA-T* (Fig. 6).

Respiratory stress activates the GABA-shunt and promotes the bud-break in grapevine buds

The respiratory stress, which is involved in the mechanism of bud-ED release in grapevines (Ophir et al. 2009; Pérez et al. 2009), disturbs the proper mitochondrial respiration, and the proper ATP production. This leads to increased production of ROS, decreased recycling of NAD/NADP and decreased production of ATP. To face this crisis, the antioxidant machinery, the alternative oxidase and glycolysis are induced to reduce the production of ROS, to allow recycling of NAD/NADP and to overcome limited production of ATP (Vergara et al. 2012a, b). Despite that GABA accumulates in most plants under respiratory stress conditions (Bouché et al. 2003), in the case of *Vitis*, it has been reported that exogenous applications of GABA to the buds down-regulated significantly the expression of genes encoding for antioxidants enzymes such as ascorbate peroxidase (APX), catalase (CAT), glutathione-peroxidase (GLPx) and superoxide dismutase (SOD) (Vergara et al. 2012a), therefore, its accumulation under respiratory stress conditions should be avoided. Effectively, in this study, it was shown that although the respiratory stress induced the expression of *VvGAD1*, the GABA content was kept constant, while Ala content increased. The lack of GABA accumulation under respiratory stress conditions, could have important physiological consequences for the survival of the grapevine bud, since on one hand, avoids the detrimental effect caused by the repression of genes related to the antioxidant defence system, and on the other, its degradation to succinate, through the GABA-shunt pathway, activates the TCA cycle, which could be of great importance for the release of buds from the ED. In kiwifruit, it has been reported that prior to bud sprouting, the respiration rate of buds increase significantly (McPherson et al. 1997), and these respiratory increases during the recess period could require a rapid activation of the TCA cycle.

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