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Differences in respiration between dormant and non-dormant buds suggest the involvement of ABA in the development of endodormancy in grapevines

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ABSTRACT

Grapevine buds (*Vitis vinifera* L) enter endodormancy (ED) after perceiving the short-day (SD) photoperiod signal and undergo metabolic changes that allow them to survive the winter temperatures. In the present study, we observed an inverse relationship between the depth of ED and the respiration rate of grapevine buds. Moreover, the respiration of dormant and non-dormant buds differed in response to temperature and glucose, two stimuli that normally increase respiration in plant tissues. While respiration in non-dormant buds rose sharply in response to both stimuli, respiration in dormant buds was only slightly affected. This suggests that a metabolic inhibitor is present. Here, we propose that the plant hormone abscisic acid (ABA) could be this inhibitor. ABA inhibits respiration in non-dormant buds and represses the expression of respiratory genes, such as *ALTERNATIVE NADH DEHYDROGENASE* (*VaND1*, *VvaND2*), *CYTOCHROME OXIDASE* (*VvCOX6*) and *CYTOCHROME C* (*VvCYTC*), and induces the expression of *VvSnRK1*, a gene encoding a member of a highly conserved family of protein kinases that act as energy sensors and regulate gene expression in response to energy depletion. In addition to inducing ED the SD-photoperiod up-regulated the expression of *VvNCED*, a gene that encodes a key enzyme in ABA synthesis. Taken together, these results suggest that ABA through the mediation of *VvSnRK1*, could play a key role in the regulation of the metabolic changes accompanying the entry into ED of grapevine buds.

1. Introduction

The growth of woody-perennial plants in temperate zones is characterized by alternating periods of growth and dormancy in response to seasonal fluctuations in environmental conditions, such as temperatures and photoperiod (Rohde and Bhalereao, 2007). Although there are many definitions of bud-dormancy, today it is considered a transient state of the meristem. During bud dormancy, the meristem suspends its activity and becomes insensitive to growth-promoting signals for a period of time before growth resumes (Rohde and Bhalereao, 2007). According to Lang et al. (1987), the lack of activity in the meristem may be due to factors located outside the bud, known as paradormancy (PD) and/or factors located within the bud, known as endodormancy (ED) or true dormancy. At the end of the growing season, the transition from PD to ED allows buds to survive winter temperatures (Morrison, 1991). In *V. vinifera* and other members of the *Vitaceae* family, the meristem of the latent bud differentiates into all of the basic elements of the shoots, including uncommitted primordium, which develop into tendrils or inflorescence (Butrosse, 1974; Mullins et al., 1992). However, all of these developmental events are interrupted by the entrance of the latent bud into ED. At the end of ED, buds resume shoot growth associated with flower formation and development (Morrison, 1991). In contrast to poplar and other tree species, *Vitis* does not set terminal buds in response to the SD-photoperiod. Upon reaching a critical day length (CDL), other hallmark phenotypic responses such as periderm development, growth cessation and bud dormancy are induced (Fennell and Hoover, 1991; Wake and Fennell 2000; Grant et al., 2013). The outgrowth of the latent bud is initially prevented by PD signals from the apex (He et al., 2012). However, these latent buds maintain cell division and differentiation activities until the SD-photoperiod signal is perceived and

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the onset of ED is triggered (Kühn et al., 2009; Grant et al., 2013). Recent works has studied the transcriptional response of grapevine buds to chilling (Mathiason et al., 2009), photoperiod (Sreekantan et al., 2010) and dormancy breaking treatments (Halaly et al., 2008; Pérez et al., 2009). In these studies, candidate genes with dual roles in flowering and dormancy have been identified (Mathiason et al., 2009; Sreekantan et al., 2010) and a role has been proposed for oxidative stress as part of the dormancy release mechanism (Halay et al., 2008; Pérez et al., 2009). Recently, Díaz-Riquelme et al. (2012) analyzed transcriptomic variation during latent grape bud development and demonstrated that major transcriptional changes were associated with para/endodormancy, endo/ecodormancy and ecodormancy/bud-break transitions. Despite these findings, the primary metabolic and biochemical changes that reduce meristem sensitivity to growth-promoting signals during ED in latent buds remain unknown.

We propose that ABA, through the mediation of the SnRK1-type kinases, could act as a potential regulator of the metabolic switch from high to low activity associated with the entry to ED in grapevine buds.

2. Material and methods

2.1. Bud dormancy status

The bud-break response of single-bud cuttings under forced conditions is a common indicator used to describe the depth of dormancy in grapevines (Koussa et al., 1994; Dennis, 2003). This system allows for work with a large number of buds and provides a proper representation of the dormancy status of a given bud population at a specific point in time during the dormancy cycle. Canes were collected every 2–3 weeks from 8-year-old (*Vitis vinifera* L cv. Thompson seedless) grown at the experimental station at the Chilean National Institute of Agriculture Research (INIA), located in Santiago (33°34′S latitude) from 11 December to mid-August of the 2012/2013 season. Detached canes, each carrying 10 buds at positions 5–14, were transferred to the laboratory. Canes were cut into single bud cuttings. Cuttings (10–12 cm length) were mounted on a polypropylene sheet and floated in tap water in a plastic container for each collection date. The cuttings were then transferred to a growth chamber set at 23 ± 2° C with a 16 h photoperiod (forced conditions). The water in the container was replaced every 5 d, and bud break was assayed every 5 d over a period of 30 d. The appearance of visible green tissue at the tip of the bud was indicative of bud break. BR_{50} is an estimate of the mean time required to reach 50% bud break under forced conditions (Pérez et al., 2007); this parameter was used to determinate the depth of dormancy.

2.2. Oxygen consumption rate of grapevine buds

The oxygen consumption rate (OCR) of single buds was measured polarographically using a Clark-type oxygen electrode (Hansatech, Norfolk, UK) in 1 ml of an air saturated water solution at 25° C. In all experiments, a piece of thread was used to suspend the bud within the tube; this was to prevent the bud from settling at the bottom and interfering with the agitator. Measurements were performed using buds of *V. vinifera* cv. Thompson seedless collected throughout the 2012/2013 season. Values are averages of three replicates, and bars represent the standard deviation. In Thompson seedless grapes grown in Santiago, Chile, the critical day-length (CDL) for transition into ED occurred at mid-January; this was indicated by increases in BR_{50}. The effects of temperature, glucose and ABA on the OCR of paradormant (collected 9 November 2012) and endodormant (collected 15 April 2013) buds of cv. Thompson seedless grapevines were studied. The effects of were studied only in endodormant buds. Temperature experiments were carried out at 5, 10, 15 and 25° C; values are averages of three replicates, and bars represent the standard deviation. For the glucose and ABA experiments, single bud cuttings were sprayed with an aqueous solution containing 2% glucose or 100 μM ABA. In both cases, water was used as a control. Subsequently, the cuttings were placed in a growth chamber set at 23 ± 1° C with a 16 h photoperiod; the OCR was measured at 25° C at the times indicated. The effect of dormancy-breaking compounds was studied by spraying single bud cuttings with 2.5% H_2CN_2 and 2% NaN_3; water was used as a control. Subsequently, the cuttings were placed in a growth chamber under the conditions described above. The OCR was measured at 25° C at the times indicated.

2.3. Photoperiod treatments

In a collaborative project with colleagues from Brazil, the effect of the photoperiod on the expression of *VvNCED1* and *VvNCED2* paralogs was analyzed in grapevine buds. These genes encode a key enzyme related to ABA biosynthesis. Photoperiod experiments were performed in Messoró, Brazil. Small variations in day length and temperature in this area made it easier to conduct this type of experiment. Cuttings of *V. vinifera* cv. Italia melhorada under rootstock IAC 572 were used as plant material for the photoperiod experiments (3 replicates per treatment). Plants were grown at the Federal University of Rural Semi-Arid (UFERSA), which is located in Messoró, Brazil (5°12′16′íS). The natural photoperiod during the entire year is (12/12 h day/night), and temperatures fluctuate between (29–31 °C). Rooted cuttings (15 per treatment) were planted into a 1:1:1 (v: v: v) mix of soil, sand and muck in 5 l pots. As growth commenced, one cane was allowed to develop on each cutting. On October 15th 2013, vines with uniform growth and 12–16 leaves were selected and randomly assigned to a photoperiod treatment for 8 weeks. Photoperiod experiments were conducted in a greenhouse under LD (14/10 h day/night) and SD (10/14 h day/night) photoperiods. The critical day length (CDL) for dormancy transition in *V. vinifera* is 13 h (Kühn et al., 2009). Supplemental light was provided automatically in the afternoon at 17:30 h using a 100 W fluorescent tube. Light restriction was imposed using a black plastic sheet in the early morning at 5:30 h. For LD treatment, lights were on from 5:30 to 19:30. For SD treatment, lights were on from 7:00 to 17:00. After treatments, buds were placed in liquid N_2, lyophilized and sent to the laboratory for gene expression analysis.

2.4. Hypoxia treatments

Canes of *V. vinifera* cv. Thompson seedless collected during ED (3 July 2013) were separated into six groups of 30 single bud cuttings each. The first group was maintained at room temperature in the dark (control). The remaining five groups were subjected to hypoxia for 3, 6, 12, 18 and 24 h. After treatment, each group was divided into two subgroups of 15 buds each (biological replicates) for gene expression analysis. To obtain a low oxygen concentration (hypoxia), single bud cuttings were placed in a glass chamber with water in the bottom. N_2 was flushed continuously at a rate of 100 ml min^{-1}. The O_2 concentration in the bulk solution of the measuring flask was recorded polarographically using a Clark-type O_2 electrode; the O_2 concentration in the control varied between 250 and 260 nmol ml^{-1}. After 3, 6, 12, 18 and 24 h of N_2 bubbling, the O_2 concentration were 162, 112, 91 and 91 nmol ml^{-1}, respectively.

2.5. Gene expression analysis in grapevine buds

For the photoperiod experiments, total RNA was extracted and purified from lyophilized buds (0.05-0.1 g) of *V. vinifera* cv. Italia

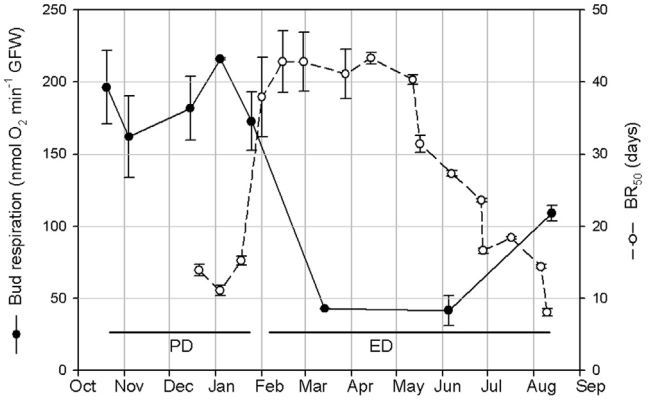

Fig. 1. Respiration rate and depth of dormancy (BR_{50}) are inversely related in grapevine buds. The depth of dormancy was determined by BR_{50} (the time required to reach 50% bud-break under forced conditions). BR_{50} values for each collection date were estimated by Probit Analysis (Minitab statistical software) using a population of 30 buds. Bars denote standard error. The oxygen consumption rate (OCR) of single buds was determined polarographically at 25 °C with a Clark type oxygen electrode. Bars denote standard deviation (n = 3). Paradormancy (PD), endodormancy (ED).

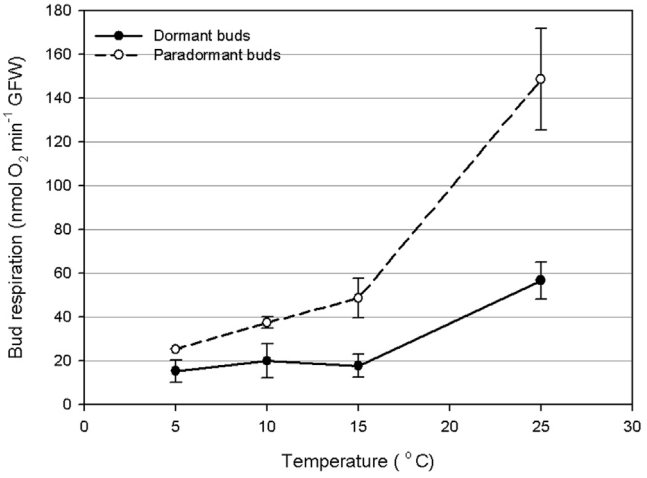

Fig. 2. Effect of temperature on the respiration rates of paradormant and endodormant buds. Single bud cuttings of *V. vinifera* cv. Thompson seedless harvested on 9 November (PD) and on 15 April (ED) were used for temperature experiments. The oxygen consumption rate (OCR) of single buds was determined polarographically at 5, 10, 15 and 25 °C using a Clark type oxygen electrode. Bars denote standard deviation (n = 3).

melhorada. For the dormancy transition, ABA and hypoxia experiments, total RNA was extracted and purified from buds of *V. vinifera* cv. Thompson seedless (0.5–0.7 GFW^{-1}). In all cases, total RNA was extracted and purified 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 (1U/μl) (Thermo Scientific, USA) at 37 °C for 30 min. First strand cDNA was synthesized from 5 μg of purified RNA using 1 μl oligo $(dT)_{12-18}$ (0.5 μg × μL^{-1}) as a primer, 1 μl dNTP mix (10 mM) and Superscript ® II reverse transcriptase (Invitrogen, CA, USA). Gene expression analysis was performed by quantitative real-time PCR using an Eco Real-Time PCR system (Illumina, Inc. SD, USA) and KAPA SYBR FAST mix (KK4602) qPCR master mix (2 X). Primers suitable for the amplification of 80–200 bp products for each gene under study were designed using the PRIMER3 software (Rozen and Skaletsky, 2000) (Suppl. Table S1) The amplification of cDNA was performed under the following conditions: denaturation at 94° C for 2 min followed by 40 cycles of 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. The induction or repression of transcript levels was calculated using the ΔΔCq method (Livak and Schmittgen, 2001); *VvUBIQUITIN* was used as a reference gene.

2.6. Statistical analysis

The depth of bud dormancy was estimated by BR_{50}, and the corresponding standard error of the mean was calculated using Probit analysis (Minitab 13.31 Minitab Inc., USA). For pairwise comparisons, the Studentís *t*-test was used with $\alpha = 0.05$.

3. Results

3.1. Dormancy depth and respiration rate are inversely related in grapevine buds

The respiration rates and the depths of dormancy (BR_{50}) of Thompson seedless grapevine buds grown in Santiago, Chile (33°31′S) were plotted throughout the 2012/2013 season (Fig. 1). The oxygen consumption rate (OCR) of paradormant buds fell sharply from 180 (nmol O_2 min^{-1}GPF) to 40 (nmol O_2 min^{-1}GPF) as the season progressed. This decrease in bud respiration coincided with an increased BR_{50}, a parameter that measures the depth of dormancy. BR_{50} is the time required to achieve 50% of bud-break under forced conditions (Pérez et al., 2007). Increasing BR_{50} values are indicative of buds transitioning from PD to ED. Throughout ED, bud respiration remained at low levels. Respiration recovered before the onset of bud sprouting (Fig. 1).

3.2. Effect of temperature on respiration in paradormant and endodormant buds

To analyze the effect of temperature on respiration in paradormant (collected on 9 November 2012) and endodormant buds (collected on 15 April 2013) of *V.vinifera* cv. Thompson seedless, the OCR at temperatures between 5 and 25° C was determined for both type of buds. At low temperatures (5–15° C), the OCR of endodormant buds remained relatively stable (20 ± 3 nmol O_2 GFW^{-1}), and in paradormant buds, the OCR increased from 25.4 ± 0.4 to 48.6 ± 8.9 nmol O_2 GFW^{-1}. At high temperatures (15–25° C), the OCR increased in endodormant and paradormant buds. However, the effect was significantly greater in paradormant compared to endodormant buds (Fig. 2).

3.3. Effect of glucose on respiration in paradormant and endodormant buds

To analyze the effect of glucose on respiration in paradormant (collected on 9 November 2012) and endodormant buds (collected on 5 April 2013) single bud cuttings of *V.vinifera* cv. Thompson seedless were sprayed with an aqueous solution of 2% glucose; water was used as a control. After treatment, cuttings were placed in a growth chamber set at 23 ± 1° C with a 16 h light cycle. Bud respiration was determined at the times indicated. The results showed that the exogenous addition of glucose significantly increased the OCR in paradormant buds at 2 and 7 days after treatment (Fig. 3A). No effect was observed for endodormant buds (Fig. 3B).

3.4. Advancing effect of dormancy-breaking compounds on the respiratory burst that occurs prior to the onset of bud-break

The OCR of endodormant buds of *V.vinifera* cv. Thompson seedless increased as the time within the growth chamber under forced conditions increased. These findings indicated that the OCR

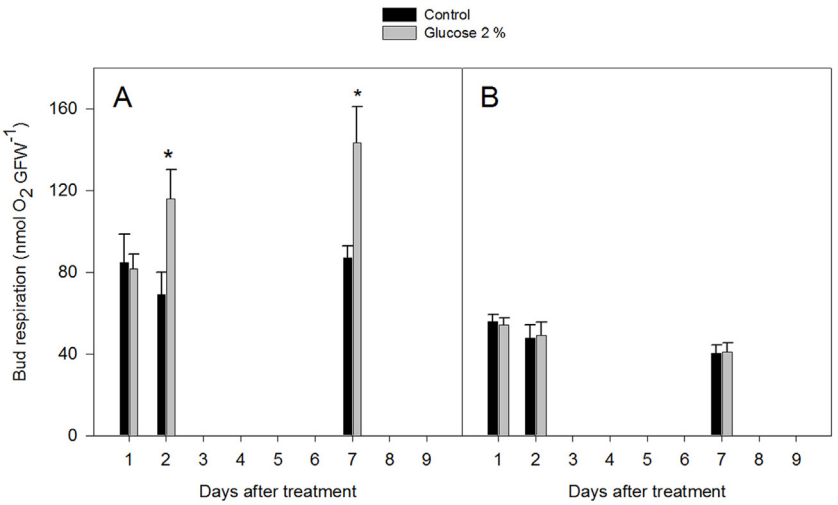

Fig. 3. Glucose increased the respiration rate of paradormant but not that of endodormant buds. Single bud cuttings of *V. vinifera* cv. Thompson seedless harvested on 9 November (PD) and on 15 April (ED) were treated with a solution of 2% glucose and water as control. The oxygen consumption rate (OCR) was measured polarographically using a Clark type oxygen electrode at 25 °C, 2 and 7 days post-treatment. Bars denote standard deviation (n = 3). Asterisk (*) indicates significant differences (Studentís *t*-test $\alpha = 0.05$).

increased as the bud is released from ED and, a respiratory burst is produced prior to bud break (Fig. 4). Applying the dormancy-breaking compounds hydrogen cyanamide (H_2CN_2) and sodium azide (NaN_3) to endodormant buds significantly advanced the respiratory burst, indicating an earlier dormancy release in the treated buds (Fig. 4).

3.5. Effect of ABA on respiration in paradormant and endodormant buds

The exogenous application of ABA significantly reduced the OCR of paradormant buds. OCR was not reduced in endodormant buds (Fig. 5A, B).

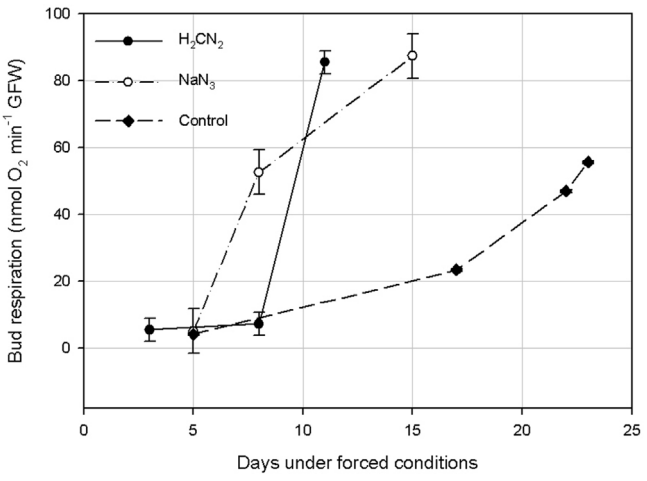

Fig. 4. Advancing effects of the dormancy breaking compounds (H_2CN_2) and (NaN_3) on the respiratory burst that occurs prior to the onset of bud-break. Single bud cuttings of *V. vinifera* cv. Thompson seedless were sprayed with an aqueous solution of 2.5% H_2CN_2, 2%, an aqueous solution of NaN_3 or water as a control. The oxygen consumption rate (OCR) of single buds was measured polarographically using a Clark type oxygen electrode at 25 °C. Bars denote standard deviation (n = 3).

3.6. Effect of ABA on the expression of respiration-related genes in non-dormant grapevine buds

To test whether the inhibition of respiration of paradormant buds by ABA is transcriptionally regulated, the expression of respiration-related genes such as *ALTERNATIVE NADH-DEHYDROGENASE* (*VvaND1*, *VvaND2*), *CYTOCHROME C* (*VvCYTC*) and the subunit 6 of *CYTOCHROME OXIDASE* (*VvCOX6*) was analyzed by RT-qPCR in paradormant buds treated or untreated with ABA. The results showed that the expression of all respiration-related genes analyzed was significantly repressed by ABA (Fig. 6).

3.7. Hypoxia, ABA and transition into ED up-regulated the expression of the VvSnRK1 genes encoding catalytic subunits in grapevine buds

Bioinformatics analysis of the *V. vinifera* homologue of SNF1, the yeast serine-threonine protein kinase, revealed the existence of two α (catalytic), two β and one γ subunit in the *V. vinifera* public database GENOSCOPE (www.genoscope.cns.fr). Primers aligning to the two catalytic subunits GSVIVT01011467001 (*VvSnRK1a*) and GSVIVT01025806001 (*VvSnRK1b*) were designed, and their specificity tested by blast analysis using the NCBI database (www.ncbi.nlm.nih.gov). The results showed that the designed primers recognized the desired sequence specifically. Moreover, phylogenetic tree analysis of SNF1 proteins sequence and their alignment revealed differences between both subunits (Suppl Fig. S1 and S2). Because the *SnRK1* transcript is up-regulated by sugar depletion and under energy deficit conditions, including darkness and hypoxia (Baena-González et al., 2007), the effect of hypoxia on the expression of *VvSnRK1a* and *VvSnRK1b* transcripts in grapevine buds was analyzed. The results showed that after 6 h of treatment, both transcripts accumulate slightly. However, after 12 h, transcript levels fell sharply. After 18 and 24 h of treatment, transcript levels significantly increased (Fig. 7A). To analyze whether ABA transcriptionally regulates the expression of *VvSnRK1a* and *VvSnRK1b*, ABA was applied exogenously to single bud cuttings and transcript expression was analyzed by RT-qPCR. The results showed that ABA up-regulated the expression of both transcripts (Fig. 7B). However, the expression of *VvSnRK1b* was more sensitive; expression was up-regulated earlier, and the level of expression was higher. The

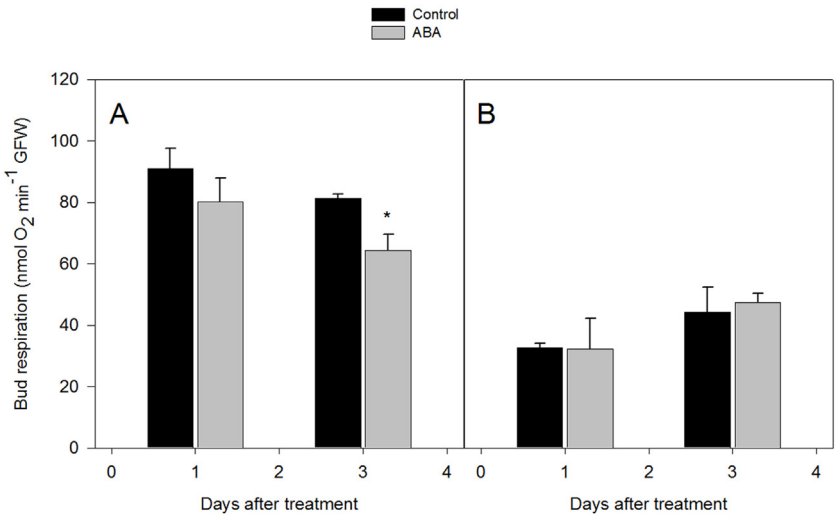

Fig. 5. ABA inhibits respiration in paradormant buds but not in endodormant buds. Experiments were carried out using single bud cuttings of *V. vinifera* cv. Thompson seedless collected on 9 November (A, paradormant) and or 15 April (B, endodormant). The cuttings were sprayed with ABA (100 μM) or water used as a control. The oxygen consumption rate (OCR) was measured polarographically using a Clark type oxygen electrode at 25 °C, 1 and 3 d after treatment. Bars denote standard deviation (n = 3). Asterisk (*) indicates significant differences (Studentís t-*t*est α = 0.05).

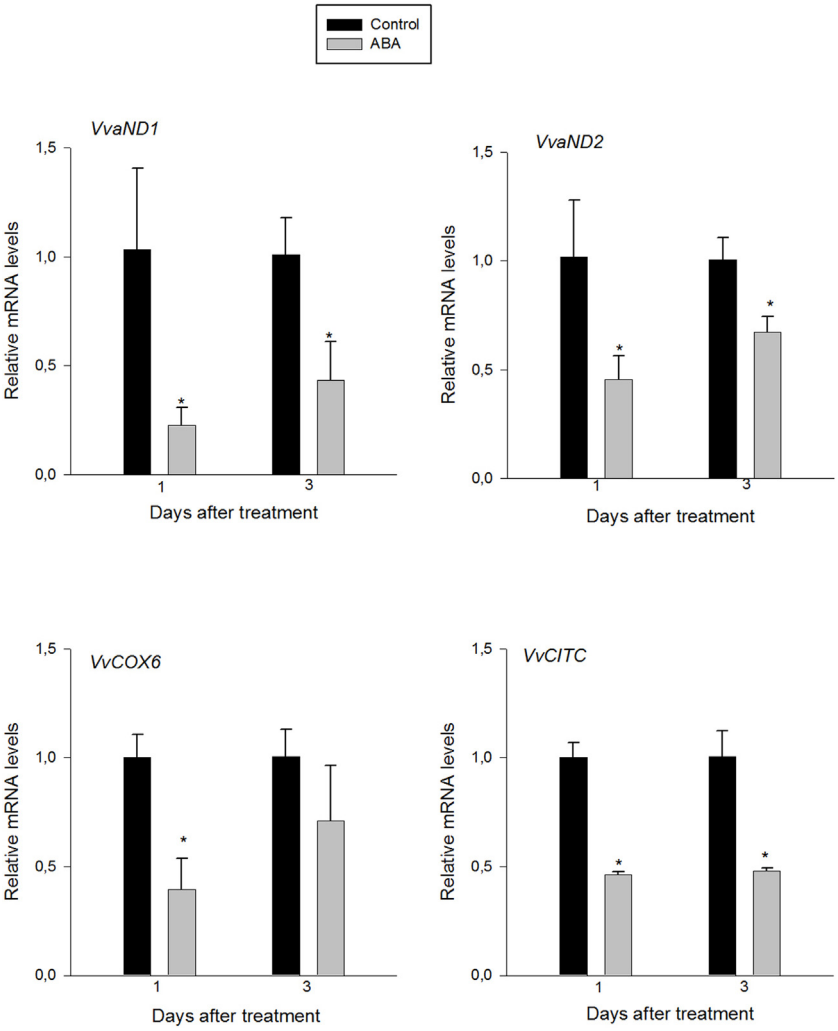

Fig. 6. ABA down-regulated the expression of respiration-related genes in non-dormant grapevine buds. Single bud cuttings of Thompson seedless grapevines were collected on 5 January during the active growth period of buds (paradormancy) and sprayed with ABA. Transcript levels of the alternative dehydrogenase genes *VvaND1* and *VvaND2* and that of the mitochondrial electron transport chain genes *VvCYTC* and *VvCOX6*. Expression was analyzed one and three days after treatment by RT-qPCR normalized against *VvUBIQUITIN*. Values are the averages of three biological replicates with three technical repetitions each. Bars denote standard deviation. Asterisk (*) indicates significant differences (Studentís *t*-test α = 0.05).

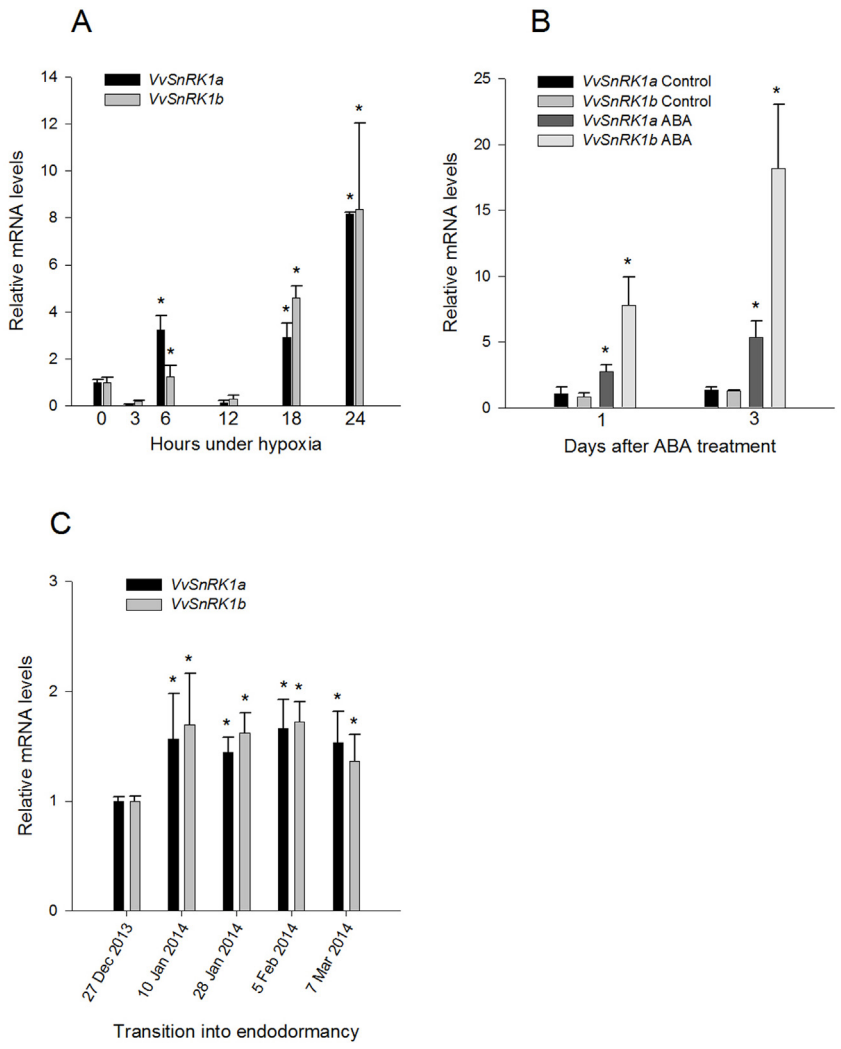

Fig. 7. Hypoxia, ABA and the transition into ED, up-regulated the expression of *VvSnRK1* genes in grapevine buds. Hypoxia (A), ABA (B) and transition into ED (C) experiments were carried out in buds of *V. vinifera* cv. Thompson seedless buds. Transcript levels of the catalytic subunit of *VvSnRK1a* and *VvSnRK1b* was determined by RT-qPCR, normalized against *VvUBIQUITIN*. Values are the averages of three biological replicates with three technical repetitions each. Bars denote standard deviation. Asterisk (*) indicates significant differences (Studentís *t*-test $\alpha = 0.05$).

transition of grape buds into ED also increased the expression of both transcripts (Fig. 7C).

3.8. The SD-photoperiod up-regulated the expression of VvNCED genes in grapevine buds

A previous bioinformatics analysis identified *three* putative homologues of *NCED* in grapevines (Young et al., 2012). *NCED* encodes the enzyme 9-*cis*-epoxycarotenoid dioxygenase, which is a key enzyme in the synthesis of ABA (Qin and Zeevart, 2002; Lefebvre et al., 2006). Three *VvNCED* genes, *VvNCED1*, *VvNCED2* and *VvNCED3*, were detected in mature grape buds. However, the transcript levels of *VvNCED3* were very low compared with those of *VvNCED1* and *VvNCED2*. The response of the three paralogs to the dormancy-breaking compound hydrogen cyanamide was similar (Zheng et al., 2015). The SD-photoperiod induces ED in *V. vinifera* (Kühn et al., 2009; Grant et al., 2013) and ABA could play a key role in the induction of ED. In this study, we analyzed the effect of the photoperiod on the expression of *VvNCED1* and *VvNCED2* in buds of *V. vinifera* cv. Italia melhorada. The results showed that the transcript levels of *VvNCED1* remained stable throughout the entire day under LD-photoperiod conditions. *VvNCED1* transcript levels increased significantly under SD-photoperiod conditions, especially at dusk (Fig. 8A). *VvNCED2* expression varied throughout the day under both LD- and SD-photoperiod conditions; under SD-photoperiod conditions, transcript levels were significantly higher at the onset and the end of the light period (Fig. 8B).

4. Discussion

4.1. Bud-respiration and the depth of endodormancy are inversely related

In this study, an inverse relationship between the depth of ED and bud respiration was revealed in *V. vinifera*. This finding indicates that important metabolic changes occur during ED in grapevine buds. How these metabolic changes decrease the meristem sensitive to growth-promoting signals and how these changes in metabolism contribute to the acquisition of cold hardiness in dormant buds remain unknown. Temperature is one of the primary factors affecting respiratory rates in plants. In general, respiration rates are usually slower when measured at lower temperatures (Kurimoto et al., 2004). Often, the change in respiration per 10° C rise in temperature (Q_{10}) is near 2.0 (Atkin and Tjoelker, 2003). The Q_{10} of plant respiration is greater when respiratory flux is limited by enzymatic capacity compared to when it is limited by substrate

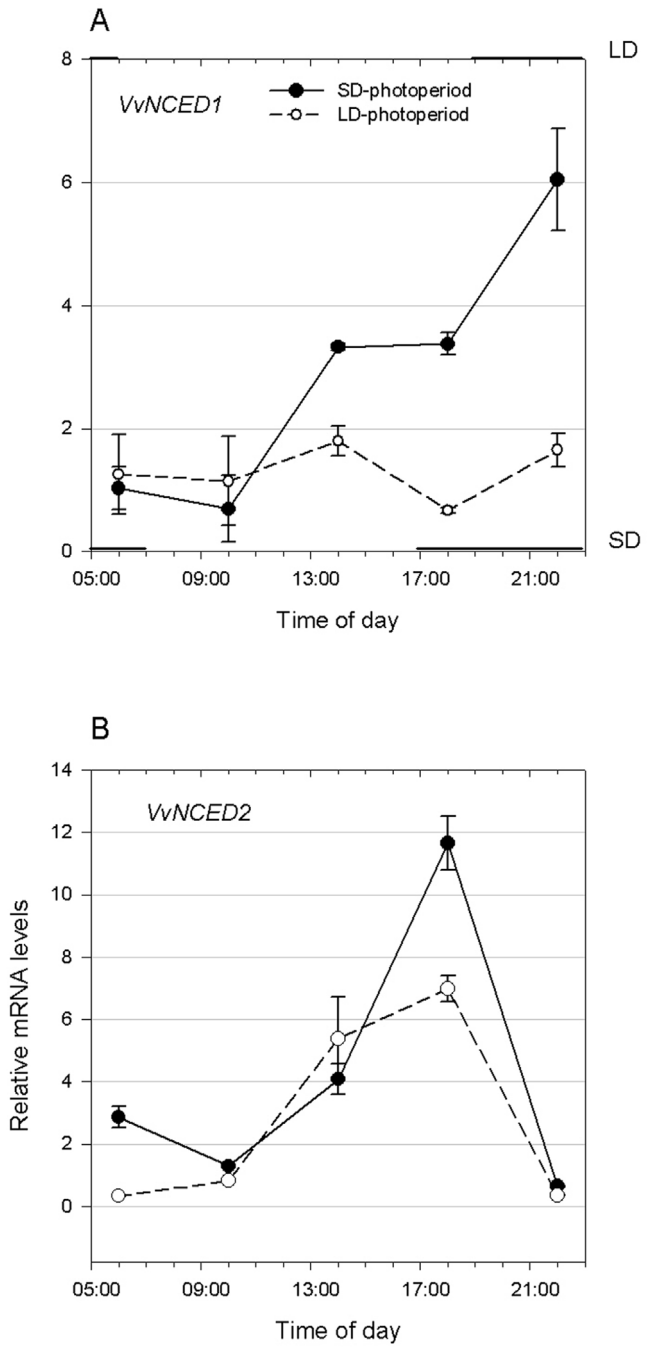

Fig. 8. SD-photoperiod up-regulated the expression of *VvNCED1* and *VvNCED2* in grapevine buds. Photoperiod experiments were carried out using *V. vinifera* cv. Italia melhorada buds in a greenhouse under LD (14/10 h day/night) and SD (10/14 h day/night) photoperiod. After 8 weeks of treatment, grapevine buds were collected at different times of the day and transcript levels of *VvNECD1* and *VvNCED2* transcript levels were determined by RT-qPCR normalized against *VvUBIQUITIN*. Values are the average of three biological replicates with three technical repetitions each. Bars denote standard deviation. Asterisk (*) indicates significant differences (Studentís *t*-test $\alpha = 0.05$).

supply. Conversely, the transition from enzymatic control to limitations imposed by substrate supply or adenylates is associated with a decline in Q_{10} (Atkin and Tjoelker, 2003). Our results demonstrated that the Q_{10} of endodormant buds is significantly lower than the Q_{10} of paradormant buds. This difference was greater under moderate-high temperatures (15–25° C) compared to low temperatures (5° C). Therefore, we hypothesize that respiration in endodormant grapevine buds is limited by the supply of substrate.

The advancing effect of hydrogen cyanamide (H_2CN_2) and sodium azide (NaN_3) on the respiratory burst in endodormant buds may be related to the ability to break dormancy. Paradoxically, both compounds transiently inhibit bud respiration in grapevines over the short term (Pérez et al., 2009; Vergara et al., 2012a). Over long periods, these compounds advance the respiratory burst prior to the onset of bud break. Therefore, the early inhibition of bud respiration by H_2CN_2 and NaN_3 can produce sub-lethal stress. This stress results in a further increase in respiration. It has previously been reported that hypoxia and H_2CN_2 induce the expression of genes encoding starch-degrading enzymes (Rubio et al., 2014), and both stimulate an increase in H_2O_2 levels in grapevine buds (Vergara et al., 2012b). H_2O_2 increases occur primarily due to interruptions in the mitochondrial electron transport chain (mETC) in grape buds (Vergara et al., 2012b), and starch hydrolysis is stimulated by respiration inhibitors (Rubio et al., 2014). It is possible that respiration inhibitors increase the OCR of grapevine buds over the long term through an increased supply of sugars, and through this mechanism promotes bud dormancy release. However, glucose and ABA do not affect the respiration in dormant buds, and ABA levels and the expression of genes that regulate its biosynthesis increase during ED (Zheng et al., 2015). It is not possible to conclude that the lack of respiratory substrate is the cause of the low rate of respiration in dormant buds, and evidence suggests that ABA could act as a metabolic inhibitor in dormant buds.

4.2. ABA as a key regulator of grape bud dormancy

The role of ABA in controlling seed dormancy has been well established (Finkelstein, 2013). However, its role in regulating bud-dormancy is not well known. In poplar, the cessation of growth and dormancy is induced by the SD-photoperiod; the ABA content and the expression of genes regulators of ABA biosynthesis (*NCED3*, *ABA1*, and *ABA2*) peaked in the apex after 3–4 weeks of exposure to the SD-photoperiod (Arora et al., 2003; Rohde and Bhalereao, 2007; Ruttink et al., 2007). In the vine, bud dormancy is also induced by the SD-photoperiod (Kühn et al., 2009; Grant et al., 2013). In addition, both the ABA content and the expression of ABA biosynthesis genes (*VvNCED1* and *VvNCED2*) increased during the induction and maintenance of ED (Zheng et al., 2015). This suggests that ABA is involved in the development of ED. In this study, we demonstrated that bud respiration is inhibited during ED. ABA inhibits respiration in non-dormant buds and down-regulates the expression of respiration-related genes such as *VvaNAD*, *VvCYTC* and *VvCOX6*, thus providing a molecular mechanism by which ABA induces bud-ED. Because the SD-photoperiod induces bud-ED in grapevines, the up-regulation of the ABA biosynthesis genes *VvNCED1* and *VvNCED2* by the SD-photoperiod is consistent with the putative role of ABA in the induction and maintenance of ED. ABA also induced the expression of an α (catalytic) subunit of sucrose-non-fermenting-1-related protein kinase (SnRK1), the plant homologue of the yeast protein SnF1 and the animal AMPK kinase. SnRK1/SnF1/AMPK kinase, acts as energy level sensor in all eukaryotes, and are activated during energy depletion or metabolic stress to inhibit growth and conserve energy for cell survival (Hardie, 2007; Baena-González and Sheen, 2008; Ghillebert et al., 2011). SnRK1 is activated by sugar depletion and under energy deficit, including darkness and hypoxia (Baena-González et al., 2007). Upon activation, SnRK1 triggers extensive transcriptional changes that restore homeostasis and promotes stress tolerance and survival. This is partly achieved through the inhibition of anabolism and the activation of catabolism (Confraria et al., 2013). GDBRPK, a transcript for an SNF1-like protein kinase that is up-regulated by hydrogen cyanamide has been identified in grapevine buds (Or et al., 2000). However, a bioinformatics analysis of the yeast SNF1 counterpart in the *V.vinifera* genome, revealed the exis-

tence of two α (catalytic) subunits of *VvSnRK1*; the sequences of these subunits do not correspond to that of GDBRPK (Suppl Fig. S1). In plants, the serine/threonine protein kinase family comprises three subfamilies (Coello et al., 2011); GDBRPK belongs to the SnRK2 subfamily. There is evidence indicating that ABA positively regulates the activity of SnRK1 by inhibiting clade A type 2C protein phosphatase (PP2Cs) (Rodríguez et al., 2013). However, there is no evidence indicating that ABA regulates the expression of *SnRK1* at the transcriptional level. Hypoxia induced the expression of *VvSnRK1a* and *VvSnRK1b*, and ABA inhibited bud respiration and the expression of respiratory-related genes in non-dormant buds. Therefore, we suggest that the up-regulation of *VvSnRK1a* and *VvSnRK1b* by ABA is indirect and could be mediated by a respiratory stress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2016.07.007.

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