

# Abscisic acid (ABA) and low temperatures synergistically increase the expression of CBF/DREB1 transcription factors and cold-hardiness in grapevine dormant buds

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- **Background and Aims** It has been reported that low temperatures (LTs) and the plant hormone abscisic acid (ABA) induce the expression of CBF/DREB1 transcription factors in vegetative tissues and seedlings of *Vitis vinifera* and *Vitis riparia* and that foliar applications of ABA to *V. vinifera* increase the freezing tolerance or cold-hardiness of dormant buds. However, the combined effect of ABA and LTs on the expression of CBF/DREB1 transcription factors and on the acquisition of freezing tolerance in dormant grapevine buds has not been investigated. The objective of this study was to analyse the combined effect of ABA and LT treatments on the expression of CBF/DREB transcription factors and the acquisition of freezing tolerance.
- **Methods** *In vitro* experiments with single-bud cuttings of grapevines were used to analyse the effect of ABA, ABA + LT and LT on the expression of CBF/DREB transcription factors, dehydrin and antioxidant genes, the acquisition of freezing tolerance and the endogenous content of ABA. Gene expression analysis was performed by quantitative real-time PCR and freezing tolerance was determined by measuring the low-temperature exotherm by differential thermal analysis. ABA levels were determined by gas chromatography coupled to an electron capture detector.
- **Key Results** The LT treatment and exogenous application of ABA to grapevine dormant buds increased the expression of the CBF/DREB1 transcription factors *VvCBF2*, *VvCBF3*, *VvCBF4* and *VvCBF6*. The joint application of LT and ABA produced a huge increase in the expression of these transcription factors, which was greater than the sum of the increases produced by them individually, which indicates the existence of a synergistic effect between ABA and LT on the activation of these transcription factors. This synergic effect was also observed on the increase in bud cold-hardiness and on the expression of antioxidant and dehydrin genes.
- **Conclusions** The synergy between ABA and LT on the expression of CBF/DREB1 transcription factors *VvCBF2*, *VvCBF3*, *VvCBF4* and *VvCBF6* plays a key role in cold acclimatization of grapevine buds. The results highlight the importance of the combination of stimuli in the improvement of genetic and physiological responses and help us to understand the adaptation of plants to complex environments.

**Key words:** Abscisic acid, cold-hardiness, CBF/DREB transcription factors, dehydrins, antioxidant genes, grapevine buds, low temperatures.

## INTRODUCTION

*Vitis vinifera* is one of the world's most important fruit crops (This *et al.*, 2006). It is cultivated mainly in temperate and semi-arid climates that can sometimes experience freezing and subfreezing temperatures, which can cause significant damage to the plants. As deciduous fruit trees, *Vitis* spp. acquire freezing tolerance or cold-hardiness before the arrival of winter, once the buds have entered endodormancy (Rubio *et al.*, 2016). Freezing tolerance is part of the cold acclimatization process that buds undergo when exposed to low, non-freezing temperatures. Cold acclimatization is a complex trait involving multiple biochemical and physiological changes and results from the induced expression or repression of a battery of regulatory and functional genes (Fennell, 2014; Wisniewski *et al.*, 2014). Among the regulatory genes, those encoding transcription factors play an important role in plant stress responses,

acting as coordinators of stress signals and orchestrating the expression of functional genes (Singh *et al.*, 2002; Wang *et al.*, 2016). It is well known that the C-repeat (CRT)-binding factor/dehydration-responsive element (DRE) binding protein 1 (CBF/DREB1) transcription factors are involved in improving cold resistance (Stockinger *et al.*, 1997; Chew and Halliday, 2011). The CBF/DREB1 proteins bind to the CRT/dehydration responsive element (CRT/DRE) of target cold-responsive genes and promote freezing tolerance (Thomashow, 2010; Theocharis *et al.*, 2012). The most well-documented cold- and CBF-regulated genes are dehydrins, a group of late embryogenesis abundant proteins (LEAs) that contain the CRT/DRE regulatory element in their promoter. Several studies have shown an increased abundance of dehydrin transcripts and proteins accumulating in woody plant buds and bark during induction of dormancy and cold acclimatization (Wisniewski *et al.*, 2014;

Rubio *et al.*, 2016). Analysis of the CBF/DREB1 pathway in grapevines has identified four CBF genes in *Vitis vinifera* and *Vitis riparia* that are upregulated in response to low temperature (LT) and abscisic acid (ABA) (Xiao *et al.*, 2006, 2008). Thus, an increase in *CBF3* and *CBF4* expression was observed in the leaves of *V. riparia* and *V. vinifera* after 1–2 d at 4 °C (Xiao *et al.*, 2006, 2008), contrasting with the quick cold induction observed in the case of *CBF1* and *CBF2* (Xiao *et al.*, 2006). Overexpression of *VvCBF4* in *V. vinifera* “Freedom” improved freezing survival and reduced freezing-induced electrolyte leakage by up to 2 °C in non-cold acclimated buds (Tillett *et al.*, 2012). Likewise, overexpression of CBF transcription factors from *V. vinifera* “Koshu” (Takuhara *et al.*, 2011) or *V. riparia* (Siddiqua and Nassuth, 2011) in *Arabidopsis* improved the freezing tolerance of transgenic plants. A total of seven CBF/DREB1 genes were cloned and sequenced from *V. riparia* and the less frost-tolerant *V. vinifera* (Carlow *et al.*, 2017). Amino acid sequence comparison and phylogenetic analysis showed two different groups of *Vitis* CBFs. One group contained *CBF1*, *CBF2*, *CBF3* and *CBF8* and the other group contained *CBF4*, *CBF5* and *CBF6* (Carlow *et al.*, 2017). However, it was reported that *CBF1* (Xiao *et al.*, 2006) was not found in the 12X *V. vinifera* genome database (Wisniewski *et al.*, 2014; Vázquez-Hernández *et al.*, 2017). A careful analysis of the data indicates that two codes entered into the NCBI database by Xiao *et al.* (2006), AY390372 and AY390376, correspond to the same gene, GIDVvT00031496001, in the *Vitis* genome database. In addition, this gene has been designated as *VvCBF2* in the work of Karimi *et al.* (2015) and Zandkarimi *et al.* (2015). So, in fact there are six CBF genes in the 12X *Vitis* genome database, which are shown in Table 1. In the present study we have analysed the effect of LT, ABA and LT + ABA on bud cold-hardiness and on the expression of CBF/DREB, dehydrin and antioxidant genes in grapevine dormant buds. The results clearly showed that bud cold-hardiness, CBF/DREB1 genes and expression of putative target genes of CBF/DREB1 were synergistically induced by combined treatment with ABA and LT.

## MATERIALS AND METHODS

### Plant material and treatments

Canes were taken from 10-year-old *Vitis vinifera* ‘Thompson Seedless’ grown at the experimental station of the Chilean National Institute of Agricultural Research (INIA, La Platina) located in the Maipo valley (33°34' S). All experiments were

carried out with grapevine dormant buds collected on 23 April and 14 May (Vergara and Pérez, 2010; Rubio *et al.*, 2016). For bud cold-hardiness and gene expression analysis, detached canes each carrying ten buds at positions 5–14 were collected from the vineyard and transferred to the laboratory and excised in single-bud cuttings. The cuttings were mounted on propylene sheets and floated in tap water in a plastic container (Supplementary Data Fig. S1) and exposed to the following treatments: (1) LT: buds were placed in a refrigerator at 4 °C in the dark; (2) ABA treatment: buds were sprayed with 100 µM ABA solution and kept in the dark at 14 °C in a growth chamber; (3) LT + ABA treatment: buds were sprayed with 100 µM ABA solution and placed in the refrigerator at 4 °C in the dark. ABA solution was prepared from 2.6 mg of ABA (Sigma–Aldrich, St Louis, MO, USA) dissolved in 1 mL of deionized water with 0.02 % Tween 20 (Sigma–Aldrich, St Louis, MO, USA). Single-node cuttings were sprayed on the bud side with the ABA solution or with water containing 0.02 % Tween-20 until runoff. To determine bud cold-hardiness and endogenous ABA content, samples collected on 14 May were harvested 0, 3, 7, 12 and 16 d after treatment. For gene expression analysis, samples were harvested after 1 and 2 weeks. To analyse the effect of ABA concentration on bud cold-hardiness, cuttings collected on 29 April were sprayed with 50, 100, 200 or 400 µM ABA solution and placed in the refrigerator at 4 °C in the dark for 1 week. To test whether hydrogen cyanamide (HC), a dormancy-breaking compound that degrades endogenous ABA in grapevine buds (Zheng *et al.*, 2015; Vergara *et al.*, 2017), affects bud cold-hardiness, two groups of 30 single-bud cuttings, each harvested on 23 April, were sprayed with 200 µM ABA solution and with 200 µM ABA plus 2.5 % (w/v) HC (Sigma–Aldrich, St Louis, MO, USA), respectively. After the treatments, both groups were placed in the refrigerator at 4 °C in the dark and bud cold-hardiness was determined after 1 week.

### Bud cold-hardiness

Bud cold-hardiness was measured in grapevine dormant buds by low temperature exotherm (LTE) detection using differential thermal analysis following the method of Mills *et al.* (2006). The differential thermal analysis was performed with a Kryoscan (Supplementary Data Fig. S2), a freezing and data acquisition device that uses Peltier elements for the cooling and detection modules (Badulescu and Ernst, 2006). Signals were recorded every 2 s, and a decrease in temperature of 4 °C

TABLE 1. List of putative *Vitis vinifera* CBF/DREB1 genes

Gene	NCBI*	<i>Vitis</i> genome <sup>†</sup>	CHROMOSOME	Reference
<i>VvCBF1</i>	AY390372	GIDVvT00031496001	-	Xiao <i>et al.</i> , 2006
<i>VvCBF2</i>	AY390376	GIDVvT00031496001	6	Xiao <i>et al.</i> , 2006
<i>VvCBF3</i>	AY390375	GIDVvT00031494001	6	Xiao <i>et al.</i> , 2006
<i>VvCBF4</i>	DQ497624	GIDVvT00040836001	16	Xiao <i>et al.</i> , 2008
<i>VvCBF5</i>	KX197193	GIDVvT00040120001	19	Carlow <i>et al.</i> , 2017
<i>VvCBF6</i>	KX197195	GIDVvT00041492001	2	Carlow <i>et al.</i> , 2017
<i>VvCBF8</i>	KX197197	GIDVvT00007002001	8	Carlow <i>et al.</i> 2017

\*NCBI: <https://www.ncbi.nlm.nih.gov>.

<sup>†</sup>*Vitis* genome 12X.v2: <https://urgi.versailles.inra.fr/species/Vitis>.

$h^{-1}$  starting at 10 °C and ending at –30 °C was programmed (Mills *et al.*, 2006). Generally, two peaks were observed, one corresponded to the high-temperature exotherm (HTE), which was assigned to the freezing point of extracellular (apoplast) water, which is non-lethal (Burke *et al.*, 1976), and the other corresponded to the LTE, which was assigned to the freezing point of intracellular water, which is lethal (Burke *et al.*, 1976). Because lethal damage to the grapevine buds occurs at temperatures equal to or lower than the LTE, the LTE value is considered the lowest temperature that the grapevine bud can resist without damage, and therefore is a measure of its cold-hardiness (Pierquet and Stushnoff, 1980; Mills *et al.*, 2006; Ferguson *et al.*, 2011). Each value corresponds to the average of 20 biological replicates of single buds.

#### ABA determinations

Fresh plant material (ten buds per harvest time) was washed with cold water before grounded with liquid nitrogen. The samples were extracted in a shaker for 1 h at 4 °C and for 10 min by ultra-sonication with 3 mL of 80 % methanol containing 1 % acetic acid and 3 ng of 2,3,5-triiodobenzoic acid (TIBA) as an internal standard (Sigma–Aldrich, USA). The extracts were centrifuged at 3000  $g$  for 10 min, and the supernatant was filtered through glass wool and a Sep-Pack  $C_{18}$  cartridge (Waters, Milford, MA, USA) that had been prewashed with 5 mL of 80 % methanol. The procedure was repeated twice, and the filtrate was evaporated to dryness. After evaporation, the residue was dissolved in 1.2 mL of ethyl acetate and 1.2 mL of 0.5 M  $KH_2PO_4$  pH 3.0 was added. The mixture was agitated with a vortex and centrifuged at 3000  $g$  for 3 min. After centrifugation, the ethyl acetate layer was removed and the aqueous phase was extracted twice with 1.2 mL of ethyl acetate. The collected ethyl acetate layers were evaporated to dryness. The dry sample was dissolved in 1 mL of ethyl acetate, and an ABA derivative was formed by reaction with pentafluorobenzyl bromide (PFB) (Sigma–Aldrich, USA), which allowed highly sensitive detection by an electron capture detector (Michler *et al.*, 1986). A Shimadzu gas chromatograph (model GC-2014) equipped with an electron capture detector (ECD-2014, Shimadzu, Kyoto, Japan) and computer integrator was used for ABA determinations. A CBP1 capillary column (25 m  $\times$  0.25 mm internal diameter) with helium as the carrier gas at a flux of 1.5 mL  $min^{-1}$  was used. The temperature of the column was initially 80 °C and after 1 min was raised to 270 °C at a rate of 20 °C  $min^{-1}$  and maintained there for 5 min. The injector was operated in the splitless mode at 225 °C, and the temperature of the detector was 300 °C. A calibration curve for ABA–PFB derivative was constructed.

#### Bud water content

For determinations of bud water content, canes collected on 12 and 19 June from ‘Thompson Seedless’ grapevines grown in the Maipo valley were excised in single-bud cuttings. Four groups of seven single-bud cuttings each were separated; two were treated with water and two with 200  $\mu M$  ABA. Of the groups treated with water, one was maintained at 14–15 °C in

the growth chamber and the other at 4–5 °C in a refrigerator for 1 week; the same was done with the ABA-treated groups. After treatments, the buds were excised and weighed before and after placing in an oven at 70 °C for 24 h. Water content was expressed as a percentage of fresh weight (FW).

#### RNA purification and cDNA synthesis

For gene expression analysis, total RNA was isolated and purified from grapevine buds (0.5 g FW) of *V. vinifera* ‘Thompson Seedless’. In all cases, total RNA was extracted and purified using a modification of the method of Chang *et al.* (1993), as described by Noriega *et al.* (2007). DNA was removed by treatment with RNase-free DNase (1 U  $\mu g^{-1}$ ) (Thermo Scientific, USA) at 37 °C for 30 min. First-strand cDNA synthesis was performed using the Superscript® II RT system (Invitrogen, CA, USA). A 1- $\mu g$  aliquot of purified RNA with 1  $\mu L$  of oligo(dT)<sub>12–18</sub> (0.5  $\mu g \mu L^{-1}$ ) was used as a primer and 1  $\mu L$  of dNTP mix (10 mM) was used for cDNA synthesis. RNA quality and quantity were reassessed with a Qubit 2.0 fluorometer (Invitrogen, USA).

#### Quantitative real-time PCR

Quantitative real-time PCR (RT-qPCR) was carried out in an Eco Real-Time PCR system (Illumina, San Diego, USA) using KAPA SYBR FAST (KK 4602) qPCR Master Mix (2 $\times$ ). Design of specific primers for *VvCBF* genes was carried out using the primer3 program (Rozen and Skaletsky, 2000). Dehydrin primers were taken from Rubio *et al.* (2016) and primers for antioxidant genes were taken from Vergara *et al.* (2012). cDNA was amplified 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. Relative changes in gene expression levels were determined using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001). Each reaction was performed in at least three biological replicates, each with three technical replicates. *VvUBIQUITIN* and *VvACTIN* were used as a reference genes for normalization.

#### Statistical analysis

Differences between treatments were analysed by ANOVA, and multiple comparison analysis was carried out using Dunnett’s test.

## RESULTS

#### *ABA and LT synergistically upregulated the expression of VvCBF transcription factors in grapevine dormant buds*

The effects of LT (4 °C in the dark), ABA (100  $\mu M$ ) and LT + ABA on the expression of *VvCBF* genes were studied by RT-qPCR in grapevine dormant buds after 1 and 2 weeks of treatment. All the treatments increased the expression of the *VvCBF* genes, both in the first and in the second week post-treatment, except for the *VvCBF2* gene, whose expression

decreased after the first week of treatment (Fig. 1). Joint application of LTs + ABA produced a greater increase in the expression of *VvCBF* genes than the application of either stimulus separately (Fig. 1). It was also observed that the effect of ABA + LT on the expression of the *VvCBF4* and *VvCBF6* genes decreased after the second week of treatment in relation to the first week, while the expression of *VvCBF2* increased and the expression of *VvCBF3* did not change (Fig. 1).

#### ABA and LT synergistically induced cold-hardiness in grapevine dormant buds

The effects of LT, ABA and LT + ABA on bud cold-hardiness were studied in grapevine dormant buds by measuring their LTE by differential thermal analysis. The samples were analysed after 0, 3, 7, 12 and 16 d of treatment. In the samples treated with ABA, the LTE values decreased until the first

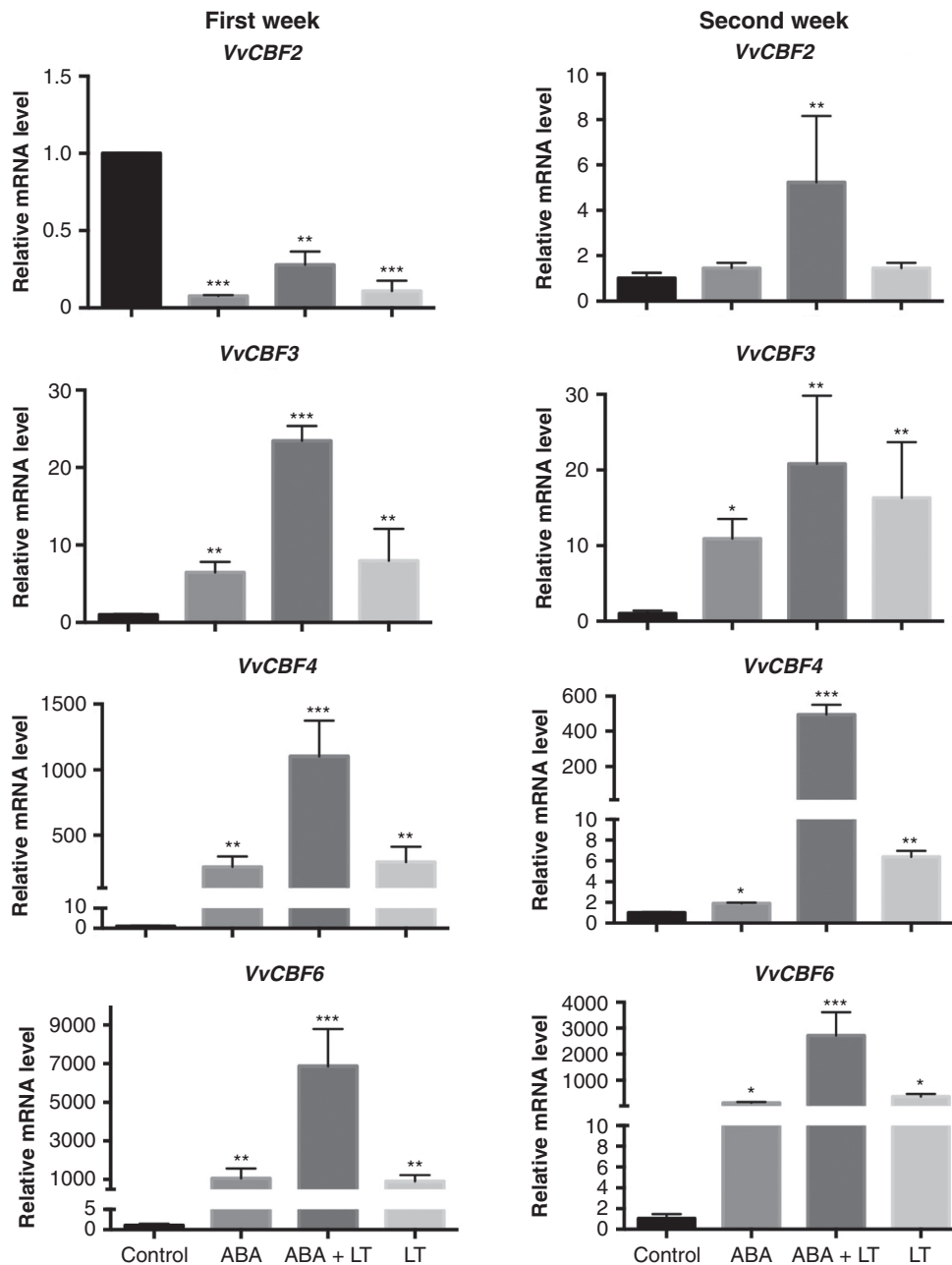


FIG. 1. ABA and low temperature (LT) synergistically induced the expression of CBF/DREB1 transcription factors in grapevine dormant buds. Effect of LT, ABA and ABA + LT on the expression of *VvCBF2*, *VvCBF3*, *VvCBF4* and *VvCBF6* in grapevine dormant buds collected on 14 May after 1 and 2 weeks of treatment. Transcript levels were determined by RT-qPCR and normalized against *VvUBIQUITIN* and *VvACTIN*. Values are averages of three biological replicates each with three technical repetitions. Bars represent  $\pm$  s.d. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$  (Dunnett's multiple comparison test).

week and thereafter recovered. However, when ABA and LT were applied together, a strong decrease in LTE was detected after 3 d of treatment, a minimum was reached after 7 d, and then LTE values recovered to reach values close to the initial value (Fig. 2A). To test whether the synergy between ABA and LT with respect to LTE depends on ABA concentration, single-bud cuttings were treated with 50, 100, 200 and 400  $\mu\text{M}$  ABA solutions, and the LTE was determined after 1 week of exposure to LT (Fig. 2B). The results showed a consistent decrease in LTE values with increasing ABA concentration; however, beyond 200  $\mu\text{M}$  ABA the LTE value remained constant, indicating a saturation effect. Because the dormancy-breaking compound HC catabolizes ABA in grapevine buds (Zheng et al., 2015; Vergara et al., 2017), we tested whether this compound would abolish the synergy between ABA and LT with respect to bud cold-hardiness. The results showed that after 1 week of exposure to LT, HC significantly increased the LTE value, indicating a reduction in its bud cold-hardiness (Fig. 2C).

#### Correlation between ABA content and cold-hardiness in grapevine dormant buds treated with ABA + LT

To analyse whether the recovery of bud cold-hardiness (LTE) after the first weeks of treatment with ABA + LT is due to a reduction in endogenous ABA content, we determined the content of ABA in grapevine dormant buds treated with ABA + LT after 0, 3, 7, 12 and 16 d of treatment (Fig. 3A), and the data were plotted against the LTE values determined at the same treatment intervals (Fig. 3B). The results showed that endogenous ABA content increased in grapevine dormant buds during the first week of treatment, and thereafter decreased (Fig. 3A). A negative correlation with  $r^2 = 0.935$  was obtained when bud LTE values were plotted against the content of endogenous ABA (Fig. 3B).

#### ABA + LT synergistically induced dehydration in grapevine dormant buds

The effects of LT, ABA and ABA + LTs on bud water content of ‘Thompson Seedless’ grapevines were analysed at two collection dates. One week after the combined application of ABA + LT, a significant reduction in the water content of the buds was observed for material collected at the two dates, while the other treatments showed no differences with respect to the control buds (Fig. 4).

#### ABA and LT synergistically induced expression of dehydrins in grapevine dormant buds

The effects of LT, ABA and LT + ABA on the expression of *VvDHN* genes in grapevine dormant buds were analysed. One week after the combined application of ABA and LT, only *VvDHN1* and *VvDHN2* expression levels were increased synergistically (Fig. 5). The expression of *VvDHN4* was induced neither by ABA nor by LT and no synergism was observed between these stimuli (Fig. 5).

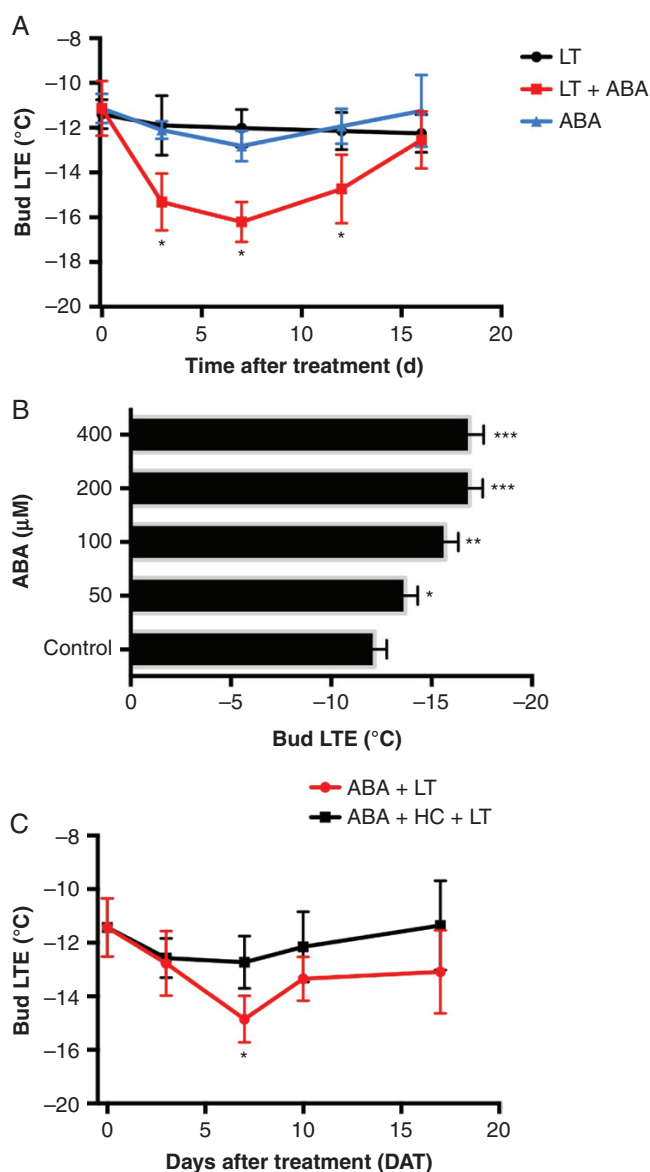


Fig. 2. ABA and LT synergistically enhanced bud cold-hardiness in grapevine dormant buds. (A) Effects of LT, ABA and ABA + LT on the low thermal exotherm (LTE) of grapevine dormant buds collected on 14 May were determined by differential thermal analysis after 0, 3, 7, 12 and 16 d of treatment. Values are averages for 20 buds and bars represent  $\pm$  s.d. \* $P \leq 0.05$  (Dunnnett's multiple comparison test). (B) Effect of ABA concentration on the LTE of grapevine buds collected on 23 April after 1 week of LT in the dark. Values are averages for 20 buds and bars represent  $\pm$  s.d. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$  (Dunnnett's multiple comparison test). (C) Effect of hydrogen cyanamide (HC) on the LTE of grapevine dormant buds treated with ABA + LT. The buds were collected on 23 April. Values are the average of 20 buds and bars represent  $\pm$  s.d. \* $P \leq 0.05$  (Dunnnett's multiple comparison test).

#### ABA and LT synergistically induced expression of antioxidant genes in grapevine dormant buds

The expression of three paralogues of ascorbate peroxidase (*VvAPX*), two of catalase (*VvCAT*), three of glutathione peroxidase (*GLPX*) and three of superoxide dismutase (*VvSOD*) were studied in grapevine dormant buds treated with ABA, LT and ABA + LT after 1 week of treatment (Fig. 6). When they were

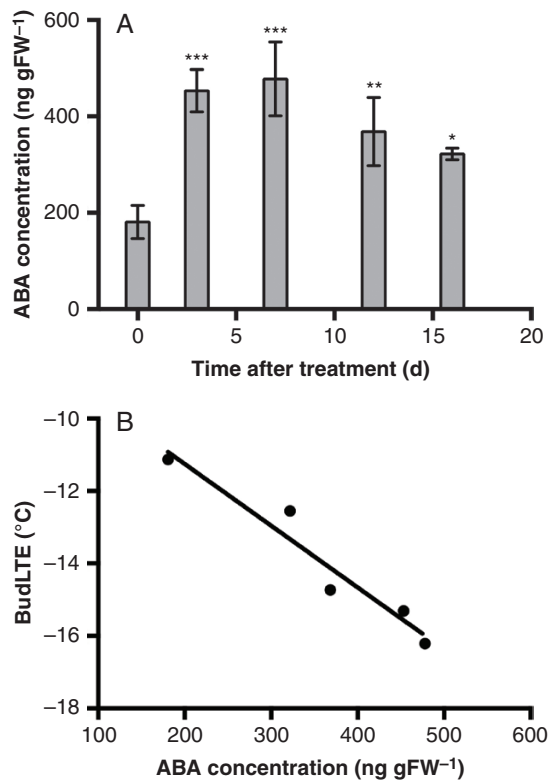


FIG. 3. Correlation between the endogenous content of ABA and the cold-hardiness of grapevine dormant buds after ABA + LT treatment. (A) ABA was determined in grapevine buds collected on 14 May after 0, 3, 7, 12 and 16 d of ABA + LT treatment by gas chromatography using an electron capture detector. Values are the average of three biological replicates and bars represent  $\pm$  s.d. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$  (Dunnnett's multiple comparison test). (B) Correlation of bud LTE (using values obtained in Fig. 2) with endogenous ABA concentration. gFW, grams fresh weight.

applied together, ABA and LTs synergistically increased the expression of *VvCAT1*, *VvGLPX3*, *VvSOD1* and *VvSOD3*. The different treatments did not significantly modify the expression of any of the *VvASPX* transcripts (Fig. 6).

## DISCUSSION

The *CBF/DREB1* transcription factors, a subfamily of the *APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF)*, have been characterized as a regulatory hub in freezing tolerance in herbaceous plant systems (Thomashow, 2010; Theocharis et al., 2012). Functional studies of *CBF* genes in woody plants have indicated that their regulation and impact on abiotic stress are more complex than in herbaceous plants (Wisniewski et al., 2014). In grapevine leaves and seedlings, Xiao et al. (2006, 2008) reported that ABA and LT upregulated the expression of *CBF/DREB1* genes. In the present study we found that LT and ABA treatments induced the expression of *VvCBF* transcription factors in grapevine dormant buds in a way similar to that in leaves and seedlings. However, when stimuli were applied together an increase in the expression of *VvCBF* genes was observed that was greater than the increase produced by them when applied individually. These results clearly indicate the existence of a synergistic induction of *VvCBF* transcription

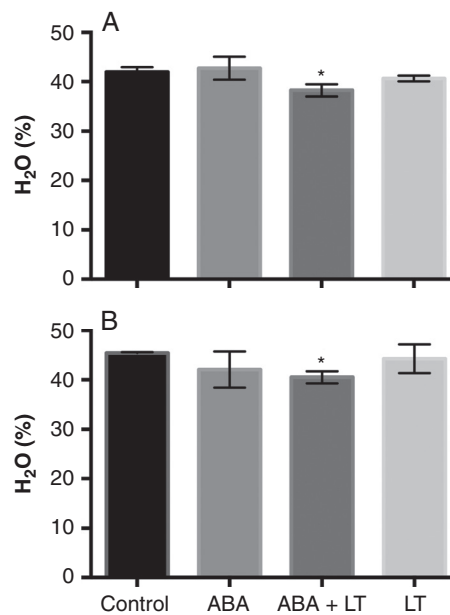


FIG. 4. ABA and LT synergistically increased dehydration in grapevine dormant buds. Effect of LT, ABA and ABA + LT on bud water content (% FW) in 'Thompson Seedless' buds collected on (A) 12 June and (B) 19 June. Bars represent  $\pm$  s.d. \* $P \leq 0.05$  (Dunnnett's multiple comparison test).

factors by the combined application of ABA and LT, a phenomenon that was not described in grapevine vegetative tissues (Xiao et al., 2006). In the grapevine, as in other temperate fruit trees, the dormant bud can develop a much greater degree of freezing tolerance than vegetative tissues, such as leaves and seedlings. Thus, while green tissues cannot withstand temperatures below 4 °C, the dormant grapevine bud can withstand temperatures of -25 °C or lower (Mullins, 1992). Therefore, it is plausible that the high expression level of *VvCBF* genes in grapevine dormant buds could be associated with high freezing tolerance, and a mechanistic relationship could exist between the two variables. The fact that the joint application of ABA and LT also synergistically increased bud cold-hardiness, and that in the buds treated with ABA + LT the expression of the *VvCBF* genes and bud cold-hardiness followed the same trend, supports the hypothesis that the level of expression of the *VvCBF* genes and the cold-hardiness of grapevine dormant buds are related. Comparison between *V. riparia* and *V. vinifera* showed that *V. riparia*, which is endemic to cold regions, had a higher expression level for all the *VvCBF* genes analysed than *V. vinifera* (Karimi et al., 2015), suggesting that the abundance of *VvCBF* transcripts might be related to the degree of cold-hardiness. Interestingly, the expression of *VvCBF2* was inversely related to the expression of *VvCBF4* and *VvCBF6* after the treatments. It has been reported that *AtCBF2* is a negative regulator of *AtCBF1* and *AtCBF3* expression and plays a central role in stress tolerance in *Arabidopsis*, suggesting that repression of *AtCBF2* would induce the expression of *AtCBF1* and *AtCBF3* (Novillo et al., 2004).

Foliar applications of ABA to grapevines increase the cold-hardiness of dormant buds (Zhang and Dami, 2012). Here we showed that in single-bud cuttings of grapevines the combined

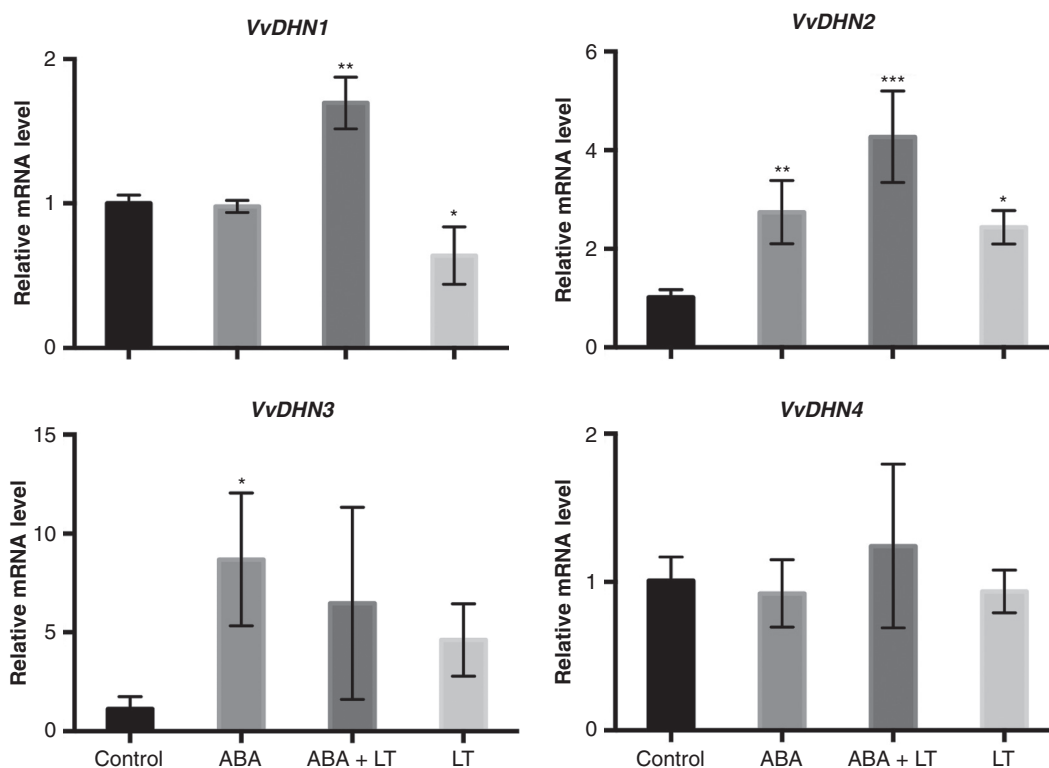


FIG. 5. ABA and LT synergistically induced the expression of dehydrins in grapevine dormant buds. The effects of LT, ABA and ABA + LT on dehydrin gene (*VvDHN*) expression in ‘Thompson Seedless’ grapevine buds collected on 23 April. Transcript levels were determined 1 week after treatment by RT-qPCR and normalized against *VvUBIQUITIN* and *VvACTIN*. Values are averages of three biological replicates each with three technical repetitions. Bars represent  $\pm$  s.d. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$  (Dunnett’s multiple comparison test).

application of ABA and LT synergistically increased bud cold-hardiness in a manner dependent on the ABA concentration. However, the effect was transient, since bud cold-hardiness started to decrease after 1 week of treatment. Moreover, the endogenous content of ABA also decreased after the first week and correlated with bud cold-hardiness, and HC, which degrades ABA in grapevine buds (Zheng *et al.*, 2015; Vergara *et al.*, 2017), abolished the synergistic effect of ABA + LT on bud cold-hardiness. All these results confirmed the hypothesis that ABA plays a crucial role in strengthening the effect of LT in the acquisition of cold-hardiness in grapevine dormant buds. Interestingly, it has been reported that under natural conditions the level of ABA increases in grapevine dormant buds just before the seasonal fall in temperature (Or *et al.*, 2000), suggesting that ABA plays a crucial role in the acquisition of cold-hardiness in grapevine dormant buds.

It has been hypothesized that in grapevines ABA enhances bud cold-hardiness by inducing bud-dehydration (Zhang and Dami, 2012). Our results support this hypothesis, since the combined application of ABA and LT synergistically enhanced bud dehydration and bud cold-hardiness. Moreover, it is well known that *VvCBF* genes are upregulated by dehydration (Yamaguchi-Shinozaki and Shinozaki, 2006). Therefore, the synergistic increase in bud dehydration caused by ABA + LT could be responsible for the increased expression of *VvCBF* genes and for the enhancement of bud cold-hardiness. Dehydrins, a subgroup of LEAs, are among the most commonly observed proteins that accumulate in plants in response

to LT and environmental factors leading to cell dehydration. The presence of DRE/CRT motifs in the promoter of several cold-regulated dehydrins suggests that they play a role in CBF/DREB-mediated signalling pathway (Gilmour *et al.*, 2004). A small family of four dehydrins genes have been identified in *V. vinifera* and their expression has been analysed (Yang *et al.*, 2012). In this study we found that the expression of *VvDHN1* and *VvDHN2*, which possess a DRE *cis*-regulatory element in their promoter (Yang *et al.*, 2012), were synergistically induced by the combined application of ABA and LT, suggesting that they are regulated by *VvCBF* transcription factors and play a role in the cold acclimatization process of grapevine dormant buds. Recently, it has been shown that *VvCBF4* transcription factor binds to the promoter of *VvDHN2* (Vázquez-Hernández *et al.*, 2017). Although *VvDHN3* gene expression was induced by ABA, no synergistic effect was observed after combined application of both stimuli, and *VvDHN4* was induced neither by ABA nor by LT.

It is important to note that not all changes in gene expression that occur during the cold acclimatization process are directly related to freezing tolerance (Wisniewsky *et al.*, 2014). Many of the biochemical and gene expression changes have to do with tissue recovery after cold stress events (Meitha *et al.*, 2015). Plants regulate the availability of oxygen and its metabolism during key transitions, including the transition from a quiescent state to an active growth state (Considine and Foyer, 2014). In this regard, the increase in the expression of antioxidant genes is an important aspect of the cold-acclimatization response. In

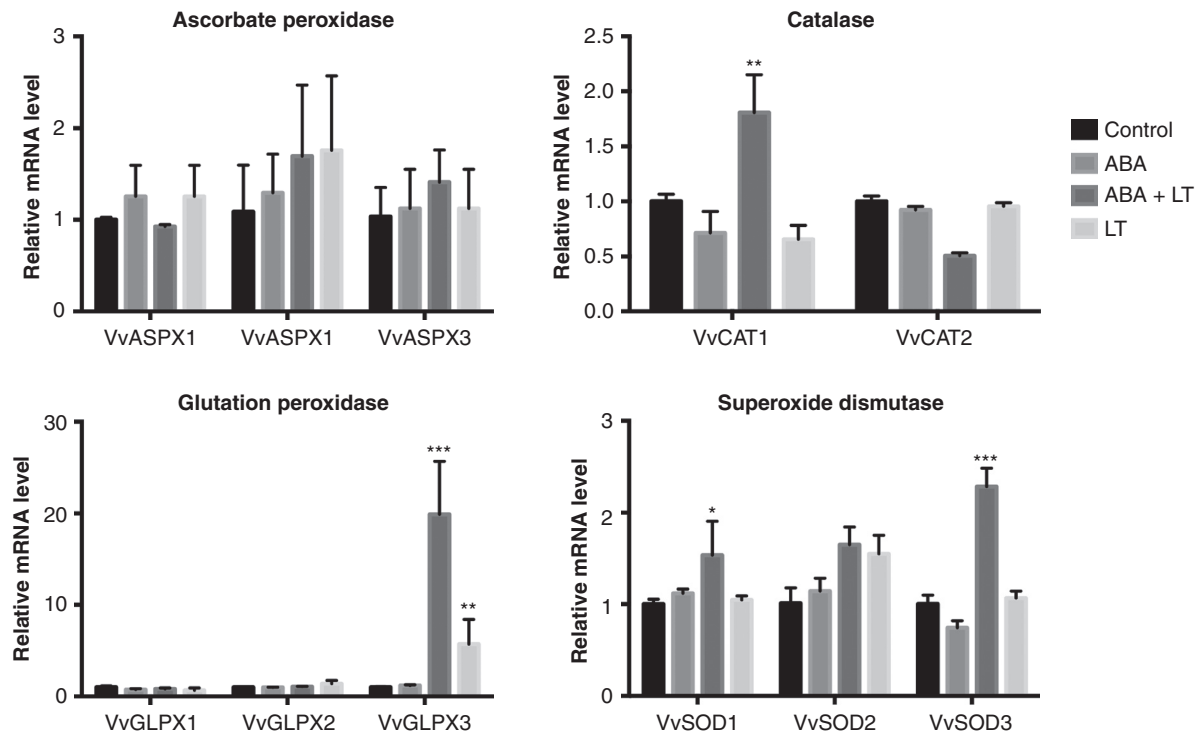


FIG. 6. ABA and LT synergistically induced the expression of antioxidant genes in grapevine dormant buds. Effect of LT, ABA and ABA + LT on the expression of *VvAPX*, *VvCAT*, *VvGPX* and *VvSOD* genes in ‘Thompson Seedless’ grapevine buds collected on 23 April. Transcript levels were determined 1 week after treatment by RT-qPCR and normalized against *VvUBIQUITIN* and *VvACTIN*. Values are averages of three biological replicates each with three technical repetitions. Bars represent  $\pm$  s.d. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$  (Dunnett’s multiple comparison test).

this study we showed that two genes coding for superoxide dismutase (*VvSOD1*, *VvSOD3*), one gene for glutathione peroxidase (*VvGPX3*) and one for catalase (*VvCAT1*) showed synergistically induced expression through the combined application of ABA and LT. These results suggest that the *VvCBF* transcription factors can control the expression of genes related to all aspects of the cold-acclimatization process in grapevine dormant buds.

### Conclusions

The huge increase in the expression of *VvCBF/DREB1* transcription factors in grapevine dormant buds as a result of the combined effect of ABA and LT, and its relationship to the increase in bud dehydration and bud cold-hardiness, together with an increase in the expression of dehydrins and antioxidant genes, highlight the importance of these transcription factors and ABA in the process of cold acclimatization in grapevine dormant buds. Additionally, the results help us to understand why, under field conditions, there is a great increase in the endogenous content of ABA in the grapevine dormant bud at the beginning of autumn, when temperatures start to drop.

### SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. Figure S1: single-bud cuttings of ‘Thompson Seedless’ grapevines mounted on

a propylene sheet and floated in tap water in a plastic container. Figure S2: the Kryoscan, a freezing and data acquisition device that uses Peltier elements for the cooling and detection modules.

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