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Relationship Between Endodormancy and Cold Hardiness in Grapevine Buds

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Abstract Endodormancy (ED) and cold hardiness (CH) are two strategies utilized by grapevine (*Vitis vinifera* L.) buds to survive unfavorable winter conditions. Each phenomenon is triggered by different environmental cues—ED by short-day (SD) photoperiod and cold hardiness (CH) by low temperatures. In grapevine buds, CH occurs mainly via the supercooling of intracellular water, a phenomenon associated with the low temperature exotherm (LTE). The seasonal dynamics of ED and CH were studied on grapevines buds by determining the BR_{50} (time required to reach 50 % of bud break under forced conditions) and the LTE, which measure the depth of ED and the level of CH, respectively. Overlapping BR_{50} and LTE curves revealed that CH began to develop in late April, when buds were fully endodormant and daily mean temperatures had started to drop below 14 °C, suggesting that ED is a prerequisite for the acquisition of full CH. Increase in starch content and thickening of the cell wall (CW) of meristematic cells which occurs in dormant buds could be involved in structural and metabolic changes that favor CH subsequent acquisition. Interestingly, the thickening of the CW and the synthesis of starch which are associated with ED were induced by a SD-photoperiod, while the hydrolysis of starch, the accumulation of soluble sugars, and the up-regulation of dehydrin genes, which are associated with CH, were induced by low temperatures. Overall, the results indicate that structural, metabolic, and transcriptional changes that occur during ED in grapevine buds are necessary for the further development of CH.

Keywords Cold hardiness · Cell wall · Dormancy · Dehydrins · Exotherms · Grapevine buds · Supercooling

Introduction

Grapevines (*Vitis vinifera* L.) develop axillary buds containing embryonic shoots from which whole branches develop after perceiving specific signals (Rohde and Bhalerao 2007). The decreasing photoperiod during late summer is the environmental signal that triggers the transition of buds into endodormancy (ED) (Fennell and Hoover 1991; Kühn and others 2009; Grant and others 2013). ED is a physiological state characterized by growth inhibition, arrest of cell division, and reduced metabolic and respiratory activity. Operationally ED is characterized by a delay in the bud-break response under forced conditions (Lang 1987; Dennis 2003). The development of ED is part of the process by which buds adapt to unfavorable winter conditions. One of the main functions of ED is to avoid bud break in response to a transient warm spell during winter, which could not only avoid further damage by frost (Jian and others 1997), but also play an important role in preparing plants for freezing temperatures (Sakai and Larcher 1987). In tree species with photoperiodically-induced dormancy such as birch (*Betula pendula*), the perception of decreasing day-length results in growth cessation, development of a terminal bud, and progression

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to a dormant and more freezing-tolerant state (Rinne and others 2001). In contrast, species of *Vitis* do not set a terminal bud in response to SD-photoperiod and the shoot apical area does not enter into ED nor cold acclimates; however, upon reaching a critical day-length (CDL), other hallmark phenotypes such as periderm development, growth cessation, and latent bud dormancy are induced (Fennell and Hoover 1991; Wake and Fennell 2000; Sreekantan and others 2010; Grant and others 2013). Freezing tolerance or cold hardiness (CH) also develops in grapevine buds in response to low non-freezing temperatures, a phenomenon known as cold acclimation (Thomashow 1999; Mills and others 2006; Ferguson and others 2011, 2014). It has been reported that the dormant buds of *Vitis riparia* (Pierquet and others 1977) exhibit deep supercooling of intracellular water, suggesting that the depression of the freezing point is the mechanism through which grapevine buds adapt to subfreezing temperatures. Differential thermal analysis (DTA) has been widely used to measure the exotherms of deep supercooled buds, two exotherms are generally observed in cold-acclimated buds, a high-temperature exotherm (HTE) and a low-temperature exotherm (LTE), which correspond to the heat released during the freezing of extracellular and intracellular water, respectively (Burke and others 1976). Lethal tissue damage takes place in buds at temperatures below LTE, indicating that LTE can serve as a measure of CH (Pierquet and Stushnoff 1980; Mills and others 2006; Ferguson and others 2011). Several factors can influence the decrease in the supercooling of water; however, the properties of a cell, tissue, or organ that allow it to undergo deep supercooling remain enigmatic, despite the prevalence of this ability in many plant species (Gusta and Wisniewski 2013).The role of sugars in the development of CH has been well documented in grapevines; total soluble sugars increase during the initial stage of CH, and it is speculated that raffinose plays an important role in cold acclimation in grapevine buds (Grant and others 2013; Hamman and others 1996). Dehydrins, a class of hydrophilic, thermostable stress proteins that belong to the late embryogenesis abundant (LEA) family, are expressed in response to drought, salinity, cold, and osmotic stress (Nylander and others 2001). In buds of *Vitis labruscana* L. cv. Concord, a heat stable 27 KD protein that accumulates in response to cold, was identified as immunologically related to dehydrins by a strong reaction with the antidehydrin antibody (Salzman and others 1996). Recently, four dehydrin genes were identified in *V. vinifera* and *V. yeshanensis*, and their responsiveness to various forms of abiotic and biotic stresses was studied (Yang and others 2012). This study examined the relationship between ED and CH in grapevine buds, and characterized partially the structural, metabolic, and transcriptional changes that occur during ED which are necessary for the further acquisition of CH at low temperatures.

Materials and Methods

Seasonal Variations on the Depth of Dormancy in Buds of *V. vinifera* cv. Thompson Seedless

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 and others 1994; Dennis 2003). This system makes it possible to work with a large number of buds, providing 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, between 11 December and mid-August 2012, from 8-year-old *V. vinifera* L cv. Thompson seedless growing at the experimental station of the Chilean National Institute of Agriculture Research (INIA), located in Santiago (33°34′S latitude). Detached canes each carrying ten buds in positions 5–14 were transferred to the laboratory, and cut into single-bud cuttings. Forty of these cuttings (10–12 cm length) were mounted on a polypropylene sheet and floated in tap water in a plastic container on each collection date. The cuttings were then transferred to a growth chamber set at 23 ± 2 °C with a 16 h photoperiod (forcing conditions). Every 5 days, water was replaced in the container and bud break was assayed for a period of 30 days. The appearance of visible green tissue at the tip of the bud was indicative of bud break. The depth of bud dormancy was determined using $BR_{50,}$ a parameter which is an estimate of the mean time required to reach 50 % bud break under forced conditions (Pérez and others 2007). The depth of dormancy has been previously determined in buds of *V. vinifera* cv. Thompson seedless in the same place and with the same methodology, obtaining similar results (Pérez and others 2007; Vergara and Pérez 2010).

Temperature Measurements

Temperature data were collected every hour from the weather station of the National Institute of Agricultural Research (INIA, La Platina) located at 33°34′S latitude 70°40′W longitude, 100 m from the vineyard.

Seasonal Variations on LTE in Buds of *V. vinifera* cv. Thompson Seedless

Canes collected weekly from field-grown *V. vinifera* cv. Thompson seedless between 22 April and 27 August 2012

were cut into single buds. Exotherms were determined in single buds by differential thermal analysis (DTA). Kryoscan, a freezing and data acquisition device that uses Peltier elements (PE) for the cooling and detection modules (Badulescu and Ernst 2006) was employed for DTA. The temperature of the cooling block was pre-chilled at 10 °C using a water bath and further chilled by a pyramidal configuration of PE connected to a temperature controller (PXR-4, Fuji electronic system, Japan). The temperature controller regulates the passing voltage through the PE according to a temperature sensor connected to the cooling block, and by the programmed temperature ramp. Miniature PE (Peltier modules series Opto Tec, Laird Technologies-Engineered Thermal Solutions, USA) instead of thermocouples were used to record exotherms, because they yield a relatively high voltage difference which is less susceptible to electrical noise, and no external zero references are needed (Badulescu and Ernst 2006). These sensitive PE detect temperature gradients generated by the exotherms and convert the thermal signal to voltage outputs. The data acquisition system (Measurement Computing USB 120BLS, USA) and DasyLab software were used to measure and collect the output voltage and the temperature. Signals were recorded every 2 s, and a decrease in temperature of 4 °C per h starting at 10 °C and ending at −30 °C was programmed (Mills and others 2006). Generally, two peaks were observed, one corresponded to HTE that was assigned to the freezing point of extracellular (apoplast) water, which is non-lethal (Burke and others 1976) and the other corresponded to LTE that was assigned to the freezing point of intracellular water, which is lethal (Burke and others 1976). Because lethal tissue damage in grapevine buds occurs at temperatures below the LTE, this value can serve as a measure of CH (Pierquet and Stushnoff 1980; Wolf and Cook 1994; Mills and others 2006). Values for each date correspond to the average of 12 biological replicates of single buds (Fig. 1). LTE measurements were repeated in the same location and with the same variety during 2013.

Effect of Temperature on LTE of Dormant and Non-dormant Buds of *V. vinifera* cv. Thompson Seedless

To analyze the effects of temperature on exotherms of dormant and non-dormant buds, single-bud cuttings of *V. vinifera* cv. Thompson seedless collected on 27 December (non-dormant) and 10 June 2012 (dormant) were exposed to low (5 °C, cooled) and room (14 °C, non-cooled) temperatures, and exotherms were measured in single buds over time (12 buds at each collection time). Dormant buds prior to harvesting were exposed in the field to approximately 200 chilling hours, and therefore, were partially cold acclimated (LTE = −15 °C) before the experiments.

Effect of Dormancy and Low Temperatures on the Starch and Soluble Sugar Content of Buds of *V. vinifera* cv. Thompson Seedless

To study the effect of dormancy on the levels of starch in grapevine buds, starch levels were determined in buds of *V. vinifera* cv. Thompson seedless collected on 27 December (non-dormant) and 10 June 2012 (dormant). To study the effect of low temperature on the starch and soluble sugar content in grapevine buds, buds of *V. vinifera* cv. Thompson seedless collected on 10 June 2012 (dormant) were used. The buds (0.2 g approx.) were ground with a mortar and pestle in liquid nitrogen and extracted 3× with 3 ml of cold acetone and 1× with a mixture of chloroform and isoamyl alcohol (24:1). The suspension was centrifuged at 13,000 rpm for 3 min, and the pellet was dried and extracted with 2 ml 80 % (v/v) ethanol for 30 min in a water bath heated to 60 °C. This extraction was repeated 3 times, and the supernatants were collected, pooled, and dried. The starch content of the pellet was determined after ethanol extraction of the soluble sugars by acid extraction using the anthrone reagent (Hansen and Moller 1975). The dried supernatant obtained from ethanol extraction was dissolved in 100 µl of pyridine and an aliquot of 15 µl was derivatized by adding 5 µl BSTFA (Sigma-Aldrich, USA); the mixture was then heated at 90 °C for 30 min. The chromatographic analyses of the derivatized samples were performed using a Shimadzu GC 2014 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a CBP1 capillary column and an FID detector. The operating conditions were as follows: injector and detector temperatures were 180 and 300 °C, respectively; carrier gas flow (helium) at 1.0 ml/min; injection volume of 1 µl with a flow splitter at a ratio of 50:5. The oven was programmed to temperatures of 60–200 °C at a rate of 30 °C min^{-1} and from 200 to 280 °C at a rate of 5 °C min^{-1}. Standard curves were constructed for the determination of the sucrose, glucose, fructose, and starch concentrations.

Influence of Dormancy and SD-Photoperiod on CW Thickness in Meristematic Cells

The thickness of the cell wall (CW) of meristematic cells of non-dormant buds (collected 27 December 20012) and dormant buds (collected 10 June 2012) of *V. vinifera* cv. Thompson seedless grown in Santiago, Chile (33°34′S 70°40′W) was examined by transmission electron microscopy (TEM). The thickness of the CW of meristematic cells of buds of *V. vinifera* cv. Italia melhorada grown in Messoró, Brazil (5′12′S) and exposed to different

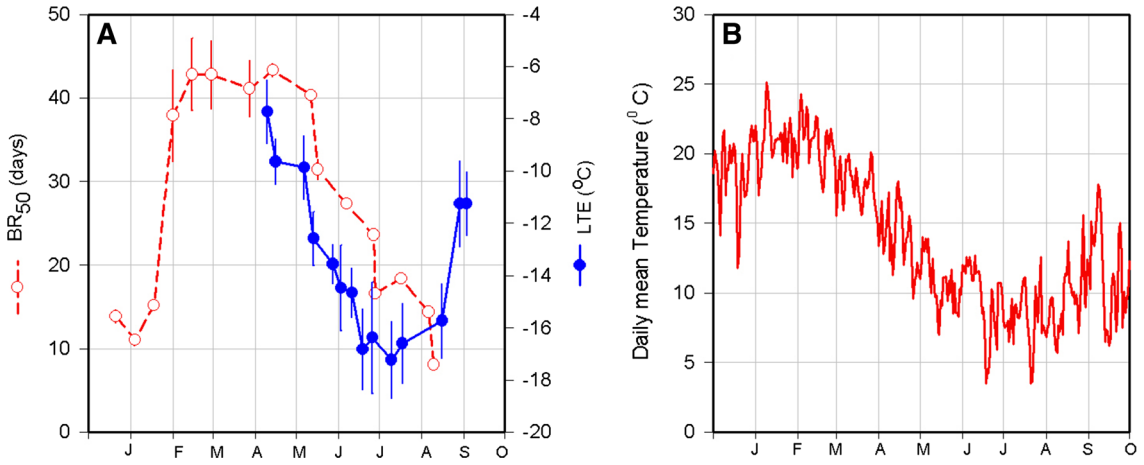

Fig. 1 Comparison of daily mean temperature, endodormancy (ED), and cold hardiness (CH) in grapevine buds. **a** Endodormancy (ED) and cold hardiness (CH) development in *V. vinifera* cv. Thompson seedless. **b** Daily mean temperatures in Santiago, Chile (33°34′S) during the year 2012. The depth of ED was determined by BR_{50} (time required to reach 50 % bud break under forced conditions). Values of BR_{50} for each collection date were determined by Probit Analysis (Minitab statistical software) and *bars* represent s.d. ($n = 40$). CH was determined by measuring the LTE by differential thermal analysis (DTA) using Peltier modules (TEM). *Bars* represent s.d. ($n = 16$)

photoperiods was also analyzed by TEM. Sections were taken from the middle of the buds and were fixed in 2 % formaldehyde, post-fixed for 2 h in 0.1 mg × ml^{-1} osmium tetra-oxide, dehydrated in a graded series of ethanol (25, 50, 70, 90, and 3 × 100 % for at least 1 h for each), and embedded in Spurr's resin. Thin sections were cut with a diamond knife, stained in uranyl acetate and lead citrate, and examined with a Jeol 100 SX electron microscope. Three buds per treatment were analyzed to determine the thickness of the CW.

Photoperiod Treatments on *V. vinifera* cv. Italia Melhorada

In a collaborative project with colleagues from Brazil, the effect of different photoperiod regimes on the thickness of the cell wall (CW) and on the expression of cellulose synthase, lacasse, and dehydrin genes was performed. Photoperiod experiments were carried out in Messoró, Brazil due to small variations in photoperiod and temperature in the area, making it easier to conduct this type of experiment. Cuttings of *V. vinifera* cv. Italia melhorada on rootstock IAC 572 grown at the Federal University of Rural Semi-Arid (UFERSA), located in Messoró, Brazil (5°12′16″S), where the natural photoperiod during the whole year is (12/12 h day/night) and temperature fluctuates between 29 and 31 °C, were used as plant material for photoperiod experiments (3 replicates per treatment). Rooted cuttings (15 per treatment) were planted into mix 1:1:1 (v: v: v) soil, sand, and muck in 5 l pots. As growth commenced, one shoot was allowed to develop on each cutting. Cuttings having uniform growth with 12–16 leaves were selected and randomly assigned to each photoperiod treatment for 8 weeks. Photoperiod experiments were conducted in a greenhouse under LD (14/10 h day/night) and SD-photoperiod (10/14 h day/night), because the critical day-length (CDL) for dormancy transition in *V. vinifera* is about 13 h (Kühn and others 2009). Supplemental light was provided automatically in the afternoon at 17:30 h using a 100 W fluorescent tube; light restriction was imposed with a black plastic sheet in the early morning at 4:30 h. After the treatments, buds were lyophilized for gene expression analysis, and fixed in 2 % formaldehyde for TEM analysis.

Photoperiod and Low-Temperature Effect on the Expression of Dehydrin Genes

For photoperiod experiments, total RNA was isolated from lyophilized buds (0.05–0.1 g) of *V. vinifera* cv. Italia melhorada. For low-temperatures experiments, total RNA was isolated from buds (0.5–0.7 g) of *V. vinifera* cv. Thompson seedless. In both cases, total RNA was extracted and purified using a modification of the method of Chang and others (1993), as described in Noriega and others (2007). DNA was removed by treatment with RNAase-free DNAase (1 U/μg) (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, USA). Gene expression analysis was performed by quantitative real-time PCR, and carried out in an Eco Real-Time PCR system (illumina, Inc. SD, USA), using KAPA SYBR FAST mix (KK 4602) and KAPA Taq

DNA Polymerase (Kapa Biosystem, USA). Primers suitable for the amplification of 100–150 bp products for each gene under study were designed using the PRIMER3 software (Table 1 supplement) (Rozen and Skaletsky 2000). The amplification of cDNA was performed under the following conditions: denaturation at 94 °C for 2 min and 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. Transcript levels were calculated by the ΔΔCq method (Livak and Schmittgen 2001) using *VvUBIQUITIN* as the reference gene. *VvUBIQUITIN* was selected as a reference gene because the transcript level was stable across treatments (Rubio and others 2014).

Statistical Analysis

The depth of bud dormancy was estimated by BR_{50} and the corresponding averages and standard deviations (s.d.) were calculated by mean of the Probit analysis (Minitab 13.31 Minitab Inc, USA). For pairwise comparison, Student's *t* test $\alpha = 0.05$ was used.

Results

Seasonal Variations of Cold Hardiness and Endodormancy in Grapevine Buds

The depth of dormancy measured as BR_{50} and the level of CH measured as LTE were determined in buds of *V. vinifera* cv. Thompson seedless throughout the year 2012 (Fig. 1a). The vines were grown in Santiago, Chile (33°34′S) and daily mean temperatures of the location are shown in Fig. 1b.

Temperature Affects LTE Values Only in Dormant Buds

Significant differences in the values of LTE were detected in cooled (exposed to 5 °C) and non-cooled (exposed to 14 °C) dormant buds. After 3 weeks of exposure to low temperatures, the value of LTE decreased from −15 to −16.5 °C, whereas in buds exposed to ambient temperature the value of LTE increased from −15 to −13 °C. Moreover, the difference among buds exposed to both treatments, increased with the progress of time, and after 6 weeks of treatment, the difference increased from 3 to

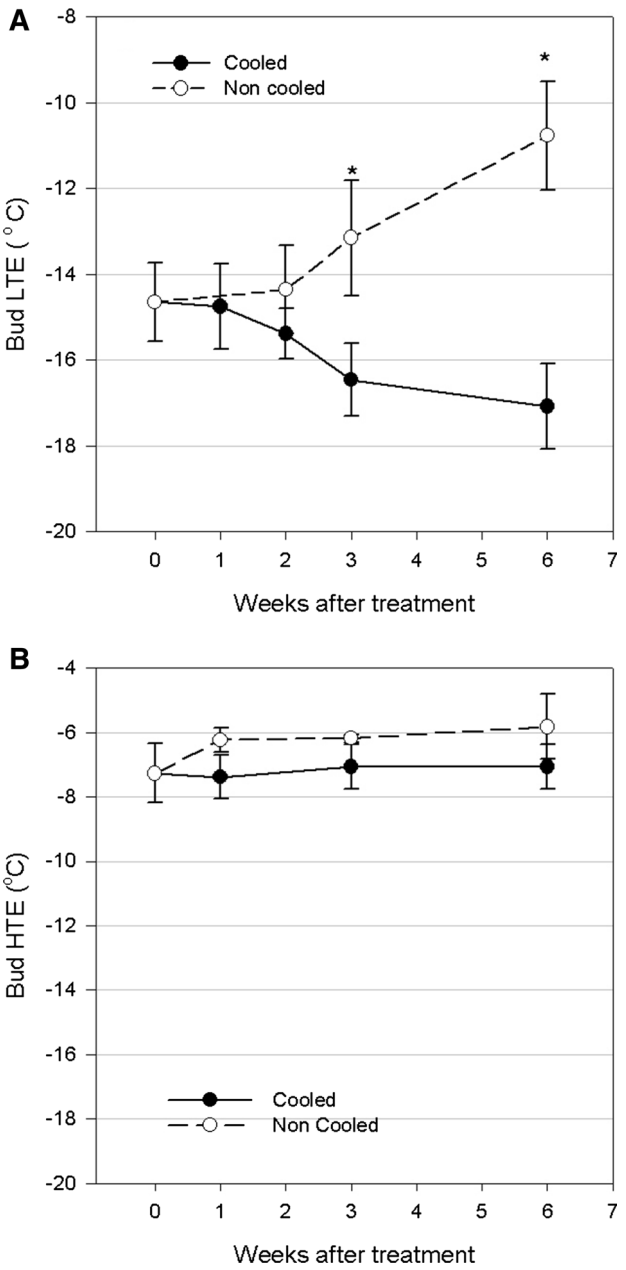

Fig. 2 Cold acclimation and deacclimation of dormant grapevine buds exposed to low (5 °C) and ambient (14 °C) temperatures. **a** Dormant buds with partial cold acclimation were collected on 10 June after being exposed to 200 chilling hours in the field. Cold acclimation was determined by measuring the low-temperature exotherm (LTE) by DTA in single buds over time. **b** Low (5 °C) and ambient (14 °C) temperature effects on the high-temperature exotherm (HTE) in non-dormant buds. Canes were collected on December 27. Values correspond to the average of 12 single and bars represent s.d. and *asterisk* indicates significant differences (Student's *t* test $\alpha = 0.05$)

6 °C (Fig. 2a). In contrast, in non-dormant buds, a broad large peak was detected at −7 ± 1 °C, which did not vary with the temperatures (Fig. 2b). This peak was interpreted as the result of an overlap between HTE and LTE.

Dormancy and SD-Photoperiod Increase the Thickness of the CW in Meristematic Cells of Grapevine Buds

Significant differences between the thickness of the CW of meristematic cells of dormant (1.4 ± 0.3 µM) and non-dormant buds (0.28 ± 0.1 µM) of *V. vinifera* cv Thompson seedless were observed (Fig. 3c, d). Because SD-photoperiod induces ED in grapevine buds (Fennell and Hoover 1991; Kühn and others 2009; Pérez and others 2009; Grant and others 2013), the effect of photoperiod on the thickening of the CW of meristematic cells of buds of *V. vinifera* cv. Italia melhorada was examined by TEM. After 8 weeks of treatment, significant differences between the thickness of the CW of meristematic cells exposed to SD (0.75 ± 0.1 µM) and LD-photoperiods (0.3 ± 0.05 µM) were observed (Fig. 3a, b).

Photoperiod Regulation of "Cellulose synthase and Laccase" Genes of Grape Buds

All cellulose synthase genes of grapevine buds analyzed were down-regulated by SD-photoperiod. After 8 weeks of treatment, the expression of *VvCSA3* and *VvCSLG* was down-regulated by SD-photoperiod, whereas the expression of *VvCSLE* was down-regulated only after 2 weeks (Table 1). Conversely, the expression of *VvLAC14*, a gene involved in laccase synthesis, was up-regulated by SD-photoperiod, whereas the expression of the other laccase genes analyzed was not affected by photoperiod (Table 1).

Dormancy Increases Starch Accumulation and Low Temperatures Increase Starch Breakdown and Soluble Sugar Content in Dormant Buds

Starch accumulates during the development of ED in grapevine buds, and the levels of starch in non-dormant buds were significantly lower than those found in dormant buds (Fig. 4a). On the other hand, low temperature (5 °C) reduced the content of starch in dormant buds. After

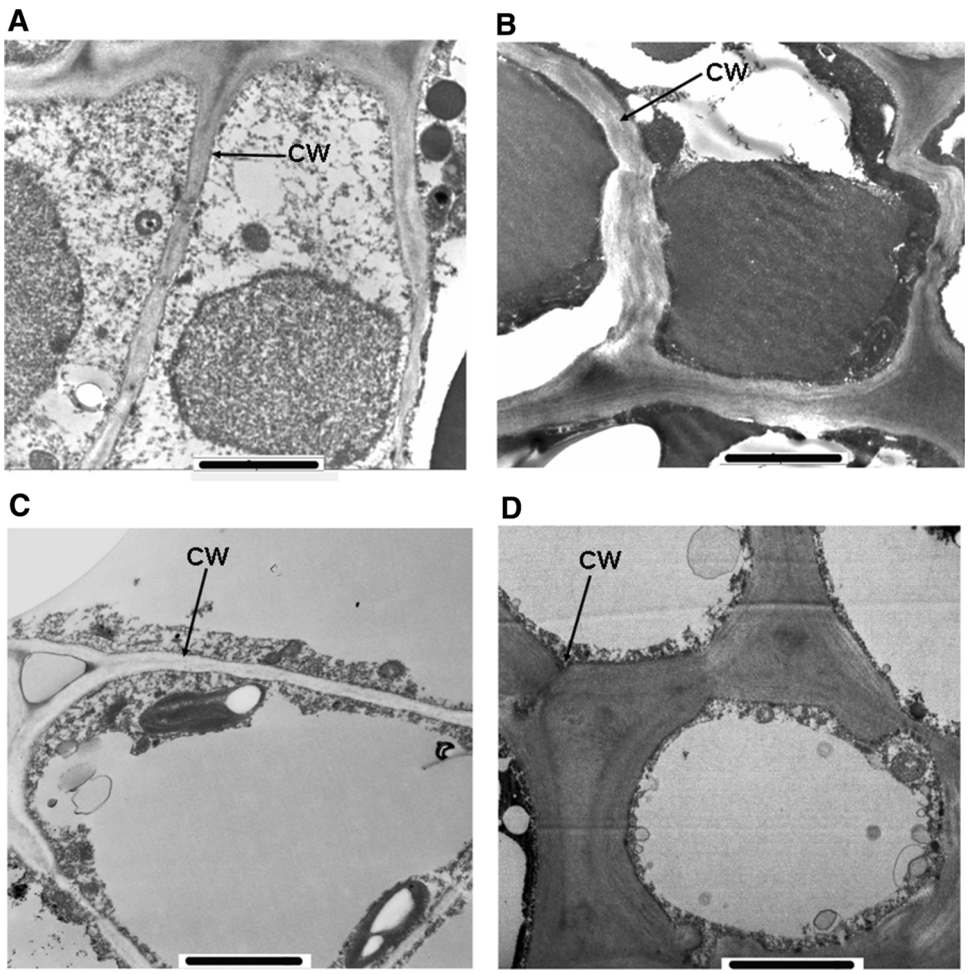

Fig. 3 Electron photomicrographs that illustrate the effect of photoperiod and endodormancy (ED) on the thickening of the cell wall (CW) of meristematic cells in buds of *V. vinifera* L cv. Italia melhorada and cv. Thompson seedless. Photoperiod studies were performed in *V. vinifera* cv. Italia melhorada exposed to **a** LD-photoperiod and **b** SD-photoperiod for 8 weeks. Endodormancy studies were performed in buds of *V. vinifera* cv. Thompson seedless collected on **c** 27 December (non-dormant), and **d** 10 June 2012 (dormant). *Scale bars* = 2 µM

Table 1 Effect of photoperiod on the expression of cellulose synthase *VvCSA3*, *VvCSLE*, *VvCSLG* and laccase *VvLAC7*, *VvLAC9*, *VvLAC14* genes in buds of *Vitis vinifera* cv

Genes	SD-photoperiod		LD-photoperiod	
	2 weeks	8 weeks	2 weeks	8 weeks
VvCSA3	1.1 ± 0.3	1.0 ± 0.1	1.0 ± 0.3	[a]2.1 ± 0.2
VvCSLE	1.1 ± 0.1	1.0 ± 0.2	[a]1.5 ± 0.2	1.1 ± 0.2
VvCSLG	1.0 ± 0.3	1.0 ± 0.4	1.1 ± 0.4	[a]1.9 ± 0.2
VvLAC7	1.1 ± 0.6	1.0 ± 0.1	0.8 ± 0.2	1.5 ± 0.6
VvLAC9	1.0 ± 0.1	1.1 ± 0.6	0.9 ± 0.1	1.4 ± 0.8
VvLAC14	1.0 ± 0.2	1.1 ± 0.1	1.0 ± 0.3	[a]0.4 ± 0.1

Samples exposed to SD-photoperiod for 2 weeks serve as controls. Values are the average of three biological replicates with three technical repetitions. Italia melhorada after 2 and 8 weeks of treatments

[a] Significant differences Student's *t* test $\alpha = 0.05$ and ±correspond to s.d

3 weeks of exposure to cold, starch content was reduced by 50 mg GFW^{-1}, whereas in buds exposed to ambient temperatures (14 °C) for the same period of time, it was reduced only by 10 mg GFW^{-1} (Fig. 4b). Accordingly low temperatures increased the content of soluble sugars, sucrose (Suc), glucose (Glc), and fructose (Fru) in dormant buds (Fig. 5).

Effect of Photoperiod and Low Temperatures on the Expression of Dehydrin Genes in Grapevine Buds

The expression of *VvDHN1*, *VvDHN2*, and *VvDHN3*, but not of *VvDHN4* was strongly up-regulated by low temperatures in buds of *V. vinifera* cv. Thompson seedless. Analysis was performed by RT-qPCR after 2 weeks of treatment (Fig. 6a). Conversely, the expression of *VvDHN1*, *VvDHN2*, and *VvDHN3* was down-regulated by SD-photoperiod, whereas the expression of *VvDHN4* was up-regulated in buds of *V. vinifera* cv. Italia melhorada (Fig. 6b).

Discussion

The present study shows that the development of dormancy in grapevine buds is a prerequisite for the acquisition of full CH; whereas bud dormancy is characterized by the thickening of the CW of meristematic cells and starch accumulation, CH is characterized by starch breakdown, soluble sugar accumulation, and up-regulation of dehydrin genes.

Overlapping BR_{50} and LTE curves of buds of *V. vinifera* cv. Thompson seedless grown in Santiago, Chile revealed

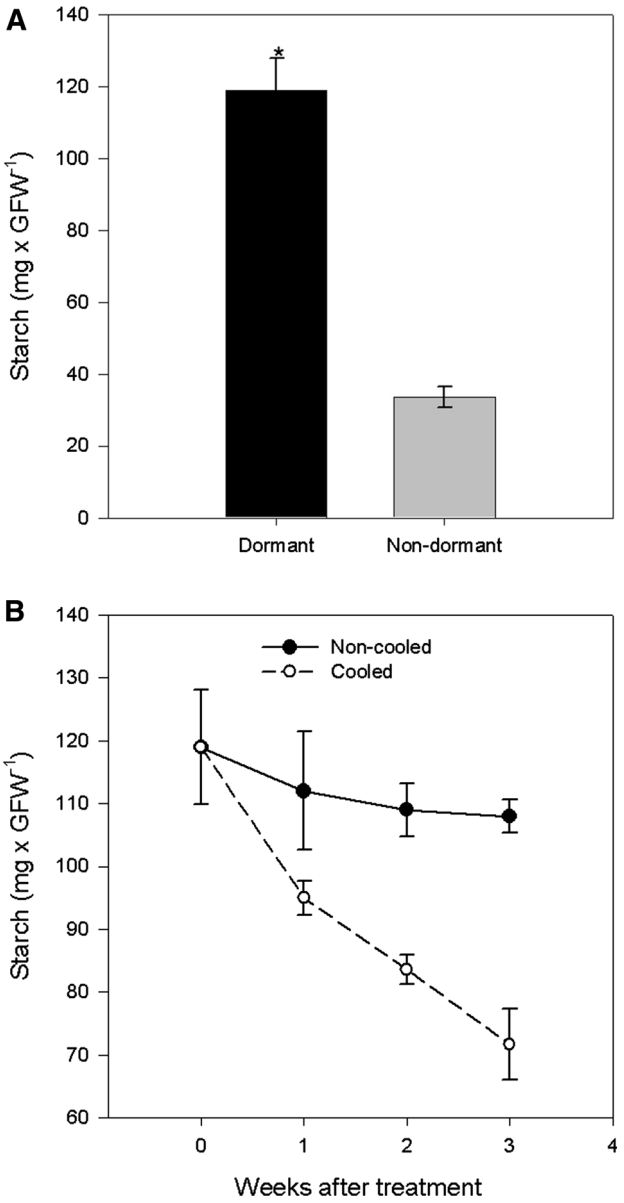

Fig. 4 Effect of dormancy and low temperature on the content of starch in buds of *V. vinifera* cv. Thompson seedless. **a** Starch content was determined in dormant buds (collected on 10 June) and in non-dormant buds (collected on 27 December). **b** Single dormant bud cuttings exposed to ambient (14 °C) (non-cooled) and low temperatures (5 °C) (cooled) were weekly analyzed for starch content for a period of 3 weeks. Values correspond to the average of three biological replicates *bars* represent s.d. and *asterisk* indicates significant differences (Student's *t* test $\alpha = 0.05$)

that CH began to develop in late April when buds were fully endodormant. However, these results do not assure whether a relationship exists between CH and ED, because the drop in temperatures, and therefore, the initiation of chilling accumulation could coincide with the stage of ED in this region. To get more insight into the existence of a relationship between dormancy and CH in grapevine buds,

temperature effects on LTE values were studied in dormant and non-dormant buds of *V. vinifera* cv. Thompson seedless. Although the experiments were carried out in single-bud cuttings in the dark, the results indicate that dormant buds can be cold acclimated or deacclimated depending on whether they were exposed to low or ambient temperatures. Conversely, non-dormant buds were not cold acclimated when they were exposed to low temperatures. Interestingly, these results are consistent with reports indicating that the buds of woody perennials cannot cold acclimate when the development of ED is prevented by over-expressing *PHYA* and *FT* genes (Olsen and others 1997; Tränker and others 2010).

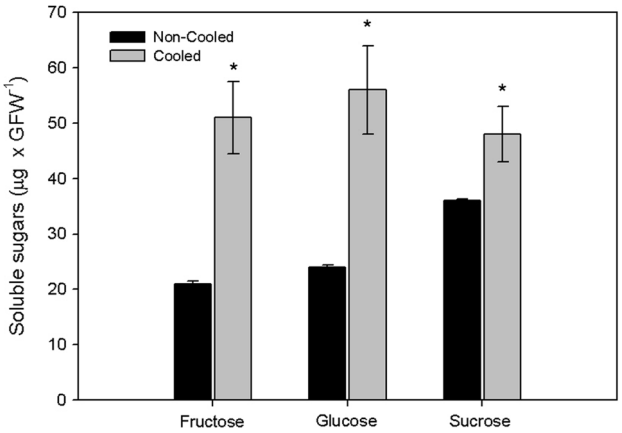

Fig. 5 Effect of low temperature on the accumulation of soluble sugars in buds of *V. vinifera* cv. Thompson seedless. Dormant buds were exposed to low (5 °C) (cooled) and ambient (14 °C) (non-cooled) temperatures for 3 weeks. Soluble sugars were extracted and measured by gas chromatography. Values are the average of three biological replicates and *bars* correspond to s.d. and *asterisk* indicates significant differences (Student's *t* test $\alpha = 0.05$)

Although SD-photoperiod induces ED in grapevine buds (Fennell and Hoover 1991; Kühn and others 2009; Grant and others 2013), its effect on LTE is very low (Grant and others 2013), indicating that CH is mainly induced by low temperatures in grapevine buds. As the thickening of the CW and starch synthesis is associated with ED, and only dormant buds are cold acclimated by low temperatures, it seems likely that CW thickening and starch accumulation that occurs during ED could play a significant role in the subsequent development of CH in dormant buds. The thickening of the CW that is triggered by SD-photoperiod does not involve an increase in the expression of cellulose synthase genes, suggesting that the synthesis of cellulose during ED is not regulated transcriptionally. However, the SD-photoperiod up-regulation of *VvLAC14* suggests that the potential increase in lignin synthesis during ED could be transcriptionally regulated.

A number of roles have been proposed for sugars in freezing tolerance, including osmotic effects (Sakai and Larcher 1987), decrease of ice nucleation point in super-cooled liquid (Gunnink 1989), cryoprotection of proteins and membranes (Ashworth and others 1993), and promotion of glass formation (Levine and Slade 1998). Therefore, it is possible sugars act in several capacities to affect

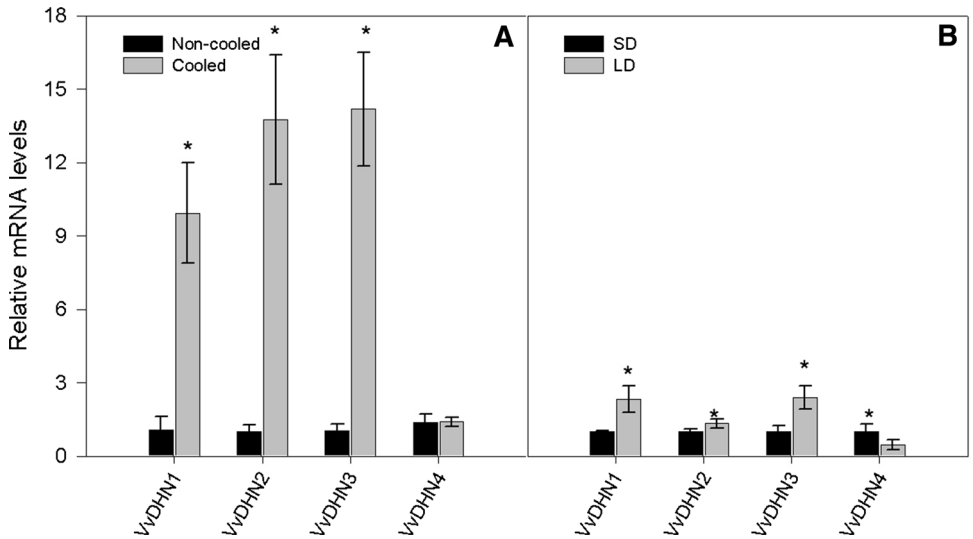

Fig. 6 Effect of low temperature and photoperiod on the expression of dehydrin genes (*VvDHNs*) in grapevine buds. **a** Single-bud cuttings of *V. vinifera* cv. Thompson seedless grapevines exposed to ambient (14 °C) (non-cooled) and low temperatures (5 °C) (cooled) for 2 weeks. **b** *V. vinifera* cv. Italia melhorada exposed to LD and SD-photoperiod for 8 weeks. Transcript levels were determined by RT-qPCR, normalized against *VvUBIQUITIN*. Samples maintained at ambient temperature 14 °C (non-cooled) serve as controls in low-temperature experiments, and vines exposed to SD-photoperiod serve as controls in the photoperiod experiment. Values are the average of three biological replicates each with three technical repetitions, *bars* represent s.d. and *asterisk* indicates significant differences (Student's *t* test $\alpha = 0.05$)

freezing tolerance depending on the tissue or conditions. An increase of total soluble sugars and a decrease in starch content have been observed coincidentally with an increase in freezing tolerance in many plant species (Levitt 1980). In this study, starch accumulation in grape buds was associated with dormancy and starch breakdown and the subsequent increase in sugar content with CH. Interestingly, it has been reported that in *V. amurensis*, a wild grapevine species with remarkable cold tolerance, the expression of genes coding for starch-degrading enzymes such as α-amylases was up-regulated by cold stress (Xin and others 2013). This result was confirmed in *V. vinifera* by Rubio and others (2014), who demonstrated that diverse isogenes coding for α-amylases was up-regulated in dormant buds exposed to low temperatures.

Recently, several studies have shown that the accumulation of dehydrins (DHNs) and other stress proteins plays an important role in the acclimation of woody plants to unfavorable temperatures (Kosova and others 2007). DHNs are a class of hydrophilic, thermostable stress proteins with a high number of charged amino acids that belong to the group II Late Embryogenesis Abundant (LEA) family. Genes that encode these proteins are expressed during late embryogenesis, as well as in vegetative tissue subjected to drought, low-temperature and high-salt conditions (Nylander and others 2001). Four dehydrin genes were identified in the genome of *V. vinifera* (*VvDHNs*), two belonging to YnSKn type *VvDHNs* (*VvDHN1*, *VvDHN4*), and two to SKn type *VvDHNs* (*VvDHN2*, *VvDHN3*), and their expression pattern and stress response varied between them (Yang and others 2012). In this study, low temperatures up-regulated, whereas SD-photoperiod down- regulated the expression of *VvDHN1, VvDHN2,* and *VvDHN3*. Because, SD-photoperiod induces ED and low temperatures induce CH in grapevine buds, it is likely that these cold-induced *VvDHNs* are associated with the acquisition of CH. Interestingly, it has been reported that *V. riparia* (*VrDHN1*) protects lactate dehydrogenase (LDH) from freeze–thaw damage more effectively than bovine serum albumin (BSA), a protein with a known cryoprotective function (Hughes and Graether 2011; Hughes and others 2013). In other woody perennials, DHN expression has been associated with both ED and CH. In blueberry, DHN proteins accumulate during cold acclimation and a relationship between the abundance of DHNs and CH has been established (Arora and others 1997). In birch (*Betula pubescens*), SD-photoperiod and low temperature induce the expression of *BPuDHN1*, whereas *BPuDHN2* was exclusively induced by low temperatures (Welling and others 2004). The potential significance of DHNs in the acquisition of CH lies in the fact that plant cells undergo dehydration during freezing stress due to the presence of ice in extracellular spaces (Levitt 1980).

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References

Arora R, Rowland LJ, Panta GR (1997) Chill-responsive dehydrins in blueberry: are they associated with cold-hardiness or dormancy transitions? Physiol Plant 101:8–16

Ashworth EN, Stirm VE, Volenec J (1993) Seasonal variations in soluble sugars and starch within woody stems of *Cornus sericea* L. Tree Physiol 13:379–388

Badulescu R, Ernst M (2006) Changes of temperature exotherms and soluble sugar in grapevine (*Vitis vinifera* L) buds during winter. J Appl Bot Food Qual Angew Bot 80:165–170

Burke MJ, Gusta LV, Quamme HA, Weiser CJ, Li PH (1976) Freezing and injury in plants. Ann Rev Plant Physiol 27:507–528

Chang S, Puryear J, Cairney JA (1993) Simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep 11:113–116

Dennis FG (2003) Problems in standardizing methods for evaluating the chilling requirements for the breaking of dormancy in buds of woody plants. Hortic Sci 38:347–350

Fennell A, Hoover E (1991) Photoperiod influences growth, bud dormancy and cold acclimation of *Vitis labruscana* and V. *riparia*. J Am Soc Hortic Sci 116:270–273

Ferguson JC, Tarara JM, Mills LJ, Grove GG, Keller M (2011) Dynamic thermal time model of cold hardiness for dormant grapevine buds. Ann Bot 107:389–396

Ferguson JC, Moyer MM, Mills LJ, Hoogenboom G, Keller M (2014) Modeling dormant bud cold hardiness and bud-break in twenty-three *Vitis* genotype reveals variation by region of origin. Am J Enol Vitic 65:59–71

Grant TNL, Gargrave J, Dami IE (2013) Morphological physiological, and biochemical changes in Vitis genotype in responses to photoperiod regimes. Am J Enol Vitic 64:466–475

Gunnink BW (1989) Critical analysis of conductometric phase transition porosimetry: i Capillary freezing and melting. Part Sci Techol 7:229–239

Gusta LV, Wisniewski M (2013) Understanding plant cold hardiness: an opinion. Plant Physiol 147:4–14

Hamman RA, Dami IE, Walsh TM, Stushnoff C (1996) Seasonal carbohydrate changes and cold hardiness of chardonnay and riesling grapevines. Am J Enol Vitic 47:31–36

Hansen J, Moller IB (1975) Percolation of starch and soluble carbohydrates from plant tissue for quantitative determination with anthrone. Anal Biochem 68:87–94

Hughes S, Graether SP (2011) Cryoprotective mechanism of a small intrinsically disordered dehydrin proteins. Prot Sci 20:42–50

Hughes S, Schart V, Malcomson J, Hogarth KA, Martynowicz DM, Tralman-Baker E, Patel SN, Graether SP (2013) The importance of size and disorder in the cryoprotective effects of dehydrins. Plant Physiol 163:1376–1386

Jian L, Li PH, Sun L, Chen TH (1997) Alterations in ultrastructure and subcellular localization of Ca^{2+} in poplar apical bud cells during the induction of dormancy. J Exp Bot 48:1195–1207

Kosova K, Vitamvas P, Prasil IT (2007) The role of dehydrins in plant response to cold. Biol Plant 51:601–617

Koussa T, Broquedis M, Bouard J (1994) Changes of abscisic acid level during the development of grape latent buds, particularly in the phase of dormancy break. Vitis 33:63–67

Kühn N, Ormeño J, Jaque-Zamora G, Pérez FJ (2009) Photoperiod modifies the diurnal expression profile of *VvPHYA* and *VvPHYB* transcript in field-grown grapevines. J Plant Physiol 166: 1172–1180

Lang GA (1987) Dormancy: a new universal terminology. Hortic Sci 22:817–820

Levine H, Slade L (1980) Thermomechanical properties of small carbohydrates-water glasses and rubbers. J Chem Soc Faraday Trans 184:2619–2633

Levitt J (1980) Freezing and high temperature stresses. Response of plants to environmental stresses, vol I. Academic Press, New York, pp 497–512

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real time quantitative PCR and the ΔΔCT method. Methods 25:402–408

Mills LJ, Ferguson JC, Keller M (2006) Cold-hardiness evaluation of grapevine buds and cane tissues. Am J Enol Vitic 57:194–200

Noriega X, Burgos B, Pérez FJ (2007) Short-day photoperiod triggers and low temperature increase expression of peroxidase RNA transcripts and basic peroxidase isoenzyme activity in grape-buds. Phytochemitry 68:1376–1383

Nylander M, Svensson J, Palva ET, Welin BV (2001) Stress-induced accumulation and tissue-specific localization of dehydrins in *Arabidopsis thaliana*. Plant Mol Biol 45:263–279

Olsen JE, Junttila O, Nilsen J, Eriksson M, Martinussen I, Olsson O, Sandberg G, Moritz T (1997) Ectopic expression of phytochrome A in hybrid aspen changes critical day-length for growth and prevents cold acclimatization. Plant J 12:1339–1350

Pérez FJ, Rubio S, Ormeño-Nuñez J (2007) Is erratic bud-break in grapevines grown in warm winter areas related to disturbance in mitochondrial respiratory capacity and oxidative metabolism? Funct Plant Biol 34:624–632

Pierquet P, Stushnoff C (1980) Relationship of low temperature exotherms to cold injury in *Vitis riparia* Michx. Am J Enol Vitic 31:1–6

Pierquet P, Stushnoff C, Low Burke MJ (1977) Temperature exotherms in stem and bud tissues of *Vitis riparia* Michx. J Am Chem Soc Hortic Sci 102:54–55

Rinne PLH, Kaikuranta P, Van der Schoot C (2001) The shoot apical meristem restores its symplastic organization during chilling-induced release from dormancy. Plant J 26:249–264

Rohde A, Bhalerao RP (2007) Plant dormancy in the perennial context. Trends Plant Sci 12:217–223

Rozen S, Skaletsky H (2000) Primer3 on the www for general users and for biologist programmers. Methods Mol Biol 132:365–386

Rubio S, Donoso A, Pérez FJ (2014) The dormancy-breaking stimuli "chilling, hypoxia and cyanamide exposure" up-regulate the expression of α-amylase genes in grapevine buds. J Plant Physiol 171:373–381

Sakai A, Larcher W (1987) Frost survival of plants: Responses and adaptations to freezing stress. Ecological studies, vol 62. Springer, Berlin

Salzman RA, Bressan RA, Hasegawa PM, Ashworth EN, Bordelon BP (1996) Programmed accumulation of LEA-like protein during desiccation and cold acclimation of overwintering grape buds. Plant Cell Environ 19:713–720

Sreekantan L, Mathiason K, Grimplet J, Schlauch K, Dickerson JA, Fennell AY (2010) Differential floral development and gene expression in grapevines during long and short photoperiods suggests a role for floral genes in dormancy transitioning. Plant Mol Biol 73:191–205

Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Ann Rev Plant Physiol Plant Mol Biol 50:571–599

Tränker C, Lehmann S, Hoenicka H, Hanke M, Fladung M, Lenhardt D, Dunemann F, Gau A, Schlangen K, Malnoy M, Flachowsky H (2010) Over-expression of and FT homologous gene of apple induces early flowering in annual and perennial plants. Planta 232:1309–1324

Vergara R, Pérez FJ (2010) Similarities between natural and chemically induced bud-endodormancy release in grapevine *Vitis vinifera* L. Sci Hortic 125:648–653

Wake CMF, Fennell A (2000) Morphological, physiological and dormancy response of three *Vitis* genotypes to short photoperiod. Physiol Plant 109:203–210

Welling A, Rinne P, Viherä-Aamio Kontunen-Soppela S, Heino P, Palva ET (2004) Photoperiod and temperature differentially regulate the expression of two dehydrin genes during overwintering of birch (*Betula pubescens* Ehrh.). J Exp Bot 55:507–516

Wolf TK, Cook MK (1994) Cold hardiness of dormant buds of grape cultivars: comparison of thermal analysis and field survival. Hortic Sci 29:453–455

Xin H, Zhu W, Wang L, Xiang Y, Fang L, Li J, Sun X, Wang N, Londo JP, Li S (2013) Genome wide transcriptional profile analysis of *Vitis amurensis* and *V. vinifera* in response to cold stress. PLoS One 8(3):e5870

Yang Y, He M, Zhu Z, Li S, Xu Y, Zhang C, Singer SD, Wang Y (2012) Identification of dehydrin gene family from grapevine species and analysis of their responsiveness to various forms of abiotic and biotic stress. BMC Plant Biol 12:140–148