Similarities between natural and chemically induced bud-endodormancy release in grapevine *Vitis vinifera* L.

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**Abstract**

Bud-break in grapevines (*Vitis vinifera* L) can be poor and uneven in locations that have warm winters with insufficient chilling. Growers in these regions use hydrogen cyanamide (HC), a dormancy-breaking compound, to increase and synchronise bud-break. Previous studies demonstrated that HC upregulates the expression of select genes encoding enzymes belonging to the ascorbate glutathione cycle (AGC) (glutathione reductase, VvGR) to the oxidative pentose phosphate pathway (oPPP) (glucose-6-phosphate dehydrogenase, VvG6PD) and a key enzyme for dormancy release (1,3-β-glucanase, VvGLU). Here, the expression of these genes was studied in grapevine buds throughout the natural progression of endodormancy (ED) and under the effects of the dormancy-breaking compounds, HC and sodium azide. Although gene expression increased with the progression of ED until mid-May, in early-June they turned off transiently and reappeared in mid-July before bud-break. The upregulation of these genes under both natural and artificial conditions suggests that dormancy-breaking compounds activate the same metabolic pathways that are naturally activated during the bud-ED release process. However, in contrast with the effects of dormancy-breaking compounds, artificial chilling did not upregulate the above genes.

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**Keywords:** Dormancy release, Grapevine buds, Hydrogencyanamide

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**A B S T R A C T**

Bud-break in grapevines (*Vitis vinifera* L) can be poor and uneven in locations that have warm winters with insufficient chilling. Growers in these regions use hydrogen cyanamide (HC), a dormancy-breaking compound, to increase and synchronise bud-break. Previous studies demonstrated that HC upregulates the expression of select genes encoding enzymes belonging to the ascorbate glutathione cycle (AGC) (glutathione reductase, VvGR) to the oxidative pentose phosphate pathway (oPPP) (glucose-6-phosphate dehydrogenase, VvG6PD) and a key enzyme for dormancy release (1,3-β-glucanase, VvGLU). Here, the expression of these genes was studied in grapevine buds throughout the natural progression of endodormancy (ED) and under the effects of the dormancy-breaking compounds, HC and sodium azide. Although gene expression increased with the progression of ED until mid-May, in early-June they turned off transiently and reappeared in mid-July before bud-break. The upregulation of these genes under both natural and artificial conditions suggests that dormancy-breaking compounds activate the same metabolic pathways that are naturally activated during the bud-ED release process. However, in contrast with the effects of dormancy-breaking compounds, artificial chilling did not upregulate the above genes.

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**1. Introduction**

Bud dormancy in woody perennials is a physiological stage that enables plants to survive long periods of adverse conditions and is characterised by growth cessation, arrest of cell division, and reduced metabolic and respiratory activity (Faust et al., 1997; Arora et al., 2003). Different degrees of dormancy found in plant meristems have been defined as paradormancy (PD), endodormancy (ED) and ecodormancy (ECD) (Lang, 1987). During bud-ED, growth cessation is due to an endogenous signal, whereas the cessation during PD is due to apical dominance and during ECD to environmental conditions (Lang, 1987). In some grape varieties, decreasing photoperiod triggers the transition of buds into ED (Fennell and Hoover, 1991; Salzman et al., 1996; Wake and Fennell, 2000; Kühn et al., 2009). Moreover, the expression of PHYA and PHYB in grapevine buds suggests a role of this embryonic organ in the perception of photoperiod (Pérez et al., 2009a). On the other hand, exposure to low temperature commonly referred to as the chilling requirement, must be fulfilled by grapevines and other deciduous fruit trees for the release of buds from ED and for their homogeneous sprouting in spring (Saure, 1985; Dokoozlian et al., 1995). Warm winters limit the productivity of the table grape in many regions (Erez, 1995), and prolonged ED, has been identified in these regions as a major obstacle for commercial production (Shulman et al., 1983; Saure, 1985; George et al., 1986; Dokoozlian et al., 1995). Applications of hydrogen cyanamide (HC) in late winter are widely used by producers in these regions to homogenise and advance bud-break to ensure commercially viable yields (Henzell et al., 1991; Erez, 1995). Understanding how bud-ED release is regulated and the role that dormancy-breaking compounds and chilling play in the process may assist in developing new dormancy-breaking strategies that do not rely on chilling, and this understanding will facilitate the searching for traits that could be used as informative markers for low chilling requirements in breeding programs.

Several recent studies in grapes have focused on bud-ED release, and HC has been used as a tool because it provides a controlled, synchronised and relatively rapid induction of dormancy release (Pang et al., 2007; Halalay et al., 2008; Pérez et al., 2009b; Ophir et al., 2009). Although the mode of action of HC is unknown, it has been demonstrated that catalase expression and activity are inhibited and H2O2 levels increased in HC-treated buds, (Shulman et al., 1983; Nir and Lavee, 1993; Or et al., 2002; Pérez and Lira, 2005). Applications of HC to grape buds, also upregulate hypoxic (PDC, ADH, SuSy) and oxidative stress-responsive genes (TrxH, GST, APX, GR) (Halalay et al., 2008; Keilin et al., 2007; Pérez et al., 2008a). Moreover, comparative studies among different dormancy-releasing stimuli led to the hypothesis that as a consequence of mitochondrial perturbation, transient respiratory and oxidative stresses are involved in...
the mechanism of dormancy release in grapevines (Keel et al., 2007; Halaly et al., 2008; Pérez et al., 2009b; Ophir et al., 2009). In this paper, we studied, under controlled conditions, the effect of HC on the bud-break response of buds from different dormancy statuses. The expression of selected genes related to the dormancy release process was analysed throughout the dormancy cycle and compared with the effect of dormancy-breaking compounds and chilling on buds from the same dormancy status.

2. Material and methods

2.1. Plant material

Plant material was collected from 8 years-old (Vitis vinifera L cv. Thompson Seedless) vineyards growing at the experimental station of the Chilean National Institute of Agriculture Research (INIA) located in Santiago (33° 34’ S). Canes were randomly collected every 3 weeks starting in April and ending in late winter (mid-August). Canes were cut off at both ends leaving the central section with 10–12 buds for further experiments.

2.2. Effect of HC application date on the bud-break response of grapevines

On each collection date two groups of 30 single-node cuttings (10–12 cm long) was prepared. One group was treated with 5% (w/v) Dormex (a commercial formulation containing 49% hydrogen cyanamide (HC), SKW, Trotsberg, Germany) and the other group was treated with water as control. Immediately after treatments, the cuttings in the plastic tray were transferred to a growth chamber set at 23°C under a 16-h photoperiod. The response of grapevines to HC varies according to the dormancy status analysis carried out previously (Pérez et al., 2007) and confirmed in this work. Canes were cut off at both ends leaving the central section with 10–12 buds for further experiments.

2.3. Chilling treatments in grapevine buds

Canes were randomly collected on 9 June during the phase of ED release in accordance with dormancy status analysis carried out previously (Pérez et al., 2007) and confirmed in this work. Canes were cut off, leaving 10–12 buds from the basal and central sections. Buds were wrapped in moist paper, placed in sealed plastic bags and moved to cold storage (4°C) for 400 h without light exposure.

2.4. Primer design

Gene-specific primers were designed using Primer3 (Rozen and Skaltsky, 2000) based on the EST database from the University of California (http://cfg.ucdavis.edu) and corroborated by the Vitis genomic database GENOSCOPE (http://www.genoscope.cns.fr/vitis). For VvGR, VvG6PD-cyt and VvβGLU22, specific primers are described in (Pérez et al., 2009b), and primers for VvβGLU78 were designed based on the sequence in the NCBI database (http://www.ncbi.nlm.nih.gov) and corroborated by the Vitis genomic database (Table 1).

2.5. RNA isolation and cDNA synthesis

Total RNA was isolated from endodormant grapevine buds (0.5–0.7 g fr. wt.) using a modification of the Chang et al. (1993) method described in Noriega et al. (2007). DNA was removed by treatment with RNase-free DNase (1 U/µL) (Invitrogen, CA, USA) at 37°C for 30 min. First-strand cDNA was synthesised 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 the Superscript™ III RNase H-RT kit (Invitrogen, CA, USA).

2.6. PCR and sequencing

Template cDNA (1 µL) was placed in a final volume of 12.5 µL in PCR tubes containing 0.25 µL of each primer (10 µM), 0.2 µL Platinum Taq DNA Polymerase (Invitrogen, CA, USA) (5 U × µL−1), 0.25 µL of dNTP Mix (10 mM), 1.25 µL of 10× PCR buffer (Tris-HCl 200 mM pH 8.4 and KCl 500 mM), 3 µL MgCl2 (50 mM) and 9.1 µL DEPC water. PCR was performed in a thermocycler (MJ Research PTC-150, Watertown, MA, USA) with, 35 cycles of 30 s at 94°C for denaturation, 30 s at 55°C for annealing and 45 s at 72°C for extension. A final extension was performed at 72°C for 10 min. PCR products were separated in 1.5% (w/v) agarose on 1× TAE buffer (50× TAE: Tris, 0.038 M, EDTA1 mM, glacial acetic acid 1.1% (v/v)) at 70 V and visualised by ethidium bromide.

For sequence analysis, PCR-amplified DNA and cDNA were purified using Montage PCR Filter Units (Millipore, Amicon USA). Sequencing reactions were performed with 2 µL of PCR- amplified product (20 ng µL−1), 2 µL DYEAMIC ET Terminator cycle kit (Amersham Bioscience) and 5 pmol of primer in 10 µL reaction volume. Sequencing fragments were separated and analysed with a 4-capillary sequencer ABI PRISM 3100-Avant genetic analyser (Applied Biosystems).

3. Results

3.1. Effect of HC application date on forced bud-break response in grapevines

The response of grapevine to HC varies according to the dormancy status of the buds (Erez, 1987; Wood, 1993; Or et al., 1999). Fig. 1 shows the effect of HC on the bud-break response of grapevine buds from different dormancy statuses. Our results confirmed the
3.2. Gene expression of VvGR and VvG6PD-cyt in grapevine buds throughout the dormancy cycle

The expression of VvGR and VvG6PD-cyt, encoding two major enzymes from the AGC and oPPP, was studied throughout the dormancy cycle in grapevine buds (Fig. 2). Under field conditions, VvGR and VvG6PD-cyt gene expression increased with the progress of the season from March to mid-May. Later in June, during the fall winter transition, both genes turned off transiently and reappeared in mid-July (Fig. 2).

3.3. Gene expression of VvβGLU78 and VvβGLU22 in grapevine buds throughout the dormancy cycle

Plant 1,3-β-glucanases (βGLU) are involved in pathogen defence and in diverse physiological and developmental processes like the release of buds from dormancy (Rinne et al., 2001) and the promotion of seed germination (Leubner-Metzger, 2003, 2005). In birch (Betula pubescens), βGLU removes the 1,3-β-glucan around plasmodesmata during the release of dormancy, a process that is stimulated by chilling (Rinne et al., 2001). To test whether these features also apply to grape buds, specific primers designed for VvβGLU were used in RT-PCR analysis, and the obtained amplicons were sequenced and designated VvβGLU78 and VvβGLU22. BLASTx analysis of both amplicons against the NCBI database is shown in (Fig. 3). The expression of VvβGLU78 and VvβGLU22 increased with the progress of the season, reaching a maximum in mid-May (Fig. 4A). Later in June, both genes turned off transiently, only to reappear in mid-July and in early August, respectively. On the other hand, applications of dormancy-breaking compounds (HC and sodium azide) induced VvβGLU22 (Pérez et al., 2009b) and VvβGLU78 expression in endodormant buds (Fig. 4B).

3.4. Chilling effect on bud dormancy release

The effects of artificial chilling on the expression of VvGR, VvG6PD-cyt, VvβGLU22 and VvβGLU78 were studied in buds harvested on 9 June. None of the genes analysed was induced after chilling treatment (Fig. 5), whereas in buds from the same dormancy status HC and sodium azide induced their expression (Pérez et al., 2009b, Fig. 4B).

4. Discussion

4.1. Evolution of BR50 and ΔBR50 parameters throughout the bud-ED period

Previous studies in grapevines cv. Thompson Seedless showed that bud-ED is triggered by short day (SD)-photoperiod, and that in plants grown in Santiago of Chile, buds enter into ED in mid-January (Kühn et al., 2009). The changes in BR50 throughout ED and the strength of HC on the bud-break response (ΔBR50) in buds from different dormancy statuses suggest that bud-ED could be divided in two phases. The first would be dormancy development that start in mid-January (Kühn et al., 2009) and ends in mid-April (deepest dormancy). The second would be dormancy release extending from mid-May to mid-July. The reduced effect of HC on buds harvested in mid-May is difficult to explain, as at that time buds were still deeply dormant. However, we conjectured that metabolic and developmental changes occurring within the bud at that time reduce the effect of HC on the bud-break response. The reduced values of BR50 and ΔBR50 during late transition stages (mid-July) indicate the end of ED and the toxicity of HC reflected by the negative ΔBR50 value detected during early August indicate that the population of buds is no longer endodormant. Although the mechanism by which HC exerts its dormancy-breaking effect is yet unknown, several pieces of evidence indicate that respiratory and oxidative stresses are produced after HC applications to grapevines (Kühn et al., 2009). Because respiratory rate and metabolism increase significantly at the end of the ED period (Pérez et al., 2007; Halalay et al., 2008; Pérez et al., 2009b), the ability of HC to induce oxidative and respiratory stress could be strongest once ED has finished, allowing it to reach toxic levels.

4.2. Expression of VvG6PD, VvGR, VvβGLU78 and VvβGLU22 genes throughout the bud-ED period

Different stimuli that induce bud-ED release in grapevines upregulate genes encoding enzymes belonging to the AGC, like
Fig. 3. Deduced amino acid sequence analysis of PCR-amplified fragments VvβGLU78 and VvβGLU22 (Blastx to NCBI database). (A) Analysis of VvβGLU78 showed 96% identity against V. riparia 1,3-β-glucanase, 96% against V. hybrid cultivar, 96% against V. vinifera unidentified protein, 94% against V. vinifera unidentified protein and 93% against V. vinifera 1,3-β-glucanase. (B) Analysis of VvβGLU22 showed 99% identity against V. vinifera 1,3-β-glucanase, 98% against V. vinifera 1,3-β-glucanase, 78% against H. brasiliensis 1,3-β-glucanase, 78% against H. brasiliensis 1,3-β-glucanase and 78% against H. brasiliensis 1,3-β-glucanase.

Ascorbate peroxidase (VvAPX) and glutathione reductase (VvGR). To the oPPP like glucose-6-phosphate dehydrogenase (VvG6PD-cyt) and key enzymes for dormancy release like 1,3-β-glucanase VvβGLU (Pacey-Miller et al., 2003; Halaly et al., 2008; Ophir et al., 2009; Pérez et al., 2009b). The AGC is a metabolic pathway that detoxifies hydrogen peroxide (H₂O₂) and involves the metabolites, ascorbate, glutathione and NADPH and the enzymes linking these metabolites (Noctor and Foyer, 1998). In non-photosynthetic tissue, the oPPP is a major source of NADPH which maintains redox potential and plays important roles in regulating cell growth (Tian et al., 1998). VvβGLU78 is reportedly upregulated in grapevine leaves after Botrytis cinerea infection, while in berries it is upregulated after mechanical wounding and berry development (Kraeva et al., 2000). Interestingly, in other species, 1,3-β-glucanase has been implicated in the release of buds from dormancy (Rinne et al., 2001).

In grapevine buds, the expression and repression of VvG6PD-cyt, VvGR, VvβGLU78 and VvβGLU22 throughout the ED period could be due to developmental or environmental factors. However, because there are not great differences in temperature and

### Table 1

<table>
<thead>
<tr>
<th>Primer used in RT-PCR.</th>
<th>Code</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>VvβGLU78</td>
<td>GSVIVT00025341001</td>
<td>5'-TGCCACCCCGCGGCAAG-3'</td>
<td>5'-CAGCTAAGTCTCGATAGGCC-3'</td>
</tr>
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</table>
Artificial chilling treatments breaking compounds induced the expression of all these genes in buds of the same dormancy status, application of dormancy-harvested according to defined conditions. Transcript analysis was carried out by semi-quantitative RT-PCR on total RNA extracted from buds harvested on the dates indicated in the figure.

4.3. Chilling effects on the release of buds from ED

Grapevine buds need to be exposed to low temperatures during winter for some period of time to achieve release from ED, the so-called chilling requirement (Weaver et al., 1974; Saure, 1985; Dokoozlian et al., 1995). However, the chilling requirement markedly varies with fruit tree species, growing area and the year among other factors (Westwood, 1978), and it has been reported to range from 50 to 400 h for grapevines (Dokoozlian, 1999). In grapevine buds, artificial chilling treatments applied during the phase of dormancy release (early-June) did not affect the expression of VvG6PD, VvGR, VvβGLU78, VvβGLU22 (Fig. 5). However, in buds of the same dormancy status, application of dormancy-breaking compounds induced the expression of all these genes (Pérez et al., 2009b and Fig. 5B). Artificial chilling treatments applied to grapevine buds (400 h at 4 °C) during the phase of dormancy release (9 June) corresponded to the chilling hours (CH) accumulated between early-June and mid-July in Santiago, Chile (Pérez et al., 2008a,b). Additionally, this duration of chilling is adequate to satisfy the chilling requirement of grapevine V. vinifera (Dokoozlian et al., 1995). However, some errors in the estimation of chilling requirements could alter the results, as the biological significance of CH calculated according to Weinberger (1950) has been questioned because it considers only temperatures between 0 and 7 °C and not the full range of temperatures (Allan, 2004). Better models have therefore been developed that are more accurate, and the best model so far is the dynamic model that quantifies chilling as chilling portions (CP) (Fishman et al., 1987; Erez et al., 1988). According to the dynamic model, approximately 26 CP are accumulated in Santiago, Chile between early-June and mid-July, while approximately 14 CP accumulate after continuous exposure to 400 h at 4 °C (Pérez et al., 2008a,b). In consequence, artificial chilling treatment only fulfilled about half of the chilling requirements of V. vinifera L, so it seems reasonable that an extension of the chilling period is necessary to stimulate the expression of genes related with the release of buds from ED. However, our results, like those reported in buds of V. riparia exposed to 500–2000 CH (Mathiason et al., 2009), indicate the down regulation of two genes encoding 1,3-β-glucanase isoforms, and genes encoding VvG6PD-cyt and VvGR, whose expression seems necessary for releasing buds from dormancy. Moreover, a careful examination of the genes differentially expressed after chilling treatments (Mathiason et al., 2009) and after treatments with dormancy-breaking compounds (Ophir et al., 2009) showed a limited correspondence between them. Thus while treatments with dormancy-breaking compounds upregulated genes related with energy and carbohydrate metabolism, which are necessary for growth resumption, chilling treatments, downregulated or had no effect on them. In addition to its dormancy release effect, chilling induces cold hardiness or freeze-tolerance in grapevine buds (Hubácková, 1996; Mills et al., 2006), so the differential genes expression observed after chilling treatments could be related either to the dormancy release or to the cold acclimation or to both processes. Therefore, following the deepness of bud-ED throughout the dormancy cycle in plants exposed and non-exposed to chilling could be a better approach to study the role of chilling in the dormancy release and in the cold acclimation processes and its interrelation with the bud-break response.

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