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Changes in the Endogenous Content and Gene Expression of Salicylic Acid Correlate with Grapevine Bud Dormancy Release

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Abstract

The accumulation of cold temperature is an important factor for the release of bud dormancy in grapevine. The stress generated by cold is related to changes in defense hormones such as salicylic acid (SA). To understand the participation of this hormone during grapevine bud dormancy release, in this study, we evaluated the effects of cold accumulation on the endogenous SA content and expression patterns of the synthesis and signaling genes of SA as well as budbreak rates. Buds were subjected to an accumulation of 900 or 600 chilling units (CUs). The budbreak percentage was determined when cold-treated buds were transferred to warm temperatures (25 °C). The percentage of budbreak was closely correlated with the amount of cumulative CUs; the percentage increased gradually with the amount of chilling applied before forcing. The increase in the expression level of the SA biosynthesis gene (*ICS2*) and the endogenous SA content were quantified in cold-treated buds, and the expression was correlated with the percentage of budbreak. These findings may indicate that in dormant grapevine buds, cold accumulation stimulates the synthesis of SA. In cold-treated buds, the cellular levels of SA led to the expression of the *NPR1* and *PR1* genes, which was mediated by the transcription factor *WRKY70*. In contrast, expression of *NPR1* and *PR1* in control buds, which maintained a basal level of SA, and expression of *ICS2* and *WRKY70* were not detected, suggesting that the constitutive expression of NPR1-SA is independent. Taken together, the results of this study suggest a possible involvement of the SA signaling pathway in grapevine bud dormancy release.

Keywords Grapevine · Flame seedless · Chilling units · Budbreak · Endogenous levels

Introduction

Bud dormancy reflects a process that is a genetic necessity for all known vine species and cultivars (Lang 1987). This physiological event occurs in cold winters, in which grapevines undergo a dormancy period that is essential for synchronizing their annual growth (Saure 1985). Cold temperatures and short day lengths control dormancy establishment, and low temperatures control growth cessation and dormancy induction (Dokoozlian et al. 1995). To complete dormancy and resume growth, prolonged periods of low temperature are required. The amount of chilling required varies depending on vine species and even cultivar (Yuri 2002). As buds advance toward dormancy release, either induced by chilling or by chemical means, the physiological and biochemical activity increase, although there is no visible growth at this time (Powell 1987). During this period, changes in respiration rates, plant growth regulators, water contents and gene expression occur (Or et al. 2000; Pérez et al. 2008; El-Shereif et al. 2005; Mohamed et al. 2012; Pacey-Miller et al. 2003). In addition, Díaz-Riquelme et al. (2012) inferred that salicylic acid (SA) participates significantly in the dormancy process. However, little is known about the metabolic pathway and how endogenous concentrations of this phytohormone vary and the extent to which this variation may support the regulatory role of SA in bud dormancy release. SA is a phenolic compound involved in regulating growth and development processes in plants, such as stomatal opening, vegetative growth, flowering, cell elongation, senescence (Rivas-San Vicente and Plasencia 2011; Dempsey et al. 2011) and the induction of

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pathogenesis-related (*PR*) genes, which confer resistance to plants (Janda and Ruelland 2015).

SA has also been recognized as a signaling molecule that mediates plant responses to abiotic stresses such as cold (Kim et al. 2013) and heat (Larkindale et al. 2005). High and low temperatures are among the major causes of abiotic stress in plants (Seyfferth and Tsuda 2014). Thus, have been reported changes in SA contents induced by heat in pea (Pan et al. 2006) and grapevine leaves (Wang and Li 2006) and those induced by low temperatures in grape berries (Wan et al. 2009) and in wheat leaves (Kosová et al. 2012). However, little is known about the endogenous content and gene expression level of this phytohormone in dormant buds. Therefore, the study of SA signaling in bud dormancy could provide valuable information to increase the knowledge of pathways that are involved in the complex process of bud dormancy release. Even though cold as a stress signal is related to both changes in the content of SA and the release of bud dormancy, it is not yet clear whether these two events are part of the same biochemical cascade that leads to bud dormancy release. Following this rationale, in this study, we evaluated the changes induced by the application of artificial cold on the endogenous content of SA and on the expression patterns of synthesis gene Isochorismate Synthase 2 (*ICS2*), signaling genes such as the receptor Non-Expressor of PR genes 1 (*NPR1*), the transcription factor WRKY70 and the response gene pathogenesis-related 1 proteins (*PR1*), and as well as the budbreak rates of dormant grapevine buds.

Materials and Methods

Plant Material

Canes with an average of five dormant buds each were randomly collected from the middle part (bud positions 3–8) of one-year-old canes of several vines. These vines were part of eight-year-old mature grape (*Vitis vinifera* L. cv. Flame seedless) vines from a commercial vineyard in Pesqueira, Sonora, Mexico (altitude 376 m, 29° 23′ N, 110° 56′ W). According to the chilling unit (CU) model of Richardson et al. (1974), at harvest, the canes accumulated 100 natural CUs on average. The canes were covered with wet sawdust and immediately transferred to the laboratory for treatment, after which they were separated into three groups. The group without low-temperature treatment (control) was immediately placed in a growth chamber under forcing conditions to break. The two other groups were placed in a chamber at 4 ± 2 °C until the canes reached an accumulation of 600 or 900 CUs, where one hour below 7 °C was equal to one CU (Richardson et al. 1974; Dokoozlian 1999). After the artificial cold treatments, the canes were placed under forcing conditions to break.

Calorimetric Measurements

Metabolic heat (Rq) was used to determine the dormancy status of the grapevine buds at collection time (Trejo-Martínez et al. 2009). The Rq was measured via a differential multicell scanning calorimeter (model 4100; Calorimetry Science Corporation, Pleasant Grove, USA) equipped with four 1 cm^3 Hastelloy ampules with removable lids. For the analysis, the buds were excised from the canes, and fresh weight was recorded. Seven buds (with an average weight of 80 mg) were placed in each ampule. To avoid dehydration, 50 µL of sterile water was added to the bottom of each ampule (Criddle et al. 1991). As part of isothermal experiments, the heat rates were measured at 25 °C for 3600 s. The instrument has a baseline noise of ± 1 µW and a working range of 30–110 °C. The temperature around the DSC chamber was maintained at 15 °C with a refrigerated circulating bath (PolyScience Corporation, USA). The data were collected at 20 s intervals. The values of the Rq are the means of three replications and were expressed on a dry weight basis (Gardea et al. 2000). The values of the Rq were analyzed by analysis of variance (ANOVA) considering a completely randomized design and the means were compared by Tukey's test ($p < 0.05$). The statistical analysis was performed using NCSS software (Number Cruncher Statistical System, version 2007, Kaysville, USA) (Hintze 2007).

Budbreak Under Forcing Conditions

To evaluate budbreak kinetics, groups of 10 cuttings of 4 buds per cutting were used, which were forced to break in a growth chamber at 25 ± 1 °C and under a 16/8 h light/dark photoperiod. The basal ends of the cuttings were immersed in water, which was changed every two days. Budbreak rates were recorded twice a week for 30 days. Buds that reached the green tip stage were considered broken (Coombe 1995). The budbreak percentage was determined at several time points during the forcing period. Endodormancy release was considered when 50% of the buds had broken. This experiment was performed in triplicate for all three treatments (control, 600 CUs and 900 CUs buds). The budbreak percentage was analyzed by analysis of variance (ANOVA) considering a completely randomized block design. The treatments included the variable factor and the time blocking factor, and the means were compared by Tukey's test ($p < 0.05$). The statistical analysis was performed using NCSS software (Number Cruncher Statistical System, version 2007, Kaysville, USA) (Hintze 2007).

Extraction and Quantification of Endogenous Salicylic Acid in Grapevine Buds

The extraction of SA was performed at 0, 3, 6, 9, 24, 48, 72, 96, 120 and 144 h after treatment. SA was extracted from frozen bud samples (2.5 g) that were ground to a fine powder in the presence of liquid nitrogen and then homogenized with 10 mL of ultrapure water with an Ultra Turrax T25 (Janke and Kunkel, IKA-Labortechnik, USA). The homogenate was then centrifuged at 10,000×*g* for 15 min (RC 5C plus centrifuge; Sorvall, USA). The pH of the supernatant was adjusted to 2.8 with 15% acetic acid, after which a double liquid–liquid extraction was carried out with equal volumes of cold diethyl ether. The aqueous phase was discarded via 1PS phase separator filter paper (Whatman; GE Healthcare Life Sciences, USA), and the two organic phases were mixed and dried under a nitrogen stream. The pellet was dissolved in 500 µL of 50% methanol (v/v) and then filtered through a 0.45 µm nylon filter (Pall Gelman Science, USA). The remaining substance was referred to as the SA bud extract.

The quantification of SA was performed following the methods of Durgbanshi et al. (2005) and Niculcea et al. (2013) by a high-performance liquid chromatography (HPLC) device (Agilent technologies Infinity 1260, Palo Alto, CA, USA) equipped with a quaternary pump coupled to diode array detector (DAD) (Agilent technologies Inc., Germany). The SA bud extracts (100 µL) were injected into an Eclipse plus 3.5 µm C-18 column (4.5 × 100 mm), which was operated at ambient temperature at a flow rate of 0.3 mL min^{-1}. The operation followed a linear gradient with mobile phases of A (100% methanol) versus B (0.2% glacial acetic acid in water) and started with 10% A and 90% B for 10 min, followed by 5 min of 50% A and then another 5 min of 100% A; these conditions were held for 25 min. The mobile phase was then returned to the initial conditions. The SA was monitored at 303 nm. An absorption spectrum of a fraction coeluting with authentic SA was obtained with a DAD detector. To quantify the SA, a standard curve was generated using diluted solutions of authentic SA (98% purity, Sigma-Aldrich, USA). A standard curve over the range of 0.02 to 1 mg mL^{-1} gave suitable values. The coefficient of determination (r^2) for the curves was greater than 0.98. The Pearson's correlation coefficient was determined using the endogenous content of SA and the budbreak percentage. The mean of the percentage values from three biological replicates of the three treatments were transformed to arc Sen, and the resulting data were correlated with the mean value of the corresponding SA concentrations.

RNA Extraction and cDNA Synthesis

Total RNA was isolated from grapevine buds following the methodology of Reid et al. (2006), with some modifications by García-Baldenegro et al. (2015), for which approximately 12 buds were used per sample, with three replicates. A total of 5 µg of RNA from each sample was treated with DNAse I (3 U 10 μg^{-1}) (Qiagen, USA). RNA quality and concentration were estimated from the absorbance ratios A260/A280 and A260/A230 using a NanoDrop 2000 (Thermo Scientific, USA). The RNA integrity was confirmed via formaldehyde in 0.1% agarose gel electrophoresis. Total RNA (2 µg) from each sample was reverse-transcribed to cDNA using oligo dTs from a Superscript II First Strand Synthesis System kit for RT-qPCR (Invitrogen, USA).

Quantitative Real-Time PCR (RT-qPCR)

Specific primers were designed using the online Primer 3 Plus software (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plusAbout.cgi) (Untergasser et al. 2007). Primers for the genes of the salicylic acid signaling pathway, *ICS2*, *NPR1*, *WRKY70* and *PR1*, were designed based on the sequences of each gene in the Grape Genome Browser (https://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/) component of the Genoscope database (Table 1). Each RT-qPCR was carried out in triplicate at a final volume of 20 µL, which comprised 5 µL of cDNA (4 ng), 10 µL of iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, USA), 1 µL (5 µM) of forward and reversal primers and water-treated DEPC at 0.1%. The annealing temperature was 60 ºC for all primer pairs and was carried out in a Step One Real-Time PCR System with a 48-well plate (Applied Biosystems Inc., USA). Dilution series of cDNA were created for each set of primers, and a calibration curve for each gene was obtained. The specificity of the individual PCR

Table 1 Primers used for qPCR

Gene	Forward primer (5′–3′)	Reverse primer (3′–5′)
VvICS2 [a](GSVIVT01008052001)	CGTGGTCCTCTAAACCGTTGC	TCCCCGCTGTTTCTTCTCCAG
VvNPR1 [a](GSVIVT01015181001)	GTGGCGGTTTTGGGGTATTTGT	GCACCTCCACCATGAAATCCAC
VvWRKY70 [a](GSVIVT01032661001)	CCAATGAACTGGGGAGCCTTG	GCACGAGGAAGCATGAGCAAA
VvPR1 [a](GSVIVT01037005001)	CTCATGTGTTGGTGGGCAATGTG	GCACCCAAGACGCACTGATTTG
VvACT [a](GSVIVT01026580001)	GCTGAGAGATTCCGTTGTCC	GCCACCACCTTGATCTTCAT

amplification was checked using a heat dissociation curve from 55 to 95 °C. Mean values and standard deviations were obtained from three biological and three technical replicates. The results obtained for each gene of interest were normalized to the expression of a reference gene, Actin (*ACT*). The relative expression levels of the genes were determined using the $2^{-\Delta\Delta CT}$ method and the mean C_T at time zero (Figs. 3, 4, 5) or time 24 (Fig. 6) was used as a baseline level for the calculation of fold change (Livak and Schmittgen 2001).

Statistical Analysis

The salicylic acid content data and the expression of the genes at different times were analyzed by one-way ANOVA, and the comparison of the means was made by the Tukey–Kramer test ($p < 0.05$). The statistical analysis was performed via NCSS software, version 2007 (Kaysville, USA) (Hintze 2007).

Results

Calorimetry

According to the calorimetric measurements, the cold-treated buds with 900 and 600 CUs presented lower Rq values compared to the control buds (Table 2). These results indicate that the buds that received artificial chilling treatments were in a state of low metabolic activity, a typical characteristic of dormant tissue (Trejo-Martínez et al. 2009).

Budbreak Under Forcing Conditions

The effects of artificial cold treatments on the budbreak response are shown in Fig. 1. Both cold treatments increased

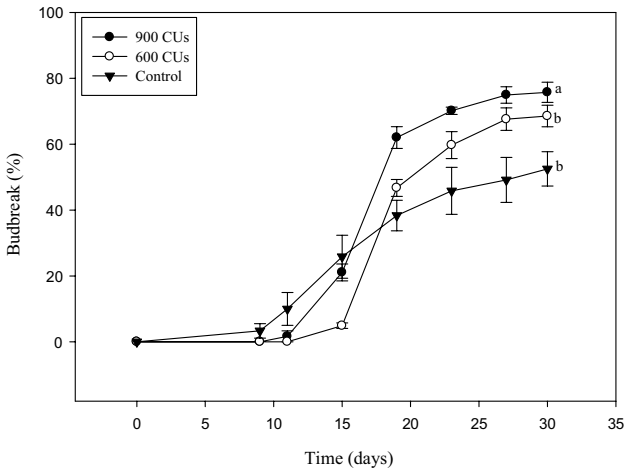

Fig. 1 Effects of cold treatment on budbreak percentage in grapevine buds of cultivar Flame Seedless. The budbreak percentages were determined during forcing conditions in a growth chamber. Each value represents the mean ± S.E. of three replicates. Bars are standard errors ($n = 3$). Different letters represent significant differences (Tukey's test ($p < 0.05$))

the percentage of budbreak with a similar pattern and timing but to different extents. The buds with 900 CUs reached the maximum percentage of budbreak (75%), while the buds with 600 CUs and the controls reached 68% and 52%, respectively. The budbreak of the 900 CU-treated buds was consistently higher than that of the 600 CU-treated buds at all time points ($p < 0.05$). The control buds started to break earlier but showed a lower percentage of budbreak throughout the subsequent sampling times. The buds with 900 CUs presented the highest rate of budbreak, reaching 50% of broken buds earlier (17 days) than buds with 600 CUs (22 days) and the controls (30 days).

Table 2 Metabolic heat (Rq) of cold-treated and control grapevine buds measured by calorimetry

Cumulative chilling units (CU)	Metabolic heat Rq (μW mg^{-1} DW)*
900	1.75 ± 0.08 a
600	1.88 ± 0.25 a
100 (Control buds)	2.16 ± 0.21 b

Rq in control buds was measured at bud collection time

The values represent the average ± standard error of three replicates. Different letters represent significant differences (Tukey's test ($p < 0.05$))

*Rq in cold-treated buds was measured immediately after the accumulation of chilling units

Endogenous Content of Salicylic Acid in Grapevine Buds

The quantification of the endogenous SA content in the buds after treatment is shown in Fig. 2. The buds treated with 900 CUs showed a significant increase in SA content at 6 h under forcing conditions and maintained this high level until 72 h. The SA content then rapidly decreased at 96 h, and a second increase at 120 h attained a peak value that was eightfold (98.21 μg g^{-1} FW) its initial value. The buds treated with 600 CUs showed an initial decline in SA content in the first 3 h and maintained this level until 24 h, after which two increases were presented at 48 and 96 h, followed by a return to the low values (18 μg g^{-1} FW). The control buds presented a steady value of SA content (an average value of 8 μg g^{-1} FW) throughout the assessment time. This value was the lowest ($p < 0.05$) of all treatments and was 12 times less than that of buds treated with 900 CUs and 7 times less

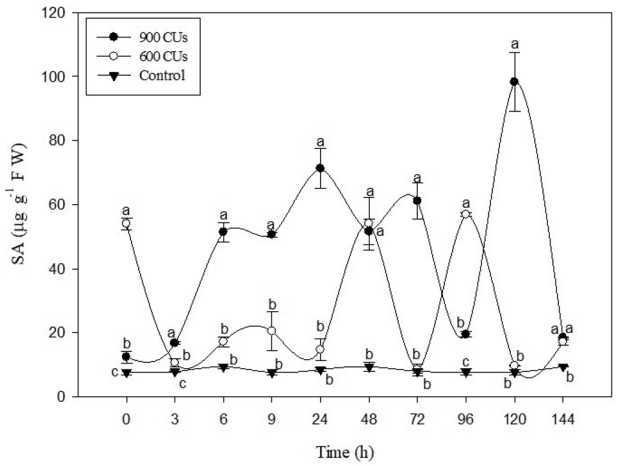

Fig. 2 Change in endogenous salicylic acid contents in grapevine buds of cultivar Flame Seedless after cold treatment and during forcing conditions of budbreak. Bars are standard errors ($n=3$). Different letters represent significant differences ($p<0.05$) between treatments according to Tukey's test

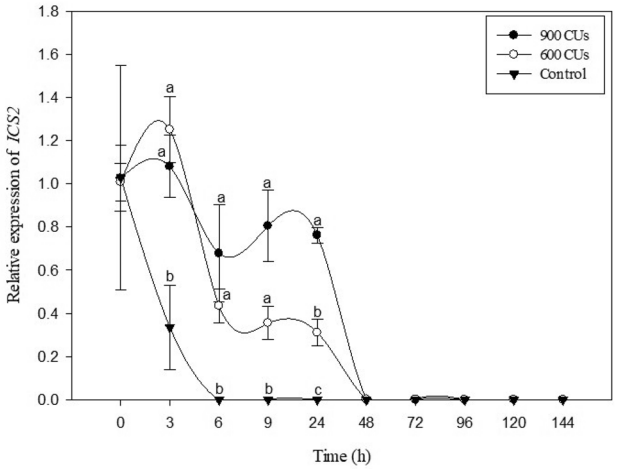

Fig. 3 Relative expression of the *ICS2* gene in grapevine buds of cultivar Flame Seedless after cold treatment and during forcing conditions. Each value represents the mean ± S.E. of three technical replicates that come of three biological replicates. Bars are standard errors ($n=3$). Different letters represent significant differences ($p<0.05$) between treatments according to Tukey's test

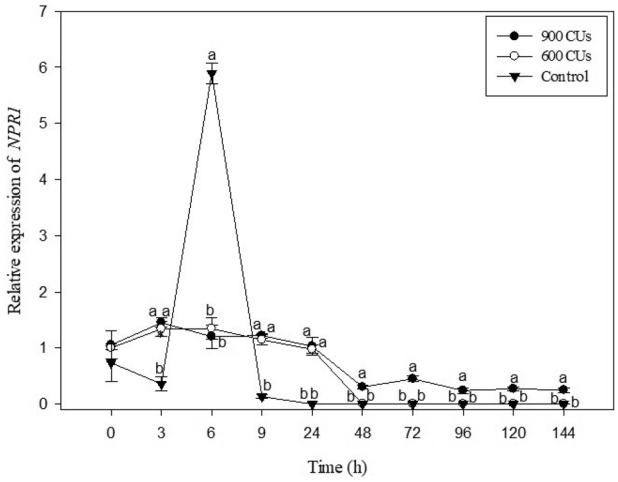

Fig. 4 Relative expression of the *NPR1* gene in grapevine buds of cultivar Flame Seedless after cold treatment and during forcing conditions. Each value represents the mean ± S.E. of three technical replicates that come of three biological replicates. Bars are standard errors ($n=3$). Different letters represent significant differences ($p<0.05$) between treatments according to Tukey's test

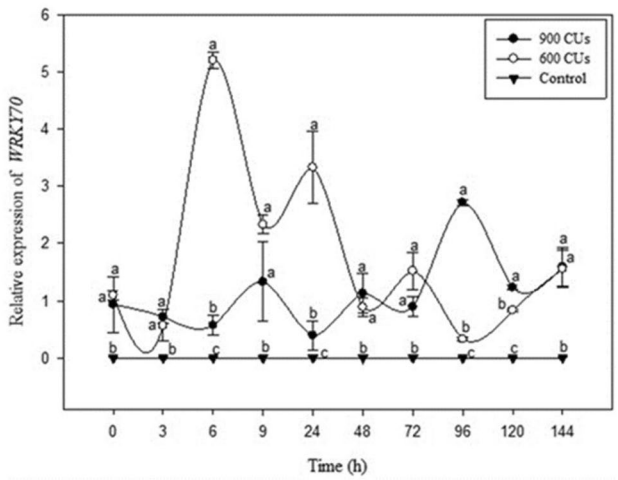

Fig. 5 Relative expression of the *WRKY70* gene in grapevine buds of cultivar Flame Seedless after cold treatment and during forcing conditions. Each value represents the mean ± S.E. of three technical replicates that come of three biological replicates. Bars are standard errors ($n=3$). Different letters represent significant differences ($p<0.05$) between treatments according to Tukey's test

than that of buds with 600 CUs. These results could indicate that in dormant grapevine buds, cold stimulates the synthesis of salicylic acid.

Expression Profiles of Salicylic Acid Signaling Genes in Dormant Grapevine Buds

To evaluate the effects of artificial cold temperature on the gene expression of SA signaling, the buds were analyzed by RT-PCR (Figs. 3, 4, 5, 6). The transcript level of the synthesis gene (Isochorismate Synthase 2, *ICS2*) was maintained in both cold treatments (Fig. 3) during the first 24 h of the assessment. In the control buds, the expression of this gene showed a sharp decrease during the first 3 h and was undetectable afterward. The signals were more intense in response to the 900 CU treatment than to the 600 CU treatment. After 48 h, the expression of this gene was reduced in the 600 CU treatment and was undetectable in the 900 CU treatment. *NPR1* gene expression was also induced by cold

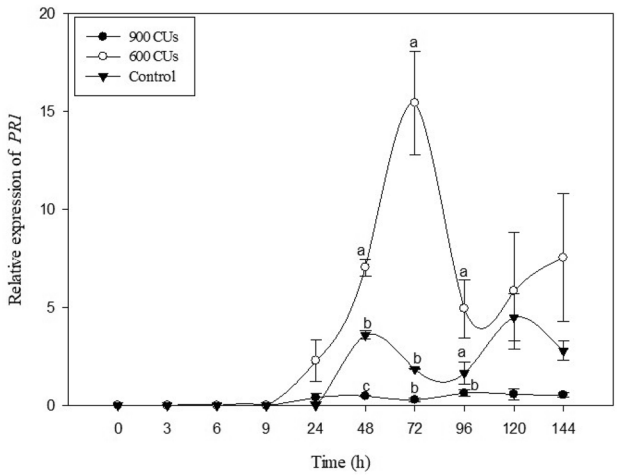

Fig. 6 Relative expression of the *PR1* gene in grapevine buds of cultivar Flame Seedless after cold treatment and during forcing conditions. Each value represents the mean ± S.E. of three technical replicates that come of three biological replicates. Bars are standard errors ($n = 3$). Different letters represent significant differences ($p < 0.05$) between treatments according to Tukey's test

temperatures (Fig. 4). In response to 900 CUs and 600 CUs, the *NPR1* transcript level increased slightly; this elevated level was maintained for 24 h, after which it decreased until 48 h but remained steady from then on. The expression profiles for both cold treatments were similar, but the extent of transcript levels differed from 48 to 144 h, where the signals were more intense ($p < 0.05$) in the buds treated with 900 CUs. In the control buds, the early and transient expression of *NPR1* peaked at 6 h; however, it decreased significantly ($p < 0.05$) at 9 h and was undetectable from then on.

The transcription factor *WRKY70*, which participates in both positive and negative regulation of SA signaling genes, was also induced in response to both cold treatments (Fig. 5). The expression profile of *WRKY70* in the buds treated with 900 and 600 CUs showed opposite temporal patterns of expression from 6 h, with a progression and more persistent induction occurring in the 600 CU treatment, in which the maximum activation was attained at 6 h with a fivefold initial value. A second activation was attained at 24 h, which reached a level threefold that of the initial value. In buds treated with 900 CUs, induction was evident at 9, 48 and 96 h after forcing conditions; its highest level occurred at 96 h, with a threefold increase in the initial value. In contrast, in the control buds, *WRKY70* expression was not detected throughout the evaluation period. Moreover, *PR1* expression was detected after 24 h in cold-treated buds and at 48 h in the control buds (Fig. 6). *PR1* expression increased following the 600 CU treatment, and its highest transcript level was reached at 72 h. The buds treated with 900 CUs maintained a low transcript level throughout the assessment. This value was the lowest of all the treatments ($p < 0.05$). The control buds showed two transient peaks: one at 48 h and the other at 120 h.

Discussion

The critical role that phytohormones play in bud dormancy regulation is becoming evident (Singh et al. 2017). The phytohormone signaling network is thought to be central to dormancy induction and release (Mornya and Cheng 2013; Zheng et al. 2015; Zhang et al. 2018). Phytohormones such as salicylic acid (SA) induce H_2O_2 accumulation (Dat et al. 1998), as do low temperatures and some dormancy-breaking agents in grapevine buds (Prasad 1996; Carvalho et al. 2015). To investigate the involvement of SA in dormancy release in grapevine buds, in this study, we evaluated the changes induced by the application of artificial cold on the endogenous SA content and the expression patterns of synthesis and SA signaling genes accompanied by budbreak patterns. This last parameter was used as an association between molecular and horticultural assessments. The Flame Seedless buds in this study were considered endodormant based on the low metabolic activity (Rq) quantified in the buds, particularly buds treated with 900 and 600 CUs (Table 2), whose Rq values were similar to those reported in buds that reached a stage of deeper endodormancy (Trejo-Martínez et al. 2009).

The percentage of budbreak was strongly correlated with the amount of cumulative cold and increased gradually with the amount of cold applied before forcing (Fig. 1), confirming the positive effects of cold accumulation on budbreak advancement (Anzanello et al. 2018). In accordance with the percentage of budbreak, the budbreak rate was highest in response to the cold treatments, confirming that the speed of budbreak and the vigor of shoot growth are a function of the amount of cold to which grapevine buds are exposed (Márquez-Cervantes et al. 2000; Melke 2015). The increase in endogenous SA content was quantified in the cold-treated buds, which reached eightfold the initial value in those treated with 900 CUs and six-fold the initial value in those with 600 CUs (Fig. 2), which correlated with the percentage of budbreak ($r = 0.922$). In addition, the relatively low SA value in the control buds was consistent with the relatively low percentage of budbreak. In accordance with these results, it has been reported that under stress conditions, SA stimulates growth in plants (Khodary 2004; Martínez et al. 2004; Li et al. 2013; Nazar et al. 2015). Particularly, in *Arabidopsis* increases in SA level was correlated with cell growth (Vanacker et al. 2001). Thus, the high content of SA observed in the cold-treated buds might favor vegetative growth, causing budbreak. Consistent with the endogenous SA quantification in the cold-treated dormant buds, a temporary increase in the expression level

of a SA synthesis gene (*ICS2*) was observed (Fig. 3). This finding could indicate that in dormant grapevine buds, cold accumulation stimulates the synthesis of SA, which is in accordance with previously reported results for other species in which stress caused by low temperatures promoted endogenous SA accumulation (Wan et al. 2009; Kosová et al. 2012; Dong et al. 2014). Increases in cellular levels of SA promote redox changes that monomerize NPR1, driving it toward nuclear localization (Mou et al. 2003; Backer et al. 2019), which leads to the expression of defense genes such as *PR* (Miura and Tada 2014). This phenomenon may be related to our data concerning cold-treated buds, where a high SA content led to the expression of the *NPR1* gene, which was observed very early; this expression was maintained for 24 h (Fig. 4) with consequent *PR1* expression after 24 h (Fig. 6) (Uquillas et al. 2004), which could be mediated by the transcription factor *WRKY70* (Fig. 5), which has recently been shown to be involved in dormancy (Craig and Ling 2014; Chen et al. 2016). *WRKY70* positively modulates the expression of genes mediated by SA as *PR* genes and negatively modulates the expression of the *ICS2* gene, maintaining the balance in the synthesis of SA and mediating the defense mechanism (Li et al. 2004, 2006; Wang et al. 2006). This notion is consistent with the finding shown here with respect to cold-treated buds, where *WRKY70* positively regulated the expression of *PR1* (Fig. 6). Even in the buds with 900 CUs that showed low expression levels of *PR1*, which is not related to the SA content or to *ICS2*, *NPR1* and *WRKY70* expression, we can infer that *WRKY70* most likely regulates the expression of other PR genes, such as *PR2* and *PR5* (Gaffney et al. 1993). *NPR1* showed an unexpected expressión in the control buds in which a basal level of SA was maintained (Fig. 2), and the expression of *ICS2* and *WRKY70* were not detected (Figs. 3, 5), suggesting a constitutive expression of *NPR1*-SA independent with consequent expression of *PR1*, which was previously reported in *Vitis vinifera* by Le Henanff et al. (2009). The lack of expression of the *WRKY70* transcription factor in the control buds is consistent with the finding that, in the absence of INA (2,6-dichloro-isonicotinic acid; an SA analog), the interaction of *NPR1* with *WRKY* is low, favoring the TGA transcription factor interaction (Pape et al. 2010). This finding may reflect the important role of *NPR1* in both basal and SA-induced resistance in grapevine (Le Henanff et al. 2011; Theocharis et al. 2012).

The expression of defense genes has been reported to play an important role during exposure to cold as mediators of biochemical and physiological changes required to plants for growth and development (Seo et al. 2010; Theocharis et al. 2012) and therefore might play a role in grapevine bud dormancy release. This hypothesis is supported by the activation of key genes in SA defense signaling pathway in dormant grapevine buds induced by cold exposure, where the relative expression of *ICS2*, *NPR1*, *WRKY70* and *PR1*, including the accumulation of SA, was confirmed. Based on the finding that grapevine has a conserved mechanism of defense mediated by *NPR1* (Le Henanff et al. 2009), its activity is controlled by cellular redox changes triggered by SA (Mou et al. 2003; Tada et al. 2008). We suspect that in grapevine buds, the differences between basal and cold-induced endogenous SA contents might differentially affect the interaction of *NPR1*, triggering SA-dependent or SA-independent defense signaling pathways. In this study, the amount of cold accumulated (600 and 900 CUs) by the buds increased the SA content, which activated the *NPR1*-SA-dependent pathway mediated by *WRKY70*. In the control buds with the lowest cold accumulation (100 CUs), in which SA did not increase, a defense signal was presented independently from *NPR1*-SA, which could be mediated by another transcription factor whose function is redundant with that of *WRKY70* or TGA (Pape et al. 2010).

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Compliance with ethical Standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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