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Short day-photoperiod triggers and low temperatures increase expression of peroxidase RNA transcripts and basic peroxidase isoenzyme activity in grapevine buds

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Abstract

Expression of peroxidase (Prx) RNA transcripts was studied in grapevine cv. Thompson seedless through use of semi-quantitative RT-PCR. In roots, the three *Vitis vinifera* Prx ESTs, reported in the genomic facility database from the University of California (http://cgf.ucdavis.edu), were expressed as RNA transcripts, and three Prx isoenzymes, two basic (PrxB1) and (PrxB2) and a neutral Prx isoenzyme (PrxN1), were detected in root extracts. In buds, although one Prx EST (CB923206) was detected as a RNA transcript, three Prx activities were found in its extract, these same being the two basic isoenzymes found in the roots, and an acidic one (PrxA1) as well. In field grown grapevines, Prx RNA transcript was expressed transiently during bud endodormancy (ED) while under controlled conditions in a growth chamber, a short day (SD) photoperiod triggered expression of the Prx transcript in bud cuttings; low temperature, enhanced its expression level. Because environmental cues that trigger and enhance Prx RNA transcript expression in bud-tissue are the same as those that induce bud-ED in *Vitis*, the transient expression of Prx EST (CB923206) identified in buds could signal the full extent of ED in grapevines.

Keywords: *Vitis vinifera*; Vitaceaceae; Peroxidase expression sequence tags (ESTs); Peroxidase isoenzymes

1. Introduction

In *Vitis*, as in other woody perennials, shortening the photoperiod and decreasing temperatures during autumn induce bud endodormancy (ED), initiates cold acclimation and, thereby, enhances bud tolerance to freezing (Fennell and Hoover, 1991; Wake and Fennell, 2000; Arora et al., 2003; Faust et al., 1997). Nevertheless, the joint effects of short days (SD) and low temperatures on the parallel development of freezing tolerance and bud-ED make it difficult to separate the two phenomena (Arora et al., 2003; Heggie and Halliday, 2005). In addition, since bud-tissue may progress from one dormancy condition to another without any phenotypical change, defining the specific dormancy stages is complicated further in perennial plants (Campbell, 2006). Thus, the search for a model from woody perennial plants in which ED can be separated from cold acclimation responses, and for molecular markers signalling dormancy, phase transitions prior to any external sign of growth are prerequisites to studying gene expression of specific dormancy phases. It has been reported that ED can be induced exclusively by a SD-photoperiod in certain *Vitis* genotypes (Fennell and Hoover, 1991; Wake and Fennell, 2000). In Thompson seedless changes in polymorphism of class III Prx have also been correlated with grapevine bud phase transition from endo to ecodormancy (Pérez and Burgos, 2004).

Class III plant Prx (EC 1.11.1.7) are heme-proteins that catalyse oxidation of hydrogen donors in the presence of

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H_2O_2. Prx can oxidize various phenolics, lignin precursors or secondary metabolites as hydrogen donors, and have been identified as multifunctional enzymes participating in a broad range of physiological processes such as lignification, suberization, cross-linking of cell-wall proteins, auxin degradation, and control of apoplastic H_2O_2levels (Passardi et al., 2005; Kawano, 2003). In grapevines, basic and acidic Prx have been reported in different plant organs such as leaf, root, pedicel, and bud (Pérez et al., 2002; Vatulescu et al., 2004; Pérez and Morales, 1999; Pérez and Burgos, 2004). In callus culture of *Vitis vinifera* cv. Touriga, a class III basic Prx responsible for the catalysis of GvP1 deposition, an abundant and ionically bound cell wall protein, was recently identified and various peptides from same were sequenced (Jackson et al., 2001).

Class III Prx expression sequence tags (ESTs) from *V. vinifera*, reported in the genomic facility database from the University of California, were used to design primers for studying the expression of Prx RNA transcripts by semi-quantitative RT-PCR. These were selected due to potential uses of class III Prx as molecular markers to signal the dormancy phase transition in Thompson seedless grapevines.

2. Results

2.1. Class III Prx ESTs and Prx isoenzymes in grapevine buds and roots

Primers designed against *V. vinifera* class III Prx ESTs, reported in the genomic facility database from the University of California (http://cgf.ucdavis.edu), were assayed by semi-quantitative RT-PCR against total RNA extracted from grapevine cv. Thompson seedless buds and roots. In Fig. 1a, lanes 2–4 show that the three *V. vinifera* Prx ESTs reported in the database (# accession AY348574, singleton); (# accession CB923206, singleton) and (# accession CTG1035712, contig) were detected as RNA-transcripts in total RNA extracted from roots. Only one RNA transcript corresponding to Prx EST (# accession CB 923206) was detected in buds (Fig. 1a, lane 3). In all RT-PCR reactions, primers designed for the grapevine actin gene (# accession AY680701) were used as an internal control (Fig. 1a, lane 5). The identity of the PCR amplified fragment (RNA transcript detected in bud) was confirmed by sequencing, and by comparing the obtained sequence with those of *V. vinifera* Prx ESTs reported in the database (Fig. 2a). A strong level of identity with Prx EST (CB 923206) was found and the amino acid sequence homology analyses (Blastx to NCBI database) showed high homology to class III Prx genes from *Gossypium hirstum* (CAE54309), *Arabidopsis thaliana* (NP194328; Q95ZH2) and *Glycine max* (AAD37375) (Fig. 2b). Analysis by non-equilibrium isoelectric focusing (NEIEF) of grapevine Prx established the presence of two highly basic isoenzymes, PrxB1 and PrxB2 in the root and bud tissues, whereas a neutral isoenzyme (PrxN1) was present only in root, and an acidic isoenzyme PrxA1 was found only in bud (Fig. 1b) tissues, respectively.

2.2. Expression of Prx EST and Px isoenzymes in grapevine buds

For field-grown grapevines, the RNA transcript corresponding to *V. vinifera* Prx EST (CB 923206) was detected transiently in buds during ED in early May (Fig. 3a, lane 3). Analysis of basic Prx isoenzyme patterns using NEIEF in buds collected on April 13 gave minor PrxB1 and more intense PrxB2 activities, whereas for buds collected in May 5, the opposite pattern was observed (Fig. 3b). Single bud-cuttings collected on April 13, exposed to 10 days of SD photoperiod (8 h light, 16 h darkness) in a growth chamber at constant temperature (23 ± 2 °C), expressed the Prx RNA transcript, whereas single bud cuttings taken for control purposes, collected on the same day and maintained in the laboratory under LD photoperiod and ambient temperature, did not (Fig. 4, lane 2).

2.3. Low temperature increases Px EST expression and basic Px isoenzyme activity

Low temperature treatment also increased expression of the RNA transcript Prx EST (CB 923206) as well as overall activity of the isoenzyme PrxB1 in buds collected on May 9. Lane 2 of Fig. 5a shows increases in Prx RNA transcript abundance in buds collected on May 9 that were held at 4 °C in a refrigerator for two weeks, in comparison to buds collected on the same date and maintained at room temperature for the same time period (control). Fig. 5b shows the effects of different exposure times to low temperature (0, 1 and 2 weeks) on Prx B1 isoenzyme activity in buds collected on May 9.

3. Discussion

3.1. Px ESTs and Px isoenzymes in grapevines bud and root

Semi-quantitative RT-PCR analysis of total RNA extracted from grapevine cv. Thompson seedless buds showed that Prx EST (CB 923206) was the only *V. vinifera* class III Prx EST of the three reported in the database (http://cgf.ucdavis.edu) expressed in bud-tissue. However, NEIEF analysis detected three Prx activities, two basic PrxB1 and PrxB2, and one acidic PrxA1 in the bud tissue extracts (Fig. 1a and b). The lack of correspondence between the number of Prx RNA transcripts expressed in bud tissue and the number of Prx isoenzymes found in bud-extracts could be due to: (a) Prx ESTs reported in the database are not related to Prx isoenzymes found in bud tissue extracts or (b) although proteins remain catalytically active in the tissue, RNA transcripts are rapidly

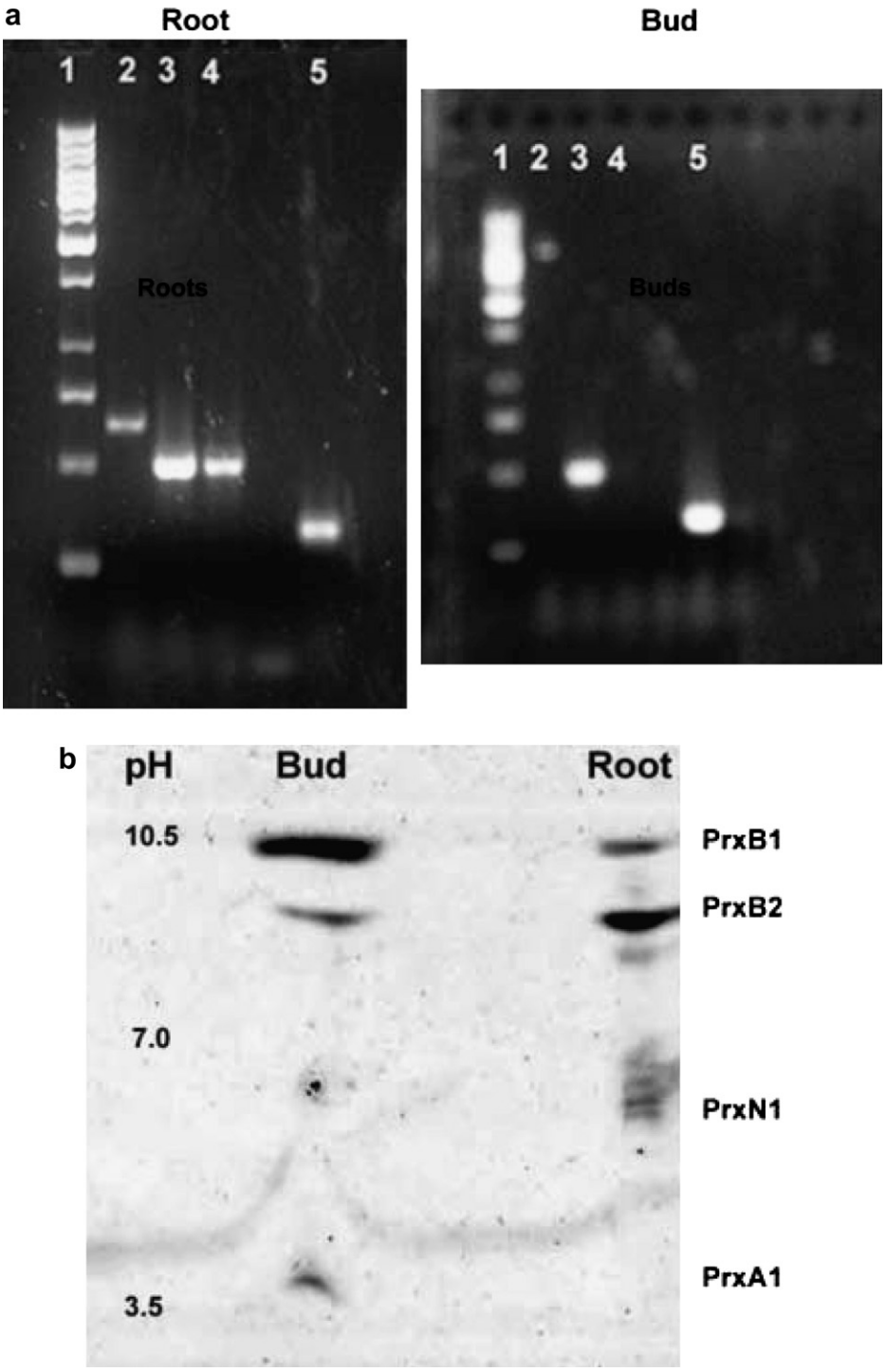

Fig. 1. (a) RT-PCR of *Vitis vinifera* class III Prx EST in total RNA extracted from Thompson seedless grapevine root and bud tissues. Lane 1 corresponds to molecular markers, lane 2 to Prx EST (AY348574) transcript, lane 3 to Prx EST (CB923206) transcript, lane 4 to Prx EST (CTG1035712) transcript and lane 5 to *V. vinifera* actin gene. In each case total RNA (1 μg) was used as template with the final PCR reaction (5 μL) loaded on to each gel lane. (b) Prx isoenzyme pattern of grapevine cv. Thompson seedless from root and bud extracts were established by isoelectric focusing with staining using 4-methoxy-α-naphthol in the presence of H_2O_2. For each lane, 3.5 U of Prx was loaded.

degraded after translation. The second possibility seems more likely, since the Prx RNA transcript was detected transiently during bud ED, while Prx isoenzyme activities remained practically constant throughout ED (Pérez and Burgos, 2004). These results show that during ED, transcriptional activity is high in bud tissue, whereas protein turnover is low. Similar findings were observed by Pacey-Miller et al. (2003) in *V. vinifera* L. cv. Purple Cornichon.

The three *V. vinifera* Prx ESTs reported in the database were identified as RNA transcripts in roots, because they

a

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CB923206    CATACCATTGGGACAACTGCATGCTTCTTCATAGAGACGAGACTCTACAACTTCACCCGAG-GCGGTGGCTCGGA 193
CB-Nor      --------TGGGACAACTGCATGCTTCTTCATAGAGACGAGACTCTACAACTTCACCCGAG-GCGGTGGCTCGGA 66
CTG1035712  CACACCATTGGAACCACAGCATGCTTCTTCATGACAGACCGGCTGTACAACTTTTTTCCGG-GTGGGGGATCCGA 212
AY348574    GGTTCTCTCAGGA--ACT-CATACC---ATAGGCAAAGCAAGATGCACAAGTTTTCGAAATCATATTTACAACGA 443

CB923206    TCCTGCAATCAACCCTGACTTCCTCCCAAAACTAAAGGCCAAGTGCCCGTTTCGGGGAGATATCAATGTCCGACT 268
CB-Nor      TCCTGCAATCAACCCTGACTTCCTCCCAAAANTAAAGGCCAAGTGCCCGTTTCGGGGAGATATCAATGTCCGACT 141
CTG1035712  TCCATCTATCAGCCCTGAATTCCTCCCAGAGCTAAAAGCAAAGTGCCCCCAGGATGGTGATGTTAATGTTCGGTT 287
AY348574    CACCGACATCGATCCAGCATTTGCAGCATCAAAACAGAAAATCTGCCCAAGATCGGGTGGAGATGATAATCTATC 518

CB923206    ACCACTTGATCCGGTGACTGAAGAAACGTTTGATGTTCAGATATTGAGGAATATCAGGGATGGATTAGCAGTGAT 343
CB-Nor      ACCACTTGATCCGGTGACTGAAGAAACGTTTGATGTTCAGATATTGAGGAATATCAGGGATGGATTAGCAGTGAT 216
CTG1035712  ACCAATGGACCAGGGAAGTGGGGAGACATTTGACAAAAAGATTCTGGAAAACATTAGGGGTGGATTTGCTGTGCT 362
AY348574    TCCTCTGGATGGAACAACTACTG---TTTTCGATAATGTTTATTTTAGGGGTTTGGAAGAAAAGAAGGGTCTGTT 590

CB923206    AGAGAGTGATGCTAGGCTTTACGATGACAGAGCAACCAAGCGGGTGGTGGACTCCTACATTGGGC-----AGCGC 413
CB-Nor      AGAGAGTGATGCTAGGCTTTACGATGACAGAGCAACCAAGCGGGTGGTGGACTCCTACATTGGGC-----AGCGC 286
CTG1035712  GCGGTCAGATGCTAGTCTTATGGAGGATGAAGCAACTAAGAGCGTAATAGACTCCTACTTCGGCCCCCTAAACTC 437
AY348574    GCACTCAGACCAGGTACTCTACAACGGTGGCTCCACAGATTCCCTAGTTAAAACCTAC-----------AGCAT 648

CB923206    GGGAGTT----CTGCATTTGGGCAGGACTTTGCAGAGGCAATGGTGAAGATGGGTAACATTGGAGTGAAGACGGG 484
CB-Nor      GGGAGTT----CTGCA------------------------------------------------------------ 298
CTG1035712  ACAATTTGGACCATCTTTTGAGGAAGATTTTGTTAACTCAATGGTGAAAATGGGCCAGATCGGTGTAGAGACAGG 512
AY348574    CGACACTG---CCACTTTCTTTACGGATGTGGCCAACGCCATGGTTAGGATGGGAGATATCAGTCCTTTAACCGG 692
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b

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G.hirsutum CAE54309        GPFYDVPTGRRDGRVSKMSLAKNLPDVDDSINVLKSKFKEKGLSDKDLVLLSGGSHTIGA 198
CB-Nor                     ----------------------------------------------------SAAHTIGT 8
A. thaliana  NP194328      GPAYEVPTGRRDGRVSNMSLAKDMPEVSDSIEILKAKFMQKGLNAKDLVLLS-AAHTIGT 238
A. thaliana  Q95ZH2        GPAYEVPTGRRDGRVSNMSLAKDMPEVSDSIEILKAKFMQKGLNAKDLVLLS-AAHTIGT 193
Glycine max AAD37375       GPAYQVPTGRRDGLVSNLSLADDMPDVSDSIELLKTKFLNKGLTVKDLVLLS-GAHTIGT 207
Oryza sativa CAH69375      GPSFDVPTGRRDGRTSSLRDADVLPDVKDSIDVLRSKFAANGLDDKDLVLLS-AAHTVGT 199
                                                                          .:**:*:

G.hirsutum CAE54309        TACFFMQKRLYNFTPGGG---SDPAINPGFLPQLKDKCPFNGDVNVRIPLDWSTQNVFDV 255
CB-Nor                     TACFFIETRLYNFTRGGG---SDPAINPDFLPKXKAKCPFRGDINVRLPLDPVTEETFDV 65
A. thaliana  NP_ 194328    TACFFMSKRLYDFLPGGQ---PDPTINPTFLPELTTQCPQNGDINVRLPIDRFSERLFDK 295
A. thaliana  Q95ZH2        TACFFMSKRLYDFLPGGQ---PDPTINPTFLPELTTQCPQNGDINVRLPIDRFSERLFDK 250
Glycine max AAD37375       TACFFMTRRLYNFFPSGEG--SDPAIRQNFLPRLKARCPQNGDVNIRLAIDEGSEQKFDI 265
Oryza sativa CAH69375      TACFFLQDRLYNFPLAGGGRGADPSIPEAFLSELQSRCAP-GDFNTRLPLDRGSEAEFDT 258
                           *****:  ***:*  .*    .**:*   **..   :*.  **.* *:.:*  ::  **

G.hirsutum CAE54309        KILRNIREGNAVIASDARLYDDRMTRQIVDSYITSS----AASFNQDFAEAMVKMGNIGA 311
CB-Nor                     QILRNIRDGLAVIESDARLYDDRATKRVVDSYIGQR----GSSA---------------- 105
A. thaliana  NP_ 194328    QILQNIKDGFAVLQTDAGLYEDVTTRQVVDSYLGMLNPFFGPTFESDFVKAIVKMGKIGV 355
A. thaliana  Q95ZH2        QILQNIKDGFAVLQTDAGLYEDVTTRQVVDSYLGMLNPFFGPTFESDFVKAIVKMGKIGV 310
Glycine max AAD37375       NILKNIREGFAVLESDARLNDDIATKNVIDSYVSPFSPMFGPSFEADFVESVVKMGQIGV 325
Oryza sativa CAH69375      SILRNIRNGFAVIASDAALYNATATVGVVDTYSSMLSAFFGPYFRQDFADAMVKMGSVGV 318
                           .**:**::* **: :** * :   *  ::*:*        ..
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Fig. 2. (a) Sequence identity analyses of cDNA fragments (CB-Nor) obtained using RT-PCR with primers designed for Prx EST (CB923206). The obtained sequence was compared to *V. vinifera* Prx ESTs sequences, reported in the genomic facility database from the University of California (http://cgf.ucdavis.edu). Identity against CB92036 was 99.7%, against CTG1035712 was 63% and against AY348574 was 39.6%. (b) Amino acid sequence homology analyses of PCR amplified fragment CB-Nor (Blastx to NCBI database) 80% against *G. hirsutum* CAE 4309, 72.5% against *A. thaliana* NP 194328, 72.5% against *A. thaliana* Q 95ZH2, 71.8% against *Glycine max* AAD37375 and 63.4% against *Oryza sativa* CAH69375.

corresponded to the specific primers used, but the presence of others Prx can not be ruled out. Moreover, PrxB1 and PrxB2, the two basic Prx activities found in bud extracts, were also found in root extracts. PrxA1, the acid Prx isoenzyme detected in buds, was not present in roots. However, the presence of an acid Prx isoenzyme that disappears 32 h after protrusion during the rooting process has been reported in grapevine cv. Touriga Nacional roots (Vatulescu et al., 2004). Thus, the lack of PrxA1 activity in Thompson seedless root-extracts might be due to the developmental stage of the root-tissue used during the preparation of the extract.

3.2. Peroxidase gene in grapevines

Assessing expression of plant Prx genes is complex, since they are regulated at different times and places by various kinds of biotic and abiotic stresses (Yoshida et al., 2003). Information on the timing and tissue specificity of Prx gene expression is necessary to establish the true function of a specific Prx isoenzyme. In grapevines, it has been proposed that Prx levels are strongly modulated during plant cell development (Kochhar et al., 1979) and in response to both biotic (Morales et al., 1998) and abiotic (Ros-Barceló et al., 2003) factors. However, little is

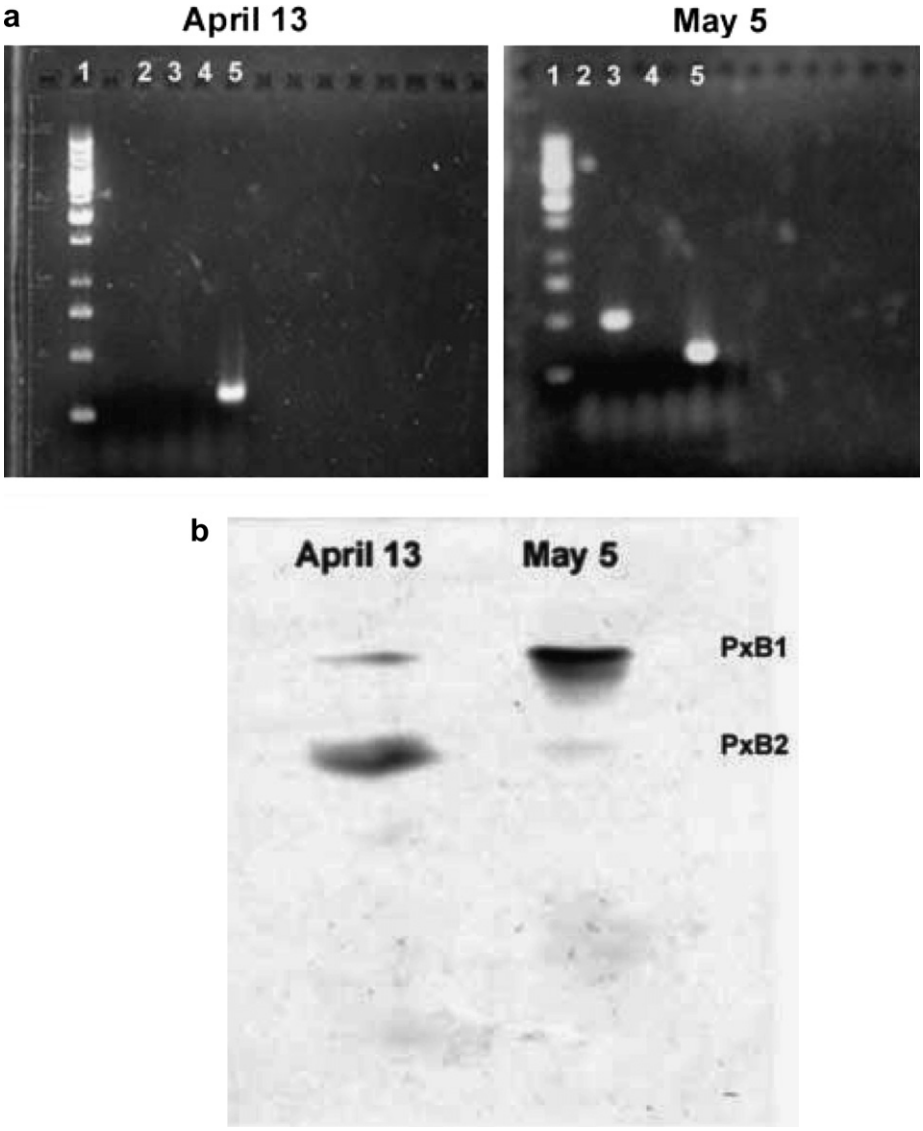

Fig. 3. RT-PCR expression as isoenzyme patterns as selected peroxidases. (a) RT-PCR expression analysis of Prx EST (CB923206) transcript in grapevine cv. Thompson seedless buds collected on April 13 and on May 5. Lane 1 corresponds to molecular markers, lane 2 to Prx EST (AY348574) transcript, lane 3 to Prx EST (CB923206) transcript, line 4 to Prx EST (CTG1035712) transcript and line 5 to *V. vinifera* actin gene. (b) Isoenzyme pattern of basic Prx from bud-extracts collected on April 13 and on May 5. Prx isoenzymes were separated by isoelectric focusing and stained with 4-methoxy-α-naphthol in the presence of H_2O_2. Eight unit of Prx activity was loaded on to each gel lane.

known about regulation of Prx genes at the transcript level in grapevines and the signal transduction pathways involved. Induction of acidic Prx isoenzyme by ultraviolet-C radiation in *Vitis rupestris* (Zapata et al., 1994) and the induction of basic Prx isoenzymes in cultured grapevine cells treated with the *Tricoderma viride* elicitor has been reported, nevertheless (Ros-Barceló et al., 1996). Amino acid sequence analyses of the amplified fragment expressed in Thompson seedless buds (blastx analysis to NCBI database) showed high homology against class III Prx from *G. hirsutum* (80%), cationic Prx from *A. thaliana* (72.5%), and Prx from *G. max* (71.8%), demonstrating the class III Prx nature of the amplified fragment (Fig. 2b).

4. Concluding remarks

4.1. Interactions between SD-photoperiod and low temperatures in the expression of Prx EST (CB 923206)

In certain *Vitis* genotypes, ED may be induced exclusively by SD photoperiod, while others require low temperatures (Fennell and Hoover, 1991; Wake and Fennell, 2000). In *V. vinifera* L. cv. White Riesling, a synergistic effect between decreasing temperatures and shortening photoperiods in prompting both cold hardiness and ED has been reported (Schnabel and Wample, 1987). Similar interactions between low temperatures and photoperiod are key in the flowering process, which is well characterised

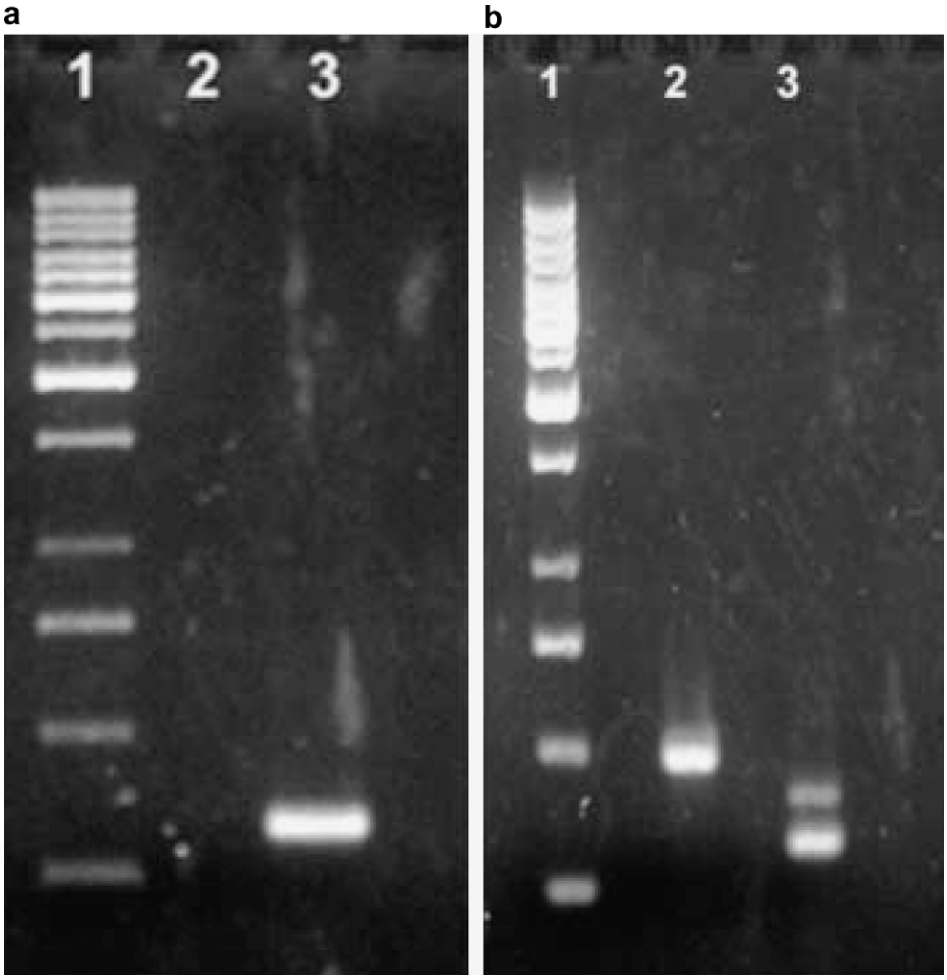

Fig. 4. Effect of SD-photoperiod on expression of Prx EST (CB923206) transcript in grapevine cv. Thompson seedless buds. (b) Buds collected in April 13 were exposed to a SD-photoperiod for 10 days in a growth chamber at 23 ± 2 °C. Total RNA was extracted with the expression of RNA transcripts analysed using RT-PCR with the corresponding primers. (a) Buds collected on the same day and maintained for the same duration under a LD photoperiod in the laboratory were used as control. Lane 1 corresponds to molecular markers, lane 2 to Prx EST (CB923206) transcript and lane 3 to the *V. vinifera* actin gene.

at the molecular level in the model plant *A. thaliana* (Scott et al., 2005), and in the less-studied dormancy process about which little is known at the molecular level (Böhlenius et al., 2006). Cold acclimation and ED are concurrent events in all species requiring low temperatures to induce ED. Consequently, it is difficult to separate changes in gene expression that are due to cold acclimation from those associated to ED. Our results, however, showed that expression of the Prx EST (CB 923206) transcript was triggered by a SD photoperiod under constant temperature (23 ± 2 °C), and that low temperatures enhanced its expression. Therefore, some interaction between SD photoperiod and low temperatures, respectively, triggering and amplifying Prx RNA transcript expression occurred. Thus, the transient expression of the Prx RNA transcript in early May, in buds of field grown grapevines, appears to be indicative of a physiological change within the bud-tissue at that stage. Earlier results with Thompson seedless found that bud-dormancy depth peaked at the beginning of May (Pérez and Lira, 2005). Thus, the Prx EST (CB 923206) RNA transcript could signal the full extent of bud-ED in Thompson seedless grapevines, and possibly in other varieties. However, since *Vitis* species demonstrate differences in responsiveness to environmental cues such as SD-photoperiod and temperature (Wake and Fennell, 2000) further characterisation of dormancy response to environmental cues and the interactions between them are necessary before general conclusions can be drawn.

5. Experimental

5.1. Plant material

Grapevine buds (*Vitis vinifera* L cv. Thompson seedless) were collected at different developmental stages from 8 year-old vines grown at the experimental station from National Institute for Agriculture research (INIA) in Santiago, Chile (33°29′S, RM). Roots were obtained from single bud-cuttings placed in a growth chamber under forcing

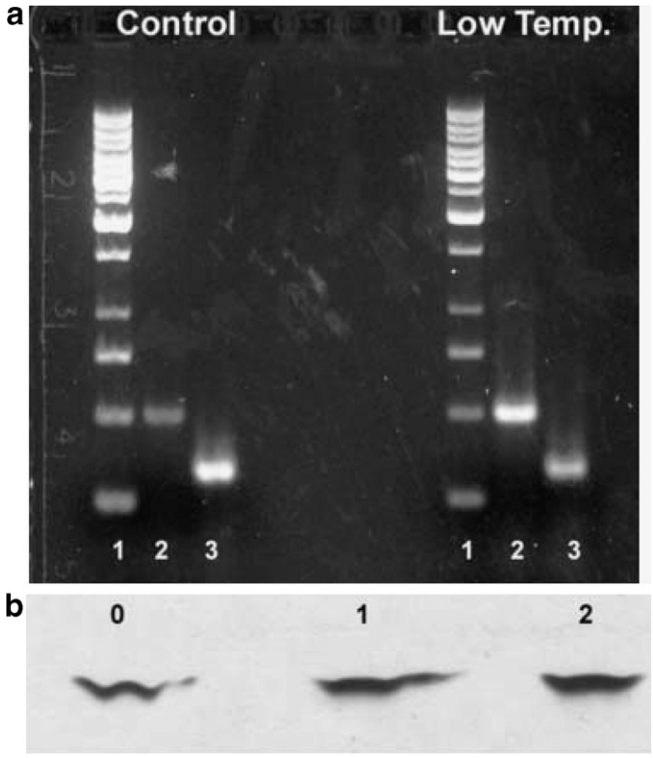

Fig. 5. (a) Effects of low temperature on expression of Prx EST (CB923206) transcript in grapevine cv. Thompson seedless bud. Buds collected on May 9 were placed in the refrigerator (4 °C) for 2 weeks (low temperature) with control buds were maintained over the same time at ambient temperature in the laboratory. Following extraction of total RNA, transcript expression was analysed by RT-PCR using the corresponding primers. Lane 1 corresponds to molecular markers, lane 2 to Prx EST (CB923206) transcript and lane 3 to *V. vinifera* actin gene. (b) Effects of low temperature on the activity of PrxB1isoenzyme. Buds collected on May 9 were kept in the refrigerator at 4 °C for 0 (control), 1 and 2 weeks, with PrxB1 activity subsequently analysed by isoelectric focusing using 4-methoxy-α-naphthol in the presence of H_2O_2. The same amount of protein (5 μg) was loaded onto each gel lane.

conditions (16 h light: 8 h dark) at 23 ± 2 °C and collected after 30 days.

5.2. Extraction and purification of RNA

Total RNA was isolated from grapevine cv. Thompson seedless buds or roots (0.5–0.7 g fresh weight) using a modification of the method described by Chang et al. (1993). Samples were ground in liquid N_2 and homogenised in buffer CTAB (CTAB 2%, Tris–HCl 25 mM, EDTA 2 mM, NaCl 2 M and PVP 2%), 2% β-mercaptoethanol was added to the homogenate immediately after buffer addition. The homogenate was vortex vigorously, heat at 65 °C for 30 min and filtered through glass wool. The filtrate was centrifuged (400$g \times 10$ min) and extracted twice with 1 vol of $CHCl_3$: isoamyl alcohol (24:1, v/v). The aqueous phase was precipitated overnight with EtOH (2.5 volumes). RNA was recovered by centrifugation (3000$g \times 20$ min) and washed with EtOH–H_2O (7:3). After solvent elimination, RNA was dried at ambient temperature and dissolved in diethyl pyrocarbonate (DEPC) H_2O. The RNA solution was incubated with DNAse (70 U/mL) at 36 °C for 30 min to eliminate DNA. RNA was purified further by binding to silica (1 vol of 6 M NaI and 0.5 vol of silica). After repeated vortexing, the supernatant was eliminated by centrifugation (3000 $g \times 1$ min). The silica was washed twice with washing buffer (500 μL, consisting of Tris–HCl 10 mM; NaCL 50 mM, EDTA 2.5 mM and EtOH 50% v/v, pH 7.5) to eliminate supernatant by centrifugation. The pellet was dried at ambient temperature, re-suspended in DEPC H_2O, with the aqueous solution containing the RNA separated by centrifugation.

5.3. Primer design and semi-quantitative RT-PCR

Specific primers for *V. vinifera* class III Prx genes were designed based on the genomic facility EST database from the University of California (http://cgf.ucdavis.edu). Six different Prx ESTs have been reported in the *Vitis* database, three of these ESTs belong to *V. vinifera* whereas the remaining three belong to other *Vitaceas* species. The three *V. vinifera* Prx ESTs, one contig (CTG1035712) and two singletons (AY348574) and (CB923206) were used for primer design, which was carried-out using the program primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)

CTG right 5′-TCC/CCA/TCA/GAA/CCT/GTC/TC-3′;
CTG left 5′-TTC/ ACT/GGC/AGC/TGA/TAT/GC-3′

CB right 5′-CCC/ GTC/ TTC/ACT/CCA/ATG/TT-3′;
CB left 5′-CAG/GCA/AAA/GGG/TCT/TTC/AG-3′

AY right 5′-GGC/CAC/ATC/CGT/AAA/GAA/AG -3′;
AY left 5′-TTA/TTG/GAT/GGC/ACT/GCA/AC C-3′

RT-PCR reactions were carried-out using the kit SuperScript™ One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen, USA) in a Thermocycler MJ Research (PTC-150). The conditions for c-DNA synthesis were 30 min at 50 °C and 2 min at 94 °C. Conditions for the 35 PCR cycles were 30 s at 94 °C for denaturation, 30 s at 55 °C for annealing and 45 s at 72 °C for extension. Final extension was performed for 10 min at 72 °C. The reaction mixture contained 1 μg of total RNA as template, 0.2 μM of each primer. RT Platinum Taq mix (1 μL) and 25 μL of 2×mix, a buffer containing (0.4 mM of each dNTP and 2.4 mM $MgSO_4$). DPC H_2O was used to bring to final volume (50 μL).

PCR products that were separated in an electrophoresis run on 1.5% agarose gel with buffer TAE (0.038 M Tris, 1 mM EDTA, 1.1% (v/v) glacial AcOH) at 50 V and visualised by staining the gel with ethidium bromide. In order to check the reliability of transcriptase and polymerase reactions, primers designed for the *V. vinifera* actin gene (# accession NCBI AY680701), which is expressed constitutively in all *Vitis* tissues, was used as internal control in each RT-PCR reaction.

5.4. Sequencing analysis

The amplified PCR fragment obtained with primers corresponding to Prx EST (CB923206) was sequenced directly after extraction from an agarose gel. Sequencing reactions were performed with 2 μL of DYENAMIC 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 analyzer (Applied Biosystems).

5.4.1. Isoelectric focusing of peroxidases

Non equilibrium isoelectric focusing (NEIEF) was performed using a 5% polyacrylmide gel containing 2% amphолites (Bio-Lyte 3/10; Bio-Rad, USA). Samples were run in a Bio-Rad 111 mini-IEF chamber according to manufacturer specifications. The same amount of protein was loaded on to the gel for each treatment. Prx activity was developed by incubating the gel in 10 mM buffer citrate pH 4.5 containing 5 mM 4-methoxy-1-naphtol and 1 mM H_2O_2 (Pérez and Burgos, 2004).

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