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Peroxiredoxins are involved in two independent signalling pathways in the abiotic stress protection in *Vitis vinifera*

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Abstract

Peroxiredoxins (Prxs) play major roles in preventing oxidative damage and their function is consistent with the presence of Prx isoforms in most, if not all, cellular compartments and their expression is dependent on environmental conditions. The aim of this study was to identify and characterize genes encoding Prxs in *Vitis vinifera*. Quantitative real time polymerase chain reaction (qRT PCR) was used to determine their response to irradiance, heat, and water stress. We identified seven *vvprx* genes, two of which were especially responsive to water stress, followed by heat stress, but no major changes were observed after high irradiance. The *vvprxIIF* targeted to mitochondria was the most responsive to water stress and it might be involved in drought tolerance through H_2O_2 signalling. The *vvprxII-2,* a putative *PrxII*, is targeted to the chloroplasts and was the most responsive to heat stress. It might be related with abscisic acid-dependent thermotolerance.

Additional key words: abscisic acid, antioxidants, grapevine, heat stress, photosynthesis, qRT PCR, water stress.

Introduction

Peroxiredoxins (Prxs) are a ubiquitous family of non-heme thiol peroxidases which catalyze the reduction of hydrogen peroxide (H_2O_2), alkylhydroperoxides, and peroxynitrite to water, alcohols, or nitrite, respectively (Rouhier and Jacquot 2005, Tripathi *et al.* 2009). These enzymes contain one or two cysteine (cys) residues in their active site and usually function as monomers or dimers. Their common catalytic mechanism involves the catalytic Cys peroxidatic (Cys_P) thiol that is oxidized by peroxides to sulfenic acid. The sulfenic acid in the majority of Prxs is reduced by a second Cys resolving (Cys_R) thiol forming an intra- or inter-molecular disulfide bond. A new catalytic cycle can only begin after the reduction of the disulfide bond using electron donors such as thioredoxins (Trxs), glutaredoxins (Grxs), or cyclophilins (Dietz *et al.* 2006). Therefore, Prxs are redox sensitive proteins that can endure reversible oxidation-reduction and thus switch 'on' and 'off' depending on the cellular redox state. Thus, they might be involved in balancing signalling cascades mediated by reactive oxygen species (ROS) and in dissipating excessively absorbed energy and protecting the photosynthetic apparatus against oxidative damage (Broin *et al.* 2002, Dietz *et al.* 2002, Konig *et al.* 2002).

In plants, there are four types of Prxs (all nuclear encoded): 1CysPrx, PrxII, 2CysPrx, and PrxQ. Each type plays specific roles according to their spatio-temporal expression patterns and subcellular localizations. Plant Prxs protect the nuclei (1CysPrx), chloroplasts (2CysPrx A, 2CysPrx B, PrxQ, and PrxIIE), cytosol (PrxIIB, PrxIIC, and PrxIID), and mitochondria (PrxIIF) against excess of ROS in stressful conditions, but they are also implicated in redox signalling (Romero-Puertas *et al.* 2007, Tripathi *et al.* 2009).

Several genetic approaches have helped to analyze the function of specific Prxs in higher plants or cyanobacteria. Plants deficient in 2CysPrx have shown inhibition of photosynthesis and plant development, decreased chlorophyll accumulation (Baier and Dietz 1999, Baier *et al.* 2000), and a lower quantum yield of

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Abbreviations: ABA - abscisic acid; APX - ascorbate peroxidase; CAT - catalase; cys - cystein; DHAR - dihydroascorbate reductase; F_v/F_m - variable to maximum chlorophyll fluorescence ratio; GOR - glutathione reductase; PPDF - photosynthetic photon flux density; Prx - peroxiredoxin; qRT-PCR - quantitative real time polymerase chain reaction; ROS - reactive oxygen species; SOD - superoxide dismutase, WS - water stress.

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photosystem II (PS II) than the wild type plants (Pulido *et al.* 2010). In the presence of a chloroplast protein synthesis inhibitor, the difference between plants deficient in 2CysPrx and wild types was enhanced which suggested that 2CysPrx can either protect PS II by localized peroxide detoxification or that the interaction between PS II and 2CysPrx affects properties of the photosystem itself, such as stability or turnover (Baier and Dietz 1999).

Plants overexpressing PrxQ are phenotypically similar to wild type plants; nevertheless, they show decreased sensitivity to oxidative stress, small increments in net photosynthetic rate, and increased tolerance to salt and cold stresess (Jing *et al.* 2006, Lamkemeyer *et al.* 2006, Petersson *et al.* 2006). In *Arabidopsis thaliana, PrxQ* transcripts are the most responsive of the four chloroplast *Prx* genes. It was thus suggested that PrxQ has a specific function in protecting photosynthesis different from that of 2CysPrx (Lamkemeyer *et al.* 2006). Under reducing conditions, *PrxQ* transcripts decrease, and likewise under H_2O_2 treatment, they increase (Horling *et al.* 2002, 2003).

The transcript of *1CysPrx* in the cyanobacterium *Synechocystis* sp. strain PCC 6803 decreased after H_2O_2 treatment and UV-B radiation (Huang *et al.* 2002, Li *et al.* 2004, Perez-Perez *et al.* 2009). In the dehydration-tolerant *Xerophyta viscosa*, *1CysPrx* transcript was not present in the fully hydrated tissue. However, increase of transcript abundance was observed under dehydration, heat, high irradiance, and abscisic acid (ABA) treatment, an indication that 1CysPrx might play a role in tolerance to extreme drought (Mowla *et al.* 2002). Using promoter deletions in *At-PER1*, it was shown that *1CysPrx* was induced by H_2O_2, leading to the hypothesis that 1CysPrx is responsive to oxidative stress (Haslekas *et al.* 2003).

PrxIIF expression was modified during an interaction between poplar and the rust fungus *Melampsora laricii* ssp. *populina* (Gama *et al.* 2007). However, poplar PrxIIF content did not substantially vary under photo-oxidative conditions or heavy metal treatments (Gama *et al.* 2007). Also, in *Arabidopsis*, *PrxIIF* was unaltered upon changes in irradiance (Horling *et al.* 2003).

Under oxidative stress, the expression of *AtPrxIIE* was not changed, however, it rised with increased irradiance and fell when plants were transferred to low irradiance or subjected to ascorbate treatment (Horling *et al.* 2002, 2003). Expression of *AtprxIIC* was strongly upregulated by salt, ascorbate, and oxidative treatments (Horling *et al.* 2002, 2003).

ABA plays a major role in the regulatory network controlling *At2cysPrx* expression (Baier *et al.* 2004). Since ABA is involved in stress adaptation (Song *et al.* 2012), interestingly it functions as a suppressor of *2cysPrx* expression as revealed from work with ABA-insensitive and ABA-deficient mutants combined with ABA feeding (Baier *et al.* 2004). This observation links 2CysPrx to a function in regular photosynthesis rather than in stress response (Dietz 2007). Promoter analyses for *cis*-regulatory elements identified the ABA-responsive element ABRE in *Arabidopsis 1CysPrxs* (*AtPER1*). In plants ectopically expressing ABI3, *AtPER1::GUS* expression was found in leaves and *AtPER1* was induced by ABA and H_2O_2 (Haslekas *et al.* 1998).

When considering the constituents of the anti-oxidative machinery, Prxs are not considered to be enzymes in the first line of defence. A prominent part of the extensive antioxidant network consists of enzymes such as superoxide dismutase (SOD) scavenging superoxide radical and ascorbate peroxidase (APX) and catalase (CAT) scavenging H_2O_2 (Asada 1999). These enzymes work in tandem or complementary, as it is the case of APX and CAT, the former with high affinity to H_2O_2, performing a crucial role in the control of ROS in each compartment, whereas the latter with its low affinity to H_2O_2 and presence mainly in peroxisomes is associated with processing H_2O_2 generated in photorespiration and in situations of excessive H_2O_2 formation (Mittler 2002, Vandenabeele *et al*. 2004). Prxs are described as interacting with these enzymes scavenging H_2O_2 and lipid peroxides and tuning their content in signalling events (Dietz 2011).

This study was designed to comprehensively analyse, for the first time in *V. vinifera,* the *Prx* gene family. For that purpose, we identified seven *Prx* genes and determined their expression under abiotic stresses. A relationship between their expression, the behaviour of other elements of the antioxidative network, and physiological responses after each abiotic stress was established. Also, the involvement of different Prxs in different stress signalling pathways was established: *vvprxIIF* in drought tolerance through H_2O_2 signalling and *vvprxII-2* in ABA involved thermotolerance. The study of the response of these genes under abiotic stress is paramount because different *Vitis* species differ in their response to stress (Upadhyay *et al.* 2012) and in a Mediterranean environment with high irradiance, high temperature, and low water supply during the summer growing season, oxidative stress is prone to occur.

Materials and methods

Cuttings from pruned wood of pre-selected grapevines (*Vitis vinifera* L.) cv. Touriga Nacional were collected from a vineyard in Pegões, 70 km SE from Lisbon. They were treated with fungicide (2 %, m/v, *Benlate*) and kept at 4 °C for 2 months and then treated again with fungicide before rooting. Rooting took place in distilled water supplemented with nutrient solution (Rhue *et al.* 1978) in the dark, until full rooting and shoot elongation. The saplings were transferred to pots filled with disinfected soil and maintained in the greenhouse under irradiance of 200 µmol m^{-2} s^{-1}, a 16-h photoperiod, day/night temperature of 25 ± 2/23 ± 2 °C and relative

humidity of *ca.* 60 %. Plants were watered with nutrient solution whenever necessary.

When plants were *ca.* 4-month-old and 70 cm high, they were exposed to different stresses: *1*) high photosynthetic photon flux density (PPFD) of 2000 μmol m^{-2} s^{-1} for 1 h (high irradiance, HI); *2*) temperature of 42 ± 1 °C for 1 h (heat stress, HS); and *3*) to water stress (WS) induced by cessation of watering till pre-dawn leaf water potential reached -0.9 MPa (pressure chamber, *Model 600*, *PMS Instruments Company*, Albany, OR, USA). The third, fourth, and fifth leaves were harvested immediately after the measurements of photosynthetic and chlorophyll fluorescence parameters and stored at -80 °C until use.

H_2O_2 production was assayed following the spectrophotometric method described by Carvalho *et al.* (2006) using the peroxide-mediated oxidation of Fe^{2+} followed by the reaction of Fe^{3+} with xylenol orange. This method produces reproducible results in the 0.1 - 1 mM H_2O_2 concentration range.

The extracts for the measurement of ABA were carried out as described by Vilela *et al.* (2007). ABA was quantified through immunoassay by indirect enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies using a commercial kit (*Olchemim Enzyme Immunoassay*, Olomouc, Czech Republic) according to the manufacturer's recommendations.

The extraction for measuring Prx activity was performed at 4 °C from 0.5 g of frozen leaf material according to Carvalho *et al.* (2006). The material was ground using a mortar and pestle with 50 % (m/v) polyvinylpolypyrrolidone. The extraction buffer was 0.2 M potassium phosphate, pH 8.0, containing 1 mM ethylenediaminetetraacetic acid (Na_2-EDTA), 1 mM dithiothreitol (DTT), 10 mM $MgCl_2$, and 0.2 mM phenylmethanesulfonylfluoride (PMSF). Extracts for all quantifications were centrifuged at 27 000 *g* and 4 °C for 10 min and the supernatants were desalted through *PD-10* columns (*GE Healthcare LifeSciences*, Buckinghamshire, UK). Protein was quantified by the method of Bradford (1976) using a commercial kit (*Bio-Rad*, Hercules, CA).

Reduction of H_2O_2 by *V. vinifera* Prxs was quantified *in vitro* using a non-enzymatic, DTT-dependent activity assay by measuring the decrease in H_2O_2 concentration in the assay solution as previously described (Horling *et al.* 2003, Aragón *et al.* 2009). The assay contained 100 mM K-Pi buffer (pH 7.0), 0.3 to 3 μM Prx, 10 mM DTT, and 100 μM H_2O_2 in a total volume of 1 cm^3. The reaction was initiated with H_2O_2 and stopped with 0.8 cm^3 of 12.5 % (m/v) trichloroacetic acid to an aliquot of 0.05 cm^3 of the assay solution. After adding 0.2 cm^3 of 10 mM $Fe(NH_4)_2(SO_4)_2$ and 0.1 cm^3 of 2.5 M KSCN, the absorbance at 480 nm was measured to quantify the H_2O_2 content of the solution, and H_2O_2 reduction rate was calculated.

Net photosynthetic rate (P_N) was measured using a *Li-6400* portable photosynthesis system fitted with a leaf chamber mounted with a light source (*LiCor*, Lincoln, Nebraska, USA). The temperature inside the leaf cuvette was set to 25 ± 2 °C. The response of P_N to irradiance (from 0 to 2000 μmol m^{-2} s^{-1}) was performed at ambient CO_2 concentration (*ca.* 350 μmol mol^{-1}). Chlorophyll fluorescence parameters were quantified with a portable modulated fluorometer (*Mini-Pam*, *Walz*, Effeltrich, Germany). The parameters measured were the minimum fluorescence of dark-adapted leaves (F_0), maximum fluorescence of dark-adapted leaves (F_m) after a saturation pulse with an irradiance of 1 800 μmol $m^{-2}s^{-1}$ to calculate the variable fluorescence ($F_v = F_m - F_0$) and the maximum quantum efficiency of PS II photochemistry in dark-adapted leaves (F_v/F_m).

Total RNA was extracted according to the modified method of Geuna *et al.* (1998), treated with *RQ1* RNase-free DNase (*Promega*, Madison, WI, USA), and reverse-transcribed using *oligo*DT_{20} and *Superscript II* RNase H-reverse transcriptase (*Invitrogen*, Carlsbad, CA, USA) according to the manufacturer's recommendations. RT qPCR was performed as previously described Coito *et al.* (2012) and the primers used are listed in Table 1.

Table 1. Primers used for the qRT PCR of the *V. vinifera* peroxiredoxins and of the ascorbate-glutathione cycle genes.

Primer	Accession No.	Sequence (5' - 3') forward	reverse
vv2cysprx01	JN392719	TCCTCTCGTCTAACCATTCCTCATC	CTCAAACTCTTCATAGCGGTCACTG
vvprxQ	JN392725	ACCTTCCTCACTCTTAATGGCTTCC	CTTTCCTCACCTTGTTTCCGTCATC
vvprxII-1	JN392721	ATCTTCCGATTTCACATCCACCATC	TCACCTGTTCCTTCGTCTTTCCTTG
vvprxII-2	JN392722	TTTCTCTCTCCACTACGATCTTGCC	CTCCTCTTGTCACGGTATCCCTTTG
vvprxIIE	JN392723	AATCTACCATAGGAATGCTCGTTGC	AATCAGACACAGGAAACCACAAACC
vvprxIIF	JN392724	CGAAGCATGATGATGAAATCAACGG	GCACCAGAAACCTTAACCTCGGATG
vv1cysprx03	JN392718	GAAACCACACATGGGAAGTTCAAAC	GGTATGACTTGTTGGATGGAAGAGC
DHAR	XM_002282363.1	TAATGACGGATCCGAGAAGG	CTGGTCAAGCTCTCAGGGAC
GOR2	XM_002285636.1	GTGCAGTCAAACTGTGCCTTAG	GAGCGAAGAACAGGCTACAGAT
CuZnSOD	XM_002274385.1	GTCATGCGGGTGACCT	AGATTGGCATGTGGTGT
MnSOD	ABX79342.1	CCTTACGATTATGGCGCATT	CTTCACTGGAAGGAGCAAGG
CAT	AF236127	ATGGATCCTTACAAGTATCGTC	GAGGTCACTCACGATGTCTC
APX3	XM_002278245.1	ATGGCTGCACCGATTGTTGATGCG	GAACGAAGAAGAGCACACTCATG
Act2	AF369525.1	TGGATTCTGGTGATGGTGTGAGTC	CAATTTCCCGTTCAGCAGTAGTGG

To compare data from different PCR runs or cDNA samples, C_T values were normalized to the C_T value of *Act2*, a gene that was found to be a good reference in similar experimental conditions (Coito *et al.* 2012).

BLASTP and *TBLASTX* searches were performed using other sequenced plant species available (*Arabidopsis thaliana, Oryza sativa*, and *Populus trichocarpa*) against *V. vinifera* in GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi; Benson *et al.* 2011) and Genoscope (www.genoscope.cns.fr). The sequences of *V.vinifera* thus obtained were screened for the conserved thioredoxin fold using the conserved domain database *CDD* (www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml; Marchler-Bauer *et al.* 2011). Each *vvprx* identified was cloned with *pMOSBlue Blunt* ended PCR cloning kit (*GE Healthcare*, *Life Sciences*) and the sequenced fragments (*STAB-Vida*, Oeiras, Portugal) compared to the expected sequences using *CLUSTALW2.0* (Larkin *et al.* 2007, http://www.ebi.ac.uk/Tools/msa/clustalw2/). The complete sequences were then submitted to GenBank and their accessions are listed in Table 2. All the other genes studied were already available in *NCBI* database.

The phylogenetic tree was calculated based on *ClustalW2.0* alignments and using the neighbor-joining method (Saitou and Nei 1987). The quality of the predicted tree was tested by calculating the bootstrap values based on 1000 replicates (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates were excluded. The distances were computed using the Dayhoff matrix based method (Schwarz and Dayhoff 1979). The analyses were conducted in *MEGA5* (Tamura *et al.* 2007).

To predict the subcellular localization sites of VvPrxs, their amino acid sequences were analysed first by *TargetP* (www.cbs.dtu.dk/services/TargetP/) followed by analysis in *WoLFPSORT* (http://wolfpsort.org/, Horton *et al.* 2007) in order to verify the results. Theoretical determination of each VvPrx molecular mass (kDa) and isoelectric point was computed in http://web.expasy.org/compute_pi/ (Gasteiger *et al.* 2005).

The following parameters were subjected to statistical analysis by one-way *ANOVA* using *GraphPad Prism v. 5.04* for *Windows* (*GraphPad Software*, La Jolla, CA, USA). Five replicates for photosynthesis and chlorophyll fluorescence, three replicates for gene expression and enzyme activity, and six replicates for ABA and H_2O_2 were used. In order to analyze the relationship between Prx activity and *Prx* gene expression with H_2O_2 content, ABA content, and F_v/F_m under each abiotic stress, R^2 from the Pearson correlation analysis was calculated and statistically analysed with *GraphPad Prism v. 5.04*.

Table 2. The seven *V. vinifera Prx* genes cloned and annotated, with their predicted protein properties.

Gene	Accession	Type	Number of amino acids	M_r [kDa]	pI
vv1cysprx03	JN392718	1 cys peroxiredoxin	183	20.22	7.85
vv2cysprx01	JN392719	2 cys peroxiredoxin	274	30.27	6.84
vvprxII-1	JN392721	type II peroxiredoxin	256	28.11	6.82
vvprxII-2	JN392722	type II peroxiredoxin	254	27.56	5.15
vvprxIIE	JN392723	type II peroxiredoxin	212	22.51	7.67
vvprxIIF	JN392724	type II peroxiredoxin	201	21.83	8.74
vvprxQ	JN392725	peroxiredoxin Q	214	23.47	9.68

Results

The *V. vinifera* Prx family includes at least seven genes (Table 2). The inclusion of these genes in their respective Prx type was confirmed by phylogentic analyses (Fig. 1). According to the number and position of conserved Cys residues, the VvPrx were classified as follows: VvPrxIIE, VvPrxIIF, VvPrxII-1, and VvPrxII-2 are type II Prx proteins, Vv1CysPrx03 is a 1CysPrx protein, Vv2CysPrx01 is a 2CysPrx protein, and VvPrxQ belongs to PrxQ (Fig. 1). The open reading frames (ORFs) of these VvPrxs encoded polypeptides of 162 - 274 amino acids with predicted molecular masses ranging from 20.22 to 30.27 kDa and pI of 5.15 to 9.68 (Table 2). Furthermore, *in silico* analyses predicted that Vv2CysPrx01, VvPrxQ, VvPrxIIE, VvPrxII-1, and VvPrxII-2 are targeted to the chloroplasts, Vv1CysPrx03 to the cytosol, and VvPrxIIF to the mitochondria.

Prxs play major roles in preventing oxidative damage and their function is consistent with the presence of Prx isoforms in most, if not all, cellular compartments. Transcription of *Prx* genes depends on environmental factors (Dietz *et al.* 2006). In *V. vinifera*, the genes encoding choroplastic (*vv2cysprx01* and *vvprxII*-1) and mitochondrial (*vvprxIIF*) Prx isoforms showed the highest expression after HS and WS.

After HI, all *vvprxs* showed low induction comparatively to the ascorbate-glutathione cycle genes. The highest transcription after HI was measured in the peroxisome, with a 12-fold induction of *APX3*, and in the mitochondria with *ca.* 2-fold induction of *MnSOD* (Table 3). In the peroxisome, *CAT* expression was unchanged. However, in the chloroplast, *CuZnSOD* was slightly repressed as well as the cytosol *GOR2*.

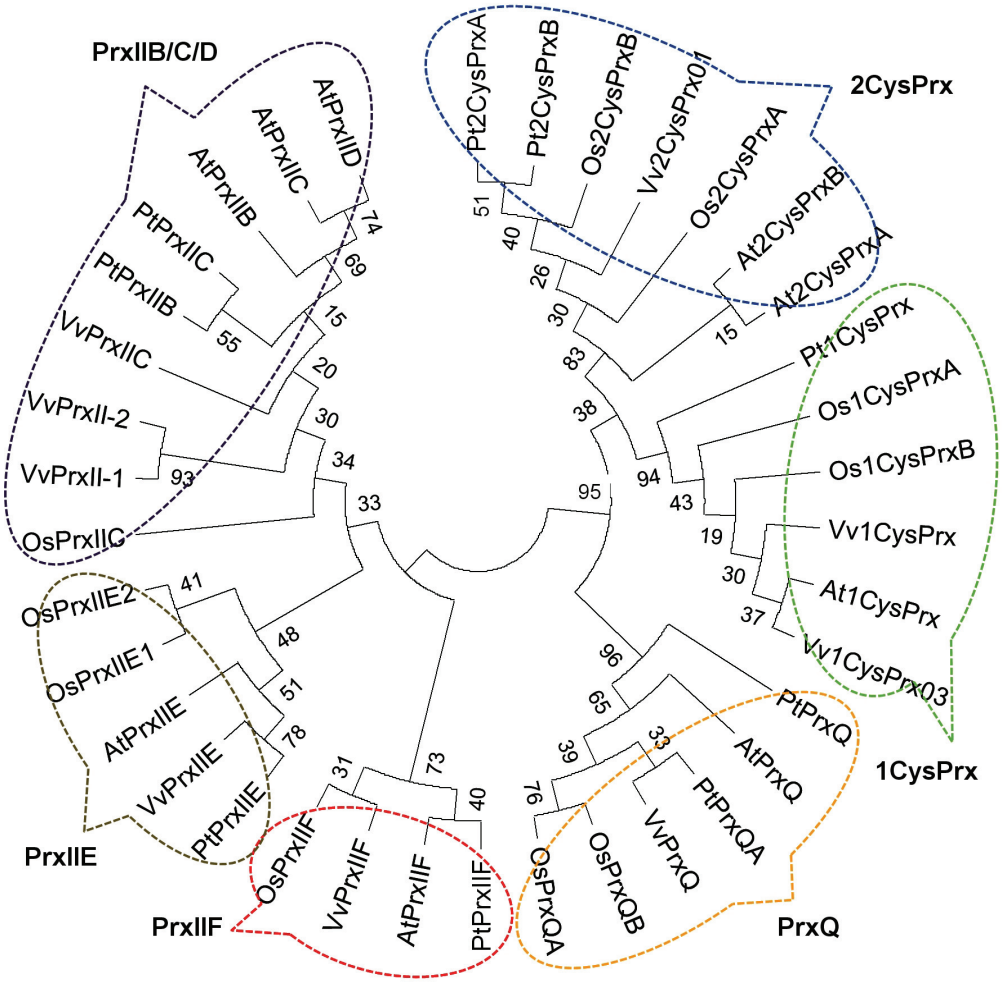

Fig. 1. Comparative analyses of *Prx* genes between *Arabidopsis thaliana* (*At*), *Populus trichocarpa* (*Pt*), *Oryza sativa* (*Os*), and *Vitis vinifera* (*Vv*), conducted in *MEGA5*.

The gene coding the chloroplast isoform VvPrxII-2 showed *ca.* 2-fold induction after HS (Table 4). Of the six genes involved in the ascorbate-glutathione cycle, five were up-regulated after HS (Table 3). The highest up-regulation was the 14-fold induction of *APX3* observed in the peroxisomes, followed by *MnSOD* with *ca.* 6-fold induction in the mitochondria, and *CAT* with *ca.* 5-fold induction in the peroxisomes. In the cytosol, *DHAR* was *ca.* 4-fold up-regulated, but *GOR2* was slightly repressed. In the chloroplast, *CuZnSOD* was up-regulated by *ca.* 2-fold.

Table 3. Relative expression of ascorbate-glutathione cycle genes after high irradiance (HI), heat stress (HS), and water stress (WS). Data are $\log_2$ expression ratios of stress treatments in relation to control conditions. Means ± SE of three independent biological replicates. * - indicate significant differences between control and treatment conditions at $P < 0.05$ (*ANOVA*).

Gene name	HI	HS	WS
CAT	0.8 ± 0.00	4.6 ± 0.00*	-0.2 ± 0.00
APX3	12.4 ± 0.00	14.0 ± 0.00*	0.4 ± 0.00
GOR2	-0.6 ± 0.02	-0.7 ± 0.01*	0.0 ± 0.00
DHAR	-0.3 ± 0.00	3.5 ± 0.00	5.7 ± 0.00*
MnSOD	1.7 ± 0.00	5.7 ± 0.00*	6.5 ± 0.00*
CuZnSOD	-0.8 ± 0.01*	1.9 ± 0.00*	-1.2 ± 0.01*

The gene coding for the mitochondrial Prx isoform showed the highest up-regulation after WS (Table 4) with 3-fold induction, followed by *vv2cysprx01, vvprxIIE,* and *vvprxII-1* in the chloroplast, and *vv1cysprx03* in the cytosol with *ca.* 2-fold induction. The transcriptions of *vvprxII-2* and *vvprxQ* were unchanged. The highest transcript levels were measured in *MnSOD* (6.5-fold) in the mitochondria and in *DHAR* in the cytosol (5.7-fold). Neither the cytosol *GOR2* nor peroxisome *CAT* changed. In the chloroplast, *CuZnSOD* was slightly down-regulated.

In the activity assay with a DTT-based non-enzymatic regeneration system, Prxs showed H_2O_2 scavenging activity decreasing after stress. Under control conditions,

Table 4. Relative expression of *vvprxs* after high irradiance (HI), heat strass (HS), and water stress (WS). For each gene, the upper-line corresponds to $\log_2$ expression ratios of stress treatments in relation to control conditions (means ± SE of three independent biological replicates) and the lower line shows correlation coefficient R^2 between gene expression and Prx activity, ABA content, and H_2O_2 content (Prx | ABA | H_2O_2). * - indicate significant differences between control and treatment at $P < 0.05$; # - indicate significant differences in the Pearson correlation at $P < 0.05$.

Gene name	HI	HS	WS
vv1cysprx03	-1.0 ± 0.13*	1.3 ± 0.05*	1.8 ± 0.15*
	0.7 | 0.7 | 0.3	0.1 | 0.1 | 0.2	0.5 | 0.8# | 0.5
vv2cysprx01	-1.0 ± 0.05	0.8 ± 0.01*	2.2 ± 0.05*
	0.2 | 0.2 | 0.8#	0.1 | 0.1 | 0.3	0.3 | 0.7 | 0.6
vvprxII-1	-0.2 ± 0.21	1.0 ± 1.03	1.8 ± 1.82
	0.8# | 0.8# | 0.2	0.0 | 0.2 | 0.1	0.3 | 0.7 | 0.4
vvprxII-2	-2.2 ± 1.15	1.8 ± 0.21*	0.9 ± 0.38*
	1.0# | 0.0 | 1.0#	1.0# | 1.0# | 0.6	0.5 | 0.1 | 0.8#
vvprxIIE	-1.3 ± 0.11	0.4 ± 0.04	1.9 ± 0.09
	0.5 | 0.4 | 0.6	0.2 | 0.1 | 0.3	0.1 | 0.5 | 0.6
vvprxIIF	-0.9 ± 0.07*	1.4 ± 0.01*	2.9 ± 0.05*
	0.1 | 0.1 | 0.9#	0.4 | 0.0 | 0.6	0.6 | 0.9# | 0.9#
vvprxQ	-1.8 ± 0.14	-1.1 ± 0.09*	0.5 ± 0.04*
	0.0 | 0.0 | 1.0#	0.1 | 0.1 | 0.2	0.7 | 0.3 | 0.5

the Prx activity was 56.4 nmol(H_2O_2) μg^{-1}(prot.) min^{-1} (Table 5). The greatest decrease was after HI (84 % decrease of total Prx activity). After HS, there was a 49 % decrease. The smallest decrease was after WS corresponding to only 3 % decrease (Table 5).

After HI, there was a decrease in both H_2O_2 and ABA content of 18 and 55 %, respectively (Table 5). However, after HS, there was a 59 % increase of ABA content but a 29 % decrease of H_2O_2 content. The highest increase of H_2O_2 content was measured after WS (51 % increase), while ABA content remained unchanged.

Plants showed higher P_N after HI and HS (Fig. 2) but no significant differences were observed in PS II efficiency in dark adapted leaves (F_v/F_m; Table 5). Actually, there were no major changes in PS II efficiency in response to the various stresses.

Table 5. Total Prx activity [nmol(H_2O_2) μg^{-1}(prot.) min^{-1}], H_2O_2 content [µmol g^{-1}(f.m.)], ABA content [nmol g^{-1}(d.m.)], and maximum quantum efficiency of PS II photochemistry (F_v/F_m) in *V. vinifera* under control conditions and after high irradiance (HI), heat stress (HS), and water stress (WS). Means ± SE of 3 - 6 independent biological replicates; * indicate significant differences between stress treatments relative to control at $P < 0.05$. A Pearson correlation analysis was made in order to analyze the relationship between Prx activity and the other data in the table, however, no significant correlations were found.

	C	HI	HS	WS
Prx	56.4 ± 0.00	9.2 ± 0.00*	28.5 ± 0.00*	54.7 ± 0.00
H_2O_2	12.9 ± 1.50	10.6 ± 1.20*	9.1 ± 0.80*	19.5 ± 2.80*
ABA	2.9 ± 0.80	1.3 ± 0.80*	4.6 ± 0.40*	2.5 ± 0.40
F_v/F_m	0.8 ± 0.01	0.8 ± 0.01	0.7 ± 0.02	0.8 ± 0.03

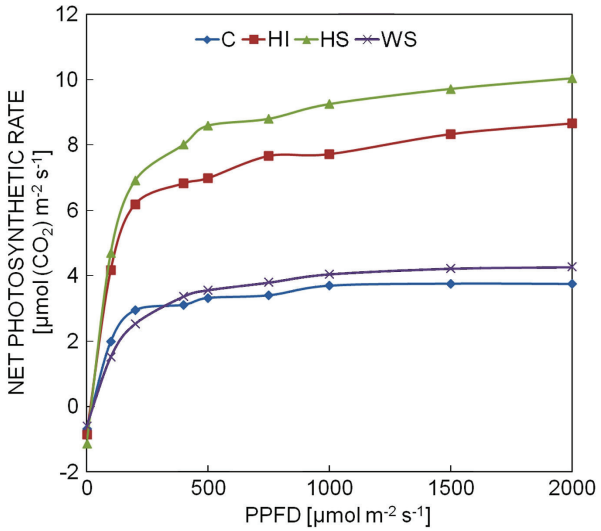

Fig. 2. Response of P_N to irradiance under control conditions (C) and after high irradiance (HI), heat stress (HS), and water stress (WS). Means of five independent biological replicates. PPFD - photosynthetic photon flux density.

Discussion

The Prx family is ubiquitous in all organisms from bacteria to higher plants. It is a small gene family with 10 genes in *Arabidopsis* and rice and 9 in poplar. In this study, seven *V. vinifera Prx* genes were cloned, one *vv1cysprx,* one *vv2cysprx*, one *vvprxQ*, and four *vvprxII* that include two putative new *vvprxs*. Most of the genes coding for Prx isoforms in *V. vinifera* were significantly up-regulated after HS and WS. As opposite to this response, the *vvprx* genes were found to be down-regulated after HI as previously observed Carvalho *et al.* (2011).

As a preliminary study to understand the function of these VvPrx*s* in grapevine under abiotic stresses, we used bioinformatic tools to predict their subcellular localization based on the N-terminal signal sequence of each protein: *vv2cysprx01, vvprxQ, vvprxIIE, vvprxII-1*, and *vvprxII-2* were targeted to the chloroplasts and *vvprxIIF* to the mitochondria. The *vv1cysprx03* was targeted to the nucleus and the cytosol consistently with *Arabidopsis 1CysPrx* (Haslekas *et al.* 2003, Pulido *et al*. 2009).

Among the seven *vvprx* genes, *vvprxQ* was repressed or did not change its transcription after the abiotic stresses

applied. However, previous studies in *Arabidopsis* showed upregulation of *AtPrxQ* after oxidative stress (Horling *et al.* 2003). PrxQ is considered the most responsive chloroplast Prx-transcript in *Arabidopsis* (Dietz 2011). In *V. vinifera* after HI, the transcription of the chloroplast genes was in general down-regulated indicating the absence of oxidative stress in the chloroplast and, therefore, explaining the repression of *vvprxQ* which showed a strong positive correlation with H_2O_2 (Table 4). The *vvprxII-2* was the gene that showed the lowest transcription which was proven by a positive correlation with Prx activity under HI.

After HS, *CuZnSOD* was 2-fold induced (Table 3) indicating a mild oxidative stress in the chloroplasts but *vvprxQ* remained repressed (Table 4), most likely due to the induction of the other chloroplast *vvprx* such as *vvprxII-2* which might replace *vvprxQ* under these conditions. The *vvprxII-2* had a strong positive correlation with Prx activity and ABA under HS (Table 4) signifying a strong influence of this gene on the activity of Prx under stress and that it also might be involved in the ABA signalling pathway. Interestingly, there was a significant increase of ABA under HS which coincided with the decrease of H_2O_2 (Table 5).

The photosynthesis of the plants after the three stresses applied in this study showed a tendency to higher rates than under control conditions (Fig. 2) although this probably reflects a transient response since these mostly short term stresses did not affect PS II (Table 5, F_v/F_m values). After HS, there was a significant increase in transcription of peroxisome genes, *CAT* and *APX3*, and of *MnSOD* in the mitochondria (Table 3). In addition, there was an 18 % decrease in H_2O_2 content which goes in concordance with the increase in transcripts of the genes coding for H_2O_2 scavenging enzymes such as CAT and APX3 and of the gene coding for MnSOD which leads to the generation of H_2O_2. There was a significant increase in ABA content after HS (Table 5) as Wang *et al.* (2005) also reported in young *V. vinifera* plants subjected to heat. These results, together with the photosynthetic response could indicate that ABA can be involved in *V. vinifera* response to HS as well as in improved thermotolerance. An *in silico* analysis revealed that the promoter of *vvprxII-2* contains the ABA-responsive element ABRE. As already mentioned, *vvprxII-2* was the most induced *vvprx* gene under HS, thus it is possible to admit that VvPrxII-2 might be involved in ABA signalling in *V. vinifera* thermotolerance.

From all the abiotic stresses applied, WS caused the most intense changes in the transcription of *vvprx* genes. Even though there was no negative effect on the photosynthetic capacity, there was a significant increase in H_2O_2 content (Table 5) accompanied by the 6-fold up-regulation of *MnSOD* (Table 3). The *vvprxIIF* sustained significantly up-regulated but no changes were observed in the peroxisome *CAT* and *APX3*. Sweetlove *et al.* (2002) showed that PrxIIF detoxifies H_2O_2 released from superoxide dismutation by MnSOD. This, together with the high transcription of *CAT* in PrxIIF knock-out plants, suggests that *vvprxIIF* could play a role in the tolerance of *V. vinifera* to drought, that is also further confirmed by the strong correlation between *vvprxIIF* expression and ABA and H_2O_2 content (Table 4). In the cytosol, *vv1cysprx03* was up-regulated as well as *vvprxII-1, vvprxIIE*, and *vv2cysprx01* in the chloroplasts indicating their roles in tolerance to drought. Similar results were observed in the dehydration-tolerant *Xerophyta viscosa* where *1CysPrx* increased under dehydration indicating that 1CysPrx also plays a role in tolerance to extreme drought (Mowla *et al.* 2002).

Prx enzymes have a low catalytic efficiency towards H_2O_2 and thus it is believed that they are only effective to eliminate low concentrations of H_2O_2 (Rhee *et al.* 2005). However, it was shown that the catalytic efficiency of the bacterial Prx (AhpC) can be as high as in other enzymes such as CAT (Parsonage *et al.* 2008). However, CAT is mainly located in peroxisomes and Prxs are present in multiple subcellular localizations. It is suggested that their ubiquitous distribution meets the requirements for local H_2O_2 detoxification in general antioxidant defence and also allows their functions as redox sensors and redox elements in signalling networks (Dietz 2011). Preliminary results using virus induced gene silencing (VIGS) in *Nicotiana benthamiana* showed that the silencing of *Prx* genes from different subcellular locations has different responses, such as a higher H_2O_2 accumulation when the chloroplast gene is silenced in comparison with the cytosol gene (data not shown).

In the current study in *V. vinifera* after WS, H_2O_2 increased but there was a decrease in Prx activity that could be due to the inactivation of the peroxidase activity of Prxs and the triggering of signalling events that led to the stress responses, such as the up-regulation of *DHAR* (Table 3). In fact, several studies have established a regulatory role of DHAR during oxidative stress tolerance and acclimation (Hossain and Fujita 2011). Actually, Wood *et al.* (2003) suggested that when H_2O_2 accumulates to large levels, inactivation of Prx through hyperoxidation occurs, thereby facilitating redox-dependent signalling, a concept known as the "floodgate" hypothesis.

In this work, we identified seven *V. vinifera Prx* genes and determined their expression under various abiotic stresses. WS produced the most extreme responses followed by HS and no changes were observed after HI. The *vvprxIIF* and *vvprxII-2* were the most responsive *Prxs* after WS and HS, respectively. It is proposed that VvPrxII-2 can be involved in thermotolerance through ABA signalling and that VvPrxIIF might play an important role in drought tolerance in *V.vinifera,* although more studies must be undertaken to confirm these hypotheses.

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