RESEARCH PAPER

Modulation of genes related to specific metabolic pathways in response to cytosolic ascorbate peroxidase knockdown in rice plants

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ABSTRACT

As a central component of the hydrogen peroxide detoxifying system in plant cells, ascorbate peroxidases (APX) play an essential role in the control of intracellular reactive oxygen species (ROS) levels. To characterise the function of cytosolic APX isoforms (*OsAPX1* and *OsAPX2*) in the mechanisms of plant defence, *OsAPX1/2* knockdown rice plants were previously obtained. *OsAPX1/2* knockdown plants (APx1/2s) exhibited a normal phenotype and development, even though they showed a global reduction of APX activity and increased hydrogen peroxide accumulation. To understand how rice plants compensate for the deficiency of cytosolic APX, expression and proteomic analyses were performed to characterise the global expression pattern of the APx1/2s mutant line compared with non-transformed plants. Our results strongly suggest that deficiencies in cytosolic APX isoforms markedly alter expression of genes associated with several key metabolic pathways, especially of genes involved in photosynthesis and antioxidant defence. These metabolic changes are compensatory because central physiological processes such as photosynthesis and growth were similar to non-transformed rice plants. Our analyses showed modulation of groups of genes and proteins related to specific metabolic pathways. Among the differentially expressed genes, the largest number corresponded to those with catalytic activity. Genes related to oxidative stress, carbohydrate metabolism, photosynthesis and transcription factor-encoding genes were also modulated. These results represent an important step toward understanding of the role played by cytosolic APX isoforms and hydrogen peroxide in the regulation of metabolism by redox modulation in monocots.

INTRODUCTION

Since the introduction of molecular oxygen into the environment through the emergence of photosynthetic organisms, reactive oxygen species (ROS) have been the uninvited companions of aerobic life (Halliwell 2006). Through a variety of reactions, molecular oxygen produces superoxide (O_2^-), which in turn leads to the formation of hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and other molecules. ROS are also produced continuously as by-products of various metabolic pathways and are localised in different cellular compartments such as chloroplasts, mitochondria and peroxisomes (Del Rio *et al.* 2006; Navrot *et al.* 2007). Hydrogen peroxide is continually generated from various sources during normal metabolism *via* the Mehler reaction in chloroplasts, electron transport in mitochondria and photorespiration in peroxisomes (Neill *et al.* 2002a), but hydrogen peroxide can

also be generated by specific enzymes. Studies have demonstrated that plasma membrane (PM)-located respiratory burst oxidase homologue (Rboh) enzymes, similar to the respiratory burst oxidase of mammalian neutrophil cells, are an essential ROS-producing system during the early stages of plant-microbe interactions (Torres *et al.* 2002; Apel & Hirt 2004). Accumulation of ROS affects many cellular functions, causing damage to proteins, lipids, carbohydrates and DNA and can result in cell death (Foyer & Noctor 2005).

Hydrogen peroxide, one of the most important ROS, is a toxic cellular metabolite, but also functions as a signalling molecule that mediates responses to various stimuli in both plant and animal cells. Therefore, hydrogen peroxide plays a dual role in plants, at low concentrations acting as a signalling molecule involved in acclimation and triggering tolerance to various biotic and abiotic stresses, and leading to cell death at high concentrations (Neill *et al.* 2002b; Quan *et al.*

2008). This molecule is also involved in a broad range of physiological processes as a key regulator in senescence, photorespiration and photosynthesis, stomatal movement, cell cycle, cell growth and plant development (Noctor & Foyer 1998; Foreman et al. 2003; Mittler et al. 2004; Peng et al. 2005; Bright et al. 2006), and hydrogen peroxide modulates the transcription of several genes encoding proteins involved in metabolism, energy, transport, cell defence, cellular organisation and biogenesis (Desikan et al. 2001).

Whether ROS will act as a damaging, protective or signalling factor depends on the delicate equilibrium between ROS production and scavenging at the correct site and time (Gratao et al. 2005). Under steady-state conditions, ROS molecules are scavenged by various antioxidative defence mechanisms (Foyer & Noctor 2005). All organisms have adaptive responses to oxidative stress, such as antioxidant defence enzymes, and these responses are induced by changes in levels of the ROS, leading to activation or silencing of genes that encode defence enzymes, transcription factors or structural proteins (Dalton et al. 1999).

Plant cells have several mechanisms to efficiently detoxify ROS. These protective mechanisms were developed by plants during evolution to control ROS levels and override this toxicity. Antioxidant molecules, enzymes or more complex systems of detoxification may be involved in cellular protection against ROS accumulation. Several regulatory enzymes prevent the toxic action of ROS on plant cells, including superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione peroxidase (GPx) (Zhang et al. 1995; Scandalios 2002).

Ascorbate peroxidase (EC 1.11.1.11) is the main enzyme responsible for hydrogen peroxide removal, and is found in the cytosol, peroxisome, mitochondria and chloroplasts of plant cells. This enzyme uses ascorbate as a specific electron donor to reduce hydrogen peroxide to water (Asada 1992; Teixeira et al. 2004, 2006; Li et al. 2007). In rice (Oryza sativa), a multigene family formed by eight genes encoding APX isoforms has been identified. Rice APXs are found in different subcellular compartments: two cytosolic isoforms (OsAPX1 and OsAPX2), two peroxisomal isoforms (OsAPX3 and OsAPX4), one mitochondrial isoform (OsAPX6), two chloroplastic isoforms (OsAPX7 and OsAPX8) and one mitochondrial/chloroplastic isoform (OsAPX5) (Teixeira et al. 2004, 2006).

Studies in Arabidopsis have demonstrated that mutants deficient in cytosolic APX are more susceptible to oxidative damage triggered by abiotic stress (Davletova et al. 2005). Rice plants were previously transformed with an RNAi construct specifically targeting OsAPX1 and OsAPX2. A preliminary characterisation of the APx1/2s lines has been described previously (Rosa et al. 2010). We have demonstrated that rice mutants with knockdown of both cytosolic APXs cope with abiotic stress such as salt, heat, high light and methyl viologen similar to the non-transformed (NT) plants (Rosa et al. 2010; Bonifacio et al. 2011). Although no major visible phenotypic alterations were observed between the knockdown and NT plants, RT-qPCR analysis showed that expression of both of the cytosolic OsAPX genes was strongly reduced in all of the APx1/2s lines compared with the NT plants, which strongly affects the entire antioxidant system. APx1/2s plants showed increased hydrogen peroxide

accumulation under both control and stress conditions (Rosa et al. 2010). In addition, analysis with T3 plants revealed that the transgenic APx1/2s line exhibited a reduction in total and cytosolic APX activities of approximately 68% and 85%, respectively, when compared with NT plants (Bonifacio et al. 2011). However, despite these differences, the knockdown rice plants had normal morphology in all of their organs. In addition, the silenced plants exhibited similar membrane damage (MD), increased levels of lipid peroxidation (TBARS) and similar photochemical efficiency parameters and CO_2 photosynthetic fixation rates as the NT plants (Bonifacio et al. 2011). The compensatory antioxidative mechanism displayed by the silenced plants was associated with higher GPx activity in the cytosolic and chloroplastic fractions. The activities of CAT and guaiacol peroxidase (GPOD; type III peroxidases) were also up-regulated. None of the six studied isoforms of OsAPX were up-regulated under normal growth conditions. Therefore, the deficiency in cytosolic APXs was effectively compensated for by up-regulation of other peroxidases.

The present study was conducted to test the hypothesis that rice plants deficient in both cytosolic APX1 and APX2, in addition to alterations in antioxidant systems, invoke global gene modulation that induces a compensatory mechanism to help the cell maintain general homeostasis. Several genes were identified with altered expression in response to the lack of OsAPX1 and OsAPX2, revealing pathways regulated by the cytosolic APX genes.

MATERIAL AND METHODS

Plant material and growth conditions

The NT and transgenic rice (Oryza sativa L. cv. Nipponbare) plants were grown at 28 °C under 12 h of light/dark (150 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in a growth chamber. APx1/2s plants were obtained as described previously (Rosa et al. 2010). The seeds were germinated in water at 28 °C in dark conditions. Four-day-old seedlings were transferred to plastic pots containing Furlani's solution (48 mg·l^{-1} N–NO$_3^-$, 12 mg·l^{-1} N–NH$_4^+$, 200 mg·l^{-1} Ca, 200 mg·l^{-1} K, 40.6 mg·l^{-1} Mg, 8.0 mg·l^{-1} P, 151 mg·l^{-1} S, 234 mg·l^{-1} Cl, 4.85 mg·l^{-1} Fe, 0.67 mg·l^{-1} Mn, 0.36 mg·l^{-1} B, 0.20 mg·l^{-1} Zn, 0.05 mg·l^{-1} Cu, 0.11 mg·l^{-1} Mo) and grown at 28 ± 2 °C with a 12-h photoperiod for 30 days for the microarray experiments. The pH of the solution was adjusted to 5.4 with 0.1 N HCl. Volume and pH of the nutrient solution were monitored daily and changed every 4 days. Thirty-day-old plants were cultivated under the same conditions for the proteomic experiments.

RNA isolation

The T1 generation of APx1/2s transgenic rice plants and control NT plants were used for microarray experiments. Leaves of 30-day-old APx1/2s and NT plants were collected and immediately frozen in liquid nitrogen. Total RNA was isolated using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and the quality of the resulting RNA was assessed by gel electrophoresis. RNA was then purified with the RNeasy Microarray Tissue Mini Kit (Qiagen, Germantown, MD, USA) and treated

with DNAse (Promega, Madison, WI, USA) according to the manufacturers' protocols.

Microarray experiments

Microarrays were performed with slides acquired from the NSF Rice Oligonucleotide Array Project (http://www.ricearray.org/nsfarray/nsfarray.shtml). Each slide consisted of 25,000 oligonucleotides that corresponded to annotated genes according to the Rice Genome Annotation Project (TIGR, http://rice.plantbiology.msu.edu/). Tissue collection from the leaves of 30-day-old APx1/2s-knockdown and NT plants was accomplished from four independent biological experiments, and each sample consisted of a pool of four plants. Dye-labelled antisense RNA was generated from these total RNA preparations and hybridised to microarrays using the Amino Allil MessageAmpII aRNA kit (Ambion, Austin, TX, USA). Dyes used for labelling RNA from the individual samples were switched in the replicate experiments to reduce dye-related artifacts. Microarrays were scanned with an Axon GenePix 4200A scanner using the Gene Pix 5.0 analysis software (Axon Instruments, Sunnyvale, CA, USA).

Microarray data analysis

Microarray image acquisition and analysis were performed with the GenePix® Pro 6 Software (Molecular Devices, Sunnyvale, CA, USA). Information was further analysed using the R/Bioconductor Limma package (Smyth 2004). All data were normalised to remove any systematic biases that may arise due to the microarray technology itself rather than differences between the probes or between RNA sample-target hybrids on the arrays. This was done using the print-tip Loess method, which corrects for dye bias and intensity within each group of adjacent spots printed by one pin (print-tip). Spots that had no signal detected or a signal-to-noise ratio below or equal to 2 were filtered out from the subsequent analysis. Differentially expressed genes were ranked based on Bayesian posterior log odds calculated with the Limma package. The empirical Bayes method was used to shrink the gene-wise sample variances towards common values, thus augmenting the degrees of freedom for the individual variances. This approach combines expression ratios and their variability between replicates to rank the genes (Smyth *et al.* 2005). Statistical significance between groups of interest was assessed for the relevant linear model contrast using moderated *t*-statistics as implemented in the Limma package. To determine which genes were significantly modulated by the knockdown, we considered differentially expressed genes according to the following comparison: knockdown rice *versus* NT rice. We used Benjamini & Hochberg's method (1995) to control the false discovery rate for the comparison. All analyses were performed using the freely available statistical software and graphics R environment (RDC 2008).

Quantitative real-time PCR (RT-qPCR)

Real-time PCR experiments were performed with cDNAs synthesised using M-MLV Reverse Transcriptase (Promega) and a 24-polyTV primer. After cDNA synthesis, the samples were diluted 100-fold in sterile H₂O. Subsequent PCR amplifications were performed using gene specific primers. Primers were designed to produce DNA fragments ranging from 100 to 200 bp (Table S1).

Quantitative real-time PCR assays were performed with biological and technical quadruplicates on a StepOnePlus Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) using two genes as internal reference controls: *OsFDH* – formaldehyde dehydrogenase (TIGR:LOC_Os02g57040) and *OseF* – elongation factor (TIGR:LOC_Os03g08020). Each experiment was repeated four times using samples collected separately. All expression data analyses were performed after comparative quantification of amplified products with the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001; Schmittgen & Livak 2008), using fluorescence detection of the SYBR-green intercalating dye. Statistical analysis was performed by Student's *t*-test to compare pair-wise differences in expression. The values were considered significantly different when $P < 0.05$.

Protein extraction

Leaves of 30-day-old NT and knockdown plants were collected and macerated in liquid nitrogen and homogenised in 0.5 M Tris–HCl, pH 8.3, 2% Triton X-100, 20 mM MgCl₂, 2% β-mercaptoethanol, 1 mM PMSF, 2.5% PEG and 1 mM EDTA buffer, and incubated at 4 °C for 2 h. The supernatant was added to a solution containing 10% trichloroacetic acid (TCA) in acetone (w/v), and the mixture was incubated for 12 h at −20 °C for protein precipitation. The resulting precipitate was washed in cold acetone and dissolved in a solution containing 7 M urea and 2 M thiourea (Acquadro *et al.* 2009). The quantification of extracted proteins was conducted according to Bradford (1976).

2-D Gel and image analysis

Approximately 500 mg of protein extract was used for 2-D electrophoresis analyses. The proteins were first separated according to their isoelectric points using a gradient of pH from 4 to 7 and subsequently separated according to their molecular weight in a 12.5% SDS-polyacrylamide gel. The gels were stained with Coomassie blue and analysed to choose the spots of interest, which were excised from the gels and digested with trypsin, according to Hellman *et al.* (1995). The solution containing the peptides was separated using a multidimensional chromatographic system. The eluted fractions were analysed in a mass spectrometer and characterised using a source of electrospray ionisation (ESI) with two mass analysers: a quadrupole (Q) associated with a tube to measure the flight time of ions (TOF) and an ion detector. Coupled with the online Q-TOF, a capillary chromatography system (Acquity UPLC) was used in which the sample was applied to a reverse-phase column. To perform MS analysis, the peptides eluted from the column were ionised, and then the ratio was determined based on the mass charge (m/z) of each. The spectra generated by MS/MS were processed using Proteinlynx (Micromass) and compared to the National Center for Biotechnology Information (NCBI) database using the MASCOT software (http://www.matrixscience.com).

Photosynthetic gas exchange and chlorophyll fluorescence analyses

Gas exchange measurements were made using an Infrared gas analyser (LI-6400XT; Li-Cor Biosciences Inc., Lincoln, NE, USA). Light was provided from a red/blue LED light source. Net photosynthesis (P_N) and stomatal conductance (g_S) were measured under ambient CO_2 conditions (400 ppm), photon irradiance of 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a constant leaf temperature of 28 °C. The Rubisco carboxylation maximum rate (V_{cmax}), maximum electron flux (J_{max}), mesophyll conductance (g_m) and light respiration (Rd) were calculated from the P_N/Ci curves obtained by varying the ambient CO_2 concentration (Ca) from 0 to 1000 ppm under a constant active photosynthetic photon flux density (PPFD) of 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$, according to the methodology of Sharkey *et al.* (2007). Photorespiration (P_r) was estimated according to the method previously described (Bagard *et al.* 2008).

Chlorophyll fluorescence was determined concurrently with gas exchange measurements using the LI-6400-40 leaf chamber fluorometer (Li-Cor Biosciences). The following parameters were assessed as proposed by Baker (2008): the maximum quantum yield of photosystem II (PSII) (Fv/Fm = (Fm − Fo)/Fm), the effective quantum yield of PSII (ΔF/Fm′ = (Fm′ − Fs)/Fm′), the photochemical quenching coefficient (qP = (Fm′ − Fs)/(Fm′ − Fo′)) and the non-photochemical quenching coefficient (NPQ = (Fm − Fm′)/Fm′). Fm and Fo are the maximum and minimum fluorescence, respectively, of dark-adapted leaves; Fm′ and Fs are the maximum and steady-state fluorescence, respectively, in the light-adapted state, and Fo′ is minimum fluorescence after far-red illumination of the previously light-exposed leaves. A saturating pulse of red light (0.8 s, 8000 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was used.

The electron transport flux (J), defined as the actual flux of photons driving photosystem II (PSII), was calculated from J = (Fm′ − Fs/Fm′) *f* I αleaf, where Fs is steady-state fluorescence (at 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$), Fm' is the maximal fluorescence during a saturating light flash, *f* is the fraction of absorbed quanta used by PSII, typically assumed to be 0.5 for C_3 plant species, I is incident photon flux density and αleaf is the leaf absorbance (0.85; according to the Li-Cor manual).

RESULTS

Plant lines with knockout or silencing of APX isoforms have been generated, resulting in different phenotypes. Knockdown or knockout plants for the cytosolic isoforms generally present altered phenotypes, such as dwarfism, stunted growth and late flowering (Davletova *et al.* 2005). The silencing of peroxisomal APX isoforms produced plants with altered panicle development and senescence (Ribeiro, unpublished results). Many studies, mostly in *Arabidopsis*, have shown that mutants deficient in cytosolic APX are more susceptible to abiotic stresses (Miller *et al.* 2007; Koussevitzky *et al.* 2008). Interestingly, APx1/2s rice plants showed a similar phenotype to NT plants, despite their higher hydrogen peroxide content (Rosa *et al.* 2010). To further demonstrate the alterations in redox homeostasis, the hydrogen peroxide content was assessed by DAB staining in silenced plants exposed to UV light (Fig. S1). The results confirmed that APx1/2s plants have less ability to scavenge hydrogen peroxide.

Microarray analyses

To understand the compensatory mechanism of the silenced plants that results in a normal phenotype, we searched for genes that are responsive to cytosolic APX silencing using global microarray and proteomic analyses of APx1/2s knockdown and NT plants. A set of 58 non-redundant genes were altered in the APx1/2s plant lines. Of these genes, 44 were up-regulated and 14 were down-regulated. The analysis also showed a reduction in transcript levels of *OsAPX1* and *OsAPX2*, confirming the knockdown of these genes in APx1/2s rice plants.

A functional classification of all differentially expressed genes is summarised in Fig. 1. The values of changes in gene expression and predicted gene function of the modulated genes (based on the Rice Genome Annotation Project: http://rice.plantbiology.msu.edu) are shown in Table S2. Among the up- and down-regulated genes, the class with the largest number of genes was that with catalytic activity, representing 22% of the induced and 28% of the repressed genes. Genes from other functional classes were also identified, including genes associated with oxidative stress, carbohydrate metabolism, photosynthesis and transcription factors. Some of the induced and repressed genes (27% and 21%, respectively) have unknown functions and likely represent the proportion of unknown genes in the entire genome. Transcription factors such as MYB, bHLH, NAM and WRKY have been previously
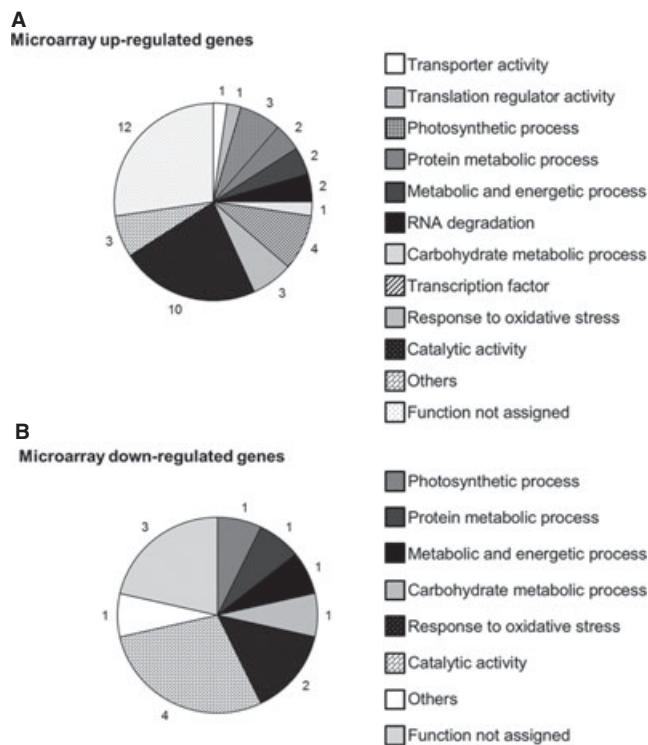

Fig. 1. Molecular functional classification of the differentially expressed genes identified in the microarray analysis. (a) Up-regulated genes; (b) Down-regulated genes. The predicted molecular functions were collected at the Rice Genome Annotation (http://rice.plantbiology.msu.edu).

described as responsive to oxidative stress (Gadjev *et al.* 2006). In addition, it has also been demonstrated that photosynthesis-related genes may have altered expression in plants lacking APX and CAT in *Arabidopsis* (Rizhsky *et al.* 2002).

To validate the microarray data, the expression of 15 genes was analysed using quantitative real-time PCR (Fig. 2). The expression patterns of all 15 genes showed a good correlation between the microarray and RT-qPCR experiments. Figure 2a–c shows validation of the down-regulated genes in knockdown plants, such as the gene encoding calcium-dependent protein kinase (Fig. 2a). Figure 2d–o shows genes with induced expression in the APx1/2s plants, including genes encoding transcription factors (Fig. 2d,c,k) and heat shock proteins (Fig. 2g). Quantitative real-time PCR analysis confirmed that the expression levels of all 15 genes were altered following knockdown of cytosolic APX genes.

Proteomic analysis

Proteomic analyses were performed to identify differentially expressed proteins in the rice cytosolic APX knockdown plants. A large number of protein spots extracted from the rice leaf were reproducibly detected through independent replicates on 2-D gels. Each protein was identified according to its molecular mass and pI. In conjunction with automated gel scanning and computer-aided image analysis, over 500 protein spots were detected and numbered on the 2-D PAGE map (data with numbered proteins not shown). To correct the variability due to staining, the spot volumes were normalised as a percentage of the total volume of all spots on the gel.

Several spots showed differences among the gels, but only the spots with coefficients of variance higher than 10% between replicates of the different groups were accepted for comparative analysis. Thus, among all protein spots detected, only 40 were selected and excised from the gels for mass spectrometry analysis. Sixteen protein spots showed no match or did not have a conclusive chromatogram when compared with the NCBI database. The 26 identified proteins were classified according to their molecular function (Table 1), forming eight groups: (i) photosynthetic process, (ii) response to oxidative stress, (iii) phosphate metabolism, (iv) carbohydrate metabolic process, (v) protein metabolic processes, (vi) assis-
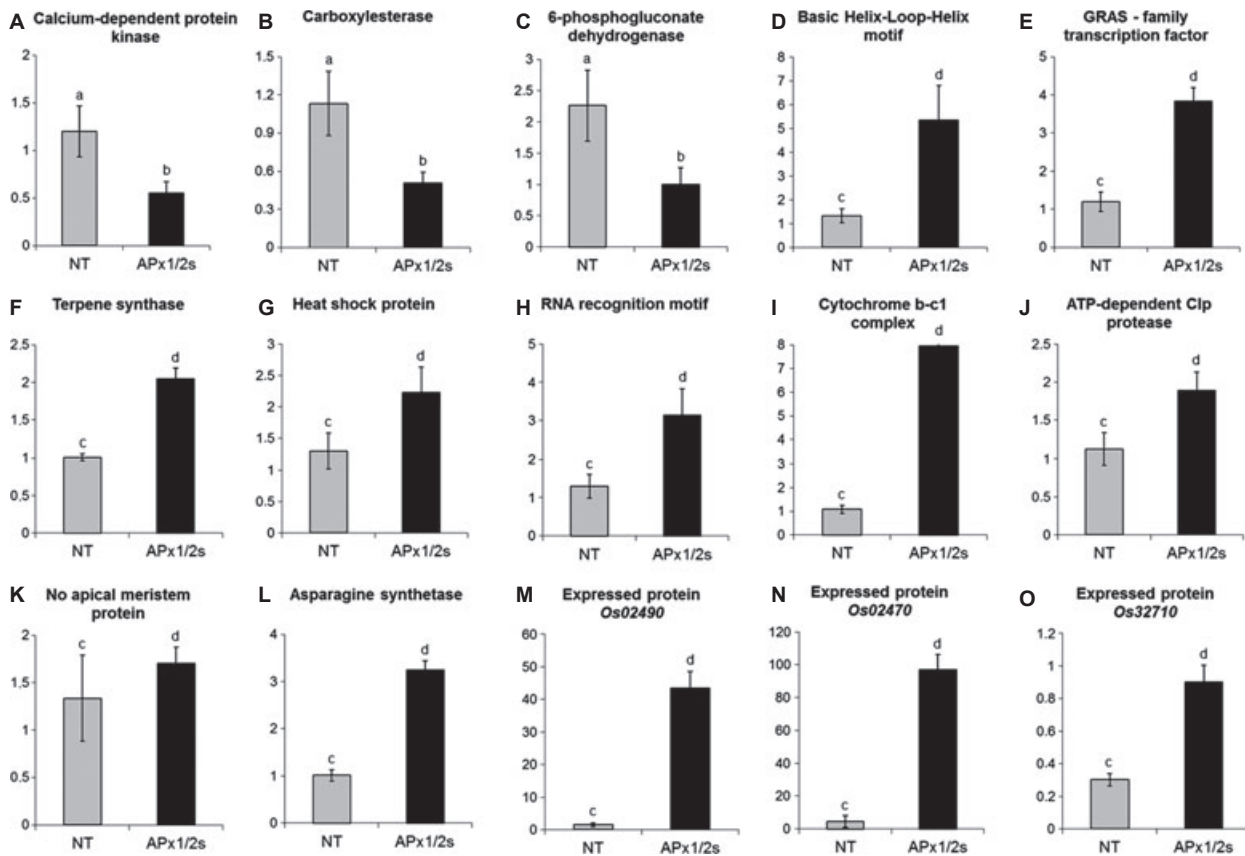

Fig. 2. Validation of differentially expressed genes in the microarray experiment by quantitative real-time PCR. This figure shows graphs of fifteen genes selected for validation. (a) Calcium-dependent protein kinase [TIGR:LOC_Os01g10890]; (b) Carboxylesterase [TIGR:LOC_Os07g34280]; (c) 6-phosphogluconate dehydrogenase [TIGR:LOC_Os06g02144]; (d) Basic Helix-Loop-Helix motif [TIGR:LOC_Os01g06640]; (e) GRAS-family transcription factor [TIGR:LOC_Os04g50060]; (f) Terpene synthase [TIGR:LOC_Os03g24760]; (g) Heat shock protein [TIGR:LOC_Os09g30412]; (h) RNA recognition motif [TIGR:LOC_Os03g18720]; (i) Cytochrome b-c1 complex [TIGR:LOC_Os04g32660]; (j) ATP-dependent Clp protease [TIGR:LOC_Os08g15270]; (k) No apical meristem protein [TIGR:LOC_Os01g01430]; (l) Asparagine synthetase [TIGR:LOC_Os03g18130]; (m) Expressed protein [TIGR:LOC_Os04g02490]; (n) Expressed protein [TIGR:LOC_Os03g02470]; and (o) Expressed protein [TIGR:LOC_Os07g32710]. RT-qPCR experiments were performed with leaves from NT and APx1/2s knockdown plants using two endogenous reference genes. The means indicated by different letters are statistically different based on the results of Student's t-test (P ≤ 0.05).

Table 1. Differentially expressed proteins in non-transformed and APx1/2s knockdown plants identified by mass spectrometry.

Functional classification	Protein id	Acc. no.[a]	Fold change[b]	SD	$pI_{(the)}$	$MM_{(the)}$	$pI_{(obs)}$	$MM_{(obs)}$	No. of peptides matched	Protein score	Sequence coverage (%)
Photosynthetic process	RuBisCO activase small isoform precursor	BAA97584	0.56	±0.24	5.80	48.0	5.25	44.5	55(22)	1143	50
	Ribulose bisphosphate/carboxylase small chain	AAA84592	1.38	±0.29	8.00	12.0	7.00	15.0	8(5)	238	46
	Putative 33 kDa oxygen evolving protein of photosystem II	NP_001043134	1.30	±0.19	6.10	35.0	5.16	36.5	84(34)	1256	60
	23 kDa polypeptide of photosystem II	BAA08564	0.66	±0.17	9.00	27.0	5.60	24.0	5(2)	93	21
	Photosystem II 10 kDa polypeptide	AAB46718	2.34	±0.33	9.00	12.0	6.01	12.7	7(2)	120	22
	Probable photosystem II oxygen-evolving complex protein 2	NP_001058863	1.35	±0.14	8.00	27.0	5.80	28.0	33(16)	734	42
	Photosystem I subunit VII	NP_001056530	1.43	±0.32	5.60	15.5	4.20	13.0	42(11)	487	46
	Plastocyanin	NP_039445	2.43	±0.53	6.50	9.4	6.60	11.7	7(3)	207	85
	Ferredoxin-1, chloroplastic	Q0J8M2	3.87	±0.51	4.40	15.2	4.00	24.0	13(4)	30	10
	Putative ferredoxin-NADP(H) oxidoreductase	BAD07827	1.88	±0.30	8.00	41.0	5.60	40.2	18(8)	433	35
	ATPase alpha subunit	NP_039380	1.47	±0.30	6.00	55.6	6.00	60.0	23(12)	635	31
	ATP synthase gamma chain	NP_001059768	1.71	±0.38	8.60	40.0	6.20	44.0	13(5)	326	27
	Phosphoglycerate kinase	EAY98560	1.18	±0.12	6.80	30.5	5.07	35.0	9(6)	508	30
	Triosephosphate isomerase, cytosolic	NP_001042016	1.45	±0.24	5.40	27.0	5.46	30.5	46(23)	953	46
	Putative glyceraldehyde-3-phosphate dehydrogenase B	NP_001048847	0.54	±0.24	6.20	47.5	5.84	47.5	25(7)	410	82
Response to oxidative stress	Gluathione s transferase	NP_001065137	2.77	±0.33	5.30	24.8	4.50	30.0	6(3)	190	27
	L-ascorbate peroxidase 1, cytosolic	NP_001049769	0.47	±0.33	5.40	27.5	5.50	30.0	20(7)	549	63
	Putative L-ascorbate peroxidase 2, cytosolic	BAA08264	0.33	±0.28	5.10	30.0	5.10	30.0	4(0)	55	26
	Putative superoxide dismutase [Cu-Zn], chloroplastic precursor	BAD09607	4.84	±0.35	5.80	20.0	5.30	24.0	9(2)	134	23
Phosphate metabolism	Putative inorganic pyrophosphatase	BAD16934	0.65	±0.17	5.80	31.0	4.90	36.5	35(10)	668	46
Carbohydrate metabolic process	Malate dehydrogenase, cytoplasmic, putative, expressed	ABG66141	1.40	±0.34	5.84	36.0	5.80	43.5	34(6)	502	41
Protein metabolic process	Putative precursor glutamine synthetase, chloroplastic	AAL87183	1.20	±0.12	6.20	49.7	5.20	48.5	54(24)	918	48
Assistant proteins	Belongs to the heat shock protein 70 family	EEE63158	0.50	±0.35	5.10	74.0	4.70	81.2	58(23)	1354	43
	Putative chaperonin 60 beta – HSP 60 family	NP_001056601	1.23	±0.15	5.60	64.0	5.10	66.5	23(7)	588	37
RNA degradation	Drought-induced S-like ribonuclease	AAL33776	3.68	±0.26	5.00	30.0	4.90	30.2	33(10)	603	48
Translation regulator activity	Elongation factor Tu	EEC73516	1.83	±0.37	6.20	50.0	5.50	50.7	40(15)	879	53

[a] NCBI accession number.
[b] Relative protein fold change in Apx1/2s leaves compared to NT.
$pI_{(the)}$, theoretical isoelectric point; $pI_{(obs)}$, observed isoelectric point; $MM_{(the)}$, theoretical molecular mass (KDa); $MM_{(obs)}$, observed molecular mass (KDa).
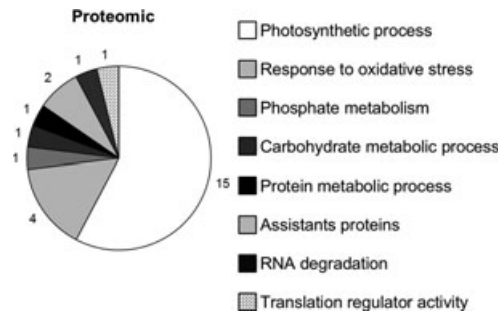
Fig. 3. Functional classification and distribution of 24 proteins with altered expression in APx1/2s plants after mass spectrometry analysis.

tant proteins, (vii) RNA degradation and (viii) translation regulator. The protein group with the highest representation in the total set of identified proteins was that associated with photosynthetic processes, with 64% of the total number of proteins, followed by proteins linked to the oxidative stress response and assistant proteins, each with 8% of the total protein. The other non-specific groups represented 4% each (Fig. 3). It was also possible to identify spots corresponding to the cytosolic APX isoforms (APX1 and APX2), confirming the knockdown of these isoforms at the protein level.

Photosynthetic and photochemical parameters

The Rubisco carboxylation maximum rate (V_{cmax}) and maximum electron flux (J_{max}) were similar in both NT and knockdown rice plants. The stomatal (g_S) and the mesophyll (g_m) conductances and the photochemical parameters such as Fv/Fm, $\Delta F/Fm'$ and qP were also similar between the different plants. The values of NPQ and J, parameters related to the dissipation of excess of energy of photosystem II and with changes in the pH gradient across the thylakoid membrane, were increased in the silenced plants. The electron transport flux (J) in photosystem II was slightly higher (approximately 10%) in the APx1/2s plants, which was associated with increased NPQ and a robust increase in the photorespiratory activity (P_r). Net CO_2 assimilation (P_N) and light respiration (R_d) rates were similar in both plant types (Table 2).

DISCUSSION

Based on the combined results of microarray and proteomic analyses, several metabolic pathways were affected by cytosolic APX silencing in rice plants. As reported in Tables 1 and S2, the major pathways affected in the mutant plant were photosynthesis, antioxidant metabolism, carbohydrate metabolism, protein metabolism and other proteins with catalytic activity. The modulation of these pathways may represent a general response to higher hydrogen peroxide accumulation in the silenced plants (Acquadro *et al.* 2009). Other studies on the hydrogen peroxide plant response corroborate our results (Yan *et al.* 2006; Wan & Liu 2008).

Differentially expressed genes associated with the photosynthetic process

The knockdown of cytosolic APX isoforms led to increasing endogenous hydrogen peroxide accumulation in rice leaves (Rosa *et al.* 2010). The photosynthetic apparatus is one of the major sources of ROS production in photosynthetic tissues, and suppression of photosynthetic activity was shown to accompany the defence response of certain plants to adverse physical conditions (Mittler *et al.* 2001). In the present analyses, 19 differentially expressed genes and proteins are associated with the photosynthetic process (Tables 1 and S2). These genes and proteins are implicated in two functional subgroups: photosynthetic photosystems I and II and the Calvin-Benson cycle.

To assess the relationship of the modulation of these genes and the acclimation processes, photosynthetic parameters were analysed. Both knockdown and NT plants presented similar maximum Rubisco carboxylation rates (V_{cmax}) and maximum electron flux (J_{max}), a photosynthetic parameter associated with the rate of ribulose 1,5-biphosphate (RuBP) regeneration (Bagard *et al.* 2008). These results were associated with similar responses in terms of gas exchange (net CO_2 assimilation and stomatal and mesophyll conductances) and also with higher Rubisco small subunit (RSS) content, as revealed by proteomic analysis (Table 1). Together, these results suggest that the photosynthetic assimilation of CO_2 in rice deficient in cytosolic APX1/2 was unchanged by silencing, in contrast to reports for other species, including Arabid-

Table 2. Photosynthetic and photochemical parameters[a].

	Photosynthetic parameters (gas exchange)						
	V_{cmax}	J_{max}	g_m	g_S	P_N	Rd	P_r
NT	174	175	6.09	0.45	19.70	7.34	0.368
APx1/2s	180	168	6.56	0.41	21.02	7.02	0.820
	Photochemical parameters						
	Fv/Fm	$\Delta F/Fm'$	qP	J	NPQ		
NT	0.727	0.651	0.936	112.6	0.079		
APx1/2s	0.730	0.643	0.935	122.0	0.119		

[a]Rubisco carboxylation maximum rate (V_{cmax}), maximum electron flux (J_{max}), mesophyll conductance (g_m), stomatal conductance (g_S), electron transport flux (J), net photosynthesis (P_N), light respiration (Rd) and photorespiration (P_r). V_{cmax}, J_{max} and g_m were obtained from P_N/Ci curves, while the other parameters were measured under [CO_2] at 400 ppm and PPFD 800 μmol m^{-2} s^{-1}.

opsis (Achard & Genschik 2009). These responses in rice indicate that silenced plants were capable of displaying similar photosynthetic efficiency, indicating the induction of an effective acclimation mechanism, as previously suggested (Achard *et al.* 2006).

The photochemical parameters such as Fv/Fm, ΔF/Fm′, qP, J and NPQ corroborate the CO_2 assimilation data, showing that changes in expression of the proteins of the photochemical system and the Calvin-Benson cycle could also be involved in the compensatory mechanism. The Fv/Fm, ΔF/Fm′ and qP parameters indicate that the functional integrity of photosystem II (the potential yield of photosystem II), (Fv/Fm), the effective quantum yield of PSII (ΔF/Fm′) and the recovery of the energy transferred as electrons (the photochemical quenching), (qP), respectively (Baker 2008), were similar in both genotypes. However, the values of J, the actual electron flux rate at photosystem II, and NPQ, a parameter related to dissipation of excess of energy of photosystem II, were increased in the silenced plants. These data suggest that silenced plants maintain a high electron flux and dissipate the excess energy, preventing damage to the photochemical apparatus (Baker 2008). The increased expression of key proteins involved in the electron flux in the chloroplast electron transport chain, such as plastocyanin, ferredoxin and ferredoxin-NADP(H)-oxidoreductase in the APx1/2s plants, suggests that these proteins may be involved in a compensatory mechanism that permits adequate electron flux to sustain photosynthesis at the same level as the NT plants.

The increase in electron flux could have increased the NADPH/NADP$^+$ ratio (Baker 2008). This hypothesis is reinforced by increased expression of ferredoxin-NADP(H)-oxidoreductase. These results were also associated with higher photorespiratory activity in the silenced plants. Thus, the higher electron flux in the silenced plants could have been dissipated as heat (higher NPQ) and higher photorespiratory activity (P$_r$) (Foyer & Noctor 2009). The increased photorespiration was associated with increased expression of chloroplastic glutamine synthetase (GS) and glycine dehydrogenase, the last two enzymes in the photorespiratory pathway. Photorespiration is involved in energy dissipation and protection against oxidative stress (Foyer & Noctor 2009). Moreover, the higher expression of plastocyanins, ferredoxin, ferredoxin-NADP(H)-oxidoreductase and chloroplastic Cu-Zn SOD isoforms could also contribute to reduce excess electrons in the photosystems, restricting potential oxidative damage.

Differentially expressed genes associated with protein metabolism

A total of ten genes related to protein metabolism showed modulation in the cytosolic APX knockdown rice plants (Tables 1 and S2). Three genes are related to protein folding and assembly (HSP60, HSP70 and HSP81-like). The contribution of heat shock proteins (HSP) to various cellular processes including signal transduction, protein folding, protein degradation and morphological evolution has been extensively studied (Nadeau *et al.* 1993). HSP60 and HSP81-like protein were up-regulated, but HSP70 was down-regulated, indicating that these proteins may respond differently in

response to hydrogen peroxide accumulation. HSPs are believed to help other proteins to maintain or regain their native conformation by stabilising partially unfolded states (Low *et al.* 2000). Current models propose that HSPs act as ATP-independent chaperones by binding to aggregating proteins and keeping them accessible for refolding by HSP70 (and co-chaperones) and, under some circumstances, by HSP100/ClpB proteins (Larkindale *et al.* 2005). In agreement with this model, expression levels of members of the HSP70 gene family have been found to be significantly elevated in maturing microspores upon exposure of plants to HS conditions (Frank *et al.* 2009). Because these proteins are related to protein folding and assembly in response to the loss of protein structural integrity (Bohler *et al.* 2007), these results suggest that the hydrogen peroxide accumulation in APx1/2s plants may induce protective responses involving HSP proteins. Indeed, one member of the AAA$^+$ family of ATPases was induced in APx1/2s-knockdown plants. This protein family is involved in resolubilising protein aggregates (Kotak *et al.* 2007). A cytosolic member of this family was found to be essential for tolerance to high temperature in plants (Hong & Vierling 2001). Among these proteins, an ATP-dependent Clp protease transcript was up-regulated in response to hydrogen peroxide accumulation. This protein is an ATP-dependent enzyme and functions as a master protease that cleaves a number of proteins (Maurizi *et al.* 1990).

Taken together, the differential expression patterns of the proteins in this group suggest that protein biosynthesis and degradation, as well as folding and assembly of proteins, are required for the survival and growth of rice plants in response to increased hydrogen peroxide.

Differentially expressed genes associated with oxidative stress

In addition to the reduced level of cytosolic APX isoforms, which confirms the silencing of these isoforms in APx1/2s knockdown plants, other antioxidant protein-encoding genes, such as the chloroplastic Cu/Zn SOD, peroxiredoxin, precursor of peroxidases, stress-related and glutathione-S-transferase genes, had altered expression. Changes in the SOD expression levels have been detected previously in mutant plants with reduced expression of APX (Rizhsky *et al.* 2002; Davletova *et al.* 2005; Rosa *et al.* 2010). In our previous study, the SOD enzymatic activity was increased in APx1/2s transgenic plants compared to NT plants (Rosa *et al.* 2010). In addition, we found that activity of the Cu/Zn SOD isoform is increased in silenced plants (Carvalho, unpublished results). Silencing of the cytosolic APX isoforms in rice plants caused hydrogen peroxide accumulation and activated the antioxidant defence system. The consistent induction of SOD, peroxiredoxin and GST is likely a consequence of antioxidative responses to the oxidative stress induced in these plants. We have previously shown that the compensatory antioxidative mechanism of the APx1/2s plants is associated with increased expression of *OsGpx*, *OsCatA*, *OsCatB* and guaiacol peroxidase (GPOD; type III peroxidases), suggesting that the deficiency in cytosolic APXs is effectively compensated for by up-regulation of other peroxidases and antioxidant enzymes (Bonifacio *et al.* 2011).

Glutathione-S-transferase (GST), which is related to oxidative stress, is also up-regulated in APx1/2s plants. The GSTs are a large group of enzymes that evolved to fulfill diverse

functions. They catalyse the S-conjugation of reactive compounds with glutathione, such as removal of lipid peroxides produced by lipid peroxidation (Foyer & Noctor 2009). GST is an antioxidative protein, and its expression is strongly enhanced by abiotic and biotic stresses (Dixon *et al.* 2002). GST expression is altered in response to hydrogen peroxide stress (Desikan *et al.* 2001; Sharkey *et al.* 2007; Koussevitzky *et al.* 2008), even in *Arabidopsis* suspension cultures (Desikan *et al.* 2000).

To further investigate other pathways related to ROS, further analyses using RT-qPCR were performed. Six genes that participate in ROS signalling, production and detoxification, including IMMUTANS (IM), NADPH oxidase (RbohD) and monodehydroascorbate reductase (MDAR), were selected for this study. The expression of these genes was analysed in the APx1/2s knockdown plants and compared with NT plants (Fig. 4).

The IM gene encodes a chloroplastic alternative oxidase protein. This enzyme has quinol oxidase activity and appears to divert electrons flowing from photosystem II to photosystem I at the plastoquinone pool, using these to reduce O_2 into water (Rizhsky *et al.* 2002). In our analysis, IM was found to be significantly repressed in APx1/2s plants (Fig. 4). The level of transcripts encoding IM was specifically induced in double APX and CAT antisense tobacco plants, APX antisense plants and CAT antisense plants, as well as in NT plants growing in high light, suggesting a role for IM in preventing ROS-related damage during high light stress in plants (Rizhsky *et al.* 2002). However, IM transcript abundance was

measured under various abiotic stresses, such as hydrogen peroxide, ozone, UV light, cold and mannitol, as well as hormones such as abscisic acid and salicylic acid, and IM transcripts remained at control levels (Rosso *et al.* 2006). *Arabidopsis* plants lacking IM have enhanced photosynthesis and increased Rubisco activity, perhaps to maximise the growth and reproductive fitness of *im* plants, as they exhibit a variegated phenotype (Aluru *et al.* 2007). Thus, IM may function to keep the PQ pool oxidised during chloroplast biogenesis and help assemble the photosynthetic apparatus. In our study, we found that the IM gene was repressed in APx1/2s plants. It is possible that in the APx1/2s plants, repression of the IM gene would allow higher activity of proteins belonging to PSI and PSII, in agreement with our proteomics analysis (Table 1).

An NADPH oxidase (Rboh, for respiratory burst oxidase homologue; RbohD) was also repressed in APx1/2s plants. The NADPH oxidase is an integral plasma membrane enzyme that oxidises cytosolic NADPH, transferring an electron across the membrane to generate superoxide in the cell wall. This superoxide can be rapidly dismutated to hydrogen peroxide, which is thought to diffuse back into the cell, providing a possible cytosolic regulator (Swanson & Gilroy 2010). Several studies have shown that RbohD is responsible for hydrogen peroxide production, as it is induced in response to different treatments, such as after cryptogein treatment of tobacco leaves (Lherminier *et al.* 2009), bacterial infection (Lina *et al.* 2010), in the guard cells of ozone-fumigated *Arabidopsis* leaves (Joo *et al.* 2005) and with salt treatment
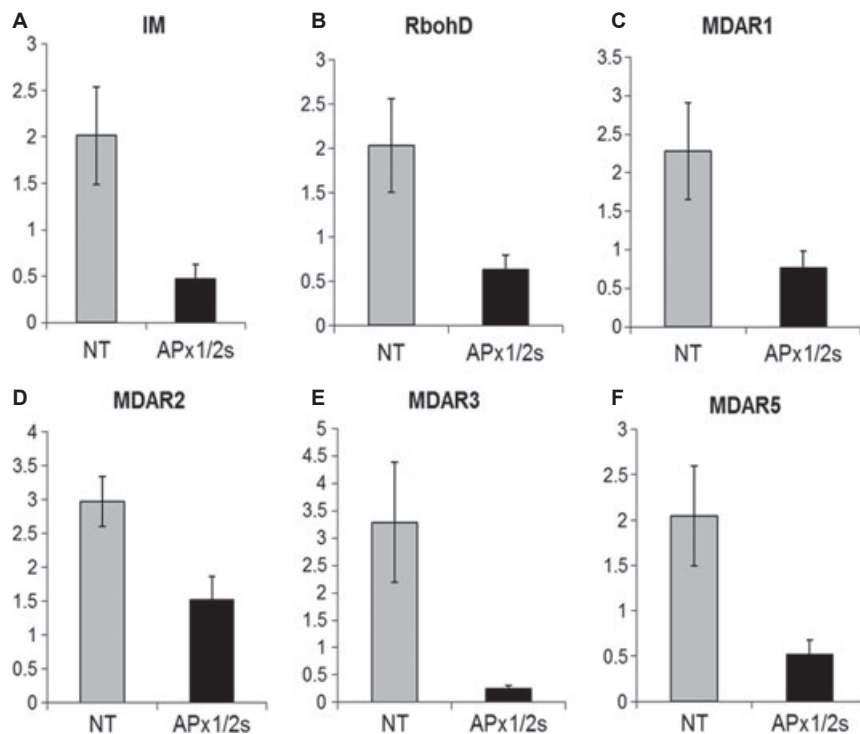

Fig. 4. Expression of the genes related to ROS. Relative expression levels were analyzed by quantitative real time PCR using leaves from NT and knockdown plants. The graphs shows the expression of (a) IMMUTANS [TIGR:LOC_Os04g57320]; (b) NADPHoxi (RbohD) [TIGR:LOC_Os11g33120]; (c) Monodehydroascorbate Reductase 1 [TIGR:LOC_Os09g39380; (d) Monodehydroascorbate Reductase 2 [TIGR:LOC_Os08g44340]; (e) Monodehydroascorbate Reductase 3 [TIGR:LOC_Os02g47800]; and (f) Monodehydroascorbate Reductase 5 [TIGR:LOC_Os08g05570].

(Achard *et al.* 2008). The silencing of cytosolic APX isoforms in rice plants caused changes in expression of the RbohD gene, which disrupts ROS homeostasis. It was also suggested that Rboh proteins function as positive amplifiers by enhancing the ROS signal generated during stress and maintaining this signal in an active state for a longer period of time (Mittler *et al.* 2004).

The monodehydroascorbate reductase family was analysed in the cytosolic APX-knockdown plants. *OsMDAR4* gene expression was not detected in our RT-qPCR experiments. The other members of this family were also analysed. In APx1/2s plants, all MDAR members were repressed. As a result, we observed a decrease in the hydrogen peroxide detoxification pathway in APx1/2s-knockdown plants.

Differentially expressed genes associated with other functions

Others genes classified in different molecular function groups were also modulated. Some of these genes should be highlighted, such as the terpene synthases, which are key enzymes responsible for biosynthesis of the complex chemical defence arsenal (i.e. terpenes) of plants and microorganisms (Greenhagen *et al.* 2006; Kampranis *et al.* 2007). Another example is malate dehydrogenase (MDH), which catalyses the interconversion of malate to oxaloacetate (Chapman *et al.* 1999), coupled to reduction or oxidation of the NAD pool. This enzyme participates in the citric acid cycle and gluconeogenesis (Tomaz *et al.* 2010) and has a crucial role in plant metabolism. MDH induction in response to stress (Becker *et al.* 2006; Bohler *et al.* 2007) and herbicides (Ahsan *et al.* 2008) has been shown in different plants species. Another enzyme involved in carbohydrate metabolism, 6-phosphogluconate dehydrogenase (6PGD), which promotes the decarboxylating reduction of 6-phosphogluconate into ribulose 5-phosphate in the presence of NADP (Broedel & Wolf 1990), was also found to be induced in the microarray analysis. The induction of NADP-MDH and 6PGD suggests that glucose catabolism could partly be due to required energy and reducing power for repair of damage caused by oxidative molecules. A carbohydrate transporter identified in the microarray analysis had reduced expression in comparison with NT plants, indicating that the carbohydrate metabolic process was affected in APx1/2s knockdown plants.

Four distinct transcription factors that are related to stress and plant development had increased expression in the APx1/2s plants: Zn finger A20/AN1, no apical meristem (NAM) protein, basic helix-loop-helix (bHLH) and a GRAS family transcription factor. The fact that genes encoding transcription factors were induced by hydrogen peroxide accumulation in APx1/2s knockdown plants might suggest that these transcription factors mediate further downstream hydrogen peroxide responses and that several other genes are likely to be induced at later times. Indeed, transcription factors have been reported to be rapidly induced during defence responses (Rushton & Somssich 1998; Durrant *et al.* 2000).

Taken together, our data demonstrate that reduced expression of cytosolic APX genes in rice can modulate expression of a specific subset of genes. The increased hydrogen peroxide accumulation in the knockdown plants altered the pattern of cellular proteins. The putative functions and changes in abundance of the 58 genes and 24 proteins identified in this work provide an overview of the complex gene network involved in oxidative stress responses. The imbalance in redox homeostasis affected essential processes of plant metabolism. In our model, some components of the photosynthetic process were affected; the plant modulated metabolism to adapt to this new condition by activating alternative pathways, such as photorespiration and glucose metabolism. Transcription factors involved in development were also modulated. Genes involved in the antioxidant defence metabolism were induced to compensate for the reduction of cytosolic APX and to promote a decrease in the amount of hydrogen peroxide accumulated. In conclusion, our findings demonstrate that the rice genome has a high degree of plasticity and responds to silencing of cytosolic APX isoforms through a very efficient compensatory mechanism.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Response of OsAPx1/2s and NT plants to UV light.

Table S1. List of primers used in RT-qPCR analysis.

Table S2. Global gene expression modulation in response to cytosolic OsApx isoform silencing.

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