

Genome-Wide Dissection of *Arabidopsis* and Rice for the Identification and Expression Analysis of Glutathione Peroxidases Reveals Their Stress-Specific and Overlapping Response Patterns

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Abstract Excessive generation of reactive oxygen species (ROS) due to environmental stresses critically effects plant development and productivity. Plants efficiently detoxify ROS by both non-enzymatic and enzymatic mechanisms. Plant glutathione peroxidases (GPXs) are non-haeme thiol peroxidases that catalyze the reduction of H₂O₂ (or organic hydroperoxides) to water or the respective alcohols using reduced glutathione or thioredoxin. Genome-wide analysis of the known GPXs from rice and *Arabidopsis* genomes revealed their gene structure, conserved motifs, localization and tissue-specific and/or organ-specific expression profiles in response to various abiotic stresses. Among the eight genes that encoded GPX proteins from *Arabidopsis*, *AtGPX3* showed two alternate spliced forms that spread over four chromosomes. Five genes encoded for rice GPX proteins, while *OsGPX1* showed three spliced variants that were distributed on five chromosomes. Utilizing the publicly available microarray and massively parallel signature sequencing (MPSS) data, the GPXs revealed stress-responsive, tissue-specific and/or organ-specific expression profiles. Presence of important cis-regulatory elements analyzed in the *GPX* promoter sequences revealed their overlapping or specific responsiveness to different abiotic stresses. Co-expression data of *Arabidopsis* *GPX* genes suggested that various protein kinase family members

and stress-responsive proteins co-expressed with the GPX proteins. Transcript profile of rice *GPX* genes by qRT-PCR validated their functional roles in signal transduction and stress pathways. Results revealed that plant GPXs play a crucial role in response to stress and significantly contribute towards their growth and development.

Keywords Peroxidase · Chromosomal synteny · Massively parallel signature sequencing · Microarray · Real-time PCR

Introduction

Environmental stresses drastically effect plant development and productivity by alteration of cellular redox homeostasis leading to excessive generation of reactive oxygen species (ROS). These ROS comprise toxic cellular metabolites like hydrogen peroxide, superoxide and hydroxyl radicals that are detrimental to biological molecules including nucleic acids, proteins and lipids. Plants have evolved efficient detoxification mechanisms both non-enzymatic and enzymatic that defend them against oxidative injury or ROS. The non-enzymatic composes of antioxidant compounds such as glutathione, ascorbate, carotenoids and tocopherols, and the enzymatic systems include superoxide dismutases, catalases, peroxidases and peroxiredoxins or thioredoxins.

Plant peroxidases (Prxs) are categorized among four known subgroups, namely 2-Cys Prx, 1-Cys Prx, Prx Q and type II Prx depending on the number and position of the conserved cysteine (Cys) residue and mechanism for catalytic peroxidase reduction (Rouhier et al. 2002). Essentially, the class 2-Cys Prx reduces the sulfenic acid (end product of

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peroxidase reduction) via synthesis of an intermolecular disulphide bridge with the resolving Cys present on the other subunit. However, the resolving Cys is situated within the same subunit forming an intramolecular disulphide bridge in Prx Q class (Rouhier et al. 2002). In other two classes, the sulfenic acid is reduced without the disulphide bond due to absence of a stringently conserved Cys in their active site. The glutaredoxin or thioredoxin systems reduce the type II Prxs, whereas the 1-Cys Prxs are reduced only by the thioredoxin system (Pedrajas et al. 2000; Finkemeier et al. 2005).

Glutathione peroxidase (GPX; EC 1.11.1.9) enzyme family comprises phylogenetically related non-haeme thiol peroxidases that catalyze the reduction of H₂O₂ (or organic hydroperoxides) to water or the respective alcohols using reduced glutathione. Numerous GPXs characterized from various kingdoms of life revealed their importance as ROS scavengers with broad substrate specificities and high affinity to H₂O₂ (Brigelius-Flohé and Flohé 2003). Majority of the animal GPXs including mammals except rats and mice (Kryukov et al. 2003) are selenocysteine GPXs (SecGPXs), whereas plants possess cysteine GPXs (CysGPXs). The catalytic site in SecGPXs was initially deciphered as a triad that comprised selenocysteine (Sec), Gln and Trp, which was eventually found to be a tetrad with an additional Asn (Tosatto 2008). In GPX family members, this tetrad is conserved with three exceptions: the Gln being replaced by Ser in mammalian GPX8 (Toppo et al. 2008) and by Glu or Gly in two GPXs from plants (Toppo et al. 2009). Catalytically active site Cys in plant CysGPXs and Sec in SecGPXs is situated in NVAx₂C (Sec) G motif at the N-terminus (Maiorino et al. 2007). An exceptional algal GPX from *Chlamydomonas reinhardtii* was reported to possess a Sec residue in its active site (Fu et al. 2002). Mammalian GPX isoforms are involved in cellular processes and physiological roles such as in spermatogenesis (Conrad et al. 2007), embryogenic development (Savaskan et al. 2007), preventing apoptosis and ROS signaling.

In plants and invertebrates, CysGPXs are GSH independent but may prefer redoxins or thioredoxins (TRX) (Herbette et al. 2007). Recently, it has been shown that in addition to plants, few yeast GPXs (Delaunay et al. 2002; Tanaka et al. 2005) may use TRX more efficiently than GSH as reductant or electron donor. Therefore, classification based on biochemical properties and not phylogeny reveals that plant GPXs or CysGPXs belong to a fifth group of Prxs (Rouhier and Jacquot 2005). Their deduced amino acid sequences possess high degree of similarity to mammalian GPX4 class of selenium-dependent phospholipid hydroperoxide GPXs. GPXs in plants or CysGPXs play a significant role in scavenging of phospholipid hydroperoxides thereby preventing peroxidation of cellular membranes (Gueta-Dahan et al. 1997). Numerous isoforms of GPX that have been identified in plants show divergence in their substrate specificity, subcellular localization and tissue-specific distribution (Jung et al. 2002). GPX transcript up-regulation has been reported in response to salinity

(Sreenivasulu et al. 2004), heavy metal toxicity (Milla et al. 2002), oxidative damage (Chen et al. 2004), hormone treatment, wounding and pathogen infection (Agrawal et al. 2002). Thus, plant GPXs perform a significant role in both abiotic and biotic stress adaptation pathways. Apart from direct scavenging of ROS, these GPX proteins may interact with other peroxide-induced proteins and thereby enhance ROS scavenging from the system. Recently, it has been proposed that GPXs could act as a signal transducer during stress conditions via activation of antioxidant genes' transcription (Miao et al. 2006). In lieu of their functional versatility in cell metabolism, an in-depth analysis of this family of proteins is crucial. Previously, *GPX* genes from *Arabidopsis* and rice have been identified and characterized (Milla et al. 2003; Chang et al. 2009). However, a genome-wide survey of GPXs and their expression profiles during abiotic stress in rice and *Arabidopsis* has not been undertaken at various developmental states. With the availability of complete genome sequences and expression data from rice and *Arabidopsis*, a genome-wide analysis of GPX gene family during abiotic stress and different developmental stages is feasible.

In the present study, we have performed in silico analysis of the known GPXs from rice and *Arabidopsis* genomes. The members were further analyzed in detail in terms of their gene structure, protein sequence and localization. Expression of GPX members was performed using publicly available microarray and massively parallel signature sequencing (MPSS) data. Further, the presence of important cis regulatory elements has been analyzed in the GPX promoter sequences. Gene expression profile of rice GPX genes was validated by qRT-PCR to highlight their functional roles in signal transduction and stress pathways.

Methodology

Screening GPX Proteins in *Arabidopsis* and Rice Genomes

Publicly available genome databases for *Arabidopsis* (TAIR9) and rice (TIGR7) were used to find the proteins with unique hidden Markov model (HMM) of GPx domain (PF00255) with an *e* value cutoff of 0.001. Co-expressed genes of *Arabidopsis* GPx were predicted by using ATTED-II (<http://atted.jp/>). The subcellular localization of *Arabidopsis* GPx proteins was retrieved from TAIR (<http://www.Arabidopsis.org/>) database. To enhance the accuracy of prediction the data were verified with experimentally proven results in SUBA database (<http://suba.plantenergy.uwa.edu.au/>). While CELLO v.2.5:sub-CELLular Localization predictor (<http://cello.life.nctu.edu.tw/>) (Yu et al. 2006) was used to identify the rice localization and results were cross-checked by using pSORT prediction software (<http://wolfsort.org/>) (Horton et al. 2007). ChloroP (<http://www.cbs.dtu.dk/services/ChloroP/>) was used to verify chloroplast localization

(Emanuelsson et al. 1999). The molecular weight, isoelectric point (PI) and number of amino acids of *Arabidopsis* and rice GPx proteins were retrieved from TAIR (<http://www.Arabidopsis.org/>) and TIGR (<http://rice.plantbiology.msu.edu/>) databases, respectively.

Chromosomal Synteny

Chromosomal map for *Arabidopsis* and rice GPX genes was constructed by using chromosome map tool available in TAIR (<http://Arabidopsis.org/jsp/ChromosomeMap/tool.jsp>) for *Arabidopsis* and *Oryza* base Integrated Science Database (<http://viewer.shigen.info/oryzavw/maptool/MapTool.do>) for rice.

Multiple Sequence Alignment and Phylogenetic Analysis

Multiple sequence alignment of GPX proteins was done using ClustalW programme with default parameters, and phylogenetic tree was generated according to neighbourhood joining method (Saitou and Nei 1987) and 1000 replicates were used for bootstrap analysis by using MEGA 5.2 software (Tamura et al. 2011).

Analysis of cis-Regulatory Elements in the Promoter Region

Upstream genomic sequences (~1 kb from transcription start) of *Arabidopsis* and rice GPX genes were retrieved from TAIR9 and TIGR database, respectively, followed by analysis using PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) (Lescot et al. 2002).

Motif Identification

Motifs present in the GPX protein families were identified by using the Multiple Expectation Maximization for Motif Elicitation (MEME) version 4.8.0 search tool (<http://meme.nbcr.net/>; Bailey et al. 2009) with default settings except that the maximum number of motifs to find was defined as 20 and the maximum width was set to 300.

Expression Analysis Using MPSS and Microarray Data

MPSS expression data of *Arabidopsis* and rice GPX genes were retrieved from MPSS project Web site (<http://mpss.udel.edu/#at>; <http://mpss.udel.edu/#rice>, respectively). The signature was considered to be significant if it uniquely identified an individual gene and showed perfect match (100 % identity over 100 % length of the tag). The expression data of *Arabidopsis* GPX genes were retrieved from the AtGenExpress (<http://jsp.weigelworld.org/expviz/expviz.jsp>) under various abiotic stresses as well as under different developmental stages as described previously (Mustafiz et al. 2011). The expression data of rice GPX genes were retrieved from Affymetrix

GeneChip Rice Genome Arrays (<http://www.ncbi.nlm.nih.gov/geo>; Gene Expression Omnibus platform accession numbers GSE6893 and GSE6901). The log₂-transformed values were used to generate heat maps, and hierarchical clustering was performed using MeV software (Eisen et al. 1998).

Expression Analysis by qRT-PCR

Rice seedlings (var. Nipponbare) were grown in growth chamber at 28±2 °C and 16 h photoperiod under normal conditions for 10 days and subjected to different stresses for the transcript analysis. Seedlings were transferred to salt solution (200 mM NaCl) for salinity stress and 10 mM H₂O₂ for oxidative stress. Seedlings were transferred to incubators at 4 and 42 °C for cold and heat stress, respectively. To mimic drought stress, seedlings were air-dried on a Whatman sheet. Seedlings germinated without any stress served as corresponding control sample. After exposure to 16 h of stress, the shoot and root tissues were collected, and RNA was isolated using TRIZOL reagent according to manufacturer's instructions (Invitrogen). The quality of the isolated RNA was assessed by gel electrophoresis, and first-strand cDNA was synthesized after DNase treatment according to manufacturer's instructions (Invitrogen). PCR primers were designed from the 3'UTR regions of genes by using Primer3 software (<http://simgene.com/Primer3>) and verified by BLAST tool of NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). qRT-PCR was conducted using the primers (Table 1) and calculated using eEF as endogenous control according to Mustafiz et al. (2011). The amplification specificity was confirmed by dissociation curve analysis and agarose gel electrophoresis. The experiment was performed in triplicate using three biological replicates for each sample. The relative expression quotient for each GPx gene was calculated using delta Ct or comparative Ct value method (Livak and Schmittgen 2001). The fold change was calculated by conversion of the relative expression value (2^{-ddCt}) into log₂ scale.

Table 1 Sequences of GPX gene primers for the real-time PCR analysis in rice

Primers' name	Sequences 5'–3'
02_OsGPX-forward	GCTTACTGCATCACTTTGCC
02_OsGPX-reverse	GCAGTCGCAGGTCTCAATAA
03_OsGPX-forward	ACTGGTTTTATATGGCTTGGCT
03_OsGPX-reverse	GTGTGCTCCCTTCTAACCAC
04_OsGPX-forward	CACAGTTGCATTGCACTA
04_OsGPX-reverse	CGAACATAACAGTGCACGC
06_OsGPX-forward	GCTTATCTGGCCCTGTAAC
06_OsGPX-reverse	CTAATGCTGCAACAGAAGTGC
11_OsGPX-forward	TGACCTACCAGGGTTCAGTT
11_OsGPX-reverse	GCTTGTGGCTAGTTGCCTAA

Heat maps for root and shoot samples were generated using MeV software package as described previously.

Results

Analysis of GPx Proteins in *Arabidopsis* and Rice

Screening based on HMM profile of GPX domain from the available protein databases revealed the presence of nine and seven GPX proteins in *Arabidopsis* and rice, respectively. Eight genes encoded the GPX proteins in *Arabidopsis*, while *AtGPX3* showed two alternate splice forms (Table 2). On the contrary, rice GPX proteins were encoded by five genes (Table 3), whereas *OsGPX1* showed three splice variants. The alternative spliced forms were named as the gene name followed by the Arabic number after dot “.”. GPX genes were distributed on different chromosomes of *Arabidopsis* and rice (Fig. 1). In case of *Arabidopsis*, the GPX genes were spread across four chromosomes. Amongst them, four genes were positioned on chromosome II followed by two genes located

on chromosome IV, while chromosomes III and I possessed one GPX gene each. On the other hand, rice GPX genes were scattered on chromosomes II, III, IV, VI and XI with one copy each (Fig. 1). A captivating pattern of chromosomal distribution and their evolutionary patterns were obtained from the rooted phylogenetic tree formed using GPX proteins from rice and *Arabidopsis*. Based on this phylogenetic analysis, four distinct clades were observed among the rice and *Arabidopsis* GPXs (Fig. 2a–d). Even though *OsGPX1* and *OsGPX3* shared high level of sequence similarity with *AtGPX6*, however, all these genes were distributed randomly in different chromosomes. Similarly, *OsGPX4* formed a clade with *AtGPX1* and *AtGPX7*; *OsGPX2* with *AtGPX4* and *AtGPX5*; and *OsGPX5* with *AtGPX2*, *AtGPX3* and *AtGPX8*. Interestingly, all these groups or clades were positioned in the same chromosome. The differential chromosomal distribution and sequence similarity pattern suggested that plant GPXs were evolutionary more divergent with different defensive roles against various stresses.

In *Arabidopsis*, GPX proteins were mainly localized in cytosol, according to TAIR and SUBA databases. A variation was observed in case of subcellular localization prediction by

Table 2 List of glutathione peroxidase (GPX) genes in *Arabidopsis* along with their chromosomal locations, alternative spliced forms, CDS and polypeptide length, localization and homologs

Gene	Protein	Chr. no	Locus	CDS (bp)	CDS coordinate (5' to 3')	Amino acids	Mass (kDa)	pI	Location	Co-expressed genes
<i>AtGPX1</i>	AtGPX1	2	AT2G25080.1	711	10668049–10670019	236	26.01	90.94	Chl	Unknown protein (At1g21065), SNG1 (At2g22990), glutaredoxin family protein (At5g18600).
<i>AtGPX2</i>	AtGPX2	2	AT2G31570.1	510	13437974–13439881	169	18.94	5.33	Cyt, Mit, Pm	RD21 (At1g47128), hydrophobic protein, low temperature and salt responsive protein (At2g24040), 3-dehydroquinase synthase (At5g66120), ATGSTU19 (At1g78380).
<i>AtGPX3</i>	AtGPX3.1	2	AT2G43350.1	621	18008642–18010600	206	23.25	9.59	Go, Cyt, Mit	Peptidase family (At3g61540), dihydropyrimidinase (At5g12200), glutathione peroxidase (At1g63460), CYP721A1 (At1g75130), NAD or NADH binding (At3g07690).
	AtGPX3.2		AT2G43350.2	621	18008482–18010606	206	23.25	9.59	Cyt	
<i>AtGPX4</i>	AtGPX4	2	AT2G48150.1	513	19687962–19689173	170	19.30	9.04	Cyt, Pm	SOLUBLE N-ETHYLMALDEIMIDE-SENSITIVE FACTOR ADAPTOR PROTEIN 30, SNAP receptor (At1g13890), protein kinase family protein (At4g34440).
<i>AtGPX5</i>	AtGPX5	3	AT3G63080.1	522	23309737–23311386	173	19.32	9.68	ER, Pm	Vesicle - associated protein 711 (At4g32150), mitogen-activated protein kinase, MAP kinase (At1g59580), avirulence-responsive protein-related (At3g28950), enzyme inhibitor (At3g47670), leucine-rich repeat family protein (At5g07910).
<i>AtGPX6</i>	AtGPX6	4	AT4G11600.1	699	7009769–7011375	232	25.58	9.85	Pm, Chl, Cyt, Mit	Zinc finger protein (At2g47890), UBX protein (At3g23605), pyruvate decarboxylase-2 (At5g54960), glucose-6-phosphate dehydrogenase 5 (At3g27300).
<i>AtGPX7</i>	AtGPX7	4	AT4G31870.1	702	15410205–15411617	233	25.58	10.07	Chl	LEA3 protein (At1g02820), β -carotene hydroxylase 2 (At5g52570), thioesterase family protein (At3g16175).
<i>AtGPX8</i>	AtGPX8	1	AT1G63460.1	504	23535016–23536467	167	19.06	4.85	Cyt, Nuc	3- β hydroxysteroid dehydrogenase protein (At2g33630), ATGPX3 (At2g43350), universal stress protein (At1g68300), Phosphofructokinase 5 (At2g22480).

Pp periplasm, *Cy* cytosol, *Ch* chloroplast, *Nu* nucleus, *Mi* mitochondria, *ER* endoplasmic reticulum, *bp* base pair, *aa* amino acid

Table 3 List of glutathione peroxidase (GPX) genes in rice along with their chromosomal locations, alternative spliced forms, CDS and polypeptide length, localization and homologs

Gene	Protein	Chro no	Locus	CDS (bp)	5'-3'CDS coordinate	Polypeptide length (aa)	Mol. Mass (kDa)	pI	localization	Orthologous genes
<i>OsGPX1</i>	OsGPX1	2	LOC_Os02g44500.1	717	26929715–26932403	239	25.83	9.88	Ppa, Chb	AT4G11600, Bradi3g51010, Bradi5g18000, GRMZM2G135893, GSVIVG00019766001, POPTR_001s09270, POPTR_003s12620, Sb04g032520, Sb06g024920
<i>OsGPX2</i>	OsGPX2	3	LOC_Os03g24380.1	510	13879054–13882526	170	19.23	8.87	Cya, b	AT2G48150, AT3G63080, Bradi1g61930, GRMZM2G329144, POPTR_0014s13490, Sb01g034870
<i>OsGPX3</i>	OsGPX3.1	4	LOC_Os04g46960.1	594	27838693–27840786	198	21.66	7.83	Oma, Chb	AT4G11600, Bradi3g51010, Bradi5g18000,
	OsGPX3.2	4	LOC_Os04g46960.2	507		169	18.48	8.42	Ppa, Oma, Chb	GRMZM2G135893, GSVIVT00019766001, POPTR_0001s09270, POPTR_0003s12620
	OsGPX3.3	4	LOC_Os04g46960.3	504		168	18.35	8.01	Ppa, Oma, Chb	Sb04g032520, Sb06g024920
	OsGPX3.4	4	LOC_Os04g46960.4	258		86	9.24	11.18	Ppa, Nub	
<i>OsGPX4</i>	OsGPX4	6	LOC_Os06g08670.1	705	4321607–4325283	235	25.07	10.00	Ppa, Chb	AT2G25080, AT4G31870, Bradi1g47140, GRMZM2G012479, GSVIVT00035981001, POPTR_0006s28120, Sb10g005820
<i>OsGPX5</i>	OsGPX5	11	LOC_Os11g18170.1	639	10232934–10230265	233	22.93	8.01	Ppa, Chb	AT2G31570, POPTR_0007s02160, Sb0010s007790

Pp periplasm, *Cy* cytosol, *Ch* chloroplast, *Nu* nucleus, *Om* outer membrane, *a* CELLO, *b* WoLF PSORT, *bp* base pair, *aa* amino acid

different tools. Some of the proteins were localized in chloroplast (AtGPX1, AtGPX4), while others were localized in mitochondria (AtGPX2, AtGPX3 and AtGPX6), plasma membrane (AtGPX2, AtGPX5 and AtGPX6), golgi bodies (AtGPX3) and nucleus (AtGPX8) (Table 2). Similarly, rice GPX proteins were localized in mitochondria, chloroplast and cytosol (Table 3). Co-expression data of *Arabidopsis* GPX genes indicated that different members of the protein kinase

family and various stress-responsive proteins co-expressed with the GPX proteins (Table 2).

Phylogenetic Analysis of GPX Proteins

To reveal the evolutionary relationship among the GPX proteins, multiple sequence alignment of all the GPX protein was done (Fig. 3), and a rooted phylogenetic tree was generated

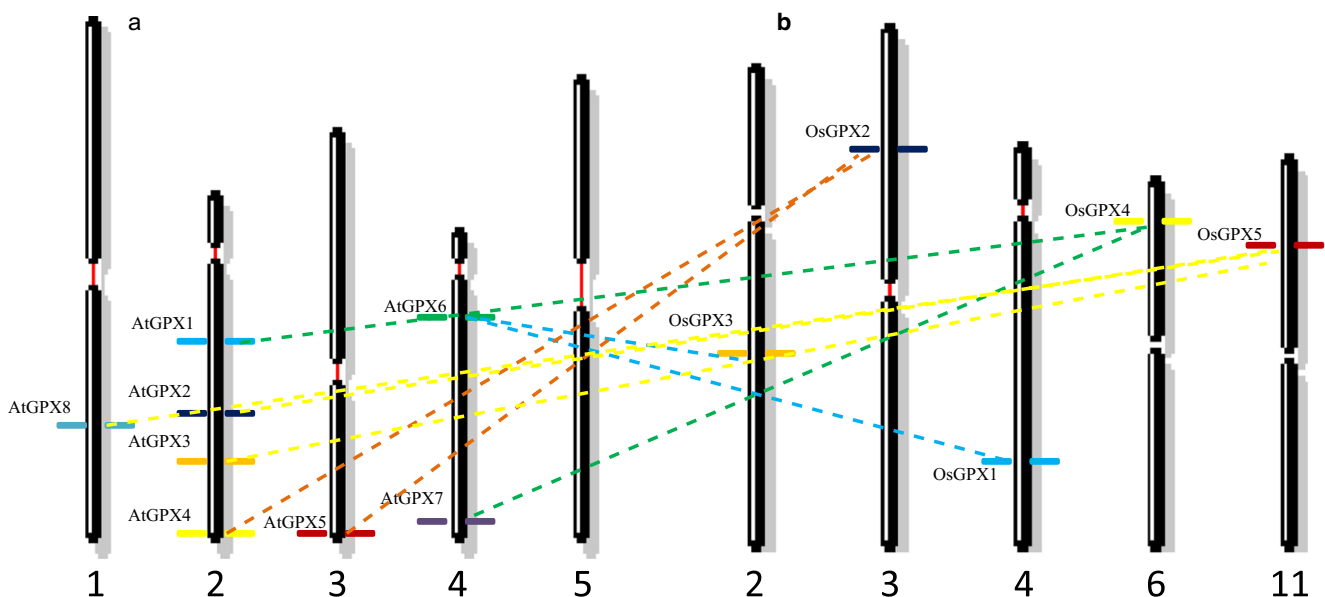


Fig. 1 Genome organization of *Arabidopsis* and rice GPX genes on four chromosomes of *Arabidopsis* and five chromosomes of rice

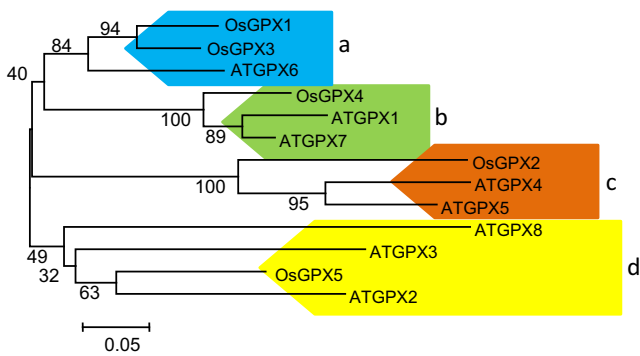


Fig. 2 A rooted phylogenetic tree of all the *Arabidopsis* and rice GPX proteins was generated to depict evolutionary convergence

using their full-length protein sequences from rice and *Arabidopsis* along with other plants, yeast and mammalian by MEGA version 5.2 (Tamura et al. 2011) with a bootstrapping of 1000 replicates (Fig. 4). Two distinct clades were observed in

the phylogenetic tree that comprised first higher plants and second lower plants and mammals. Interestingly, the mammalian and higher plant GPXs fell in to the higher eukaryotic clade, whereas yeast, fungi, bacteria and diatomic GPXs were grouped into the lower eukaryotic clade. Result indicated that the different evolutionary trends of GPXs for plants and animals may have emerged due to the selenium-dependent and -independent nature of their catalytically active sites.

Motif Identification of GPX Proteins

The MEME motif search was utilized to identify the motifs shared among the GPX family of rice and *Arabidopsis* (Bailey and Elkan 1995). Apart from the presence of other motifs, seven motifs were found to be widespread in *Arabidopsis* and rice GPXs. The identified motifs and their sequences are shown in Table 4. Amongst the seven common motifs, five

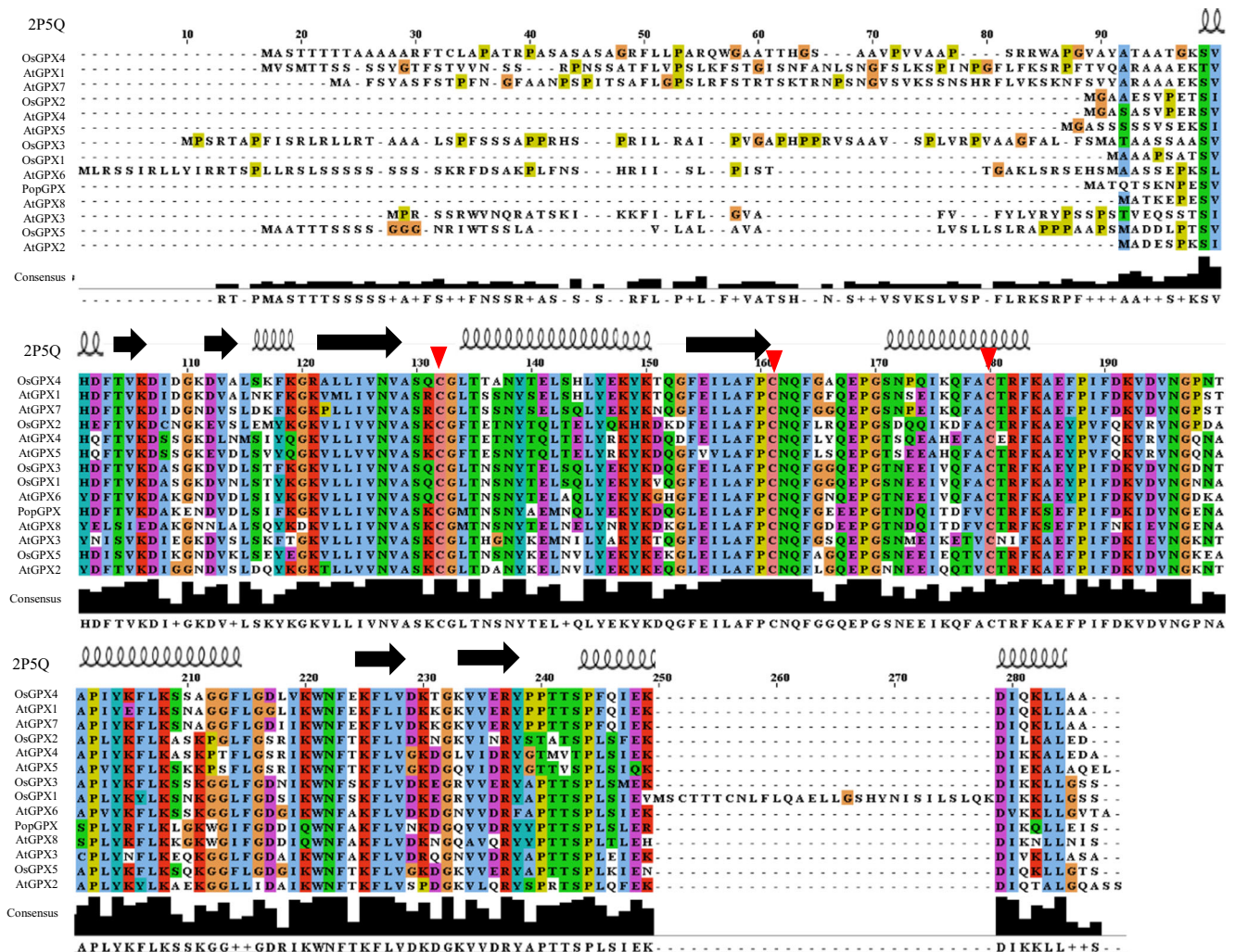
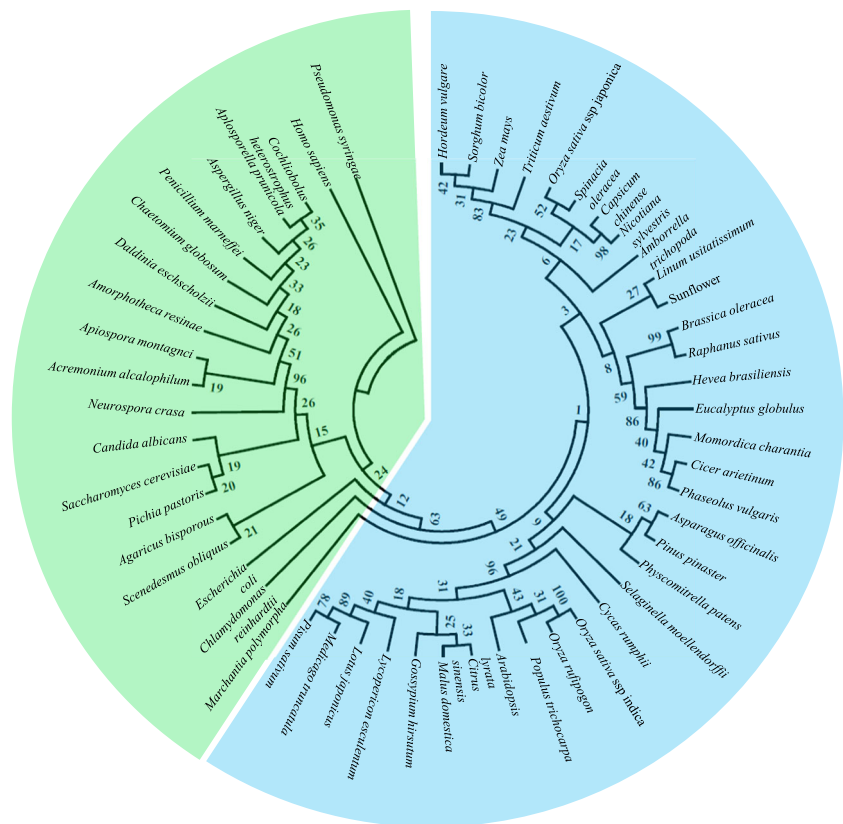


Fig. 3 Multiple sequence alignment of deduced amino acid sequences of various GPXs from rice, *Arabidopsis* and the available secondary structure from *Populus trichocarpa* GPX-5 (PDB code 2P5Q). Secondary structure comprised α helices and β strands represented as *black coils*

and *black arrows*, respectively. The three conserved cysteine residues that play important role in the enzyme activity have been marked by *red triangle*

Fig. 4 Phylogenetic analysis of *GPX* genes of rice and *Arabidopsis* with other reported *GPX* genes from diverse genera. Construction of the tree was based on neighbour-joining method of Mega software using 1000 bootstrap replicates



were prevalent among GPX proteins (e.g. motifs 1–5). Interestingly, certain motifs were specific to rice or *Arabidopsis*. Different patterns of motifs in the protein sequences may have an implication on gene efficacy in different plants. In addition, exceedingly comparable motif dispersion in the proteins also highlighted their evolutionary proximity.

In Silico Analysis of Promoters of *Arabidopsis* and Rice GPX Genes








Differential expression of the GPX transcripts was observed in response to different stresses that intrigued us to analyze their promoters. To reveal the correlation of transcriptional regulation amongst the GPX genes from *Arabidopsis* and rice, we have retrieved the 1 kb upstream regions or putative promoter sequences and analyzed them using PlantCARE. A number of important cis-regulatory elements and stress-responsive motifs were identified (Fig. 5). A few known stress-related cis-regulatory elements (CREs) for instance MYB-binding site (MBS), ABA-response element (ABRE), anoxia-response element (ARE), heat shock element (HSE), salicylic acid responsive element (SARE), fungal elicitor responsive element (Box-W1), wound-responsive element (WUN-motif), defense and stress-responsive elements (TC-rich repeats) were present in the promoters of GPX genes. Presence of these stress-related motifs in the promoters of GPXs showed direct correlation to the altered gene expression under stress.

Expression Profiling of GPX Genes from *Arabidopsis* and Rice by MPSS Analysis

GPX expression was analyzed using publicly available MPSS data of *Arabidopsis* (<http://mpss.udel.edu/at>) and rice (<http://mpss.udel.edu/rice>) under various developmental stages. Elaborate analysis of *Arabidopsis* GPXs expression profile (Fig. 6a) revealed that both *AtGPX1* and *AtGPX6* showed high level of constitutive expression in all the tissues tested. Precisely, *AtGPX1* showed significant level of expression in silique, root and inflorescence stage, which suggested its involvement in major developmental stages. Similarly, when we checked the expression of *AtGPX6*, it showed predominant expression as *AtGPX1* in similar developmental stages securing its role in developmental pathways, whereas expression of *AtGPX5* and *AtGPX2* was also found to be constitutive with some exceptions as in seed (GSE) and silique (CAS) tissues. However, *AtGPX2* had dynamically higher expression in callus, inflorescence and leaves signifying its potential role in plant development. On the contrary, *AtGPX3*, *AtGPX7* and *AtGPX8* were expressed at low levels in most of the stages, with moderate expression in some specific stages/tissues that indicated their stage-specific role. Expression of *AtGPX4* gene was not analyzed due to the lack of specific probe against it (Fig. 6a).

Similarly, analysis of rice MPSS data (Fig. 6b) revealed that *OsGPX1* and *OsGPX2* transcripts were the most abundant forms of GPXs in all the analyzed tissues. When

Table 4 Conserved motif sequences in the deduced amino acid sequences of GPXs from *Arabidopsis* and rice

Motif	Width	E-value	Multilevel Consensus Sequences
1	30	3.5e-263	
2	30	1.2e-224	
3	30	1.3e-220	
4	30	2.1e-198	
5	28	9.4e-177	
6	8	9.1e-020	
7	6	6.4e-007	

we analyzed the expression pattern, it was observed that *OsGPX1* and *OsGPX2* constitutively expressed in all the stages of development except during seed development, their expression levels turn quite interesting. At one specific stage in seed development, expression of both isoforms remained absent, whilst at other times, their expression was low. A similar pattern of high transcript abundance was observed in case of *OsGPX3*, *OsGPX4* and *OsGPX5* with some exceptions where the expression became very low. Expression level of *OsGPX3* is quite similar with *OSGPX1* and *OsGPX2*. This may support the observation of their close homologous similarity. On the other hand, *OsGPX4* and *OsGPX5* showed their predominant expression in all the stages of root development. Interestingly, low level of transcript abundance for all the rice GPXs was observed in various stages of seed development. This data denoted the key information regarding the function of GPX genes during the developmental stages of plant.

Microarray-Based Expression Analysis of *Arabidopsis* GPX Genes

Expressions of *Arabidopsis* GPXs were further validated by utilization of the available microarray data (Fig. 7) that ratified the findings from the MPSS data. It was observed that three of the *AtGPXs*, *AtGPX1*, *AtGPX2* and *AtGPX6*, exhibited high levels of transcript expression at all the developmental stages including roots, hypocotyls, cotyledons, leaves, flowering stages and seed developmental stages. Interestingly, *AtGPX1* and *AtGPX2* were not expressed in mature pollen stage. However, *AtGPX3*, *AtGPX5*, *AtGPX7* and *AtGPX8* transcript expression ranged from low to medium in all stages of development. *AtGPX4* was expressed negligibly in all the developmental stages, except in stamens and pollen, which highlighted its significant role in plant reproductive organ development.

The role of GPXs in the various developmental stages led us to investigate their expression patterns during stress. *Arabidopsis* GPX genes were expressed in both root and shoot

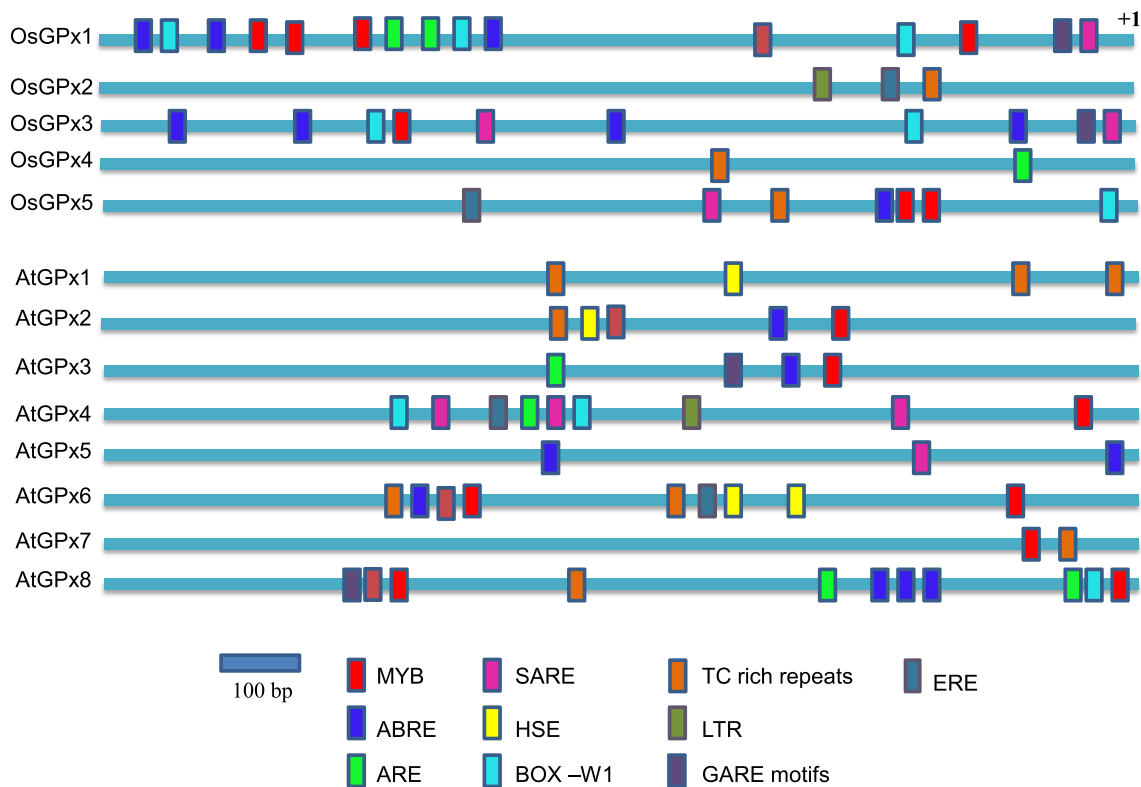


Fig. 5 Putative cis-regulatory elements identified in the promoter regions of *GPX* genes from *Arabidopsis* and rice. The approximate positions of putative cis-regulatory elements were predicted in the 1 kb upstream regions of the *GPX* genes by PlantCARE database

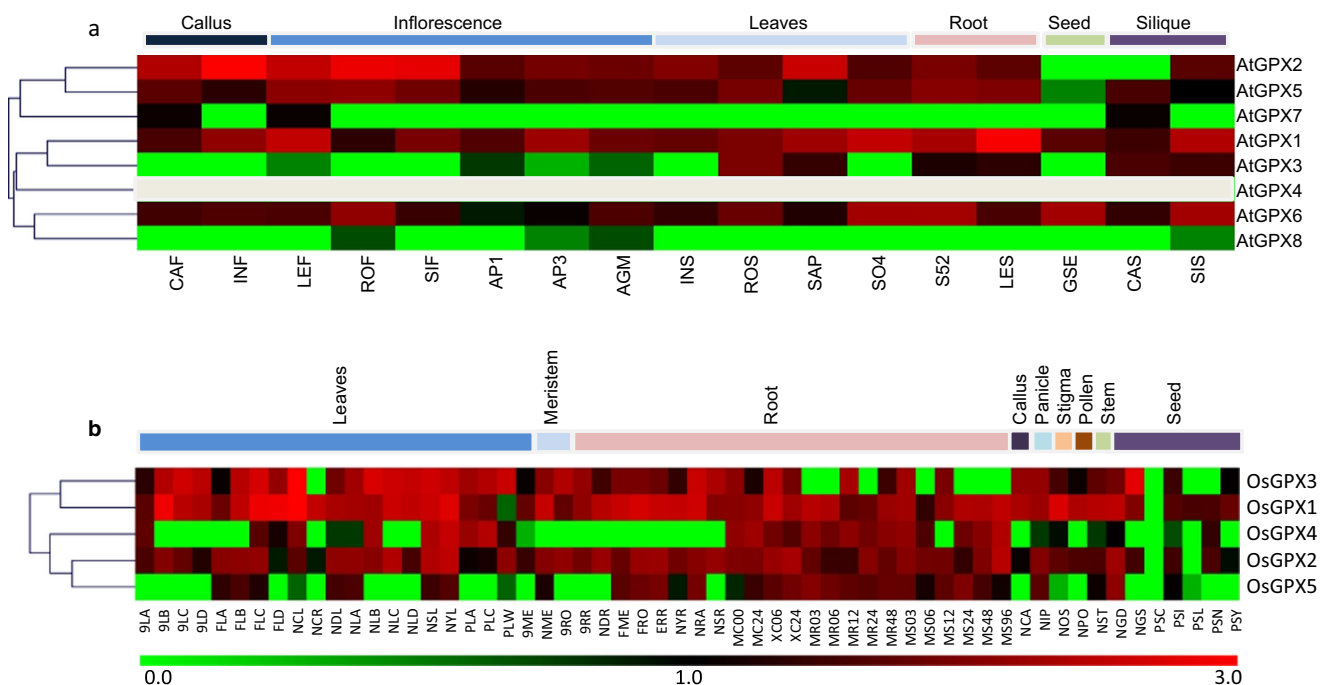
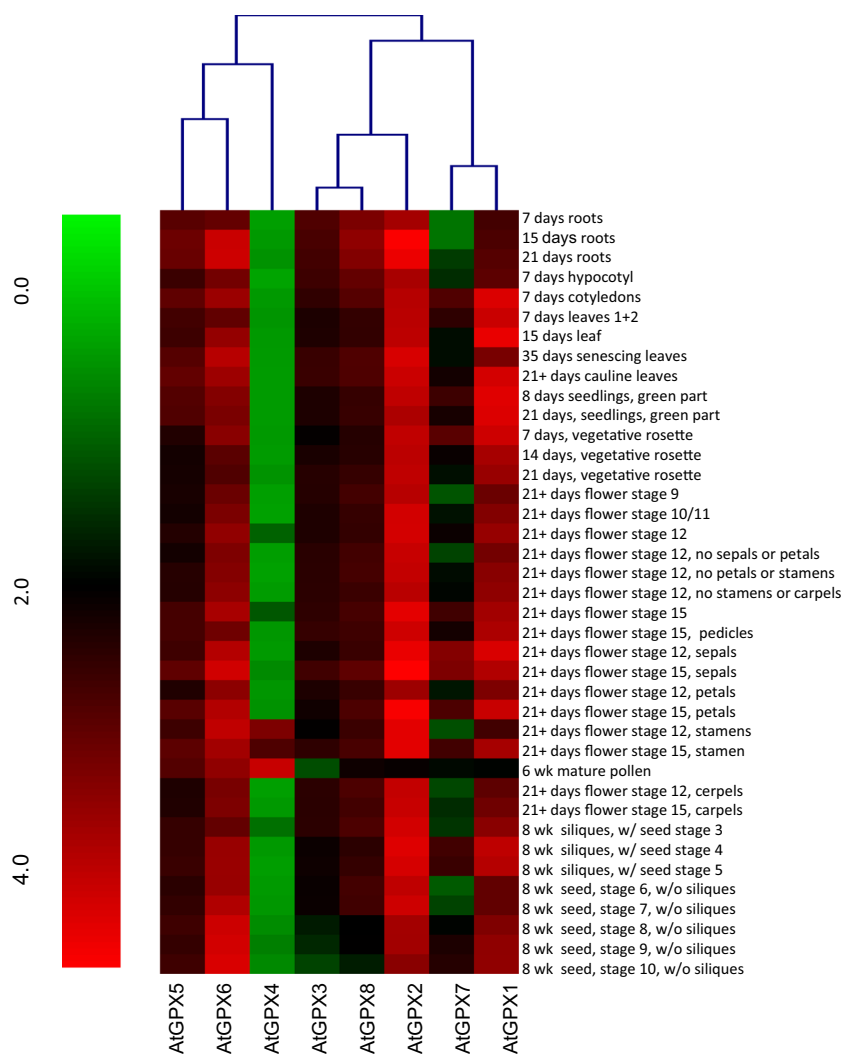


Fig. 6 Expression analysis of *Arabidopsis thaliana* (a) and *Oryza sativa* (b) *GPXs* from MPSS data. The name of the various MPSS libraries has been labelled below each heat map according to their expression in different tissues or organs. Description of these MPSS libraries may be availed from the Web site mentioned in the “Methodology” section. Heat

maps represented hierarchical clustering of average log signal values of *GPX* genes in various tissues/organs that were generated by TIGR MeV software package. The colour bar below represents relative expression values, wherein green represents lowest, black represents medium and red signifies highest expression levels

Fig. 7 Expression profiling of *GPX* genes of *Arabidopsis* at different developmental stages using microarray data. Heat map and hierarchical cluster display differential expression profile for *GPX* genes. Developmental stages used for expression profiling are mentioned on the right side. Various stages are listed in the temporal order of development. The colour bar below represents relative expression values, wherein green represents lowest, black represents medium and red signifies highest expression levels



tissues under different abiotic stresses. A divergent pattern of expression was observed depending on the type and period of stress exposure (Fig. 8a, b). *AtGPX* genes were maximally expressed in both shoot and root tissues in response to various stresses. It was noted that *AtGPX6* was highly up-regulated in response to cold, osmotic stress, salt, oxidative stress and UV-B exposure, in both shoot and root at an early time point and expressed for 24 h. High expression of *AtGPX7* in response to cold, UV-B, wounding and heat was an early response to stress that continued up to 24 h of exposure, whereas its expression sharply down-regulated at different time points in response to drought and genotoxic stresses in shoot and root tissues. Expression of *AtGPX1* in shoots was induced during prolonged stress (6 to 12 h) in response to cold, but moderately induced in response to wounding and heat. However, its expression in the roots was induced in response to all stresses such as at 6 h of oxidative and osmotic stresses, whereas an early response was observed in case of heat and wounding stresses. However, once expressed, the expression continued up to 24 h, in case of all stresses. Taken together, expression

data indicated that expression of *GPX* genes was correlated to incidence of various stresses, which in turn was responsible for meeting the physiological demands.

Microarray-Based Expression Analysis of Rice *GPX* Genes

Likewise, expression of rice *GPX* genes was analyzed using publicly available microarray data from gene investigator. The retrieved data was analyzed in varied developmental stages (Fig. 9a), tissues (Fig. 9b) and abiotic stress conditions (Fig. 9c). *OsGPX4* and *OsGPX1* showed high levels of expression at all the developmental stages (Fig. 9a). This indicated their imperative role in the plant growth and development. Further expression of genes was analyzed in various tissues that revealed their tissue-specific functions. Two rice *GPX*s, *OsGPX1* and *OsGPX2*, showed very high transcript abundance in all the analyzed tissues (Fig. 9b). On the other hand, *OsGPX3*, *OsGPX4* and *OsGPX5* exhibited low to moderate expression in all tissues. As *GPX*s were directly involved in the stress-induced ROS scavenging, their expression was

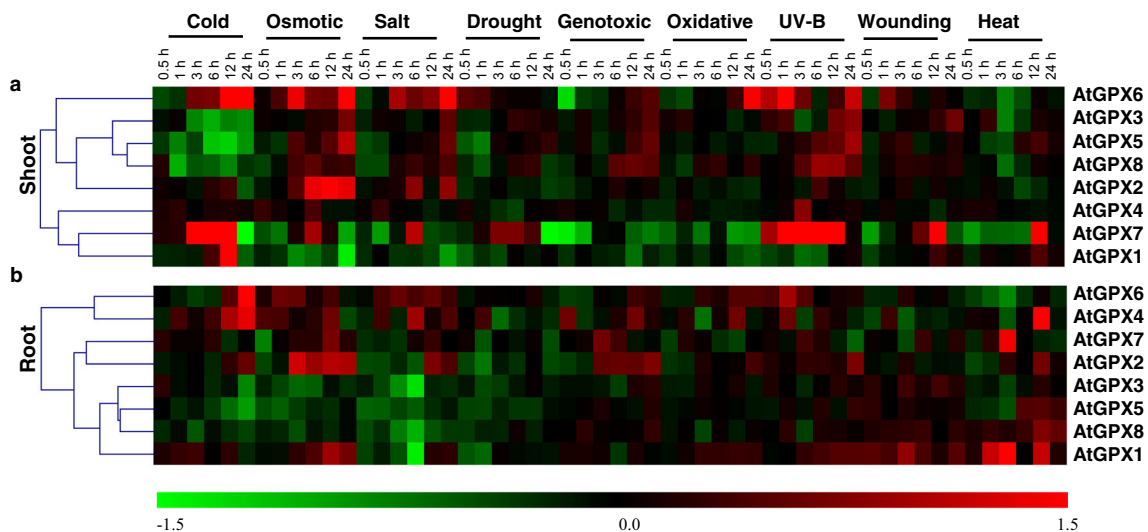


Fig. 8 Expression analysis of *Arabidopsis* GPX genes in response to stress conditions in shoot (a) and root (b) tissues. Microarray expression data for *AtGPX* genes was retrieved from TAIR (ver 9) during various abiotic stresses, i.e. salt, drought, osmotic, cold, heat, oxidative, genotoxic, wounding and UV/B stress. The datasets obtained for various time points of stress, namely 0.5, 1, 3, 6, 12 and 24 h, were analyzed with

respect to the control. The colour bar below represents relative expression values, wherein green represents lowest, black represents medium and red signifies highest expression levels. The hierarchical clustering is performed, and heat maps have been generated using TIGR MeV software package

analyzed in response to varied abiotic stresses (Fig. 9c). An up-regulation in expression of *OsGPX4* was observed in response to heat, while an up-regulation in expression of *OsGPX2* and *OsGPX3* was observed in response to salinity and drought. Further, *OsGPX5* expression was down-regulated during all the stresses. Overall data confirmed the differential expression of GPX transcripts at various developmental stages, tissue and environmental stimuli.

qRT-PCR to Validate the Expression Data

Expression profiles of rice GPX genes were validated in response to salinity, oxidative, heat, cold and drought stresses (Fig. 10) in both root and shoot tissues. Expression profiles of GPX family members as determined by real-time PCR positively validated the microarray and MPSS data. Expression analysis data revealed that all the *OsGPX* genes, except for *OsGPX5*, highly up-regulated in response to oxidative stress in case of shoot tissues. However, in root tissues, *OsGPX5* showed up-regulation during all stresses, except in response to cold stress wherein it showed down-regulation. Incidentally, *OsGPX5* showed down-regulation in response to all the analyzed stresses in shoot tissues as monitored by qRT-PCR and microarray data. This suggested that *OsGPX5* might be confined to root-specific stress tolerance or responsiveness. Among all the genes, *OsGPX1* and *OsGPX3* consistently up-regulated in both shoot and root tissues in response to all stress treatments, precisely in oxidative stress, heat and

oxidative stresses in root tissues. Moreover, in shoot tissues, *OsGPX2* and *OsGPX4* showed similar patterns of up-regulation in response to drought and oxidative stress, whereas down-regulation in response to the salinity, heat and cold. In case of root tissues, *OsGPX2* and *OsGPX4* showed up-regulation during all stresses except cold stress, suggesting their crucial role in imparting stress tolerance to rice plants. From the above results, we inferred that *OsGPX1*, *OsGPX2* and *OsGPX3* might be the most crucial set of genes for execution of oxidative stress response in rice, since they were up-regulated by oxidative stress in both shoots and roots. Similarly, *OsGPX4* may also play an important role in drought and salt response in rice. The functional validation of the role of *OsGPXs* in conferring stress tolerance can be further studied using transgenic approach.

Discussion

Generation of ROS is the common response of plants exposed to any unfavourable environmental stimuli (Desikan et al. 2005). Plants have evolved a cellular machinery to neutralize excess ROS. Among them, GPXs are considered to be the most potential ROS scavengers due to their broad substrate specificities and strong affinity for H_2O_2 (Brigelius-Flohé and Flohé 2003). Evidence from various studies on GPXs from various species further proved their imperative physiological and developmental roles. For instance, *AtGPX3* has been

reported to be involved in stomatal closure during drought stress by changing the leaf and chloroplast morphology (Chang et al. 2009). Considering their significant role, a genome-wide-based analysis was performed in rice and *Arabidopsis* to identify the GPX family members and their expression patterns in response to stress. An interesting pattern of evolutionary relationship was observed for GPXs from lower and higher plants and mammals. We analyzed all GPX proteins from evolutionary diverse species of *Escherichia coli* to *Homo sapiens*. The rooted phylogram (Fig. 4) showed two distinct clades with high bootstrap values: One clade represented the higher plant GPXs, and the other comprised lower plant GPXs including fungi, algae, diatoms as well as mammals, i.e. *H. sapiens*. This clearly indicated a divergence of GPXs in the course of their evolution. Major differences observed between the two clades of GPXs were in terms of their catalytic residues and mechanism of action.

Different pattern of sub-cellular localization was observed for this multi-member plant gene family (Mustafiz et al. 2011; Zhai et al. 2013). Localization studies performed for some of these GPX proteins by in vitro and in vivo experiments showed correlation with the predicted data. For instance, AtGPX1 was localized in the thylakoid membrane of chloroplast (Ferro et al. 2003; Peltier et al. 2004), AtGPX3 was localized in cytoplasm (Miao et al. 2006) and AtGPX8 was localized in both the nucleus and cytoplasm (Gaber et al. 2012). Similar patterns of diverse sub-cellular localizations

were predicted for rice and *Arabidopsis* GPX members by our study. However, mitochondrial, chloroplastic and cytosolic GPXs were the major classes observed in rice (Passaia et al. 2013) and in poplar (Navrot et al. 2006).

The co-expression data of *Arabidopsis* GPXs revealed a number of stress and signaling proteins such as MAP kinase signaling pathway proteins, avirulence-responsive proteins, leucine-rich repeat family proteins, pectin-esterase inhibitors, LEA3 family proteins, salt and drought-responsive proteins and disease resistance proteins (Table 2). Current data suggests that GPXs play an important role in various signal transduction and abiotic stress response pathways. Expression of several PHGPxs was found in different tissues, cellular compartments (Yang et al. 2006). The expression profiling of GPX genes has revealed their induction in response to a variety of abiotic and biotic stresses (Chen et al. 2004; Miao et al. 2006). For instance, AtGPX1 transcripts were reported to be up-regulated in response to salinity and heat stress (Milla et al. 2003). AtGPX3 is involved in stomatal closure during drought stress by utilizing the Ca^{2+} channel (Miao et al. 2006). Reports reveal that AtGPXs are involved in alteration of leaf and chloroplast morphology (Chang et al. 2009). Therefore, we investigated the expression profiles of rice and *Arabidopsis* GPX genes in response to various environmental factors likely abiotic stresses, at different time points and in different vegetative and reproductive developmental stages. Our results support the observed up-regulation of *AtGPX1* and

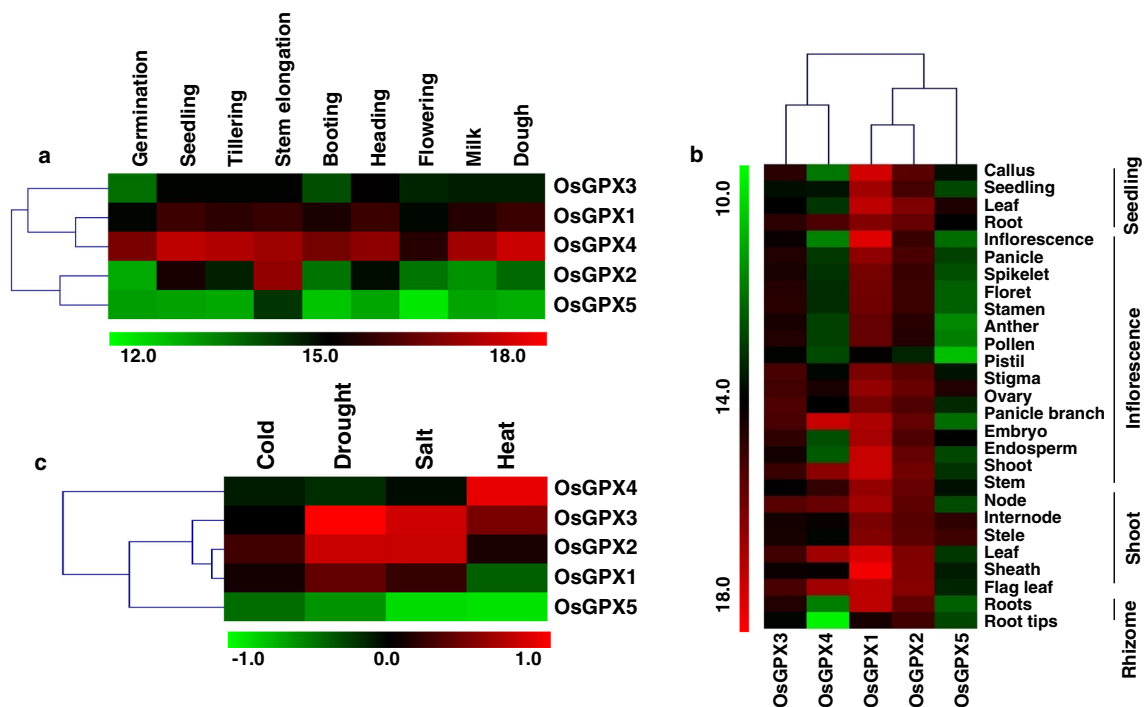


Fig. 9 Expression analysis of rice *GPX* genes at different developmental stages (a), tissues (b) and various abiotic stress conditions (c) using microarray data. The colour bar below represented relative expression values, wherein green represents lowest, black represents medium and

red signifies highest expression levels. Developmental stages, tissues and abiotic stress used for expression profiling are mentioned. The description of these stages has been given in the “Methodology” section

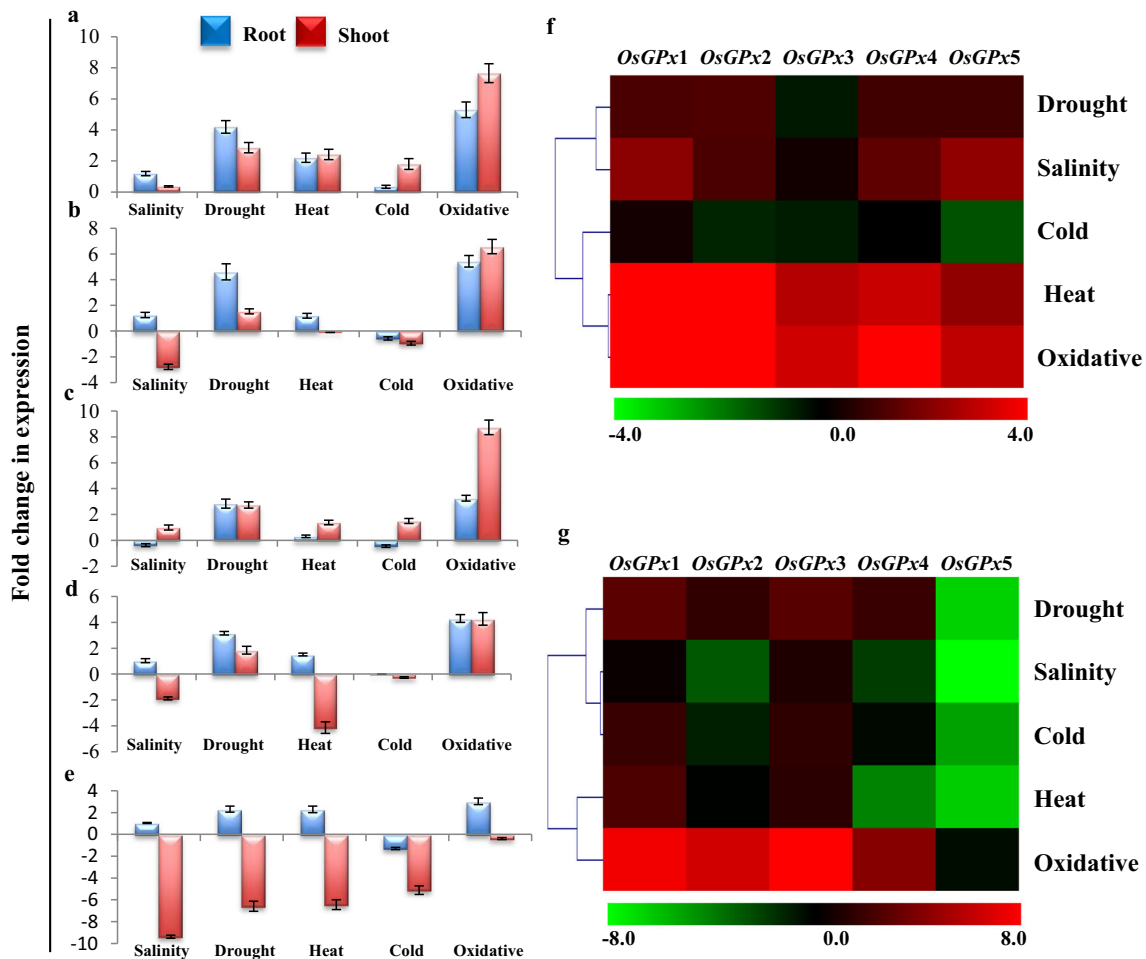


Fig. 10 Relative transcript abundance of *OsGPX* genes based on qRT-PCR analysis. Real-time PCR analysis used cDNA templates from shoot (red bar) and root (blue bar) tissues subjected to various abiotic stresses and their respective controls. Histograms represent the fold change in expression of the particular *OsGPX* at various stress conditions, such as *OsGPX1* (a), *OsGPX2* (b), *OsGPX3* (c), *OsGPX4* (d) and *OsGPX5* (e).

AtGPX7 in response to genotoxic stress, cold, wounding and heat stresses, in both shoot and root tissues (Fig. 6). Although our study on gene expression in *Arabidopsis* does not include GPX responses to DNA damage, however, expression of GPX genes was found to be up-regulated under osmotic, salt, genotoxic and UV/B, whereas moderately induced in drought and oxidative stress that corroborates with the previous studies. Reports have shown that *AtGPX2*, *AtGPX5* and *AtGPX6* recombinant proteins reduce H₂O₂ and lipid hydroperoxides (Iqbal et al. 2006) and provide protection against ROS-, salt- and copper-induced stresses (Milla et al. 2003). Significantly high expression of *AtGPX4* in stamen and pollen stages in comparison to other stages indicates its role in reproductive machinery development.

The possible role of GPX in the ROS detoxification in cells and during redox signaling has been demonstrated in many organisms including plants (Delaunay et al. 2002; Vivancos et al. 2005). Expression analysis of rice *OsGPXs* by qRT-PCR

revealed the up-regulation of GPX transcripts during various abiotic stresses, especially in response to oxidative stress or ROS in both shoot and root. *OsGPX5* exhibited up-regulation in response to all stresses, except in response to cold stress wherein it showed down-regulation. Evidentially, *OsGPX5* showed down-regulation in response to all the analyzed stresses in shoot tissues as monitored by microarray data and qRT-PCR. Thereby, we conclude that *OsGPX5* may play a significant role in root-specific stress tolerance or responsiveness. On the basis of our expression study and previous reports, it could be inferred that *OsGPX1*, *OsGPX2* and *OsGPX3* are the key players under oxidative stress. Thus, we infer that GPX genes exhibit developmental and stress-regulated expression patterns in both *Arabidopsis* and rice. Certainly, there is a need for the functional validation of these GPX genes, which exhibit ‘induced pattern of expression’ during multiple stresses. Our study rivets attention to the functional traits of these GPX proteins highlighting their possible roles in the face of

various developmental and abiotic stresses. Genetic manipulation may further aid in identification of potential candidate genes with defined features for designing of abiotic stress-tolerant plants using molecular plant breeding techniques. Besides, low expression or down-regulation has often been observed even in stress-tolerant plants during abiotic stresses, and our study predicts several targets that may be utilized to bridge this “gap”. Future studies may exploit these targets or stress-regulated genes by their overexpression or knockdown to impart oxidative stress tolerance during normal and abiotic stress conditions. Coincidentally, we know that promoters maneuver the regulation of gene expression utilizing inherent signal motifs to induce tissue-, development- or stress-specific expression. Two ROS responsive elements (ORE1 and ORE2) have been reported in the promoter region of the human GPX (Cowan et al. 1993). Moreover, several studies have shown the presence of singlet oxygen responsive (Leisinger et al. 2001), dehydration-responsive element (DREB) (Kizis and Pagès 2002), ABA-responsive element (ABRE) (Hattori et al. 2002) and plant defence-related element W-Box (Yu et al. 2001) in the promoter regions of various GPXs from *Arabidopsis*. Our analysis of the upstream sequences of GPX genes or their putative promoters reveals the presence of elements that regulate their stress-inducible expression. Several multiple stress-inducible cis-regulating elements such as MYB-binding site (MBS), W-box motif, ABRE and CRT/DREB motif have been found in the promoters, which may direct the up-regulation of GPXs in response to stress. In addition, several heat shock element (HSE), wound-responsive elements, salicylic acid-responsive elements (SARE) and defence-related or stress-responsive element (TC-rich repeats) are also present in their promoters. Presence of these multiple stress-inducible elements may induce an alteration in GPX gene expression in response to environmental and developmental changes.

Conclusions

In the present study, an epigrammatic analysis of GPX gene family of rice and *Arabidopsis* was carried out to enhance our knowledge about the regulation of the individual genes and their elementary traits in stress responses. Although a lot of work has been done on mammalian GPX proteins, the study on plant counterparts lacks extensively. Hence, comprehension of the coordinate expression pattern of plant GPX genes under various adverse conditions is imperative for highlighting the underlying mechanisms of their stress responsiveness. A comprehensive analysis on gene expression may provide a better assessment of the prospective gene(s) for plant improvement. Our study shall aid to analyze various functional aspects of the members of GPX gene family. Functional

genomics based on transgenic approach may be utilized to validate its potential candidature for crop improvement.

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