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Genome-Wide Survey and Expression Analysis Suggest Diverse Roles of Glutaredoxin Gene Family Members During Development and Response to Various Stimuli in Rice

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Abstract

Glutaredoxins (GRXs) are glutathione-dependent oxidoreductase enzymes involved in a variety of cellular processes. In this study, our analysis revealed the presence of 48 genes encoding GRX proteins in the rice genome. GRX proteins could be classified into four classes, namely CC-, CGFS-, CPYC- and GRL-type, based on phylogenetic analysis. The classification was supported with organization of predicted conserved putative motifs in GRX proteins. We found that expansion of this gene family has occurred largely via whole genome duplication events in a species-specific manner. We explored rice oligonucleotide array data to gain insights into the function of GRX gene family members during various stages of development and in response to environmental stimuli. The comprehensive expression analysis suggested diverse roles of GRX genes during growth and development in rice. Some of the GRX genes were expressed in specific organs/developmental stages only. The expression of many of rice GRX genes was influenced by various phytohormones, abiotic and biotic stress conditions, suggesting an important role of GRX proteins in response to these stimuli. The identification of GRX genes showing differential expression in specific tissues or in response to environmental stimuli provide a new avenue for in-depth characterization of selected genes of importance.

Key words: glutaredoxin; rice; development; stress; gene family

1. Introduction

Glutaredoxins (GRXs) are ubiquitous oxidoreductase enzymes that mediate reversible reduction of disulphide bonds of their substrate proteins in the presence of glutathione (GSH) via a dithiol or monothiol mechanism. GRXs generally contain a conserved cys-x-x-cys (dithiol GRXs) or cys-x-x-ser (monothiol GRXs) active-site motif, which is involved in reduction reaction. GRXs maintain and regulate cellular redox state and redox-dependent signaling pathways.[1] These enzymes are involved in diverse cellular processes and play an important role in defense against oxidative stress.[2] GRXs are present in a number of isoforms in prokaryotes and eukaryotes.

Several cDNAs encoding GRX proteins have been isolated from various plant species.[3,4] In *Arabidopsis*, at least 31 GRX genes have been predicted and divided into three major classes, namely CPYC-, CGFS- and CC-type, based on their predicted amino acid sequences and the composition of active-site motifs.[3–5] Among these, CPYC- and CGFS-type classes exist in all organisms from prokaryotes to eukaryotes including plants, whereas the CC-type class has so far been identified only in land plants. The information on biochemical activity, structure and function of plant GRXs is very limited. Plant GRX isoforms possess the ability to reduce dehydroascorbate and type II peroxiredoxin.[6,7] Recently, structure–function analyses indicated that poplar GrxC1 contains a {2Fe–2S} cluster in the holo form, suggesting a potential role of this Grx in sensing oxidative stress and iron–sulfur biosynthesis.[8] The

chloroplast-targeted AtGRXcp and AtGRX4 have been shown to play a critical role in oxidative stress response and redox regulation.[9,10] Molecular genetic evidence suggests that *ROXY1*, encoding a CC-type GRX, is required for petal development in *Arabidopsis*.[11] Further, it has been shown that ROXY1 together with its closest homolog, ROXY2, play a major role in control of male gametogenesis.[12] Recently, the function of their closest homologs in rice have also been found to be conserved.[13] It has been demonstrated that GRX interacts with TGA class of bZIP transcription factors and is involved in salicylic acid/jasmonic acid cross-talk.[14] The probable role of plant GRXs in long-distance signaling has also been proposed due to their detection in phloem sieve tubes.[3,15]

Although some of the members of GRX gene family have been identified, cloned and characterized in rice,[13,16,17] the model monocot plant, a systematic genomic analysis at a genome-wide level is lacking. Previously, the presence of a total of 27 GRX genes has been reported in rice.[5] In this study, we have identified 48 members of GRX gene family in rice and divided them into four classes based on the phylogenetic analysis and sequence conservation. The gene structure and putative conserved motifs have been analyzed. The expression studies indicated that GRXs exhibit a variety of gene expression patterns during development and in response to various environmental stimuli. Our analysis provides the framework for future studies to dissect functions of this important gene family.

2. Materials and methods

2.1. Identification of GRX proteins

A preliminary search for GRX proteins was performed using keyword 'GRX' in the Rice Genome Annotation Project (RGAP) database for rice and TAIR database for *Arabidopsis*. Another approach used was to search for the proteins containing PFam domain PF00462, which encodes GRX domain. Further, the rice and *Arabidopsis* proteomes downloaded from RGAP (version 6.1) and TAIR (version 9), respectively, were searched using the hidden Markov model (HMM) profile (build 2.3.2) for GRX domain (PF00462) downloaded from the PFam database. The results obtained from all the above approaches were pooled together and redundancy was removed. Further, all the proteins were searched in the SMART and PFam databases to confirm the presence of GRX domain. The GRX proteins in *Populus trichocarpa*, *Glycine max* and *Zea mays* were identified by BLASTP searches of all the identified rice and *Arabidopsis* GRX proteins against their annotated proteomes followed by confirmation of the presence of GRX domain in the SMART and PFam databases.

2.2. Protein sequence alignment and phylogenetic analysis

Multiple sequence alignments of amino acid sequences were performed using ClustalX (version 1.83) program and were corrected manually. The unrooted phylogenetic trees were generated by the neighbor-joining (NJ) method using ClustalX and Phylogeny Inference Package (PHYLIP v3.69) programs with default parameters and displayed using NJPlot program. Bootstrap analysis was performed with 1000 replicates to obtain a support value for each branch.

2.3. Sequence analysis

The amino acid sequences of all the predicted GRX proteins were analyzed for subcellular localization by TargetP1.1.[18] The putative conserved motifs were identified using MEME (version 4.1.0) program with default parameters except the minimum number of sites for a motif to find was defined as five.[19]

2.4. Chromosomal localization and gene duplication

All the GRX genes were positioned on the rice and *Arabidopsis* chromosomes using BLASTN. The GRX genes present on duplicated chromosomal segments were identified by segmental genome duplication of rice available at RGAP with the maximum length distance permitted between collinear gene pairs of 500 kb. The presence of *Arabidopsis* GRX genes on duplicated chromosomal segments were investigated using 'Paralogons in Arabidopsis' (http://wolfe.gen.tcd.ie/athal/dup). The GRX genes separated by a maximum of five genes were identified as tandemly duplicated genes.

2.5. Expression analysis using cDNA/EST and massively parallel signature sequencing data

To find cDNA and EST sequences corresponding to *OsGRX* genes, BLASTN search was performed in non-redundant and EST databases at NCBI. The gene expression evidence search page available at RGAP database was also used to find the availability of corresponding FL-cDNA and/or EST evidences. The MPSS (massively parallel signature sequencing) data were searched at Rice MPSS project for 20-base signatures from 22 mRNA libraries representing 18 different tissues/organs in rice for expression evidence analysis.[20] Only the signatures, which uniquely identify an individual gene and show perfect match (100% identity over 100% of the length of the tag), were used for the analysis. The normalized abundance (tags per million, tpm) of these signatures for a given gene in a given library represents the quantitative estimate of the expression of that gene.

2.6. *Expression analysis using microarray data*

For rice, the microarray data available at Gene Expression Omnibus (GEO) database under the series accession numbers GSE6893 (various stages of development),[21] GSE6901 (abiotic stress),[21] GSE4471 (arsenate stress),[22] GSE5167 (auxin and cytokinin treatment),[23] GSE7256 (virulent infection by *Magnaporthe grisea*)[24] and GSE10373 (interaction with the parasitic plant *Striga hermonthica*)[25] were used for expression analysis of *OsGRX* genes. In addition, microarray analysis was performed for oxidative stress treatments with methyl viologen and hydrogen peroxide, and hormone treatments including ABA, ACC, SA and JA using Affymetrix whole genome rice arrays as described earlier.[21] For *Arabidopsis*, the microarray data for various stages of developments, hormone treatments and abiotic stress treatments corresponding to those analyzed for rice were downloaded from GEO (series accession numbers GSE5620, GSE5621, GSE5623, GSE5624 and GSE5629–GSE5634) and AtGenExpress (http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp) databases. The Affymetrix CEL files were imported into Genespring GX (version 11) software (Agilent Technologies). The normalization and probe summarization was performed by the Gene Chip Robust Multi Array (GCRMA) method. A stringent statistical analysis consisting of one-way ANOVA over all the samples in a series was performed and the Benjamini–Hoschberg multiple testing correction was applied to the data ($P \leq 0.05$).

The IDs of probe sets present on the Affymetrix rice genome array representing the *OsGRX* and *AtGRX* genes were identified. The data for only one probe set for each GRX gene were used for expression analysis. This resulted in identification of probe sets for 40 *OsGRX* and 38 *AtGRX* genes that were represented on the rice and *Arabidopsis* Affymetrix arrays, respectively. A fold change of at least 2-fold with a *P*-value of at least 0.05 was considered to be significant to identify differentially expressed genes. The tab-delimited files for the average log signal values for development data and fold-change values for abiotic stress, biotic stress and hormone treatments were imported into TIGR MultiExperiment Viewer (MeV) to generate heatmaps based on hierarchical clustering.

2.7. *Real-time PCR analysis*

The real-time PCR analysis was performed using gene-specific primers as described earlier.[26] All the primer sequences used in this study are listed in Supplementary Table S1. At least two biological replicates of each sample and three technical replicates of each biological replicate were analyzed for real-time PCR analysis. The expression of each gene in different RNA samples was normalized with the expression of the suitable internal control gene, *UBQ5*,[26] to ensure the equal amount of cDNA used for individual reactions. The relative mRNA levels for each candidate gene in different tissue samples were calculated using the $\triangle\triangle C_T$ method.

2.8. *Structure model*

To identify the best template structures, all the OsGRX proteins were searched by BLASTP in all the GRX proteins for which a three-dimensional structure is known in Protein Data Bank (PDB). The crystal structures of *Populus* GRX S12 (PDB ID 3fza) and GRXC1 (PDB ID 2e7p) were used as templates for constructing the structure models of rice CPYC- and CC-type class proteins. Similarly, crystal structures of human GRX5 (PDB ID 2wul) and *Escherichia coli* GRX4 (PDB ID 2wci) were used as templates for constructing the structure models of rice CGFS-type class proteins. The sequences of template and representative OsGRX proteins were aligned by align2D_Mult program in the MODELLER package.[27] On the basis of the alignment, structures were built using Model_Mult of MODELLER package. The stereochemical properties of all the optimized generated models were assessed using PROCHECK to select the best model for a given protein.[28] Dock (6.2) program was used for docking of GSH to the GRX proteins.

2.9. *Plant growth*

For salt, desiccation, cold, arsenate, methyl viologen and hydrogen peroxide stress treatments, 7-day-old light-grown rice (*Oryza sativa* L. ssp. *indica* var. IR64) seedlings were transferred to a beaker containing 200 mM NaCl solution, dried between folds of tissue paper at 28 ± 1°C, kept at 4 ± 1°C, transferred to a beaker containing 50 μM sodium arsenate solution, transferred to a beaker containing 100 μM methyl viologen solution and transferred to a beaker containing 10 mM hydrogen peroxide solution, respectively, each for 3 h. Likewise, 7-day-old light-grown rice seedlings were transferred to a beaker containing 50 μM solutions of indole-3-acetic acid and benzyl aminopurine and 100 μM solutions of abscisic acid, 1-aminocyclopropane-1-carboxylic acid (ACC), salicylic acid and jasmonic acid for different hormone treatments. The control seedlings were kept in water for 3 h, at 28 ± 1°C.

3. Results and discussion

3.1. *Identification of GRXs in rice and* Arabidopsis

A total of 31, 36 and 27 GRX proteins have been identified in *Arabidopsis*, *Populus* and rice, respectively, and divided into three classes.[3,4] Further, the presence of 21, 22 and 17 plant-specific CC-type GRXs has

been reported in *Arabidopsis*, *Populus* and rice, respectively.[5,13,29] In addition, 15 non-canonical GRX-like (GRL) proteins have been identified in *Arabidopsis*.[30] In this study, multiple searches followed by confirming the presence of GRX domain using SMART and PFam, resulted in the identification of 48 loci in the rice genome that encode for putative GRX proteins. We identified an identical number (48) of GRX genes in the *Arabidopsis* genome as well. Despite the difference in genome sizes between *Arabidopsis* and rice, the two plant species have identical number of genes encoding GRX proteins. Phylogenetic tree among the rice and *Arabidopsis* GRX proteins was constructed by the NJ method using ClustalX and PHYLIP programs. The 96 GRXs were grouped into four distinct groups with high bootstrap support using ClustalX program (Fig. 1A). An identical grouping of GRX proteins was observed in the phylogenetic tree constructed using PHYLIP program (Supplementary Fig. S1). The classes include CC-type (17 in rice and 21 in *Arabidopsis*), CGFS-type (5 in rice and 5 in *Arabidopsis*), CPYC-type (7 each in rice and *Arabidopsis*) and GRL-type (19 in rice and 16 in *Arabidopsis*) (Fig. 1A; Table 1; Supplementary Tables S2 and S3). The number of CC-type GRXs identified in this study is identical as that reported earlier.[13] The members of CC-type, CPYC-type and CGFS-type classes contain a conserved active-site motif and were designated as *OsGRX* in rice and *AtGRX* in *Arabidopsis*, followed by a number according to their position on rice or *Arabidopsis* chromosomes from top to bottom (Table 1; Supplementary Tables S2 and S3). The members included in GRL-type class showed low homology to classical GRXs and do not harbor conserved active-site motif as reported earlier.[30] To distinguish these proteins from classical GRXs, these proteins were designated as *OsGRL* in rice and *AtGRL* in *Arabidopsis* followed by a number according to their position on rice or *Arabidopsis* chromosomes from top to bottom (Table 1; Supplementary Tables S2 and S3). The detailed genomic information about all the predicted GRX genes in rice and *Arabidopsis* is given in Supplementary Tables S2 and S3.

A comparison of the coding sequence with the corresponding genomic DNA sequences showed that quite a large number of GRX genes (69% in rice and 71% in *Arabidopsis*) are intronless (Fig. 1B), which is very large number as compared at the whole genome level (20%).[31] Striking gene structure conservation was found within the members of each class. Notably, all but one member of CC-type class and most of members of GRL-type class GRX genes are intronless. Such intronless class/family of genes can evolve rapidly by gene duplication or reverse transcription followed by integration in the genome.[31–34] This might explain the larger number of members of these classes of proteins, which may perform diverse plant-specific functions. There are only few GRX genes found in other organisms. The large number of GRX genes present in rice and *Arabidopsis* genome highlights the importance of these genes in plants. Although CGFS-type and CPYC-type class GRX proteins represent ancestral genes from prokaryotes to eukaryotes, the plant-specific CC-type and GRL-type class GRX proteins are more in number both in rice and *Arabidopsis*, further indicating their importance. Earlier, a comparative analysis in various plant species, including *Physcomitrella patens*, *Pinus taeda*, *O. sativa*, *P. trichocarpa* and *Arabidopsis thaliana* revealed that the number of CC-type class GRXs has expanded during plant evolution.[29] On the contrary, the number of CPYC- and CGFS-type class members remained constant. For example, only two CC-type GRXs have been found in *Physcomitrella*,[29] whereas 17 in rice and 21 in *Arabidopsis*. On the basis of this observation, a possible function of CC-type class GRXs in contributing to the evolution of land plants that forms organs of higher complexity has been proposed.[29]

3.2. *Genomic organization and expansion of GRX gene family*

The physical locations of all the 48 *OsGRX*s were assigned to the 12 rice chromosomes (Fig. 2). The distribution of GRX genes among the chromosomes appears to be uneven; chromosome 9 harbors no GRX genes, whereas chromosome 1 harbors nine GRX genes. Other chromosomes have 2–5 GRX genes localized on them. Only two clusters of tandemly arranged *GRX* genes were found in rice; one of four GRXs on chromosome 11 and other of two GRXs on chromosome 12. On the other hand, we found 10 pairs (20 members) of GRX genes were located on duplicated chromosomal segments in rice. The distribution of GRX genes relative to duplicated chromosomal segments in rice is illustrated in Fig. 2. In *Arabidopsis*, the 48 GRXs were localized on all the five chromosomes ranging from 7 to 13 in number (Supplementary Fig. S2). Three clusters were observed, one of two GRXs on chromosome 2; second of three GRXs on chromosome 3 and third of five GRXs on chromosome 4. Seven pairs (14 members) of GRX genes were located on duplicated chromosomal segments in *Arabidopsis*. Further analysis revealed that a similar set of genes were found to be present in the proximity of GRX gene pairs predicted to be located on duplicated chromosomal segments in rice and *Arabidopsis*. The details of the genes present in the proximity of the GRX gene pairs located on chromosomal segments in rice and *Arabidopsis*, including their gene identifiers, description,

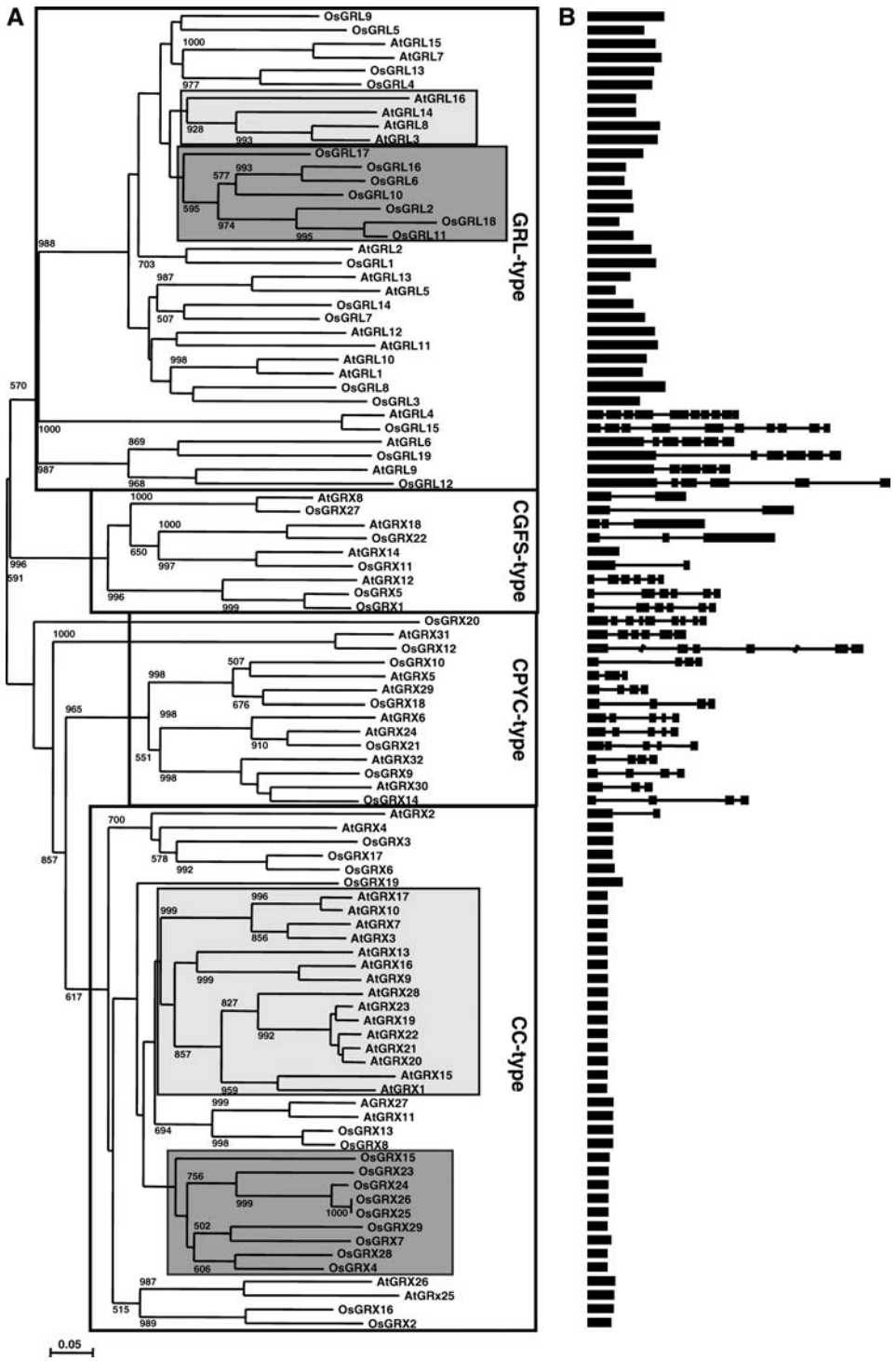


Figure 1. Phylogenetic relationship among rice and *Arabidopsis* GRXs, their classification and gene structure. (A) The unrooted tree was constructed using ClustalX program based on the multiple sequence alignment of the GRX domain sequences by the NJ method. The number at the nodes represent the bootstrap values (>50%) from 1000 replicates. Different types of GRXs have been indicated. Rice- and *Arabidopsis*-specific clades have been indicated in different grey-shaded boxes. (B) The gene structure (exon–intron organization) of the coding sequence of GRXs is shown. Exons are represented by black boxes and introns as lines.

chromosomal location and *E*-value are given in Supplementary Tables S4 and S5, respectively.

Taken together, it appears that the chromosomal segment duplications have mainly contributed to the expansion giving rise to 42 and 29% of GRX genes in rice and *Arabidopsis*, respectively. However, tandem or local gene duplication occurred less frequently in *Arabidopsis* and rice. Further, it is also

Table 1. GRX genes predicted in rice

Class	Gene name[a]	Locus[b]	Active site[c]
CC type	*OsGRX2*	Os01g09830	CYMA
	OsGRX3	Os01g13950	CCMA
	OsGRX4	Os01g27140	CCMC
	OsGRX6	Os01g47760	CCLI
	OsGRX7	Os01g70990	CFMC
	OsGRX8	Os02g30850	CCMC
	OsGRX13	Os04g32300	CCMC
	OsGRX15	Os05g05730	CGMS
	OsGRX16	Os05g10930	CCMC
	OsGRX17	Os05g48930	CCLS
	OsGRX19	Os07g05630	CCMC
	OsGRX23	Os11g43520	CGMC
	OsGRX24	Os11g43530	CCMC
	OsGRX25	Os11g43550	CCMC
	OsGRX26	Os11g43580	CCMC
	OsGRX28	Os12g35330	CCMC
	OsGRX29	Os12g35340	CPMC
CGFS type	*OsGRX1*	Os01g07950	CGFS
	OsGRX5	Os01g34620	CGFS
	OsGRX11	Os03g63420	CGFS
	OsGRX22	Os10g35720	CGFS
	OsGRX27	Os12g07650	CGFS
CPYC type	*OsGRX9*	Os02g40500	CPFC
	OsGRX10	Os02g43180	CPYS
	OsGRX12	Os04g17050	CPFC
	OsGRX14	Os04g42930	CPFC
	OsGRX18	Os06g44910	CPYC
	OsGRX20	Os08g44400	CPFC
	OsGRX21	Os08g45140	CSYC
GRL type	*OsGRL1*	Os01g13480	
	OsGRL2	Os01g61350	
	OsGRL3	Os02g01200	
	OsGRL4	Os02g51370	
	OsGRL5	Os03g07470	
	OsGRL6	Os03g24030	
	OsGRL7	Os03g44650	
	OsGRL8	Os04g33680	
	OsGRL9	Os04g54860	
	OsGRL10	Os05g28530	
	OsGRL11	Os05g39450	
	OsGRL12	Os06g12030	
	OsGRL13	Os06g12190	
	OsGRL14	Os07g06600	
	OsGRL15	Os07g46410	
	OsGRL16	Os07g46570	
	OsGRL17	Os08g07450	
	OsGRL18	Os08g44070	
	OsGRL19	Os10g34170	

[a]Systematic name given to GRX genes identified in rice.
[b]Locus identified from RGAP.
[c]Conserved active-site motif in the predicted GRX protein sequences.

clear that chromosomal segment duplication has contributed more for expansion of GRX genes in rice when compared with *Arabidopsis*. In addition, in the phylogenetic tree constructed from rice and *Arabidopsis* GRX domain sequences, we observed some species-specific clades within a class (Fig. 1A, Supplementary Fig. S1). This indicates that the expansion of different classes has occurred independently in a species-specific manner in rice and *Arabidopsis*. This observation was further validated by constructing a phylogenetic tree among the GRX domain sequences from a total of 259 GRX proteins identified from various plant species, including rice (48), *Arabidopsis* (48), *Populus* (45), *G. max* (82) and *Z. mays* (36) using ClustalX (Supplementary Fig. S3) and PHYLIP (data not shown) programs. We found both lineage-specific (monocot- and dicot-specific) and species-specific clades in these phylogenetic trees as well, further confirming the expansion of GRX gene family in the monocots and dicots independently. Recently, the expansion of CC-type class GRX proteins in angiosperms has also been proposed mainly through paleo-polyploidy duplication events after the monocot–dicot split.[35]

3.3. *Sequence analysis*

The length of GRX proteins varied greatly ranging from minimum of 103 (OsGRX4 and OsGRX28) to 711 amino acids (OsGRL12) in rice. The pairwise comparison of GRX protein sequences showed considerable sequence diversity. However, relatively more identity was found among the members of the same class. The multiple sequence alignments of full-length GRX protein sequences showed that the core region, which represents the GRX domain, is most conserved. All the members of CGFS-type, CPYC-type and CC-type class GRX proteins harbor the conserved active site (Table 1). The cysteine at first position of the active site was invariably conserved in all the proteins; however, variations were found at other positions. All the five members of CGFS-type class contain perfectly conserved CGFS as an active site. However, only one member harbors characteristic CPYC motif in CPYC-type class, other

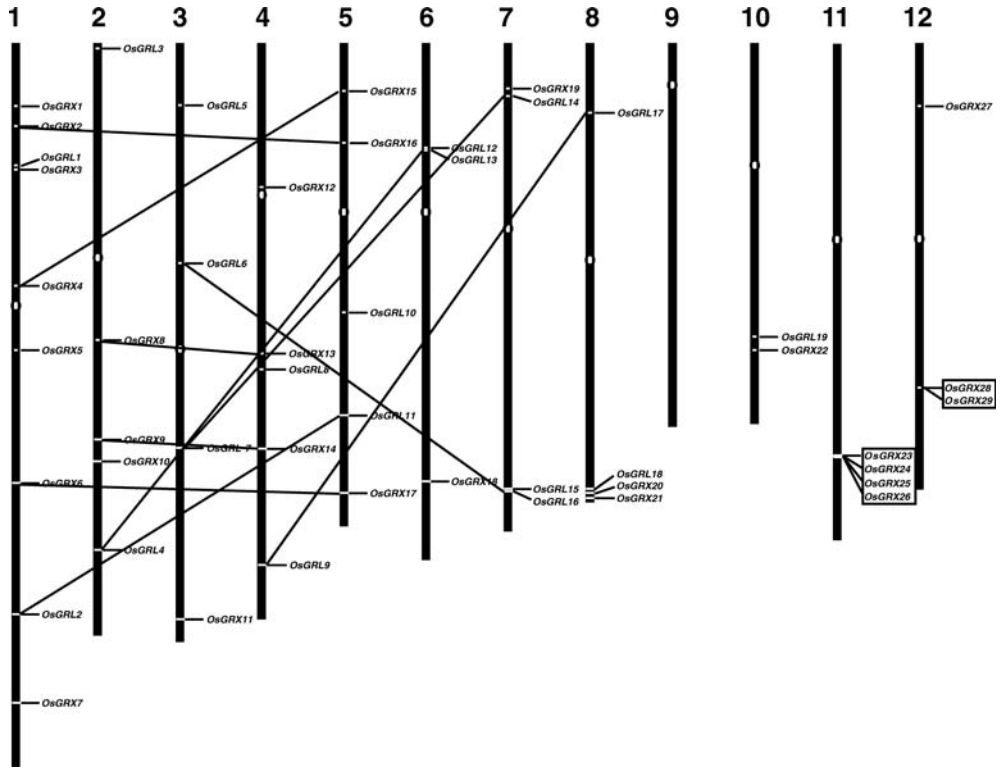


Figure 2. Chromosomal distribution and segmental duplication events of *OsGRX* genes. The chromosome number is indicated on the top of each bar. The GRX genes localized on duplicated chromosomal segments are connected by lines. The clusters of tandemly arranged GRX genes are indicated in boxes.

members have CPFC (four), CPYS (one) and CSYC (one) as active site. In CC-type class GRX proteins, nine members harbor CCMC as active site. The characteristic two cysteine residues were conserved in all CC-type GRXs except for five. In fact, the second cysteine residue has not been found crucial for the activity of CC-type GRXs in *Arabidopsis*.[11] In addition, most of the members of CC-type class rice GRX proteins harbor four C-terminal residues, A(L/I)W(L/V), which were found to be essential for their *in planta* function and physical interaction with target proteins.[36] Most of the members of GRL-type class proteins harbor eight conserved cysteines, arranged in two CxxCx7CxxC clusters in rice at the C-terminal end. Similar pattern of conserved cysteines has been reported in *Arabidopsis* GRL proteins as well.[30]

To identify additional putative conserved motifs in GRX proteins, all the 96 GRX proteins from rice and *Arabidopsis* were searched using MEME program. Ten highly significant ($<e-50$) putative motifs of at least six amino acids in length conserved in more than 10 GRX proteins were identified (Table 2; Supplementary Fig. S4). Notably, all the putative motifs identified are conserved in rice and *Arabidopsis*. Some of the motifs were present in the members of more than one class. However, others were specific to a particular class. For example, motif 1, which represents a part of GRX domain, was present in most of the members of all classes. Motif 2 was present in CPYC-type and CC-type GRX proteins. Motifs 3, 5 and 7 were present only in GRL type of GRX proteins. Motif 4 was present in CC-type and CPYC-type proteins only. Motifs 9 and 10 were present in CC-type proteins only, whereas motifs 6 and 8 were present in CGFS type only. Overall, the organization of putative conserved motifs coincided with the grouping of GRXs in various classes and may be responsible for functional specificity among GRX proteins of various class types.

GRXs have been predicted to localize in all cellular components except nucleus using prediction software programs.[3] However, recently, it has been shown that nuclear activity of one of CC-type GRX, ROXY1, is required for its role in petal development.[36] We also analyzed the putative subcellular localization of OsGRX proteins using TargetP program.[18] Using TargetP, 11, 15 and 10 of GRXs were predicted to localize to chloroplast, mitochondria and secretory pathway, respectively, other 12 were predicted to localize to any other location. Further, TMHMM predicted the presence of transmembrane α-helix in only two of GRX proteins (one predicted in secretory pathway and second in any other location).

Table 2. List of putative conserved motifs in rice and *Arabidopsis* GRX proteins predicted by MEME

Motif no.	Motif sequence	Number of sites in rice	Number of sites in *Arabidopsis*	Length	*E*-value	Class of GRX proteins
1	PQVFIGGKHIGGCDQVMSMHENGELVPMLK	45	48	30	3.3e-1153	CC-type, CGFS-type, CPYC-type and GRL-type
2	VIFSKSYCCMCHTVKTLFQDL	22	27	21	2.2e-474	CC-type and CPYC-type
3	RVVLYFTSLRGIRKTYEDCCAVRAILRGHRVWVDERDVSMH	16	13	41	4.9e-636	GRL-type
4	GVNPTIHELDQDPDGWEIQRALAQWGCQP	15	21	29	1.8e-372	CC-type and CPYC-type
5	GWRRCPGCNENRFVPCPNC	28	25	19	4.4e-362	GRL-type
6	RLEQLVNSHPVMLFMKGTPDEPQCGFSQKVVQILKQYNVPFGSFDILTDE	7	6	50	1.6e-222	CGFS-type
7	TPPNEPEVINTWELMAGLDD	6	7	20	3.8e-082	GRL-type
8	ELRQGLKNYSNWPTF	7	6	15	2.7e-073	CGFS-type
9	AGALWL	15	15	6	3.5e-065	CC-type
10	MDMVQRMASEK	10	13	11	2.0e-058	CC-type

Although these results provide a clue toward putative subcellular localization of GRX proteins, experimental evidences are required to validate the results.

3.4. *Structure modeling*

Although the structure of a number of GRXs have been determined by X-ray and NMR from various organisms, the structure of only two plant GRXs (from *Populus*) have been determined so far.[8,35] We made an attempt to study the structure of representative members of different classes of GRXs based on homology modeling. One representative member from each of CGFS-type, CPYC-type and CC-type classes showing best homology with known proteins in PDB database was selected and analyzed. Rice GRL-type class proteins did not show any significant homology to known GRX proteins in PDB and thus were not analyzed. The three-dimensional structure modeling of representative GRX proteins from CGFS-type, CPYC-type and CC-type classes showed that, in general, the structure of OsGRXs are conserved (Fig. 3A). The structure examination showed that GRX protein shared the same conformation of structural elements of α-helices and β-sheets as that of templates (six α-helices and four β-sheets for CC- and CPYC-type, and six α-helices and three β-sheets for CGFS-type). However, structural differences are present in the variable loop regions, where sequence conservation is relatively weak. In the modeled structures, the positions of three conserved regions (constituted by WCSY, TVP and GST residues in *Populus*),[37] representing GSH-binding sites, were marked and found to be conserved structurally in the members of CPYC-type, whereas the modeled structures of CC-type and CGFS-type exhibited slight variability. However, these variations were not significant as depicted by root mean-square deviation calculations of modeled structures and templates using Chimera tool (Supplementary Table S6). Further, the Ramachandran plot analysis revealed that 98−100% of residues lie within allowed regions, only 1−2% being in disallowed regions in the case of CGFS type (Supplementary Table S6). Overall, the structures of CPYC-type and CC-type class members were more similar than that of CGFS-type. It has been proposed that the CC-type GRXs probably arose by diversification from the CPYC-type.[35] The molecular docking of GSH was done with representative OsGRX proteins based on the known structure of *Populus* GRX S12 complexed with GSH (PDB ID 3fza). The analysis showed that in the structure of OsGRX proteins GSH could fit well in the groove constituted by conserved active site residues (Fig. 3B). The thermodynamically stable binding of ligand GSH was revealed by calculation of grid score (Supplementary Table S6). The negative energy values indicated thermodynamic stability of the binding. The stability of GSH complexed OsGRX proteins was further substantiated using consensus scoring function X-score (Supplementary Table S6). Taken together, the overall structure including GSH-binding site of OsGRX proteins seems to be conserved as that of known GRX proteins, suggesting their similar biochemical properties.

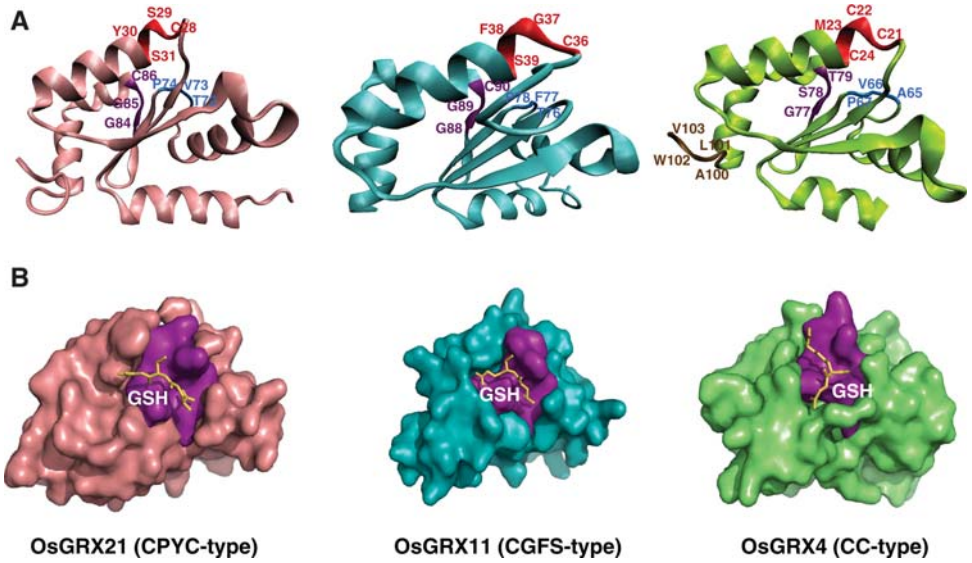


Figure 3. The structure of a representative CPYC-type, CGFS-type and CC-type GRX protein from rice. (A) Predicted structure of OsGRX21, OsGRX11 and OsGRX4. The residues of GSH-binding sites are marked in different colors. (B) Surf structure of OsGRX21, OsGRX11 and OsGRX4 showing surface topology complexed with GSH. The location of residues of the GSH-binding site is indicated in purple color.

3.5. *Differential expression of OsGRX genes in various organs/developmental stages*

The gene expression patterns can provide important clues for the gene function. We looked for the expression evidence for all the *OsGRX* genes in cDNA, EST and MPSS databases. This analysis revealed that one or more cDNA and/or EST sequences were available for 39 (81%) of *OsGRX*s, providing strong indication for expression of most *OsGRX*s (Fig. 4A). However, the frequency of ESTs for GRX genes varied and also the ESTs were derived from various tissues/organs, indicating their differential expression. Further, we surveyed Rice MPSS Project database for each of *OsGRX* gene to collect the expression evidence in terms of MPSS tags. We found significant unique 20 b signatures corresponding to at least 31 *OsGRX* genes (Fig. 4A). This included MPSS signatures for three GRX genes, for which no cDNA and/or EST evidence was available. The number of tags (in tpm) also varied significantly in various tissues/organs indicating differential expression of *OsGRX* genes (Supplementary Table S7). Taken together, we found expression evidence for at least 42 (88%) *OsGRX* genes in terms of availability of cDNA, EST and/or MPSS tags.

In addition to sequence-based expression analysis methods, we previewed the expression profiles of *OsGRX* genes during various stages of development using microarray data. The microarray data from 54 arrays representing 18 stages of development throughout the life cycle of rice, including root, mature leaf, Y leaf, shoot apical meristem (SAM), stages of panicle development (P1-I to P1-III, P1 to P6) and stages of seed development (S1 to S5) was analyzed. The average log signal values for all the 40 *OsGRX* genes represented on Affymetrix Rice Genome array were analyzed. Following whole chip data processing, the average log signal values were extracted (Supplementary Table S8). The hierarchical cluster analysis based on average log signal values indicated that *OsGRX* genes display diverse expression patterns (Fig. 4B). Most of the 40 GRX genes are expressed in at least one of the 18 rice tissues investigated in this study. Most diverse expression patterns were found in the members of CC-type GRX genes, indicating that this class of proteins are most evolved with specialized function. *OsGRX8* has a significantly higher expression in SAM and stages of panicle development than in other tissues, whereas *OsGRX3* seems to be predominantly expressed in late stage of panicle development (P6) and stages of seed development. *OsGRX28* exhibited specific expression during S1 stage of seed development only. *OsGRX13* and *OsGRX19* were also expressed preferentially in SAM and early stages of panicle development. Two members of CGFS-type class, *OsGRX11* and *OsGRX27* were expressed at lower level in SAM and stages of panicle development as compared to other tissues/developmental stages. *OsGRX20*, a CPYC-class member, was expressed at higher level in mature and Y leaf. At least six members of GRL type showed very low level of expression in all the tissues examined. *OsGRL1*, a GRL-type GRX, showed maximum expression levels in mature leaf and stages of seed development. *OsGRL19* also exhibited similar expression profile with some expression in late stages of panicle development. However, *OsGRL16* showed distinct very low level of expression in SAM as compared to all other tissues/developmental stages analyzed. Similar to rice, the expression patterns of CC-type GRX genes were most

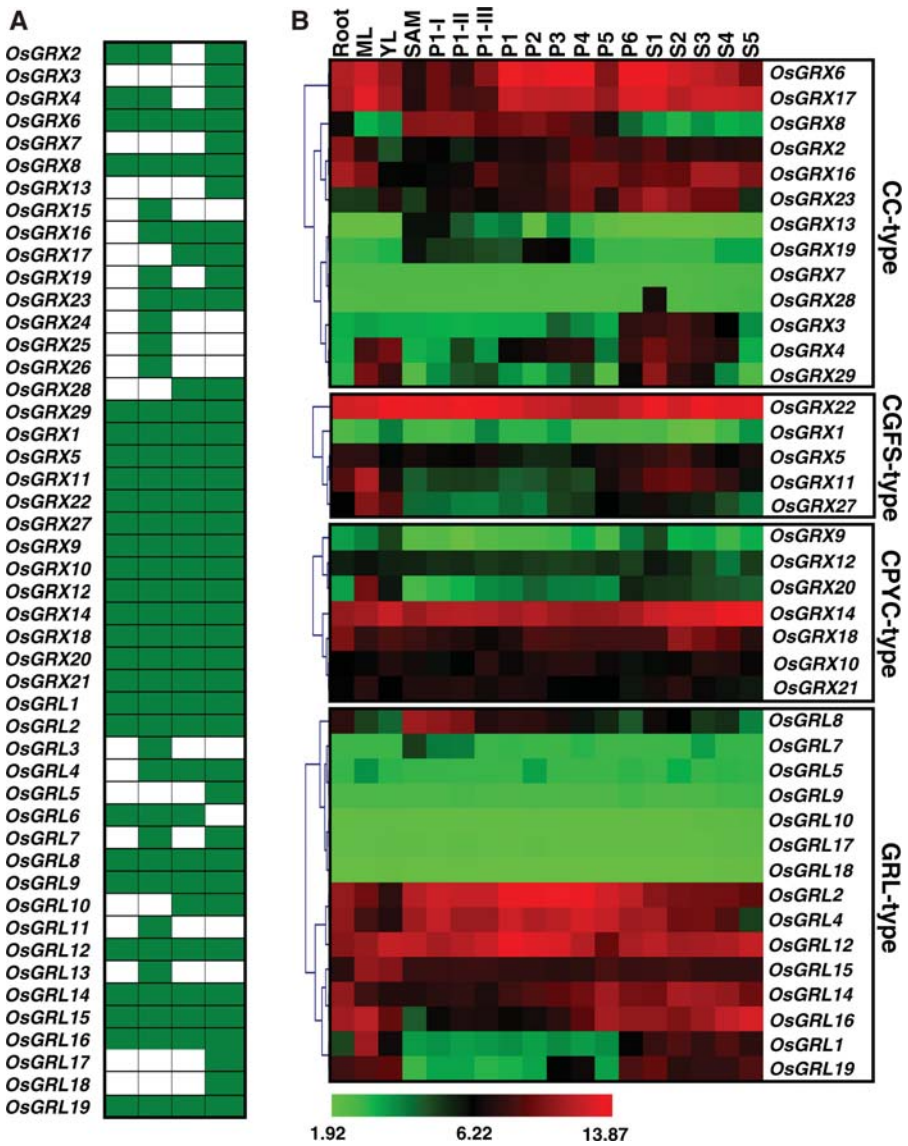


Figure 4. Expression profiles of *OsGRX* genes in various tissues. (A) The green box represents detection of cDNA (column 1), EST (column 2), MPSS signature (column 3) and/or microarray data (column 4) gene expression evidence. (B) Heatmap showing hierarchical clustering of 40 *OsGRX* genes based on their average log signal values in various tissues/developmental stages analyzed. ML, mature leaf; YL, Y leaf; SAM, shoot apical meristem; P1-I to P1-III and P1 to P6, stages of panicle development; S1–S5, stages of seed development. The color scale is given at the bottom.

diverse in *Arabidopsis* as well, showing differential and/or specific expression in various tissues/developmental stages analyzed (Supplementary Fig. S5A). The members of CGFS- and CPYC-type classes were expressed at high level in all the tissue samples analyzed, whereas few genes of GRL-type class also exhibited tissue-/developmental stage-specific expression patterns (Supplementary Fig. S5A).

Although the expression profiles of GRXs have been reported in various plant species,[15,16,38,39] the evidence for their role in plant growth and development is lacking except for floral organ development. The molecular genetic analysis revealed the role of two *Arabidopsis* CC-type GRXs named as ROXY1 and ROXY2, in petal and anther development.[11,12] It has been demonstrated that nuclear localization of ROXY1 protein is crucial for its role in petal development.[36] In a yeast two-hybrid screen, ROXY1 was found to interact with TGA family of transcription factors.[36] Recently, complementation analysis revealed the conserved function in floral organ development of orthologs of ROXY1 and ROXY2 in rice as well.[13] In our study also, we found the preferential expression of *OsROXY1* (*OsGRX8*) during stages of panicle development, which further validates its role in floral organ development. Similarly, other *OsGRX* genes expressed preferentially in specific tissue/developmental stage might perform specific function crucial for the development.

3.6. *Differential expression of OsGRX genes in response to hormone treatments*

We also studied the expression profiles of *OsGRX* genes in rice seedlings subjected to various hormone treatments auxin (IAA), cytokinin (BAP), abscisic acid (ABA), ethylene derivative (ACC), salicylic acid (SA) and jasmonic acid (JA)). A total of 11 GRX genes showed differential expression more than 2-fold in at least one hormone treatment as compared to control (Fig. 5A). Six (*OsGRX3*, *GRX6*, *GRX8*, *GRX9*, *GRX14* and *GRX28*) genes showed up-regulation, while three (*OsGRX2*, *GRX27* and *GRX29*) genes showed down-regulation in different hormone treatments as compared to control. *OsGRX23* showed down-regulation in response to IAA, ABA, SA and JA, however, up-regulation in response to BAP. *OsGRX16* was up-regulated in response to ABA, but down-regulated in response to BAP. Five of *OsGRX* genes showed response to specific hormone treatments only, while others were responsive to two or more hormone treatments. The fold-change values of *OsGRX* genes showing differential expression is given in Supplementary Table S9. The real-time PCR results of selected differentially expressed genes under various hormone treatments were consistent with the microarray data (Fig. 5B). In *Arabidopsis* also, at least eight GRX genes (five CC type and three GRL type) showed significant differential expression in response to various plant hormones (Supplementary Fig. S5B; Supplementary Table S10). A very little information is available regarding the direct link between GRX and hormonal response so far. One of the TGA-interacting GRX protein has been proposed as a potential regulatory component of SA/JA antagonism.[14] Our results clearly show that GRXs might play important role in response to various plant hormones.

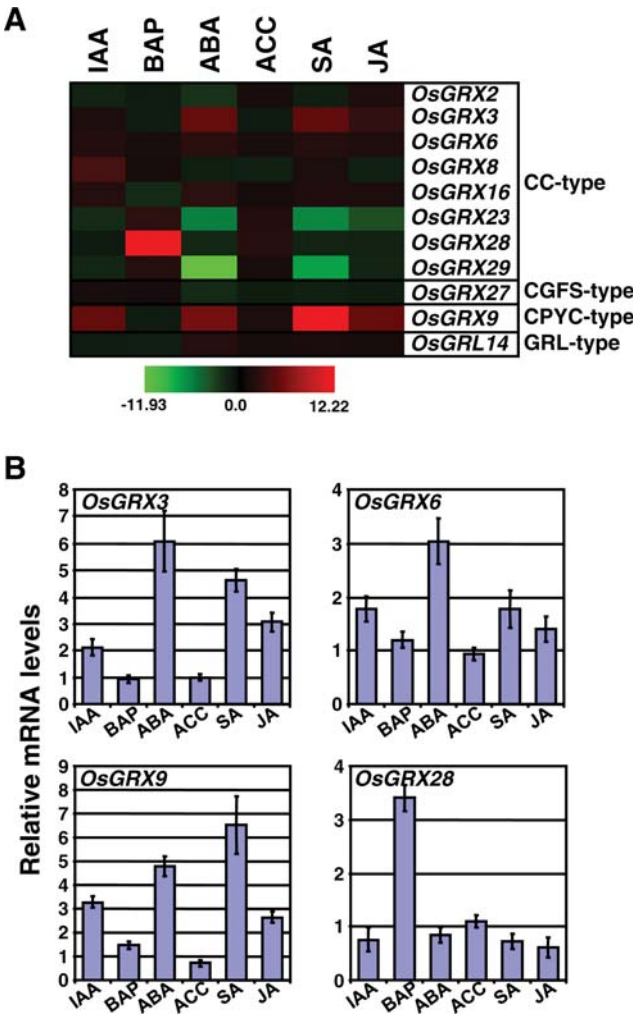
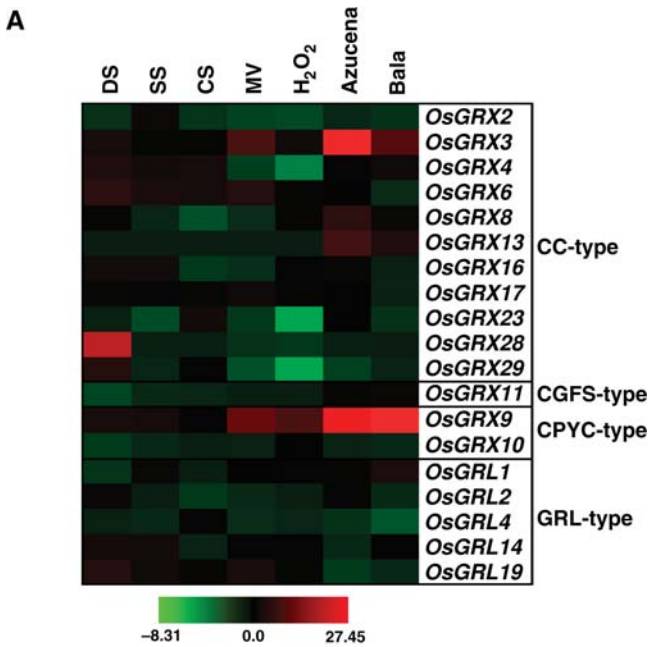


Figure 5. Expression profiles of *OsGRX* genes showing differential expression in response to various hormone treatments. (A) Heatmap showing the differential expression of *OsGRX* genes in response to various hormone treatments. The heatmap has been generated based on the fold-change values in the treated sample when compared with its mock-treated control sample. The color scale for fold-change values is shown at the bottom. (B) Validation of differential expression of representative GRX genes in response to various hormone treatments. Relative mRNA levels of GRX genes in response to various hormone treatments when compared with its mock-treated control sample are shown. IAA, auxin; BAP, cytokinin; ABA, abscisic acid; ACC, ethylene derivative; SA, salicylic acid; JA, jasmonic acid.

3.7. *Differential expression of OsGRX genes in response to different stresses*

To gain insight into the role of *OsGRX* genes during various abiotic stress conditions, their expression patterns were investigated in rice seedlings subjected to desiccation, salt, cold, methyl viologen, hydrogen peroxide and arsenate stress. A total of 19 (40%) GRX genes showed differential expression under at least one of the abiotic stress conditions analyzed (Fig. 6A). Notably, 11 of these genes belong to CC-type and five from GRL-type class. Some of them were regulated by a specific stress condition only; however, others were regulated by more than one stress condition. The fold-change values of *OsGRX* genes showing differential expression is given in Supplementary Table S9. The validation of expression profiles of some of the selected genes by real-time PCR analysis confirmed their differential expression (Fig. 6B). Likewise, a total of 23 (48%) of *AtGRX* genes, including 14 CC type, 1 CGFS type, 3 CPYC type and 5 GRL type, were differentially expressed in root and shoot tissues subjected to various abiotic stress conditions (Supplementary Fig. S5C; Supplementary Table S10).

We also analyzed the response of *OsGRX* genes to infection of fungus *M. grisea* and obligate root hemiparasite *S. hermonthica*, which cause severe loss to rice yield. For this analysis, we used microarray data from two earlier studies on transcriptome analysis of rice (Nipponbare) after infection with *M. grisea* and gene



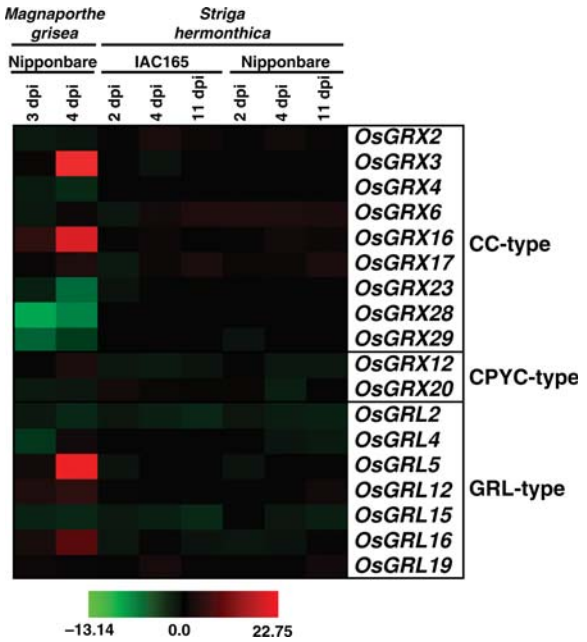


Figure 7. Expression profiles of *OsGRX* genes showing differential expression in response to various biotic stress treatments. Heatmap showing the differential expression of *OsGRX* genes in response to various biotic stress treatments. The heatmap has been generated based on the fold-change values in the treated sample when compared with its mock-treated control sample. The color scale for fold-change values in shown at the bottom. dpi, days post inoculation.

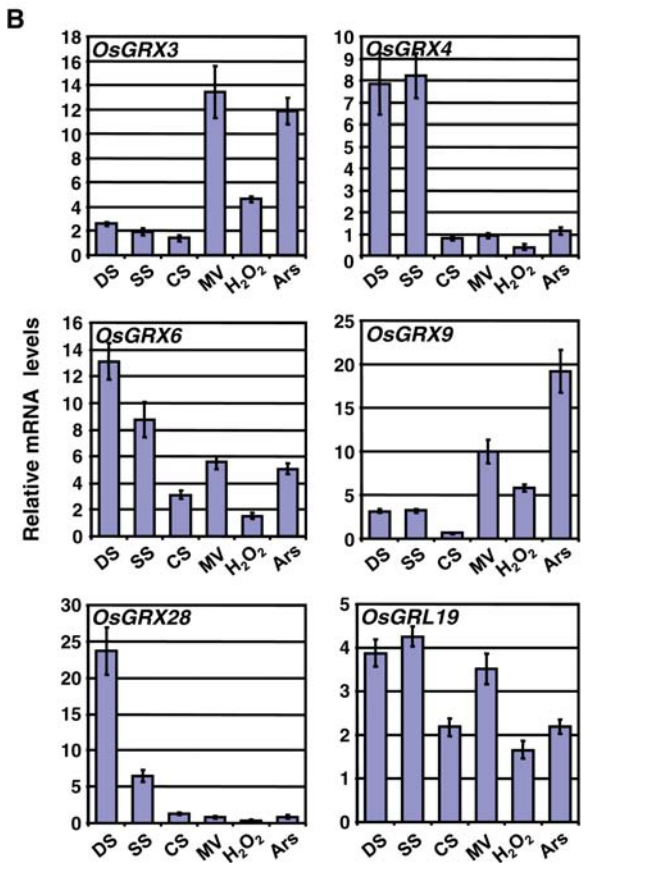


Figure 6. Expression profiles of *OsGRX* genes showing differential expression in response to various abiotic stress treatments. (A) Heatmap showing the differential expression of *OsGRX* genes in response to various abiotic stress treatments. The heatmap has been generated based on the fold-change values in the treated sample when compared with its mock-treated control sample. The color scale for fold-change values in shown at the bottom. (B) Validation of differential expression of representative GRX genes in response to various abiotic stress treatments. Relative mRNA levels of GRX genes in response to various abiotic stress treatments when compared with its mock-treated control sample are shown. DS, desiccation; SS, salt; CS, cold; MV, methyl viologen; H_2O_2, hydrogen peroxide, Ars, arsenate stress. Azucena and Bala represent arsenate-sensitive and arsenate-resistant rice varieties, respectively.

expression profiling in roots of susceptible (IAC165) and highly resistant (Nipponbare) cultivars after infection with *S. hermonthica*.[24,25] The data analysis revealed that a total of 18 *OsGRX* genes showed response to biotic stress conditions (Fig. 7). Among these, nine genes belong to CC-type class. The fold-change values of *OsGRX* genes showing differential expression is given in Supplementary Table S9. These results further confirm that members of CC-type class GRX genes are more functionally evolved as compared to other classes.

The role of GRXs in stress responses has been proposed in many studies. The *Arabidopsis* chloroplastic GRXs, *AtGRXcp* and *AtGRX4*, were induced by various stresses and suppressed the yeast *grx5* mutant sensitivity to oxidants.[9,10] The *atgrxcp* mutant plants showed defects in early seedling growth under oxidative stress suggesting the biological role in protecting cells against oxidative damage.[9] The overexpression of *Pteris vittata* GRX, *PvGRX5*, improved arsenic tolerance in transgenic *Arabidopsis* plants via decreasing arsenic accumulation.[40] The analysis of transgenic plants

overexpressing *OsWRKY13* revealed that OsWRKY-associated disease resistance pathway positively interact with GSH/GRX system to monitor redox homeostatsis.[41] Recently, it has been reported that the overexpression of rice CC-type GRXs in *Arabidopsis* led to an increased accumulation of H_2O_2 levels and hyper-susceptibility to infection from necrotrophic pathogen *Botrytis cinerea*.[13] Several plant GRX targets have been identified which are involved in various cellular processes and located in different subcellular compartments. The study suggested that GRXs have a broad capacity to reduce oxidized disulfides and to provide electrons to many enzymatic reduction reactions maintaining redox homeostasis.[42] Taken together, the differential expression of several *OsGRX* genes indicates their important role in various abiotic and biotic stress responses and they might be involved in maintaining redox homeostasis during stress conditions and overexpression of few of these might impart stress tolerance in transgenic plants.

3.8. *Duplicated GRX genes exhibit redundant and divergent expression patterns*

Gene duplication is an important event, which gives rise to functional redundancy and diversification. Gene duplication may lead to sub- or neo-functionalization in addition to non- or hypo-functionalization.[43] We found many segmentally duplicated and a few tandemly duplicated *OsGRX* genes. We investigated differences in the expression patterns of duplicated *OsGRX* genes to reveal the role of gene duplication in functional diversification. The expression data were available for six gene pairs present on duplicated chromosomal segments and one gene pair localized in tandem. Among the six gene pairs, three, *OsGRX2/16*, *OsGRX6/17* and *OsGRX8/13*, showed similar expression patterns with high (>0.75) Pearson correlation coefficient (PCC). Both the genes in gene pair *OsGRL9/17* showed very low expression levels in all the tissues examined. Two gene pairs *OsGRX9/14* and *OsGRL7/14* showed highly divergent expression patterns with PCC value of 0.19 and -0.45, respectively. One gene pair (*OsGRX28/29*), which is tandemly localized, also showed divergent expression patterns with PCC value of 0.56. The gene pairs with divergent expression patterns might represent the events of sub- or neo-functionalization, suggesting their functional diversification during evolution.

3.9. *Conclusions*

In this study, we have identified 48 GRX genes in rice and compared with those of *Arabidopsis*. The GRX proteins formed four classes as supported by phylogeny and motif organization. Gene duplication analysis revealed that expansion of this gene family has occurred independently in rice and *Arabidopsis* via whole genome duplication events. Further, the phylogenetic analysis showed that GRX gene family has expanded in species-specific manner after monocot–dicot split. The expression analysis revealed the differential temporal and spatial expression patterns of *OsGRX* genes, which suggested their role in regulating plant growth and development throughout the lifecycle of plant. Further, we found the expression of *OsGRX* genes is influenced by several environmental stimuli, including hormones, abiotic stress and biotic stress conditions indicating their role in hormonal and stress responses. Although from our expression analysis results in rice and *Arabidopsis* (Figs 4–7; Supplementary Fig. S5), the GRX proteins from different classes could not be linked to a specific function and/or response pattern, it is clear that CC-type class GRX proteins are most functionally evolved and are involved in diverse biological processes, including development and response to various environmental stimuli (hormone, abiotic and biotic stress). However, the GRL-type class GRX proteins appear to be more specifically involved in response to various environmental stimuli. Previously, the members of CGFS and CPYC type have been linked only to response to oxidative stress. Our results also support their role in various stress responses. However, the functional validation and biochemical characterization of various members will only provide definitive clues about the specific roles of different classes of GRX proteins. The data provided in this study will serve as useful information for further in-depth functional analysis of *GRX* genes in rice and *Arabidopsis*.

Supplementary Data: Supplementary Data are available at www.dnaresearch.oxfordjournals.org.

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