Identification and Comparative Analysis of H_{2}O_{2}-Scavenging Enzymes (Ascorbate Peroxidase and Glutathione Peroxidase) in Selected Plants Employing Bioinformatics Approaches

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Among major reactive oxygen species (ROS), hydrogen peroxide (H_{2}O_{2}) exhibits dual roles in plant metabolism. Low levels of H_{2}O_{2} modulate many biological/physiological processes in plants; whereas, its high level can cause damage to cell structures, having severe consequences. Thus, steady-state level of cellular H_{2}O_{2} must be tightly regulated. Glutathione peroxidases (GPX) and ascorbate peroxidase (APX) are two major ROS-scavenging enzymes which catalyze the reduction of H_{2}O_{2} in order to prevent potential H_{2}O_{2}-derived cellular damage. Employing bioinformatics approaches, this study presents a comparative evaluation of both GPX and APX in 18 different plant species, and provides valuable insights into the nature and complex regulation of these enzymes. Herein, (a) potential GPX and APX genes/proteins from 18 different plant species were identified, (b) their exon/intron organization were analyzed, (c) detailed information about their physicochemical properties were provided, (d) conserved motif signatures of GPX and APX were identified, (e) their phylogenetic trees and 3D models were constructed, (f) protein-protein interaction networks were generated, and finally (g) GPX and APX gene expression profiles were analyzed. Study outcomes enlightened GPX and APX as major H_{2}O_{2}-scavenging enzymes at their structural and functional levels, which could be used in future studies in the current direction.

Keywords: ROS, signal transduction, antioxidant, peroxisome, chloroplast, mitochondria
INTRODUCTION

Reactive oxygen species (ROS), once perceived as toxic by-products, were known to cause oxidative damage in cells (Mittler et al., 2004; Suzuki and Mittler, 2006). Later, novel regulatory roles of these species were revealed in a wide range of biological processes such as cell signaling, growth, development, programmed cell death, and plant responses to various biotic/abiotic stress factors (Mullineaux and Karpinski, 2002; Uzilday et al., 2014). \( \text{H}_2\text{O}_2 \) is an endogenous ROS species known to play a dual role in plants, where it is beneficial at low concentrations but lethal at higher levels (Petrov and Van Breusegem, 2012). Nevertheless, at steady state levels, \( \text{H}_2\text{O}_2 \) acts as signaling molecule inducing the signal transduction mechanism to produce various cellular responses. Interestingly, pre-treatment of plants with \( \text{H}_2\text{O}_2 \) makes them more tolerant to biotic/abiotic stresses (Hossain et al., 2015). \( \text{H}_2\text{O}_2 \) was also noted for its regulatory functions in photosynthesis, cell cycle, development, senescence, and apoptosis (Mittler et al., 2004; Petrov and Van Breusegem, 2012). \( \text{H}_2\text{O}_2 \) has been accepted as a central component of signal transduction pathways in plant-adaptation to altered environmental conditions as it is both the only ROS with high permeability across membranes (that enables the transport of signals to distant sites) and its high stability when compared to other ROS with \( \sim 1 \text{ms} \) half-life (Bienert et al., 2007; Dynowski et al., 2008; Petrov and Van Breusegem, 2012). On the other hand, when the delicate balance between production and scavenging of \( \text{H}_2\text{O}_2 \) is disturbed, its overproduction results in significant damage to cell structures (Anjum et al., 2015; Sofo et al., 2015). To overcome \( \text{H}_2\text{O}_2 \)-related cellular damage, aerobic organisms have developed various antioxidant machineries with enzymatic and non-enzymatic components. Ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT) are the main enzymes responsible for suppressing toxic levels of \( \text{H}_2\text{O}_2 \) (Apel and Hirt, 2004). However, APX may have pivotal roles in ROS-scavenging because even very low concentrations are sufficient for \( \text{H}_2\text{O}_2 \) decomposition (Anjum et al., 2014; Sofo et al., 2015).

APX (EC, 1.11.1.11) belongs to the plant-type heme peroxidase superfamily in plants (Lazzarotto et al., 2011). Genome-wide studies demonstrated that APX in higher plants is encoded by multigenic families. Arabidopsis was reported to contain nine APX genes; whereas, rice has eight and tomato seven (Chew et al., 2003; Teixeira et al., 2004; Najami et al., 2008). Different isoforms are classified into sub-families according to their subcellular localization. Transmembrane domains in N- and C-terminal regions, as well as organelle-specific target molecules are the primary determinants in target localization of APXs (Ishikawa et al., 1998; Negi, 2011). Among nine APX genes identified in Arabidopsis, three were found to be encoded in cytosol whereas the other six were distributed in stroma, thylakoid, and peroxisome (Chew et al., 2003; Mittler et al., 2004). In rice, chloroplastic isoforms were expressed by three genes, cytosolic and peroxisomal forms were both encoded by two genes, and one gene was for the mitochondrial APX (Teixeira et al., 2006; Anjum et al., 2014). APX activity was also reported to increase under various stress conditions. For example, APX is differentially upregulated in response to heavy metal, drought, water, and heat stress (Sharma and Dubey, 2005; Koussevitzky et al., 2008; Yang et al., 2008; Anjum et al., 2014). In a previous study, Arg-38, Glu-65, Asn-71, and Asp208 residues were reported to be conserved among the entire APX family and known to be important in ligand (heme)-binding (Welinder, 1992). In addition to enzymatic properties, structural investigations on catalytic domains of the enzymes have been also performed. Three-dimensional structures of cAPX, sAPX, and their substrates showed the relationship between loop structure and stability in the absence of ascorbate (AsA; Yabuta et al., 2000; Anjum et al., 2014). The mitochondrial and chloroplastic APXs (<30 s) have shorter half inactivation times (>1 h) compared to cytosolic and peroxisomal isoforms, which makes them more sensitive in either low concentrations or the absence of AsA (Caverzan et al., 2012; Anjum et al., 2014). Another important enzyme in \( \text{H}_2\text{O}_2 \)-scavenging is the GPX from the non-heme containing peroxidase family (Bela et al., 2015). In Arabidopsis, eight GPX genes were reported (Milla et al., 2003; Koua et al., 2009). Based on in silico analysis, GPXs were predicted in chloroplast, mitochondria, cytosol, and ER localizations (Rouhier and Jacquot, 2005), and demonstrated high level of sequence similarity with strictly conserved cysteines and motifs (Dietz, 2011). Plant GPXs have cysteine residue in their active site (Koua et al., 2009), which is functional in both glutathione (GSH) and thiol peroxidase classes of the non-heme family. GPXs were also reported to be involved in stress responses. Many studies have demonstrated the significant increase in mRNA levels of GPXs under various abiotic/abiotic stress conditions such as oxidative stress, pathogen attack, metal, cold, drought, and salt (Navrot et al., 2006; Diao et al., 2014; Fu, 2014; Gao et al., 2014). For example, GPX genes were found to be upregulated under excess \( \text{H}_2\text{O}_2 \) and cold stresses in rice (Passaia et al., 2013). Transcriptome analysis indicated high level of GPX transcripts in dehydrated Glycine max samples (Criqui et al., 1992; Ferreira Neto et al., 2013). Several transgenic studies also supported the proposed function of GPXs. For example, the overexpression of GPX in its transgenic tomato resulted in higher tolerance against abiotic stress (Herbette et al., 2011). In addition to stress response, GPXs are also thought to regulate cellular redox homeostasis by modulating the thiol-disulfide balance (Bela et al., 2015). GPX expression was found to be highly upregulated to maintain redox homeostasis under oxidative stress which helped Brassica rapa to adapt long-term spaceflight (Sugimoto et al., 2014).

A scan of contemporary literature reveals a paucity of information on the identification and comparative analysis of GPX and APX in model and economically important food crops. Given the above, employing bioinformatics approaches, efforts were made in this study (a) to identify potential GPX and APX genes/proteins from 18 different plant species, (b) to analyze their exon/intron organization, (c) to provide detailed information about their physico-chemical properties, (d) to identify conserved motif signatures of GPX and APX, (e) to construct their phylogenetic trees and 3D models, (f) to generate protein-protein interaction networks, and finally (g) to analyze GPX and APX gene expression profiles.
MATERIALS AND METHODS

Retrieval of GPX and APX Genes/Proteins

Eight Arabidopsis GPX reference protein sequences such as GPX1 (P52032.2), GPX2 (O04922.1), GPX3 (O22850.1), GPX4 (Q8L103.1), GPX5 (Q9LYB4.1), GPX6 (O48646.2), GPX7 (Q9S5Z4.2), and GPX8 (Q8LB2U.1), as well as eight Arabidopsis APX reference sequences such as APX1 (Q05431.2), APX2 (Q1PER6.3), APX3 (Q42564.1), APX4 (P82281.2), APX5 (Q7XZP5.2), APX6 (Q8GY91.1), APX7 (Q42593.2), and APX5 (Q42592.2) were obtained from UniProtKB/Swiss-Prot database of NCBI (Romiti, 2006). These reference sequences were queried in proteome datasets of selected 18 plant species: Arabidopsis thaliana (L.) Heynh., Brachypodium distachyon (L.) P. Beauv., Brassica rapa L., Chlamydomonas reinhardtii P. A. Dang., Cucumis sativus L., Eucalyptus grandis W. Hill ex Maiden, Glycine max (L.) Merr., Gossypium raimondii Ulbr., Medicago truncatula Gaertn., Oryza sativa L., Phaseolus vulgaris L., Physcomitrella patens (Hedw.) Bruch & Schimp., Populus trichocarpa Torr. & A.Gray ex. Hook., Prunus persica (L.) Batsch, Solanum lycopersicum L., Sorghum bicolor (L.) Moench, Vitis vinifera L., and Zea mays L., all found in the Phytozome v.10.3 database (Goodstein et al., 2012). After sequences were obtained, the Hidden Markov Model (HMM) search of protein sequences were performed by Pfam (http://pfam.sanger.ac.uk) to confirm the protein domain families (Finn et al., 2016). Species were arbitrarily selected to represent the main plant groups such as monocots, dicots, and lower plants.

Analysis of GPX and APX Genes/Proteins

Physicochemical properties of GPX and APX proteins were determined by using ProtParam tool (Gasteiger et al., 2005). Sub-cellular localization was predicted by CELLO (Yu et al., 2006) and WoLF PSORT (Horton et al., 2007) servers. Exon-intron organization of GPX/APX genes was analyzed by using a GSDS server (Hu et al., 2014). The Conserved motif structure of GPX/APX sequences was analyzed using the MEME tool with the following parameter settings: maximum number of motifs to find, 5; minimum width of motif, 6 and maximum width of motif, 50 (Bailey et al., 2009). Protein sequences were aligned by ClustalW (Thompson et al., 1994) and phylogenies were constructed by MEGA 6 (Tamura et al., 2013) with the maximum likelihood (ML) method for 1,000 bootstraps. The gene duplication events were detected using the following criteria: (a) length of alignable sequence covers >75% of the longer gene, and (b) similarity of aligned regions is >75% (Gu et al., 2002). The expression data of APX and GPX genes at anatomical and developmental levels were retrieved from the Genevestigator database (Hruz et al., 2008). 3D models of APX/GPXs were predicted by using the Phyre² server (Kelley and Sternberg, 2009). Model validation was performed by Rampage Ramachandran plot analysis (Lovell et al., 2003). 3D structure comparisons were done by calculating RMSD values of models using the CLICK server employing α-carbon superposition (Nguyen et al., 2011). Putative interaction partners of APX/GPXs were predicted with the STRING server (Franceschini et al., 2013) and an interactome network was generated using cytoscape (Smoot et al., 2011).

RESULTS AND DISCUSSION

H₂O₂ plays double roles in plants and modulates various crucial metabolic processes (Petrov and Van Breusegem, 2012). However, its increased levels can cause severe damage to cell structures; hence, steady-state level of cellular H₂O₂ is required to be tightly regulated (Anjum et al., 2014, 2015; Sofo et al., 2015). GPX and APX are two major ROS-scavenging enzymes which catalyze the reduction of H₂O₂ to prevent H₂O₂-derived cellular damage. In order to understand the structural, functional as well as evolutionary aspects of GPX and APX, employing bioinformatics approaches, this study attempted to present comparative analyses of putative GPX and APX homologs identified from 18 plant species.

Analysis of GPXs

Retrieval of GPX Genes/Proteins

Eight potential Arabidopsis GPX protein sequences, namely GPX1-8, obtained from the UniProtKB/Swiss-Prot database of NCBI were used as queries in Phytozome database to retrieve the very close homologs of GPX sequences in 18 plant species. In the selection of GPX homologs from blastp hits, very strict criteria (only the highest hit sequence) was applied to avoid the redundant sequences and alternative splices of the same gene. A total of 87 GPX sequences were identified from the protein datasets of 18 plant species. These include; 8 genes for A. thaliana, 4 genes for B. distachyon, 8 genes for B. rapa, 1 gene for C. reinhardtii, 6 genes for C. sativus, 5 genes for E. grandis, 5 genes for G. max, 6 genes for G. raimondii, 5 genes for M. truncatula, 5 genes for O. sativa, 5 genes for P. vulgaris, 2 genes for P. patens, 5 genes for P. trichocarpa, 5 genes for P. persica, 5 genes for S. lycopersicum, 4 genes for S. bicolor, 5 genes for V. vinifera, and 3 genes for Z. mays (Table 1). Then, genomic, transcript, CDS, and protein sequences of identified 87 GPX sequences were retrieved for further analyses.

Sequence Analysis of GPX Genes/Proteins

A total of 87 GPX homologs were identified in the protein datasets of 18 plant species using Arabidopsis GPX1-8 for homology search. Identified GPX homologs belonged to the GSHPx (PF00255) protein family. They encoded a polypeptide of 166–262 amino acids residues (average length 197.5) and 18.4–29.7 kDa molecular weight with 4.59–9.60 pI value. The sequence variations in analyzed GPXs primarily derived from the very close homologs of GPX sequences in 18 plant species. These include: 8 genes for A. thaliana, 4 genes for B. distachyon, 8 genes for B. rapa, 1 gene for C. reinhardtii, 6 genes for C. sativus, 5 genes for E. grandis, 5 genes for G. max, 6 genes for G. raimondii, 5 genes for M. truncatula, 5 genes for O. sativa, 5 genes for P. vulgaris, 2 genes for P. patens, 5 genes for P. trichocarpa, 5 genes for P. persica, 5 genes for S. lycopersicum, 4 genes for S. bicolor, 5 genes for V. vinifera, and 3 genes for Z. mays (Table 1). Studies of molecular cloning and sequencing in A. thaliana have reported that chloroplastic GPX1 and GPX7 consists of 236 and 233 amino acids, respectively; the first 1–64 residues in GPX1 and 1–69 residues in GPX7 from N-terminal site contained the transit peptide (Mullineaux et al., 1998; Lin et al., 1999; Mayer et al., 1999). Arabidopsis GPX2 and GPX4 were reported to be 169 and 170 residues, respectively with cytosolic localization: thereby, they did not contain any transit peptide (Lin et al., 1999). Arabidopsis GPX3 and GPX6 were 206
| Species name                          | Phytozome gene ID   | Protein domain family\(^a\) | Domain family description | Exon no. | Protein length (KDa) | MW Theor. | Localization CELLO\(^b\) | Localization WoLF PSORT\(^b\) |
|--------------------------------------|---------------------|-----------------------------|---------------------------|----------|----------------------|-----------|--------------------------|-------------------------------|
| Arabidopsis thaliana (L.) Heynh.     | AT1G63460           | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 167                  | 19.0      | Cyto                     | Cyto                          |
|                                      | AT2G25080           | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 236                  | 26.0      | Chlo/Mito                | Chlo                          |
|                                      | AT2G31570           | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 169                  | 18.9      | Cyto                     | Chlo                          |
|                                      | AT2G43350           | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 206                  | 23.2      | Mito/Plas                | Chlo/Mito                     |
|                                      | AT2G48150           | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 170                  | 19.3      | Cyto                     | Mito                          |
|                                      | AT3G63080           | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 173                  | 19.3      | Extr/Chlo/Nuc            | Chlo                          |
|                                      | AT4G31600           | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 232                  | 25.5      | Mito/Chlo                | Mito                          |
|                                      | AT4G31870           | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 233                  | 25.7      | Chlo                     | Chlo                          |
| Brachypodium distachyon (L.) P.Beauv.| Brad1g47140         | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 226                  | 24.4      | Chlo                     | Chlo                          |
|                                      | Brad1g61930         | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 198                  | 22.4      | Cyto                     | Chlo                          |
|                                      | Brad3g51010         | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 240                  | 25.9      | Chlo                     | Chlo                          |
|                                      | Brad5g18000         | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 168                  | 18.4      | Cyto/Chlo/Nuc            | Chlo                          |
| Brasica rapa L.                      | Brara.B02692        | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 229                  | 25.2      | Mito                    | Chlo/Mito                     |
|                                      | Brara.C02198        | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 197                  | 21.9      | Extr/Plas               | Extr                          |
|                                      | Brara.E00003        | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 170                  | 19.2      | Extr/Cyto               | Cyto                          |
|                                      | Brara.G01994        | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 176                  | 19.5      | Extr/Cyto/Nuc           | Chlo                          |
|                                      | Brara.I01234        | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 167                  | 18.9      | Cyto                   | Cyto                          |
|                                      | Brara.I04448        | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 233                  | 25.8      | Mito/Chlo/Extr          | Chlo                          |
|                                      | Brara.K00392        | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 232                  | 25.9      | Mito/Extr               | Chlo                          |
| Chlamydomonas reinhardtii P.A.Dang.  | Cre03.g197750       | GSHPx (PF00255)             | Glutathione peroxidase    | 7        | 200                  | 21.9      | Mito                   | Chlo                          |
| Cucumis sativus L.                   | Cucsa.084960        | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 176                  | 19.7      | Cyto                   | Chlo                          |
|                                      | Cucsa.094950        | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 204                  | 23.4      | Plas/Extr               | Chlo                          |
|                                      | Cucsa.184280        | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 170                  | 19.0      | Cyto/Extr              | Chlo                          |
|                                      | Cucsa.271420        | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 241                  | 26.4      | Chlo                   | Chlo                          |
|                                      | Cucsa.303050        | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 241                  | 26.8      | Mito                   | Chlo/Chlo                     |
|                                      | Cucsa.303070        | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 170                  | 19.2      | Cyto                   | Cyto                          |
| Eucalyptus grandis W. Hill ex Maiden | Eucgr.A00257        | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 202                  | 22.8      | Extr/Chlo              | Chlo/Vacu                     |
|                                      | Eucgr.C02602        | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 247                  | 26.9      | Chlo                   | Chlo                          |
|                                      | Eucgr.D01856        | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 170                  | 19.4      | Cyto                   | Cyto                          |
|                                      | Eucgr.E00579        | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 250                  | 27.3      | Chlo                   | Chlo                          |
|                                      | Eucgr.K03399        | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 170                  | 18.9      | Cyto                   | Chlo/Chlo                     |
| Glycine max (L.) Merr.               | Glyma.03G151500     | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 170                  | 19.0      | Mito/Cyto             | Chlo                          |
|                                      | Glyma.05G240100     | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 199                  | 22.7      | Extr                  | Chlo                          |
|                                      | Glyma.08G013900     | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 167                  | 18.9      | Cyto                | Chlo                          |
|                                      | Glyma.11G024100     | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 167                  | 18.5      | Cyto               | Chlo                          |
|                                      | Glyma.17G223900     | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 234                  | 26.3      | Mito/Chlo        | Chlo                          |
| Gossypium raimondii Ubr.             | Gorai.001G038600    | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 242                  | 26.6      | Chlo                   | Chlo                          |
|                                      | Gorai.004G083200    | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 171                  | 19.1      | Nucl/Cyto/Extr          | Nucl                          |
|                                      | Gorai.004G087300    | GSHPx (PF00255)             | Glutathione peroxidase    | 6        | 208                  | 23.6      | Extr                  | Extr                          |

(Continued)
| Species name | Phytozome gene ID | Protein domain family | Domain family description | Exon | Protein length (KDa) | MW Theor. pl | Localization CELLO | Localization WoLF PSORT |
|--------------|------------------|-----------------------|---------------------------|------|----------------------|-------------|-------------------|------------------------|
| Gorai.004G211400 | GSHPx (PF00255) | Glutathione peroxidase | 5 | 166 | 18.4 | 6.73 | Cyto | Chlo |
| Gorai.006G186100 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 168 | 18.7 | 6.73 | Cyto | Chlo |
| Gorai.008G246600 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 168 | 19.1 | 4.59 | Cyto | Chlo |
| Zea mays | GRMZM2G012479 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 230 | 24.9 | 9.55 | Mito | Chlo |
| Vitis vinifera | GSVIVG01010737001 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 167 | 18.6 | 5.53 | Cyto | Chlo |
| Oryza sativa | LOC_Os02g44500 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 238 | 25.8 | 9.42 | Chlo | Chlo |
|  | LOC_Os03924380 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 169 | 19.2 | 8.80 | Cyto | Chlo |
|  | LOC_Os06946960 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 168 | 18.4 | 8.33 | Cyto | Chlo |
|  | LOC_Os06909760 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 234 | 25.8 | 9.51 | Mito/Chlo | Chlo |
|  | LOC_Os1118170 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 212 | 22.9 | 7.62 | Chlo/Extr | Chlo |
| Medicago truncatula | Medtr1g014210 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 236 | 26.4 | 9.32 | Mito/Chlo | Chlo |
|  | Medtr7g0944600 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 170 | 19.2 | 9.18 | Cyto/Mito | Nucl |
|  | Medtr8g098400 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 172 | 19.3 | 4.82 | Cyto | Chlo |
|  | Medtr8g098410 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 233 | 25.8 | 9.27 | Mito | Chlo |
|  | Medtr8g105630 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 167 | 18.9 | 8.32 | Plas | Chlo |
| Physcomitrella patens (Hedw.) Bruch & Schimp. | Phpat.004G3103100 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 247 | 26.7 | 9.24 | Chlo/Extr | Chlo |
|  | Phpat.017G045400 | GSHPx (PF00255) | Glutathione peroxidase | 1 | 170 | 19.1 | 8.30 | Cyto | Chlo |
| Phaseolus vulgaris L. | Phvul.001G041100 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 262 | 29.7 | 9.68 | Mito | Chlo |
|  | Phvul.001G190900 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 168 | 18.8 | 9.31 | Cyto/Nucl | Nucl |
|  | Phvul.002G157200 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 170 | 19.0 | 4.97 | Cyto | Chlo/Nucl |
|  | Phvul.002G288700 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 230 | 25.6 | 8.76 | Chlo/Mito | Chlo |
|  | Phvul.002G322400 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 198 | 22.5 | 5.94 | Extr | Extr |
| Populus trichocarpa Torr. & A.Gray ex. Hook. | Popul.001G105100 | GSHPx (PF00255) | Glutathione peroxidase | 5 | 170 | 19.3 | 4.78 | Cyto | Chlo |
|  | Popul.003G126100 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 238 | 26.2 | 9.29 | Mito/Chlo | Chlo |
|  | Popul.006G265400 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 232 | 25.3 | 9.48 | Chlo/Mito | Chlo |
|  | Popul.007G126600 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 203 | 22.8 | 8.63 | Extr | Extr/Vacu |
|  | Popul.014G138800 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 170 | 18.9 | 9.15 | Cyto/Extr | Chlo/Extr |
| Prunus persica (L.) Batsch | ppa010584m.g | GSHPx (PF00255) | Glutathione peroxidase | 6 | 244 | 26.7 | 9.33 | Chlo | Chlo |
|  | ppa010771m.g | GSHPx (PF00255) | Glutathione peroxidase | 6 | 237 | 25.9 | 9.20 | Mito | Chlo |
|  | ppa01168m.g | GSHPx (PF00255) | Glutathione peroxidase | 6 | 200 | 22.7 | 8.27 | Extr/Cyto | Extr |
|  | ppa012378m.g | GSHPx (PF00255) | Glutathione peroxidase | 6 | 172 | 19.4 | 8.97 | Cyto | Nucl/Cyto |
|  | ppa012416m.g | GSHPx (PF00255) | Glutathione peroxidase | 6 | 170 | 19.4 | 4.86 | Cyto | Chlo |
| Sorghum bicolor (L.) Moench | Sobic.001G365800 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 171 | 19.3 | 8.79 | Cyto | Chlo |
|  | Sobic.006G173900 | GSHPx (PF00255) | Glutathione peroxidase | 6 | 168 | 18.4 | 6.58 | Cyto | Chlo/Nucl |

(Continued)
TABLE 1 | Continued

| Species name          | Phytozome gene ID | Protein domain family\(^a\) | Domain family description | Exon no. | Protein length (KDa) | MW [kDa] | Theor. pl | Localization CELLO\(^b\) | Localization WoLF PSORT\(^b\) |
|-----------------------|-------------------|-----------------------------|---------------------------|----------|----------------------|----------|----------|--------------------------|-------------------------------|
| Sobic.010G0671000     | GSHPx (PF00255)   | Glutathione peroxidase       | 6                         | 232      | 24.9                 | 9.50     | Cyto     | Mito/Chlo                |                               |
| Sobic.K022000         | GSHPx (PF00255)   | Glutathione peroxidase       | 6                         | 205      | 22.6                 | 5.68     | Cyto     | Mito/Chlo                |                               |
| Solanum lycopersicum  |                   |                             |                           |          |                      |          |          |                          |                               |
| Solyco06g073460.2     | GSHPx (PF00255)   | Glutathione peroxidase       | 6                         | 167      | 18.9                 | 6.37     | Cyto     | Chlo                     |                               |
| Solyco08g006720.2     | GSHPx (PF00255)   | Glutathione peroxidase       | 6                         | 238      | 26.2                 | 9.18     | Chlo     | Chlo                     |                               |
| Solyco08g080840.2     | GSHPx (PF00255)   | Glutathione peroxidase       | 6                         | 239      | 26.7                 | 9.16     | Mito     | Chlo                     |                               |
| Solyco09g064850.2     | GSHPx (PF00255)   | Glutathione peroxidase       | 6                         | 170      | 19.0                 | 9.33     | Mito     | Mito/Extr                |                               |
| Solyco12g056240.1     | GSHPx (PF00255)   | Glutathione peroxidase       | 6                         | 170      | 19.3                 | 4.97     | Cyto     | Cyto                     |                               |

\(^a\)Protein domain families were searched in Pfam database.

\(^b\)Cyto, Cytosolic; Chlo, Chloroplastic; Mito, Mitochondrial; Vacu, Vacuolar; Nucl, Nuclear; Extr, Extracellular; Plas, Plasma membrane.

More than one localization specified in a single column also shows the significance of other entries in order.

and 232 residues, respectively, with mitochondrial localizations; the first 1–12 amino acids in GPX3 and 1–54 residues in GPX6 covered the transit peptide (Lin et al., 1999; Mayer et al., 1999). Arabidopsis GPX5 was 173 residues with probable ER or Plasma membrane localization, without transit peptide (Erfle et al., 2000). Arabidopsis GPX8 comprised of 167 amino acids with cytosolic or nuclear localization, without transit peptide (Theologis et al., 2000). In the present study, alignment analysis revealed that in chloroplastic/mitochondrial-related GPXs, the transit peptide sequences formed the first 50–90 amino acid residues from the N-terminal site while in extra cellular/plasma membrane-related GPXs, residues of the first 20–50 amino acid from N-terminal region contained the putative transit peptide. However, cytosolic sequences lacked of any putative transit residues (Supplementary Figure S1). Thus, analyzed GPX sequences were roughly categorized in three main groups based on their sequence length; the chloroplastic/mitochondrial related GPXs comprised the longer sequences (i), extra cellular/plasma membrane related GPXs formed the medium-sized sequences (ii), and cytosolic related GPXs included the shorter sequences (iii). In addition, the regions corresponding to the transit peptide sites in analyzed sequences did not demonstrate any particular patterns. The less-conserved transit peptide residues could be related with the functional diversities of GPXs at various targets. However, despite the variations in sequence length and transit peptide residues, transcripts of GPX homologs mainly contained the six exons. Therefore, it is reasonable to claim that analyzed GPX sequences could have highly-conserved protein sequences, preserved during the formation of various GPXs. The alignment analysis of 87 GPX protein homologs also confirmed this claim, demonstrating the presence of more conserved residues in the main sites of the active enzyme (Supplementary Figure S2). Moreover, to discern the conserved motif patterns in GPX sequences, the most conserved five motif sequences were searched in sets of 87 GPX homologs using MEME tool (Table 2). Motif 1 and 3 were the 50 amino acid residues, while the motif 2 was 41, motif 4 was 15, and motif 5 was 6 residues in length. Motif 1 and 3 were related with the GSHPx (PF00255) protein family and present in almost all GPX homologs. The presence of long conserved residues and their relation with the GSHPx family could indicate the highly conserved structures of GPX sequences at these sites between/among species.

Furthermore, alignment analysis also demonstrated that Asn (N), Gln (Q), Lys (K), Trp (W), and Cys (C) residues that correspond to these catalytic sites in other analyzed sequences were found to be strictly conserved (Supplementary Figure S3). This shows that catalytic residues for Arabidopsis GPX1-8: GPX1 (Cys-111, Gln-146, Trp-200), GPX2 (Cys-41, Gln-76, Thr-130), GPX3 (Cys-80, Gln-115, Thr-169), GPX4 (Cys-44, Gln-79, Trp-133), GPX5 (Cys-46, Gln-81, Trp-135), GPX6 (Cys-105, Gln-140, Trp-194), GPX7 (Cys-108, Gln-143, Trp-197), and GPX8 (Cys-41, Gln-76, Trp-130). Interestingly, residues that correspond to these catalytic sites in other analyzed sequences were found to be strictly conserved (Supplementary Figure S3). This shows that active sites of the enzyme are considerably conserved between species.

**Phylogenetic Analysis of GPXs**

The evolutionary relationships between identified GPX sequences were analyzed by MEGA 6 using the Maximum Likelihood (ML) method with 1000 bootstraps. The constructed phylogeny included all 87 GPX homologs to discover the phylogenetic distribution of sequences (Figure 1). The tree was divided into six major groups based on the tree topology, and each group was indicated with a different color segment. The red segment included cytosolic, extra cellular, and plasma membrane localization.
TABLE 2 | Most conserved five motifs of glutathione peroxidase (GPX) homologs in 18 plant species.

| Motif | Width | Identified site no. | Sequence | Protein domain family\(^a\) |
|-------|-------|---------------------|----------|---------------------------|
| 1     | 50    | 87 of 87            | KYKDQGFEILAFPCNQFGGQEPGTNEEIQFACTRFKAEMYIPFDKVDVNG | GSHPx (PF00255) |
| 2     | 41    | 87 of 87            | FGDRIRKWNFTKFVLDEQHHVNDRLYAPTTSLQIEKDIQKL | Not found |
| 3     | 50    | 86 of 87            | KSHFDFTVDIRGNODVLHSHKYLIVNVSQOGMTSNYNTELNHLYE | GSHPx (PF00255) |
| 4     | 15    | 87 of 87            | NAAPLYKFLKSSKKG | Not found |
| 5     | 6     | 63 of 87            | MAASHS   | Not found |

\(^a\)Protein domain families have been searched in Pfam database.

FIGURE 1 | Phylogenetic tree of glutathione peroxidase (GPX) homologs from 18 plant species. Tree was constructed by MEGA 6 using Maximum likelihood (ML) method with 1000 bootstraps. Segment classification based on the consensus of two subcellular localization servers, CELLO and WoLF PSORT as well as tree topology for ambiguous sequences. Red segment includes cytosolic, extracellular, and plasma membrane related GPXs, green segment contains mitochondrial and chloroplast related GPXs, blue segment only have cytosolic GPXs, cyan segment includes cytosolic and chloroplast/mitochondrial related GPXs, yellow segment contains cytosolic/nuclear related GPXs, and non-colored segment has lower plant Chlamydomonas GPX with chloroplast/mitochondrial relation.
related GPXs, the green segment contained mitochondrial and chloroplast related GPXs, the blue segment only had cytosolic GPXs, the cyan segment included cytosolic and chloroplast/mitochondrial related GPXs, the yellow segment contained cytosolic/nuclear related GPXs, and the non-colored segment had lower plant Chlamydomonas GPX with a chloroplastic/mitochondrial relation. Annotation of each segment based on the consensus of two subcellular localization servers, CELLO and WoLF PSORT, as well as tree topology for ambiguous sequences. Mainly cytosolic, nuclear, extra cellular and plasma membrane related GPXs were clustered together, while chloroplast/mitochondrial related GPXs also cluster together. Therefore, the presence or absence of transit peptide residues was the main contributing entity in the phylogenetic distribution of GPX sequences. In addition, the presence of sequences with different subcellular localizations in the same group inferred the possibility of gene duplication events in the formation of various GPX sequences. Duplications in plant genomes could be either as small-scale such as tandem and segmental duplications, or as large-scale such as whole-genome duplications (Ramsey and Schemske, 1998). The segmental duplications are observed in different chromosomes whereas tandem duplications occur in the same chromosome (Liu et al., 2011). Several segmental duplications were identified between GPX pairs (Table 3). The presence of segmental duplications, particularly between sequences with various subcellular localizations may partly explain the possibility of gene duplication events in GPX formations.

**Expression Profile Analysis of GPXs**

The potential expression profile of GPX genes was analyzed at 105 anatomical parts and 10 developmental stage levels using model organism A. thaliana GPXs from Genevestigator platform (Figure 2). Eight Arabidopsis genes, namely GPX1 (AT2G25080), GPX2 (AT2G31570), GPX3 (AT2G43350), GPX4 (AT2G48150), GPX5 (AT3G63080), GPX6 (AT4G11600), GPX7 (AT4G31870), and GPX8 (AT1G63460) were retrieved from the “Affymetrix Arabidopsis ATH1 Genome Array” platform using the Genevestigator interface, and conditions and genes associated with the metabolic state of the cells. Therefore, it seems that the expression profiles of GPX1, 2, 3, 4, 5, 6, 7, and 8 demonstrate relatively similar expression profiles compared to those of GPX1, 2, 3, 4, and 7. At the developmental level (Figure 2B), the expression profiles of Arabidopsis GPX1-8 genes were analyzed at 10 developmental stages, including senescence, mature siliques, flowers and siliques, developed flower, young rosette, germinated seed, seedling, bolting, young flower, and developed rosette. GPX1-8 were relatively expressed in all developmental stages. However, the expression of GPX8 in the senescence stage demonstrated slightly different patterns, particularly the mitochondrial GPX6 gene had the highest expression profile compared to other developmental stages. This may have been caused by senescence-related cellular deteriorations, leading to the substantial metabolic or physiological changes that significantly affect the overall metabolic energy consumption. Therefore, it seems that the expression profiles of GPXs are highly associated with the metabolic state of the cells.

### Table 3 | The segmental duplication events in some glutathione peroxidase (GPX) pairs.

| Species name          | Segmental duplication pairs                                                                 |
|-----------------------|---------------------------------------------------------------------------------------------|
| Arabidopsis thaliana  | AT2G25080-AT4G31870                                                                        |
| Brachypodium distachyon | AT2G48150-AT3G63080                                                                       |
| Brassica rapa         | AT4G31870-AT4G51010                                                                         |
| Gossypium raimondii   | AT4G31870-AT4G51010                                                                         |
| Vitis vinifera        | AT4G31870-AT4G51010                                                                         |
| Oryza sativa          | AT4G31870-AT4G51010                                                                         |
| Prunus persica        | AT4G31870-AT4G51010                                                                         |

**3D Structure Analysis of GPXs**

3D models of putative GPXs were constructed by using Phyre2 server for eight identified Arabidopsis GPX1-8 gene sequences (Figure 3). These sequences were: AT2G25080.1 (GPX1), AT2G31570.1 (GPX2), AT2G43350.1 (GPX3), AT2G48150.1 (GPX4), AT3G63080.1 (GPX5), AT4G11600.1 (GPX6), AT4G11600.1 (GPX7), and AT1G63460.1 (GPX8).
FIGURE 2 | Expression profile of *Arabidopsis* glutathione peroxidase GPX1-8 genes at 105 anatomical parts (A) and 10 developmental stage levels (B). Genes and conditions with similar profiles were comparatively analyzed using hierarchical clustering tool with Euclidean distance method at Genevestigator platform.
(GPX6), AT4G31870.1 (GPX7), and AT1G63460.1 (GPX8). In modeling, three templates such as 2F8A:A (GPX1, GPX3, GPX6, and GPX7), 2V1M:A (GPX2 and GPX5), and 2P5Q:A (GPX4 and GPX8) were used to maximize the alignment coverage, percentage identity and confidence for submitted sequences. Predicted models demonstrated the ≥98% of residues in allowed region in Ramachandran plot analysis, indicating that constructed models were fairly in good quality. To find out the structural divergence/similarity in generated models, the structures were superposed to calculate the percentage of structural overlap and RMSD values (Table 4). GPX1-GPX3, GPX4-GPX8, and GPX6-GPX7 pairs demonstrated the highly conserved structural overlap (100%) with 0.14, 0.00, and 0.03 RMSD values, respectively. The each designated pair also belonged to either chloroplastic/mitochondrial or cytosolic form, indicating their functional similarities with minor specifications. In addition, GPX1-GPX6 and 7, GPX2-GPX5, and GPX3-GPX6 and 7 pairs showed very high structural similarity with ≥94 structural overlaps. Despite the highly conserved structures of Arabidopsis GPX members, some minor variations were also present. It seems that these divergences in GPXs may not change the protein-3D structure, however, they could attribute the new functional roles to catalytic activities.

**Interaction Partner Analysis of GPXs**

The interactome network was constructed for 10 putative interactors of Arabidopsis cytosolic GPX2, using Cytoscape with STRING data (Figure 4). APX1 (L-ascorbate peroxidase), GSH2 (glutathione synthetase), GSTF6 (glutathione S-transferase F6), GSTT1 (glutathione S-transferase THETA 1), PER1 (1-Cys peroxiredoxin PER1), AT1G65820 (glutathione S-transferase), GSTF12 (glutathione S-transferase phi 12), GSTF2 (glutathione S-transferase F2), GSTF8 (glutathione S-transferase F8), and GSTU19 (glutathione S-transferase U19) proteins were predicted as the main interaction partners of Arabidopsis cytosolic GPX2. APX1 is a type of H$_2$O$_2$-scavenging enzyme and a central component in the reactive oxygen gene network (Storozhenko et al., 1998; Fourcroy et al., 2004). GSH2 involves in the second step of the glutathione synthesis pathway from L-cysteine and L-glutamate (Wang and Oliver, 1996). GSTF6 functions in camalexin biosynthesis, is involved in the conjugation of reduced glutathione to various exogenous/endogenous hydrophobic electrophiles, and has a detoxification role for certain herbicides (Su et al., 2011). GSTT1, GSTF8, and GSTU19 are reported to have glutathione S-transferase or peroxidase activity. They further conjugate the reduced glutathione to various exogenous/endogenous hydrophobic electrophiles and play a detoxification role for certain herbicides (Wagner et al., 2002). PER1 is an antioxidant protein involved in the inhibition of germination under stress (Haslekås et al., 1998). AT1G65820 is a glutathione S-transferase. GSTF12 is involved in the transport of anthocyanins and proanthocyanidins into the vacuole (Kitamura et al., 2004). GSTF2 plays a role in binding and transport of small bioactive products and defense-related compounds under stress (Smith et al., 2003). The analysis indicated that cytosolic GPX2 enzyme is closely related with various pathways involving in antioxidant and secondary metabolite metabolisms, thereby supporting the functional role of GPXs in H$_2$O$_2$-scavenging and plant defense.

**Analysis of APXs**

**Retrieval of APX Genes/Proteins**

Eight potential Arabidopsis APX protein sequences such as APX1-6, APXT, and APXS, obtained from the UniProtKB / Swiss-Prot database of NCBI, were used as queries in Phytozome database to retrieve the very close homologs of APX sequences in
18 plant species. In the selection of APX homologs from blastp hits, a very strict criterion (only the highest hit sequence) was applied to avoid redundant sequences and alternative splices of the same gene. A total of 120 APX sequences were identified from the protein datasets of 18 plant species. These were 8 genes for A. thaliana, 7 genes for B. distachyon, 8 genes for B. rapa, 4 genes for C. reinhardtii, 5 genes for C. sativus, 7 genes for E. grandis, 7 genes for G. max, 8 genes for G. raimondii, 7 genes for M. truncatula, 6 genes for O. sativa, 6 genes for P. vulgaris, 5 genes for P. patens, 7 genes for P. trichocarpa, 6 genes for P. persica, 7 genes for S. lycopersicum, 8 genes for S. bicolor, 6 genes for V. vinifera, and 8 genes for Z. mays (Table 5). Then, genomic, transcript, CDS, and protein sequences of 120 identified APX sequences were retrieved for further analyses.

**Sequence Analysis of APX Genes/Proteins**

A total of 120 APX homologs were identified in protein datasets of 18 plant species using Arabidopsis APX1-6, APXT, and APXS sequences by homology search. Identified APX sequences contained the peroxidase (PF00141) protein family domain. They encoded a protein of 197–478 amino acids residues (average length 323.9) and 23.7–52.1 kDa molecular weight with 5.03–9.23 pI value. The sequence variations in analyzed APXs demonstrated a correlation with their putative localizations,
| Species name                                | Phytozome gene ID | Gene/protein features of GPX sequences |
|--------------------------------------------|------------------|--------------------------------------|
| Arabidopsis thaliana (L.) Heynh.           |                  |                                      |
| AT1G07890                                  | Peroxidase (PF00141) | Peroxidase 8 250 27.5 5.72 Cyto Cyto |
| AT1G77490                                  | Peroxidase (PF00141) | Peroxidase 12 426 46.0 6.81 Chlo Chlo |
| AT3G09640                                  | Peroxidase (PF00141) | Peroxidase 9 251 28.0 5.87 Cyto Cyto |
| AT4G08390                                  | Peroxidase (PF00141) | Peroxidase 10 372 40.4 8.31 Chlo Chlo |
| AT4G09010                                  | Peroxidase (PF00141) | Peroxidase 10 349 37.9 8.59 Chlo/Mito Chlo |
| AT4G32320                                  | Peroxidase (PF00141) | Peroxidase 10 329 36.2 8.99 Chlo Chlo |
| AT4G35000                                  | Peroxidase (PF00141) | Peroxidase 9 287 31.5 6.47 Cyto Cyto |
| AT4G35970                                  | Peroxidase (PF00141) | Peroxidase 9 279 30.8 8.80 Cyto/Nucl Cyto |
| Brachypodium distachyon (L.) P. Beauv.     |                  |                                      |
| Bradi1g16510                               | Peroxidase (PF00141) | Peroxidase 9 256 27.7 5.28 Cyto Cyto |
| Bradi1g65820                               | Peroxidase (PF00141) | Peroxidase 9 250 27.4 5.71 Cyto Cyto |
| Bradi3g40330                               | Peroxidase (PF00141) | Peroxidase 11 329 35.4 6.36 Chlo Chlo |
| Bradi3g42340                               | Peroxidase (PF00141) | Peroxidase 9 289 31.5 7.70 Cyto/Chlo Cyto |
| Bradi3g45700                               | Peroxidase (PF00141) | Peroxidase 12 439 47.3 5.61 Chlo Chlo |
| Bradi5g10490                               | Peroxidase (PF00141) | Peroxidase 11 345 37.4 8.77 Chlo/Mito Chlo |
| Bradi5g20670                               | Peroxidase (PF00141) | Peroxidase 10 333 36.1 8.71 Mito Mito |
| Brasa rapa L.                              |                  |                                      |
| Brara.A00250                               | Peroxidase (PF00141) | Peroxidase 8 280 31.0 7.69 Cyto Cyto |
| Brara.A03521                               | Peroxidase (PF00141) | Peroxidase 9 251 28.1 6.41 Cyto Cyto |
| Brara.C02583                               | Peroxidase (PF00141) | Peroxidase 9 348 37.9 8.59 Chlo/Mito Chlo |
| Brara.G00648                               | Peroxidase (PF00141) | Peroxidase 10 439 47.5 7.70 Chlo Chlo |
| Cucumis sativus L.                         |                  |                                      |
| Cucsa.060660                                | Peroxidase (PF00141) | Peroxidase 11 413 44.8 7.09 Chlo Chlo |
| Cucsa.162470                                | Peroxidase (PF00141) | Peroxidase 8 249 27.3 7.74 Chlo/Cyto Nucl |
| Cucsa.213340                                | Peroxidase (PF00141) | Peroxidase 9 249 27.3 5.43 Cyto Cyto |
| Cucsa.311620                                | Peroxidase (PF00141) | Peroxidase 11 368 40.2 7.67 Chlo Chlo |
| Cucsa.370590                                | Peroxidase (PF00141) | Peroxidase 9 286 31.4 6.41 Cyto Cyto |
| Eucalyptus grandis W. Hill ex Maiden        |                  |                                      |
| Euogr.A01180                                | Peroxidase (PF00141) | Peroxidase 9 249 27.4 6.07 Cyto Cyto |
| Euogr.B02456                                | Peroxidase (PF00141) | Peroxidase 9 249 27.2 5.29 Cyto Cyto |
| Euogr.C01740                                | Peroxidase (PF00141) | Peroxidase 9 369 39.6 8.44 Chlo Chlo |
| Euogr.F00373                                | Peroxidase (PF00141) | Peroxidase 11 356 38.3 6.50 Chlo Chlo |
| Euogr.F04344                                | Peroxidase (PF00141) | Peroxidase 12 446 48.2 8.71 Chlo Chlo |
| Euogr.F04344                                | Peroxidase (PF00141) | Peroxidase 11 397 42.8 8.60 Chlo Chlo |
| Glycine max (L.) Merr.                      |                  |                                      |
| Glyma.06G088200                             | Peroxidase (PF00141) | Peroxidase 10 319 34.2 7.56 Chlo Chlo |
| Glyma.06G114400                             | Peroxidase (PF00141) | Peroxidase 12 432 47.0 7.13 Chlo Chlo |

(Continued)
| Species name | Phytozome gene ID | Protein domain family<sup>a</sup> | Domain family description | Exon no. | Protein length | MW (KDa) | Theor. pI | Localization CELLO<sup>b</sup> | Localization WoLF PSORT<sup>b</sup> |
|--------------|------------------|----------------------------------|---------------------------|---------|----------------|---------|----------|----------------|----------------|
| Glyma.11G078400 | Peroxidase (PF00141) | Peroxidase | 9 | 280 | 31.1 | 9.08 | Cyto/Mito | Cyto |
| Glyma.12G032300 | Peroxidase (PF00141) | Peroxidase | 9 | 287 | 31.7 | 6.27 | Cyto | Cyto |
| Glyma.12G073100 | Peroxidase (PF00141) | Peroxidase | 9 | 250 | 27.1 | 5.65 | Cyto | Cyto |
| Glyma.14G177200 | Peroxidase (PF00141) | Peroxidase | 10 | 347 | 37.9 | 6.76 | Cyto | Cyto |
| Gossypium raimondii Ulbr. | | | | | |
| Gorai.002G196800 | Peroxidase (PF00141) | Peroxidase | 9 | 288 | 31.7 | 5.64 | Cyto | Cyto |
| Gorai.005G254100 | Peroxidase (PF00141) | Peroxidase | 9 | 288 | 31.9 | 6.67 | Cyto | Cyto |
| Gorai.009G104500 | Peroxidase (PF00141) | Peroxidase | 9 | 250 | 27.5 | 5.73 | Cyto | Cyto |
| Gorai.009G246900 | Peroxidase (PF00141) | Peroxidase | 11 | 385 | 41.7 | 8.89 | Chlo | Chlo |
| Gorai.010G038200 | Peroxidase (PF00141) | Peroxidase | 11 | 355 | 38.8 | 7.53 | Chlo | Chlo |
| Gorai.010G051400 | Peroxidase (PF00141) | Peroxidase | 12 | 422 | 48.0 | 6.77 | Chlo | Chlo |
| Gorai.010G115200 | Peroxidase (PF00141) | Peroxidase | 10 | 334 | 36.2 | 8.17 | Chlo | Chlo |
| Zea mays L. | GRMZM2G004211 | Peroxidase (PF00141) | Peroxidase | 9 | 290 | 32.0 | 7.72 | Cyto/Mito | Cyto |
| GRMZM2G006791 | Peroxidase (PF00141) | Peroxidase | 12 | 451 | 48.9 | 5.60 | Chlo | Chlo |
| GRMZM2G047968 | Peroxidase (PF00141) | Peroxidase | 7 | 223 | 23.7 | 9.01 | Chlo/Cyto | Mito/Chlo |
| GRMZM2G054300 | Peroxidase (PF00141) | Peroxidase | 9 | 250 | 27.3 | 5.56 | Cyto | Cyto |
| GRMZM2G120517 | Peroxidase (PF00141) | Peroxidase | 11 | 339 | 37.0 | 8.86 | Mito | Chlo |
| GRMZM2G137839 | Peroxidase (PF00141) | Peroxidase | 9 | 250 | 27.3 | 5.64 | Cyto | Cyto |
| GRMZM2G156227 | Peroxidase (PF00141) | Peroxidase | 10 | 351 | 38.3 | 6.62 | Mito | Chlo |
| GRMZM2G460406 | Peroxidase (PF00141) | Peroxidase | 8 | 289 | 31.6 | 7.73 | Cyto | Chlo |
| Vitis vinifera L. | GSVVG010098846001 | Peroxidase (PF00141) | Peroxidase | 11 | 372 | 40 | 7.10 | Chlo | Chlo |
| GSVVG010099709001 | Peroxidase (PF00141) | Peroxidase | 10 | 344 | 37.4 | 6.66 | Extr/Chlo | Chlo |
| GSVVG01024035001 | Peroxidase (PF00141) | Peroxidase | 9 | 289 | 31.7 | 7.72 | Chlo/Cyto | Cyto |
| GSVVG01025104001 | Peroxidase (PF00141) | Peroxidase | 9 | 250 | 27.5 | 5.71 | Cyto | Cyto |
| GSVVG0102551001 | Peroxidase (PF00141) | Peroxidase | 9 | 253 | 27.9 | 5.43 | Cyto | Cyto |
| GSVVG01038858001 | Peroxidase (PF00141) | Peroxidase | 10 | 330 | 35.9 | 6.47 | Chlo/Cyto | Chlo |
| Oryza sativa L. | LOC_Os02g34810 | Peroxidase (PF00141) | Peroxidase | 12 | 478 | 51.1 | 5.36 | Chlo | Chlo |
| LOC_Os04g35520 | Peroxidase (PF00141) | Peroxidase | 11 | 359 | 38.3 | 8.76 | Chlo | Chlo |
| LOC_Os04g51300 | Peroxidase (PF00141) | Peroxidase | 11 | 353 | 38.1 | 8.67 | Mito/Chlo | Chlo |
| LOC_Os07g49400 | Peroxidase (PF00141) | Peroxidase | 9 | 251 | 27.1 | 5.18 | Cyto | Cyto |
| LOC_Os08g41090 | Peroxidase (PF00141) | Peroxidase | 10 | 331 | 35.5 | 6.95 | Chlo | Chlo |
| LOC_Os08g43560 | Peroxidase (PF00141) | Peroxidase | 9 | 291 | 31.7 | 7.74 | Chlo/Cyto | Mito |
| Medicago truncatula Gaertn. | Medtr3g088160 | Peroxidase (PF00141) | Peroxidase | 11 | 436 | 47.4 | 9.02 | Chlo | Chlo |
| Medtr3g088160 | Peroxidase (PF00141) | Peroxidase | 10 | 387 | 42.0 | 8.73 | Chlo | Chlo |
| Medtr3g107060 | Peroxidase (PF00141) | Peroxidase | 10 | 320 | 34.7 | 8.08 | Chlo | Mito/Chlo |
| Medtr4g061140 | Peroxidase (PF00141) | Peroxidase | 9 | 250 | 27.1 | 5.52 | Cyto | Cyto |
| Medtr4g073410 | Peroxidase (PF00141) | Peroxidase | 9 | 287 | 31.7 | 6.26 | Chlo/Cyto | Chlo |
| Medtr5g022510 | Peroxidase (PF00141) | Peroxidase | 9 | 281 | 31.4 | 8.74 | Cyto | Cyto |
| Medtr5g064810 | Peroxidase (PF00141) | Peroxidase | 10 | 353 | 38.9 | 8.18 | Mito/Nucl | Chlo |
| Physcomitrella patens (Hedw.) Bruch & Schimp. | Phpat.001G070500 | Peroxidase (PF00141) | Peroxidase | 11 | 358 | 38.4 | 7.56 | Chlo | Chlo |
| Phpat.001G040400 | Peroxidase (PF00141) | Peroxidase | 9 | 300 | 32.6 | 7.01 | Chlo | Cyto |
| Phpat.001G162800 | Peroxidase (PF00141) | Peroxidase | 2 | 440 | 48.2 | 8.11 | Chlo | Chlo |
| Phpat.017G025400 | Peroxidase (PF00141) | Peroxidase | 11 | 357 | 38.4 | 6.15 | Chlo | Chlo |
| Phpat.020G01100 | Peroxidase (PF00141) | Peroxidase | 9 | 250 | 27.6 | 5.66 | Cyto | Cyto |

(Continued)
| Species name | Phytozone gene ID | Gene/protein features of GPX sequences |
|--------------|------------------|---------------------------------------|
|              |                  | Protein domain family<sup>a</sup> | Domain family description | Exon no. | Protein length (KDa) | MW (KDa) | Theor. pI | Localization CELLO<sup>b</sup> | Localization WoLF PSORT<sup>b</sup> |
| Phaseolus vulgaris L. | Phvul.008G176700 | Peroxidase (PF00141) | Peroxidase | 10 | 347 | 37.6 | 6.06 | Chlo/Extr | Chlo |
| | Phvul.009G090000 | Peroxidase (PF00141) | Peroxidase | 10 | 317 | 34.2 | 8.38 | Chlo | Chlo |
| | Phvul.009G126500 | Peroxidase (PF00141) | Peroxidase | 12 | 436 | 47.8 | 8.67 | Chlo | Chlo |
| | Phvul.009G126500 | Peroxidase (PF00141) | Peroxidase | 11 | 387 | 42.4 | 8.51 | Chlo | Chlo |
| | Phvul.011G035000 | Peroxidase (PF00141) | Peroxidase | 9 | 287 | 31.6 | 7.10 | Cyto | Cyto |
| | Phvul.011G071300 | Peroxidase (PF00141) | Peroxidase | 9 | 250 | 27 | 5.54 | Cyto | Cyto |
| Populus trichocarpa Torr. & A.Gray ex. Hook. | Potri.004G174500 | Peroxidase (PF00141) | Peroxidase | 9 | 286 | 31.5 | 6.67 | Cyto | Cyto |
| | Potri.005G161900 | Peroxidase (PF00141) | Peroxidase | 10 | 347 | 37.8 | 7.59 | Chlo/Mito | Chlo |
| | Potri.005G179200 | Peroxidase (PF00141) | Peroxidase | 10 | 345 | 37.8 | 5.98 | Chlo | Chlo/Mito |
| | Potri.006G132200 | Peroxidase (PF00141) | Peroxidase | 9 | 249 | 27.4 | 5.27 | Cyto | Cyto |
| | Potri.006G254500 | Peroxidase (PF00141) | Peroxidase | 10 | 337 | 36.7 | 8.44 | Chlo | Chlo |
| | Potri.009G015400 | Peroxidase (PF00141) | Peroxidase | 9 | 249 | 27.3 | 5.53 | Cyto | Cyto |
| | Potri.009G134100 | Peroxidase (PF00141) | Peroxidase | 9 | 286 | 31.4 | 7.06 | Cyto | Cyto |
| Prunus persica (L.) Batsch | ppa006270m | Peroxidase (PF00141) | Peroxidase | 11 | 420 | 45.4 | 8.48 | Chlo | Chlo |
| | ppa008080m | Peroxidase (PF00141) | Peroxidase | 10 | 349 | 38.4 | 6.09 | Mito/Chlo/Extr | Chlo |
| | ppa009582m | Peroxidase (PF00141) | Peroxidase | 9 | 286 | 31.4 | 6.21 | Cyto | Cyto |
| | ppa010426m | Peroxidase (PF00141) | Peroxidase | 9 | 250 | 27.6 | 5.37 | Cyto | Cyto |
| | ppa015878m | Peroxidase (PF00141) | Peroxidase | 10 | 319 | 34.3 | 6.24 | Chlo | Chlo |
| Sorghum bicolor (L.) Moench | Sobic.001G410200 | Peroxidase (PF00141) | Peroxidase | 9 | 250 | 27.2 | 5.55 | Cyto | Cyto |
| | Sobic.002G431100 | Peroxidase (PF00141) | Peroxidase | 9 | 250 | 27.1 | 5.18 | Cyto | Cyto |
| | Sobic.004G175500 | Peroxidase (PF00141) | Peroxidase | 13 | 473 | 51.1 | 5.03 | Chlo | Chlo |
| | Sobic.006G021100 | Peroxidase (PF00141) | Peroxidase | 9 | 476 | 52.1 | 8.97 | Nucl | Chlo |
| | Sobic.006G084400 | Peroxidase (PF00141) | Peroxidase | 11 | 344 | 37.2 | 8.60 | Mito/Chlo | Chlo |
| | Sobic.006G204000 | Peroxidase (PF00141) | Peroxidase | 11 | 395 | 42.9 | 8.74 | Mito/Chlo | Chlo |
| | Sobic.007G177000 | Peroxidase (PF00141) | Peroxidase | 8 | 289 | 31.5 | 7.73 | Cyto | Cyto |
| | Sobic.007G205600 | Peroxidase (PF00141) | Peroxidase | 10 | 333 | 36.2 | 7.58 | Chlo | Chlo |
| Solanum lycopersicum L. | Solyc01g111510 | Peroxidase (PF00141) | Peroxidase | 8 | 287 | 31.6 | 7.10 | Cyto | Cyto |
| | Solyc04g74640 | Peroxidase (PF00141) | Peroxidase | 10 | 345 | 37.6 | 7.60 | Chlo/Mito | Chlo |
| | Solyc09g005150 | Peroxidase (PF00141) | Peroxidase | 9 | 250 | 27.3 | 5.86 | Cyto | Cyto |
| | Solyc09g060260 | Peroxidase (PF00141) | Peroxidase | 10 | 345 | 37.8 | 8.48 | Chlo | Chlo |
| | Solyc08g059760 | Peroxidase (PF00141) | Peroxidase | 10 | 326 | 35.4 | 5.65 | Chlo | Chlo |
| | Solyc09g072700 | Peroxidase (PF00141) | Peroxidase | 9 | 250 | 27.6 | 5.63 | Cyto | Cyto |
| | Solyc11g018550 | Peroxidase (PF00141) | Peroxidase | 10 | 421 | 46.0 | 8.20 | Chlo | Chlo |

<sup>a</sup>Protein domain families were searched in Pfam database.

<sup>b</sup>Cyto, Cytosolic; Chlo, Chloroplastic; Mito, Mitochondrial; Nucl, Nuclear; Extr, Extracellular.

More than one localization specified in a single column also shows the significance of other entries in order.

thereby indicated the presence of transit residues (Table 5). Molecular cloning studies from *A. thaliana* have demonstrated that APX1, APX2, and APX6 are polypeptides of 250, 251, and 329 amino acids, respectively, with cytosolic localizations but without transit peptide (Davletova et al., 2005; Jones et al., 2009; Aryal et al., 2011). APX3 and APX5 consisted of 287 and 279 amino acids, respectively, with peroxisomal localizations; however, sites of transit peptide residues are not precisely specified (Panchuk et al., 2002; Narendra et al., 2006; Bienvenut et al., 2012). APX4 is a 349 amino acids protein with chloroplastic localization, including 1–82 residues as transit peptide from the N-terminal site (Kieselbach et al., 2000; Panchuk et al., 2005; Aryal et al., 2011). APXT is a 426 amino acids chloroplastic protein, including 1–78 residues of transit peptide (Theologis et al., 2000; Panchuk et al., 2005). APXS consists of 372 amino acids with chloroplastic and/or mitochondrial localizations, but...
the exact site of the transit peptide is not specified (Jespersen
et al., 1997; Mayer et al., 1999; Chew et al., 2003). In the
present study, multiple-alignment of APX sequences revealed
that chloroplastic/mitochondrial-related APxs contained the
transit peptide residues in approximately 1–90 amino acids from
the N-terminal site while cytosolic APxs did not have any
putative transit peptide (Supplemental Figure S4). Thus, the
analyzed APX sequences were gathered in two main groups based
on subcellular localizations, such as chloroplastic/mitochondrial
APxs (i) and cytosolic APxs (ii).

In addition, the regions corresponding to the transit peptide
sites in analyzed sequences did not demonstrate any particular
pattern. This could indicate that less conservancy in transit
peptides may be associated with the functional diversities
of APxs at various targets. Besides, APX transcripts mainly
consisted of 8–12 exons, supporting the relatively less conserved
structure of APXs compared to GPxs. However, alignment
analysis also demonstrated the presence of a considerable
degree of conserved residues in the main sites of enzyme
(Supplementary Figure S5). Moreover, to analyze the availability
of any conserved motif pattern/s in APX sequences, the most
conserved five motif sequences of APX homologs were searched
using MEME tool (Table 6). Motif 1 was 29 residues long, motif 2
and 4 were 21 residues long, motif 3 was 32 residues long, and motif 5
was 25 residues in length. However, only motifs 2 and 3 had a relation
with the peroxidase (PF00141) protein family, and in this case
were present in most of the sequences. This could indicate the
highly conserved structures of APX sequences at those sites
with peroxidase activity.

Furthermore, alignment analysis also demonstrated that Asp
(D) and Gly-Gly (GG) residues are strictly conserved in all
aligned sequences, indicating their potential functions in enzyme
activity and/or stability (Supplementary Figure S6). To infer a
functional relationship between these conserved residues and
APX sequences, we searched for the known binding residues of
model organism Arabidopsis APxs in the UniProtKB database
(http://www.uniprot.org/uniprot/). The following residues were
reported as potential active and metal binding residues for
Arabidopsis GPX1-6, APXT, and APX5: APX1 (Arg-38, His-
42, His-163, Thr-164, Thr-180, Asn-182, Ile-185, Asp-187),
APX2 (Arg-39, His-43, His-163, Thr-164, Thr-180, Asn-182,
Ile-185, Asp-187), APX3 (Arg-36, His-40, His-160, Thr-161,
Thr-177, Asp-184), APX5 (Arg-35, His-39, His-158, Thr-159,
Thr-175, Asp-182), APX6 (Arg-119, His-123, His-224), APXT
(Arg-108, His-112, His-241, Thr-242, Thr-274, Asp-281), and
APXS (Arg-129, His-133, His-262, Thr-263, Thr-295, Asp-302).
These active and metal binding residues did not correspond to
any of the strictly conserved residues in analyzed APX sequences
but they were found to be conserved at considerable rates.
However, when taken into consideration that some of the strictly
conserved residues in analyzed GPX sequences correspond to the
catalytic sites of the enzymes, we can make an inference that
these strictly conserved residues in APX sequences may also be
attributed to the various functional diversities of the enzyme.

**Phylogenetic Analysis of APxs**

To analyze the evolutionary relationship between identified APX
homologs, the phylogenetic tree was constructed by MEGA 6
using the Maximum Likelihood (ML) method with 1000
bootstraps (Figure 5). The constructed tree was divided into
five major groups based on the tree topology, and each group
was indicated with a different color segment. Blue, red, and
green segments included the chloroplast/mitochondria-related
APxs with relatively longer, medium and short sequences,
respectively, whereas cyan and yellow segments mainly contained
longer and shorter cytosolic APX sequences, respectively.
Annotation of each segment was based on the consensus of
two subcellular localization servers, CELLO and WoLF
PSORT, as well as tree topology for ambiguous sequences. Overall,
the trees were observed that cytosolic-related APxs clustered together,
while alternatively chloroplast/mitochondrial-related APxs were
together. In addition, in clustering of sequences at sub-branches
was primarily based on the sequence length and monocot/dicot
separation. However, there were considerable variations between
sequences, even those belonging to the same subcellular
localization. It is thought that these sequence variations could
be attributed to the various functional diversities of APxs
and/or be associated with different subcellular localizations.
Moreover, some sequences were also available with different
subcellular localizations in the same clade, indicating the
possibility of gene duplication events in formation of some
APX genes. The gene duplication events were searched based on
the previously designated protocol (Gu et al., 2002). In
doing so, several segmental and tandem duplications were
identified between some APX pairs (Table 7). The identified
segmental or tandem duplications in APX genes were observed
between either chloroplastic and chloroplastic, or cytosolic
and cytosolic forms. This could indicate the possibility of
gene duplication events in the formation of close APX
homologs.

| Motif | Width | Identified site no. | Sequence | Protein domain family |
|-------|-------|---------------------|----------|----------------------|
| 1     | 29    | 120 of 120          | CHPIMLRLAWHDAGTYKNTKWGPNGSI | Not found |
| 2     | 21    | 101 of 120          | MGLNDCQIVALSGGHTLGRCH   | Peroxidase (PF00141) |
| 3     | 32    | 119 of 120          | IITYADLYQLAGVVAVEVCGGPTIPMHCGRND | Peroxidase (PF00141) |
| 4     | 21    | 118 of 120          | DPEFRPWEKYAEDQDAFFRD  | Not found |
| 5     | 25    | 84 of 120           | ERSQEQPWTVNLKFDNSYFKEIL | Not found |

*Protein domain families have been searched in Pfam database.*
Expression Profile Analysis of APXs
The gene expression profiles of APXs were analyzed at 105 anatomical parts and 10 developmental stage levels using model organism Arabidopsis thaliana APXs from Genevestigator platform (Figure 6). Eight Arabidopsis genes, namely APX1 (AT1G07890), APX2 (AT3G09640), APX3 (AT4G35000), APX4 (AT4G09010), APX5 (AT4G35970), APX6 (AT4G32320), TAPX (AT1G77490), and SAPX (AT4G08390), were retrieved from the “Affymetrix Arabidopsis ATH1 Genome Array” platform using the Genevestigator interface. Thereafter, conditions and genes with similar profiles were comparatively analyzed using Hierarchical clustering tool with Euclidean distance method.

At the anatomical level (Figure 6A), APX genes were expressed in almost all analyzed tissues of Arabidopsis with various folds. It was clear that the expression levels of genes were closely related with the expressed tissue type/s. For example, both cytosolic APX1 and chloroplastic/mitochondrial SAPX had significantly higher expression in actively growing zones, as well as many root and root protoplast-related structures. APX3, APX4, APX6, and TAPX were expressed in various shoot, bud, leaf, flower and seed related tissues at considerable rates. All these indicated that stress factors, actively growing tissues as well as normal physiological and metabolic changes could induce the expression of APX genes in tissue-dependent way. All these
metabolic activities or their related consequences could exert the stresses to the cells. Many studies have further demonstrated that abiotic/abiotic stress factors such as heavy metal, drought, water, heat, cellular H$_2$O$_2$ level, oxidative state of the cell could increase the expression of APX genes to either suppress or eliminate the stressors (Ishikawa and Shigeoka, 2008; Koussevitzky et al., 2008; Petrov and Van Breusegem, 2012) and oxidative state of the cell could increase the expression of APX genes to either suppress or eliminate the stressors (Ishikawa and Shigeoka, 2008; Koussevitzky et al., 2008; Petrov and Van Breusegem, 2012).

### 3D Structure Analysis of APXs

3D models of eight identified *Arabidopsis* APX sequences were constructed by using Phyre2 server (Figure 7). The modeled sequences were AT1G07890.1 (APX1), AT3G09640.1 (APX2), AT4G35000.1 (APX3), AT4G09010.1 (APX4), AT4G35970.1 (APX5), AT4G32320.1 (APX6), AT1G77490.1 (APXT), and AT4G08390.1 (APXS). In modeling, six templates such as 1APX:A (APX1), 1OAF:A (APX2 and APX5), 3RRW:B (APX4), 1BGP:A (APX6), 1ITK:B (APXT), and 1IYN:A (APXS) were used to maximize the alignment coverage, percentage identity, and confidence for the submitted sequences. Predicted models showed the ≥96% of residues were within the allowed region in Ramachandran plot, indicating that structures were acceptably high in quality. To analyze the divergence or similarity in generated models, the structures were superposed in order to calculate the percentage of structural overlap and RMSD values (Table 8). The superposition of APX sequences demonstrated that APX2-APX3, APX2-APX5, and APX3-APX5 pairs have highly conserved structural overlap (100%) with 0.00, 0.38, and 0.38 RMSD values, respectively. These conserved pairs primarily shared the cytosolic and/or peroxisomal localizations, inferring the possibility of a functional relationship between them. In addition, the APX1-APX2, 3, and 5 pairs had very high structural similarity with ≥99 structural overlaps. Therefore, it could be deduced that APX members topologically demonstrated highly conserved structures, despite their functional diversities in different cellular compartments.

### Interaction Partner Analysis of APXs

The interactome network was constructed for 10 putative interactors of *Arabidopsis* cytotoxic APX1 using Cytoscape with STRING data (Figure 8). MDHAR (monodehydroascorbate reductase), GPX2 (glutathione peroxidase 2), DHAR1 (dehydroascorbate reductase), MDAR1 (monodehydroascorbate reductase 1), RHL41 (zinc finger protein ZAT12), ATFQ (ATP synthase subunit d), FBP (fructose-1,6-bisphosphatase), ATMDAR2 [monodehydroascorbate reductase (NADH)],...
FIGURE 6 | Expression profile of Arabidopsis ascorbate peroxidase APX1-6, TAPX and SAPX genes at 105 anatomical parts (A) and 10 developmental stage levels (B). Genes and conditions with similar profiles were comparatively analyzed using hierarchical clustering tool with Euclidean distance method at Genevestigator platform.
FIGURE 7 | 3D models of predicted *Arabidopsis* ascorbate peroxidase APX1-6, APXT, and APXS sequences. Models were constructed by using Phyre² server for AT1G07890.1 (APX1), AT3G09640.1 (APX2), AT4G35000.1 (APX3), AT4G09010.1 (APX4), AT4G35970.1 (APX5), AT4G32320.1 (APX6), AT1G77490.1 (APXT), and AT4G08390.1 (APXS) sequences, and colored by rainbow from N- to C-terminus.

TABLE 8 | Structural overlap (%)/RMSD values in superposed *Arabidopsis* ascorbate peroxidases (APXs).

|       | APX1  | APX2  | APX3  | APX4  | APX5  | APX6  | APXS | APXT  |
|-------|-------|-------|-------|-------|-------|-------|------|-------|
| APX1  | –     | 99.19/0.43 | 99.59/0.41 | 75.10/1.75 | 99.58/0.51 | 81.53/1.52 | 95.18/0.95 | 89.16/1.49 |
| APX2  | 99.19/0.41 | – | 100.00/0.00 | 75.00/1.78 | 100.00/0.35 | 81.45/1.58 | 95.16/0.86 | 87.90/1.35 |
| APX3  | 99.59/0.41 | 100.00/0.00 | – | 75.31/1.85 | 100.00/0.38 | 82.30/1.56 | 97.12/0.86 | 88.48/1.38 |
| APX4  | 75.10/1.75 | 75.40/1.73 | 73.66/1.91 | – | 73.22/1.92 | 72.29/1.88 | 75.79/1.72 | 66.27/1.83 |
| APX5  | 99.58/0.48 | 100.00/0.38 | 100.00/0.38 | 74.48/1.85 | – | 81.17/1.67 | 97.49/0.90 | 89.12/1.31 |
| APX6  | 82.73/1.57 | 82.26/1.70 | 83.13/1.68 | 69.88/1.82 | 84.10/1.70 | – | 81.12/1.50 | 73.90/1.66 |
| APXS  | 95.18/1.00 | 95.97/0.97 | 97.12/1.00 | 75.00/1.73 | 97.49/1.05 | 82.73/1.55 | – | 83.52/1.38 |
| APXT  | 87.55/1.47 | 89.52/1.36 | 89.71/1.32 | 67.46/1.87 | 90.79/1.32 | 74.70/1.80 | 82.42/1.34 | – |

Red-color highlighted pairs show the highly conserved structural overlaps.

CYTC-1 (cytochrome c-1), and CYTC-2 (cytochrome c-2) proteins were predicted as the main interaction partners of *Arabidopsis* cytosolic APX1. MDHAR, MDAR1 and ATMDAR2 catalyze the conversion of monodehydroascorbate to ascorbate (Chew et al., 2003). GPX2 is a type of H₂O₂-scavenging enzyme and a crucial component in reactive oxygen network (Tanaka et al., 2005). DHAR1 has dual functions: soluble protein, it demonstrates GSH-dependent thiol transferase and dehydroascorbate (DHA) reductase activities, and is involved in redox homeostasis. As a peripheral membrane protein, it functions as voltage-gated ion channel (Dixon et al., 2002; Sasaki-Sekimoto et al., 2005). RHL41 affects in modulation of light acclimation, and cold and oxidative stress responses (Rizhsky et al., 2004; Davletova et al., 2005). ATPQ functions in ATP production (Carraro et al., 2014). FBP is reported to be a key component in photosynthetic sucrose synthesis (Cho et al., 2012). CYTC-1 and CYTC-2 are electron carrier proteins related with mitochondrial electron transport chain (Welchen and Gonzalez, 2005). In light of putative interaction partner analysis, it was apparent that *Arabidopsis* cytosolic APX1 is either directly or indirectly associated with redox homeostasis, stress adaptation and photosynthesis/respiration-related pathways. This could also help in better understanding the functional role of APX1 in various plant defense mechanisms.

Comparison of APX and GPX Sequences
A strict homology search of *Arabidopsis* GPX1-8 sequences in proteome datasets of 18 specified plant species has given a total of 87 putative GPX sequences; however, homology search of *Arabidopsis* APX1-6, APXT, and APXS in proteome...
datasets of these species identified a total of 120 putative APXs (Tables 1, 5). Sequences of GPX homologs contained the GPX (PF00255) protein family domain while APX homologs included the peroxidase (PF00141) domain. GPX genes encoded a protein of 166–262 residues with 18.4–29.7 kDa molecular weight and 4.59–9.60 pI value, while APXs encoded a polypeptide of 197–478 residues with 23.7–52.1 kDa molecular weight and 5.03–9.23 pI value. GPX transcripts mainly contained six exons; whereas, APX usually had 8–12 exons, implicating the relatively less conserved structure of APXs compared to GPXs. Sequence variations in GPX and APX homologs primarily derived from the “transit peptide” residues between organelle and non-organelle related sequences. Besides, regions corresponding to transit peptide sites in GPX/APX sequences did not demonstrate any particular pattern, indicating the less conserved structure of transit peptides thereby the functional diversities of APXs/GPXs at various targets. In addition, multiple-alignment analyses demonstrated the presence of a considerable degree of conserved residues in main sites of both enzymes. In GPX phylogeny, cytosolic-, nuclear-, extra cellular-, and plasma membrane-related GPXs were relatedly clustered while chloroplast/mitochondrial-related GPXs grouped together. APX phylogeny also showed similar clustering pattern, in which cytosolic-related APXs were relatedly clustered while chloroplast/mitochondrial-related APXs were together. This indicates that presence/absence of “transit peptide” residues was the main determinant in phylogenetic distribution of APX/GPX sequences. Moreover, presence of sequences with different subcellular localizations in the same phylogenetic group inferred the possibility of gene duplication events in formation of some APX/GPX sequences. Several segmental duplications were identified in some GPX pairs, while several segmental and tandem duplications were available in some APX pairs. Expression profiles of GPX and APX genes in model organism Arabidopsis indicated that stress factors, actively growing tissues, even normal physiological, and metabolic changes could induce the expression of APX/GPX genes. Interactome analyses of Arabidopsis cytosolic APX1 and GPX2 also implicated that both enzymes are closely related with antioxidant and redox homeostasis, secondary metabolite metabolisms and stress adaptation thereby supporting the functional roles of APXs/GPXs in H$_2$O$_2$-scavenging and plant defense. Despite of some minor variations, APX and GPX members, they topologically demonstrated highly conserved structure.

**CONCLUSIONS**

The presence or absence of transit peptide residues are the main contributing factors in subcellular localization and phylogenetic distribution of APX/GPXs. The APX/GPX expression is highly associated with the metabolic state of the cells. In addition, there are grounds for belief that these two enzymes work together in various pathways such as antioxidant and secondary metabolite metabolisms, redox homeostasis, stress adaptation,
and photosynthesis/respiration. This also supports the functional role of these enzymes in H₂O₂-scavenging, thereby implicating their importance in the plant defense. However, further molecular and physiological studies are required to elucidate the various functional roles of APX/GPX isoforms.

AUTHOR CONTRIBUTIONS

IK and EF contributed to the study conception and design. KK performed experiments and collected data. Data analysis and interpretation were performed by RV. IK, EF, KK, and RV prepared, and NA performed critical reading and revision of the manuscript. IO and EF supervised and MO coordinated this work. All the authors read and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00301

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