Molecular characterization and transcriptional profiling suggested structural and functional divergence in ascorbate peroxidase (APX) and APX-related (APX-R) genes in *Triticum aestivum* L.

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Abstract

Background: Ascorbate peroxidases (APXs) are heme-dependent H₂O₂ scavenging enzymes providing the antioxidant defense and regulating myriad biological processes. APX-related (APX-R) is an additional form of APX protein which lacks peroxidase activity but form dimer with the chloroplastic APX (chlAPX). Despite of tremendous their functions, a systematic and comparative study of these genes is limited in *Triticum aestivum* L. **Results:** Herein, a total of 21 TaAPX and six TaAPX-R genes were identified from the A, B and D sub genomes of *Triticum aestivum*, which form nine homeologous groups. These identified genes consisted of three pairs of paralogous genes which confronted pre-hybridization gene duplication under purifying selection. An unequal evolutionary rate after gene duplication suggested functional divergence between paralogous genes. The phylogenetic study classified TaAPXs into four distinct clades, where TaAPX-R proteins were clustered in clade I. The members of each clade showed similarity in gene and protein architecture, motifs distribution, sub-cellular localization that suggested their conserved nature. The tertiary structure analysis of three TaAPXs and one TaAPX-R revealed the presence of helices and coils, and residues involved in ligand binding. Transcriptional profiling of TaAPX and TaAPX-R genes suggested their specific role during development and abiotic and biotic stress response. Modulated transcript expression and APX enzyme activity during heat, drought and salt stress confirmed their role in these stress conditions. Co-expression analyses predicted interaction of these genes with the other genes involved in numerous biological processes. These genes were also found to be regulated by miRNAs associated with various developmental pathways and/or stress response. Further, they also exhibited interaction with the mediator compounds of Halliwell-Asada cycle. **Conclusions:** Overall, the present study reported numerous molecular and structural features of TaAPXs and TaAPX-Rs, and their role in plant development and stress response. It provides a platform for detailed functional characterization of TaAPX and TaAPX-R genes in future studies.

Background

Ascorbate peroxidases (APXs) are class I heme peroxidases that belong to the non-animal peroxidase superfamily. These are abundant in the plant kingdom and metabolize hydrogen peroxide (H₂O₂) into water (H₂O) and oxygen (O₂) within the plant cell [1]. Moreover, they are also reported from *Trypanosoma cruzi* and bovine eye [2, 3]. These are key regulatory enzymes of Halliwell-Asada or ascorbate-glutathione pathway that equilibrates the reactive oxygen species (ROS) concentration within the cell [4]. This pathway occurs in numerous sub-cellular organelles but it is crucial in chloroplast and mitochondria due to continuous production of ROS during photosynthesis and respiration [5, 6].

In a plant cell, APXs may occur in soluble or membrane-bound forms, and localized in various sub-cellular compartments [7]. They may be cytosolic (cAPX), stromal (sAPX) and mitochondrial (miAPX) in soluble form, while peroxisomal/glyoxisomal (mAPX) and thylakoidal (tAPX) in membrane-bound form [8]. A new form of APX called APX-related (APX-R) protein is also known, which forms a dimer with the chloroplastic APX (chlAPX) but lacks peroxidase activity [9]. They are conserved in most of the plant species and share high structural and functional homology. APXs show high specificity toward ascorbate (AsA) which acts as an electron donor during H₂O₂ reduction. The concentration of AsA within the cell extremely affects the APXs activity [8, 10]. Moreover, cAPX and miAPX isoforms display comparable affinity toward other electron

donors such as guaiacol or pyrogallol in certain conditions [1, 11], which suggests the occurrence of two kinetically compatible binding sites in these isoforms [11].

In higher plants, *APX* genes are known for regulating the normal plant developmental processes and abiotic and biotic stresses as well by controlling the signal flux of H_2O_2 . Under normal conditions, reduction in photosynthesis and growth rate, and delayed flowering has been observed in a *cAPX1* mutant of *Arabidopsis thaliana* in comparison of wild type [12]. However, *AtAPX3* mutant has no effect on growth and development [13]. Further, a *APX-R* gene i.e. *AtAPX6* provides protection to seeds in *A. thaliana* from oxidative stress during desiccation, at the time of maturation and germination [14]. The function of *OsAPX1* in seed development has been reported in *Oryza sativa* where mutant lines showed a reduction in panicle size and weight and produced ~58% aborted seeds. Moreover, the pattern of growth and development before flowering and number of seeds per panicle were similar to the wild-type [15]. The *OsAPX2* knockout mutant has both pleiotropic effects on growth and development, and reduced tolerance to abiotic stress [16]. Higher *APX* activity is observed in a tolerant genotype of sweet potato after 24 and 48 h of salinity stress [8]. Further, an elevated expression of *AtAPX2* is reported at high light intensity and temperature stress [14]. Similarly, peroxisomal *APX* of *Populus*, *OsAPX1*, *OsAPX2*, and *OsAPX5-OsAPX7* of *O. sativa*, and cytosolic *APX2* of *Vigna unguiculata* exhibited elevated expression during drought stress [8]. Over expression of *tAPX* of *Solanum lycopersicum* provided enhanced tolerance to salt and osmotic stress in transgenic tobacco plants [8].

Despite their significant roles, a systematic characterization of *APX* genes (*TaAPXs*) has not been carried out in the staple crop *Triticum aestivum* (bread wheat). In the current study, we performed genome-wide analysis of *TaAPX* and *TaAPX-R* genes in *T. aestivum*. The identified genes and proteins were subjected to a systematic *in-silico* characterization to explore their physicochemical and structural properties. The phylogenetic and syntenic relationships, gene duplication events and divergence time of paralogous genes were estimated to understand the evolution of *APX* and *APX-R*. The *cis*-regulatory elements, miRNA targets, and expression and co-expression patterns were studied to reveal the functions of these genes in growth and development and stress conditions. Further, the expression of randomly selected one gene from each phylogenetic clade was confirmed by qRT PCR. The *APX* enzyme assay was also performed after heat, drought and salinity stress treatments to validate their role in these stresses.

Results

Identification and chromosomal localization of *TaAPX* and *TaAPX-R* genes

A total of 21 *TaAPX* and six *TaAPX-R* genes were identified from the A, B and D sub-genomes of *T. aestivum*, which were clustered into nine homeologous groups (HGs) (Additional file 1). These HGs included seven *TaAPX* (*TaAPX-1* to *TaAPX-3*, *TaAPX-5* to *TaAPX-7*, and *TaAPX-9*) and two *TaAPX-R* (*TaAPX-4* and *TaAPX-8*) group genes. A maximum of four genes (three *TaAPXs* and one *TaAPX-R*) were localized on chromosome 2, which was followed by chromosome 7 with two genes on each A, B and D sub-genome. The remaining chromosomes consisted of one gene each except chromosome 1 and 3 (Fig. 1a, Additional file 2).

Insight into the gene duplication events and their divergence

The study of paralogous genes suggested the occurrence of three segmental gene duplication events (*TaAPX2-A/TaAPX5-A*, *TaAPX2-B/TaAPX5-B*, and *TaAPX2-D/TaAPX5-D*) with more than 5 Mb distance between paralogous gene pairs (Fig. 1a, Table 1). The Ka/Ks ratio of each duplicated gene pair was <0.1, which indicated purifying selection. Analysis of divergence time indicated the occurrence gene duplication around 79 to 93 mya (million years ago) (Table 1). The evolutionary rate per year between the duplicated gene pairs was compared using Tajima's relative rate test. The results showed a significant value (30- 44) of χ^2 for all duplicated gene pairs (Table 2). Further, a diverse expression pattern was observed between the duplicated genes (Fig. 1b).

Phylogeny and synteny analysis

A total of eight APX genes in each *A. thaliana*, *Brachypodium distachyon*, and *Hordeum vulgare*, and ten in each *O. sativa*, and *Zea mays* were identified through extensive data mining and used for the analysis of syntenic relationship with *TaAPX* and *TaAPX-R* genes (Fig. 2a-e). The results indicated variable distribution of these genes on various chromosomes in each plant species, and a close evolutionary relationship among the orthologs of different plant species. The APX protein sequences of these orthologs were used for the phylogenetic analysis (Fig. 3). Tree topology indicated the grouping of APXs into four distinct clades, which were further divided into sub-clades (Fig. 3). Based on the homology with the earlier reported APX proteins of *A. thaliana* and *O. sativa*, it was presumed that the clade I APXs were mostly chloroplastic, and specific to a special group of APX-related (APX-R) proteins [9, 17]. However, APXs of clade II, III and IV were predicted as cytoplasmic, peroxisomal and chloroplastic/mitochondrial, respectively.

Intron/exon distribution and splice variants

Gene structure of all *TaAPXs* and *TaAPX-Rs* and their splice variants were investigated for exon/intron organization and intron phases. The number of exons ranged from 7 to 12, however, 46% of genes were having 11 exons (Fig. 4a). Among all the genes, ~60% introns were in 0 intron phase, followed by 23% and 17 % in phase 1 and 2, respectively. *TaAPX-3*, *TaAPX-6* and *TaAPX-7* group genes predominantly consisted of 0 phase introns, while intron phase 1 and 2 were leading in case of *TaAPX-8* group genes. *TaAPXs* also shared the conserved pattern in the number and length of various exons across the tightly clustered genes in phylogeny. For instance, the *TaAPX1*, *TaAPX2*, *TaAPX5* and *TaAPX9* group genes, and *TaAPX3*, *TaAPX6* and *TaAPX7* group genes shared a similar number and length of exons. However, *TaAPX4* and *TaAPX8* group genes of *TaAPX-R* exhibited different genomic structure as compared to the other group genes. The length of exon 2, 4, 5, 6 and 7 was conserved with 125, 49, 83, 80 and 103 bp, respectively. However, the 3rd exon (116 bp) of *TaAPX-1* was assumed to be split between 3rd (50 bp) and 4th (66 bp) exons of *TaAPX-9* group genes, or vice versa. Similarly, 2nd (125 bp) and 3rd (50 bp) exons of *TaAPX9* were probably merged to form 2nd exon (175 bp) of *TaAPX-2* and *TaAPX-5* group genes or vice versa. Moreover, the length of other exons was conserved in these genes. On the basis of variation in the number of exons or exon length, a total of 13 and three splice variants were identified, which were encoded by nine *TaAPX* and three *TaAPX-R* genes, respectively (Fig. 4a, Additional file 1). The TSA BLAST analysis suggested the existence of most of the splice variants at transcript stage (Additional file 3). Nine splice variants differed in exon length, while seven in exon number. The difference in length of the 2nd exon was observed in most *TaAPX-6* group genes and their splice variants. *TaAPX3-A.1* and *TaAPX3-A.2* showed the difference of three nucleotides in the length of

the 9th exon. However, a few have differences in higher nucleotide numbers such as 1st exon of *TaAPX7-B.1* and *TaAPX7-B.2* differed in 24 nucleotides. In case of variable number of exons, the events of exon skipping such as in *TaAPX9-D.2* or intron retention such as in *TaAPX7-B.3* were observed. However, the other variants differed in the number of exons such as *TaAPX5-A.1*, *TaAPX8-A.1*, *TaAPX8-D.2* etc. (Fig. 4a).

Physicochemical and structural properties

The length of TaAPXs and TaAPX-Rs ranged from 227 to 469 amino acid (AA) residues (Table 3, Additional file 2). The average molecular weight (MW) of clade I (TaAPX4 and TaAPX8), clade II (TaAPX2 and TaAPX5), clade III (TaAPX1 and TaAPX9) group proteins were 35, 29 and 31 kDa, respectively. In clade IV, the average MW of TaAPX3 and TaAPX6 group proteins was 35 kDa, while TaAPX7 group proteins was 47 kDa. Latter consisted of an additional transmembrane helix (TM). The presence of TM was also predicted in clade III proteins or mAPXs except TaAPX1-B and TaAPX9-D.2. The pI ranged from 4.9 to 9.25, though the pI of maximum proteins was above 7. The grand average hydropathy (GRAVY) value was ranged from -0.522 to -0.108. The minutiae of protein sequences were analyzed for conserved motifs using the MEME server as well as multiple sequence alignments (Fig. 4b and 5a-d). The TaAPX proteins showed a conserved pattern in various AA residues involved in catalysis and ligands binding such as Lys-30, Cys-32, Arg-172, Trp-179, His-163 etc. However, in case of TaAPX-Rs most of these residues such as Cys-32, Arg-172, Trp-179 etc. were either absent or substituted with the other AA residues. Further, TaAPX-4 and TaAPX-8 group proteins were also varied from each other in the primary structure at the active-site domain (ASD), heme-binding domain (HBD) and cation-binding domain (CBD) (Fig. 5d). The MEME server identified a total of ten conserved motifs in the TaAPX proteins (Fig. 4b, Additional fig. 1). Motifs 1 to 9 were found conserved in all chlTaAPX proteins (TaAPX3, TaAPX4 and TaAPX7), while motif 10 was additionally present in TaAPX7 group proteins (Fig. 4b). In mAPX (TaAPX1 and TaAPX9) and cAPX (TaAPX2 and TaAPX5), all the motifs were conserved, except motifs 6 and 10. Motifs 2, 5, 7 and 8 were conserved in TaAPX-R (TaAPX4 and TaAPX8) proteins. Analyses of AA residues of motifs 2, 3 and 6 showed that they represent ASD, HBD, and CBD, respectively. The BLAST search of the other motifs sequence revealed the presences of heme-dependent peroxidase domain. Further, two conserved signature motifs; chloroplastic domain 1 (K-N-I-[EKNS]-[ED]-W-P) near ASD, and chloroplastic domain 2 (E-T-K-Y-T-K-[DNTE]-G-P-G-[AE]-[PA]-G-G-Q-S) near HBD were also detected in chlTaAPX proteins.

The secondary and tertiary structure analyses revealed the prominence of helices and loops in TaAPX proteins (Fig. 5e-5h). The percentage of helices was higher than loops in TaAPX1-A (mAPX) and TaAPX2-B (cAPX) in contrast to TaAPX3-A.1 (chlAPX) and TaAPX8-B.1 (APX-R). Primarily, helices A to J were present in all the structures, however, few small helices such as A1', B', D' etc. were also identified adjacent to some helices. Further, TaAPX1-A, TaAPX2-B and TaAPX8-B.1 have common best PDB hit (2vcfX, cAPX of soybean), but TaAPX2-B showed high percentage structural homology with 2vcfX than others, indicating the sub-cellular localization based structural relatedness. Besides this, TaAPX3-A.1 showed high homology with PDB hit 1iynA, a chlAPX from the tobacco plant. Various structural parameters and ligand binding residues have been mentioned in Table 4. Further, TaAPX3 and TaAPX7 group proteins and TaAPX-R (TaAPX4 and TaAPX8) proteins consisted of cTP while the TaAPX6 group proteins consisted of mTP (Additional file 2). TaAPX1 and TaAPX9 group proteins consisted of an alanine and valine rich region along with SRK domain (Fig. 5b) which suggested their peroxisomal localization [18, 19].

Promoter analysis

The promoter region of all *TaAPX* and *TaAPX-R* genes were analysed for the presence of various *cis*-regulatory elements except *TaAPX4-B* and *TaAPX8-A* due to unavailability of the promoter sequence. They have been broadly categorized into growth and development related, hormone, light, and stress-responsive *cis*-acting elements (Additional table 1). The growth and development responsive elements such as CCGTCC-box and CCGTCC-motif related to meristem-specific activation were predominant among these genes. The *cis*-acting elements such as circadian, AC-I, AC-II, CAT-box, O₂-site etc., were also identified in the promoter region of various genes and known for regulating different plant developmental processes. However, none of the growth and development responsive elements has been identified in *TaAPX5-A* and *TaAPX8-D*. The hormone responsive elements such as CGTCA-motif and TGACG- motif involved in the MeJA responsiveness, and MYC involved in jasmonic and abscisic acid signalling were prominent in the majority of the promoter. Besides this, AuxRR-core and TGA-element (auxin-responsive elements), and GARE-motif, P-box and TATC-box (gibberellin-responsive element) were also found in the promoter region of certain *TaAPX* genes. Various kind of light responsive *cis*-acting elements such as ATC-motif, AE-box, Box III, Box 4, G-box, GATA-motif, Gap-box, GATT-motif, TCCC-motif, TCT-motif etc. have also been found in *TaAPXs* promoters. Additionally, several stress-responsive *cis*-acting elements were identified including ABRE (abscisic acid responsive element), as-1 (involved in stress response related to auxin, salicylic acid, and methyl jasmonate), MYB (drought inducibility), MBS (MYB binding site; involved in drought-inducibility), STRE (heat, osmotic, starvation etc. stress responsive), W box (defence against pathogen), WRE3 (wound-response element) etc.

Transcriptional profiling in tissue developmental stages and stress conditions

The transcriptional profiling was performed to analyze the putative role of *TaAPX* and *TaAPX-R* genes during plant development and in the presence of various biotic and abiotic stress conditions. A significant correlation (R^2 0.98) between the replicates suggested consistent expression value in replicates (Fig. 6a, b). During plant development, *TaAPX1*, *TaAPX2*, *TaAPX3* and *TaAPX5* group genes showed spatiotemporal expression in different developmental stages of root, stem, spike and grain (Fig. 6c), in which former three were highly expressed in spike, grain and root tissues, respectively. However, *TaAPX7* and *TaAPX9*, and *TaAPX-R* (*TaAPX4* and *TaAPX8*) group genes were predominantly expressed in all the leaf developmental stages and stem_z65. Moreover, high expression of *TaAPX1*, *TaAPX2* and few other genes were observed in spike and grain developmental stages.

During biotic stress, the expression of a total of 25 transcripts including splice variants was affected (Fig. 6d). *TaAPX3-B*, *TaAPX3-D* and *TaAPX5-A.2* were consistently up-regulated after *Bgt* infestation, but less affected after *Pst* infestation. However, *TaAPX6-A.1*, *TaAPX6-B.1*, *TaAPX6-B.3* and *TaAPX8-A.2* were upregulated after the infestation of both the fungal pathogens. A few genes were found to be either early or late responsive. For instance, *TaAPX3-A.2* and *TaAPX7-B.2* were upregulated after 24 h, while *TaAPX6-B.1* and *TaAPX8-A.2* were upregulated after 72 h of infestation of *Bgt* and *Pst*, separately. Further, a few genes were found to be pathogen-specific such as *TaAPX5-D* and *TaAPX1-B* were exclusively up-regulated after *Bgt* and *Pst* infestation, respectively.

A total of 13 transcripts exhibited differential expression in the presence of salt stress, and most of them showed temporal differentiation (Fig. 6e). For instance, *TaAPX6-B.3* and *TaAPX6-D.2*; *TaAPX3-B*; *TaAPX5-B.2* and *TaAPX6-D.3*; *TaAPX1-A*, *TaAPX5-A.1* and *TaAPX6-A.1* were selectively upregulated at 6, 12, 24 and 48 h of salt treatment, respectively. *TaAPX1-B* and *TaAPX8-D.2* were found upregulated at both 24 and 48 h, and *TaAPX8-A.1* at 6 and 24 h of salt treatment. However, *TaAPX6-D.1* and *TaAPX6-D.2* were down-regulated at 24 and 48 h of salt treatment.

The transcriptional profiling of *TaAPX* genes was also performed after heat (HS), drought (DS) and their combination (HD) stress. Expression of a total of 29 transcripts was affected during these stresses (Fig. 6f). The majority of *TaAPX* and *TaAPX-R* genes were downregulated after HS, DS and HD, while *TaAPX1-A*, *TaAPX1-B*, *TaAPX1-D*, *TaAPX7-B.3* and *TaAPX8-D.2* were upregulated in one or more stress. *TaAPX7-B.2* was upregulated in each treatment except DS_1h, while *TaAPX3-D* was downregulated in all treatments.

The validation of expression profile of one randomly selected gene from each phylogenetic clade such as *TaAPX3-B*, *TaAPX5-B*, *TaAPX8-D* and *TaAPX9-A* was performed by qRT PCR under similar abiotic stress conditions. The results were mostly found consistent with the expression pattern obtained from the RNA seq data (Additional Fig. 2, Additional table 2).

Co-expression analysis

The co-expression analyses of *TaAPX* and *TaAPX-R* genes were performed to explore their putative *modus-operandi* in tissue developmental stages and stress conditions, as well. A total of 51 genes were found co-expressed with *TaAPX6-D.3* and 5 genes with *TaAPX6-A.1* in different tissue developmental stages (Fig. 7a). However, under biotic, salt, and heat, drought and their combination stress in total 74, 130 and 105 genes were co-expressed with 7, 16, 18 *TaAPX* and *TaAPX-R* genes, respectively (Fig. 7b-d). The co-expressed genes encode various development related and stress-responsive proteins such as wall-associated receptor kinase (WAK), E3 ubiquitin-protein ligase (E3-Ubl), F-box, chaperone protein DnaJ, mitogen- activated protein kinase kinase 3-like isoform X1 (MAPKK3), receptor-like cytoplasmic kinase 176 (RLCK176), dehydrin 7 (dhn7), auxin response factor 17 (ARF17), histone proteins, serine/threonine-protein kinase (STK), WRKY transcription factor 72 (WRKY72), GDSL esterase/lipase (GDSLE), blue copper protein (BCP) etc. (Additional file 4). The gene ontology (GO) mapping of these co-expressed genes suggested their role in the biosynthetic process, reproduction, cell differentiation, growth, response to stress, protein kinase activity, thiol-dependent ubiquitin-specific protease activity, protein hetero-dimerization activity, defence response to fungus etc. (Additional file 4).

miRNAs and chemical interaction

A total of 51 known miRNAs of *T. aestivum* showed interaction with 29 *TaAPX* and nine *TaAPX-R* transcripts including splice variants, where 44 miRNAs function through cleavage while six miRNAs by translation inhibition (Fig. 7e, Additional file 5). Moreover, tae- miR319b functions by both mechanisms, it regulates the expression of *TaAPX6-B* and *TaAPX6-D* by cleavage, and *TaAPX4-D* and *TaAPX4-A* by translation inhibition. A few miRNAs including tae-miR2026a, tae-miR1211a, tae-miR444b etc. target single gene, while the majority of miRNAs interacted with more than one *TaAPX* genes. For instance, tae- miR319b and tae-miR396g

targeted *TaAPX4-A*, *TaAPX4-D*, *TaAPX6-B* and *TaAPX6-D*, and *TaAPX4-A*, *TaAPX4-B*, *TaAPX4-D*, *TaAPX5-A*, *TaAPX5-B* and *TaAPX5-D*, respectively.

The chemical interaction analysis showed the association of TaAPX and TaAPX-R proteins with most of the mediator compounds in Halliwell-Asada or ascorbate-glutathione cycle such as H₂O₂, ascorbic acid, catalase, ascorbate radical, reduced glutathione, dehydroascorbic acid, superoxide anion, GSSG etc. [4]. Besides this, the association with other inorganic compounds like calcium ions, water, manganese, sodium, ammonia, oxygen was also observed (Fig. 7f, Additional file 6).

Ascorbate peroxidase assay

The effect of HS, DS, HD and salt stress on APX activity was analyzed in root and shoot tissues of *T. aestivum*. The APX activity in shoot tissue was significantly increased by ~2 fold at 1 h of HS and DS treatments, while ~1.5 fold in HD 1 h (Fig. 8a). However, the activity was ~1.5 fold higher at 6 h of HS, DS and HD treatments. The activity in root tissue was significantly increased at DS 6 h, whereas, it was not much affected in case of HS and HD (Fig. 8b).

In case of salt stress, the activity in shoot tissue was increased by 1.3 fold at 12 h and 24 h but remained unaffected at 6 h. However, at 48 h the activity decreased by 1.2 fold (Fig. 8c). Similarly, the APX activity in root tissue was increased ~1.3 fold in 6, 12 and 24 h of treatment while the activity declined by ~1.2 fold at 48 h of treatment (Fig. 8d).

Discussion

APX provides antioxidant defense response and regulates various biological pathways by maintaining H₂O₂ homeostasis within the plant cell. The present study focuses on the molecular characterization of *TaAPX* and *TaAPX-R* genes and proteins in the genome of *T. aestivum*. It reinforces the knowledge about the structure, phylogenetic and syntenic relationships, and transcriptional profiling of *TaAPXs* and *TaAPX-R*. The APX assay was also performed to examine the change in enzyme activity under various abiotic stress treatments.

The studies in different plants showed the occurrence of six to eight APX genes in most of the diploid plants such as in *A. thaliana*, *Gossypium raimondii*, *O. sativa*, etc. [19–21], while 26 *GhAPX* genes in tetraploid *G. hirsutum* [22]. In lower plants, the occurrence of four and five genes were reported in *Chlamydomonas reinhardtii* and *Physcomitrella patens*, respectively [21]. In the present study, 27 *TaAPX* genes including *TaAPX-R* are identified in *T. aestivum*. The occurrence of about thrice the number of APX genes in the *T. aestivum* genome as compared to the diploid plant species might be attributed to their allohexaploid nature. The results suggested an analogy in the number of APX genes and the complexity of the plant genome. On the contrary, typically one *TaAPX-R* gene has been reported in most of the plants and a few consisted of two genes [23]. This might be attributed to gene loss during the course of evolution.

In addition to the ploidy level, the expansion of gene families occurs due to the phenomenon of gene duplication, which is important for genetic diversity. Certain duplicated genes showed functional divergence

while others remain conserved based on their evolutionary rate [22]. In the various plant species, one to three segmental DEs are reported in *APX* gene family [21] as observed in the case of *T. aestivum*. However, a few tandem, or both segmental and tandem DEs were also described in certain plants such as *B. distachyon*, *G. raimondii*, *Z. mays* etc. [21]. The *TaAPX-R* genes faced strong negative selection pressure; therefore, gene duplication among them is uncommon [23]. Similar to the *TaAPXs*, the paralogous *APX* genes in other plant species such as *G. hirsutum* were also under purifying selection pressure, which indicated conserved nature of this gene family [22]. The hybridization events among the progenitors of A, B and D sub genomes of *T. aestivum* were predicted from 0.5 to 6 mya [24], which suggested pre-hybridization DEs among *TaAPX* genes. The significant value of χ^2 within duplicated genes indicated that the genes were evolved at an unequal rate. It leads to the rejection of the molecular evolutionary clock hypothesis which articulate a similar evolutionary rate for the duplicated gene [25]. The deviations in the expression profiles of duplicated *TaAPX* gene pairs also supports the incidence of functional divergence or neo-functionalization among the paralogous genes. In the case of *G. hirsutum*, *GhAPX10A/11A/12A* duplicated genes were significantly evolved at an unequal rate suggesting the occurrence of functional divergence. However, *GhAPX10D/11D/12D* duplicated genes showed insignificant evolutionary rate [22]. The synteny and phylogenetic analysis indicated a close evolutionary relationship among the orthologous genes of various plant species. The phylogenetic clustering of *APXs* with respect to their sub-cellular localization has also been discussed in several plant species such as *O. sativa* and *G. hirsutum* [19, 22]. *APX-R* proteins were separately clustered and are known to control the activity of other *APXs* especially *chlAPXs* through physical protein-protein interaction and regulate antioxidant responses [10].

The occurrence of comparable intron/exon organization and similar exon length in the *APX* genes of various plant species such as *A. thaliana*, *O. Sativa*, *Pisum sativum*, *T. aestivum* and other plants suggested conserved gene architecture in higher plants [19, 21, 22]. However, variations in the structure of *TaAPX-R* genes suggested their divergent nature in comparison to the *TaAPX* genes. The analysis of splice variants indicated the events of exonization or pseudo-exonization during the course of evolution of this gene family. The variation in exon length in splice variants was probably introduced due to the availability of additional 5' or 3' splice sites. The occurrence of splice variants may facilitate structural and functional divergence among *TaAPX* genes.

The physicochemical and structural properties of *TaAPX* and *TaAPX-R* proteins show consistency with other plant species such as *A. thaliana*, *B. distachyon*, *G. max*, *O. sativa*, *Z. mays* etc. [19, 21, 22]. Further, the low GRAVY value of *TaAPXs* suggested their hydrophilic nature and better interactions with water. *APX* also shares highly conserved motifs and domains such as ASD, HBD and CBD which are important for the peroxidase activity. These domains are substituted with new sets of conserved amino-acid residues in *APX-R* [9]. In addition, the two signature motifs specific for *chlAPXs* play a significant role in determining the substrate specificity, but they are not related to peroxidase activity [19]. The occurrence of motif 10 at C-terminus of *chlTaAPX7* and N-terminus of *TaAPX8* group proteins suggested their possible interaction as shown in *chlAPX* and *APX-R* of *O. sativa* [9]. The localization of *APXs* into different subcellular compartments mainly depends on the presence of a type of sorting peptide such as cTP and mTP for chloroplast and mitochondria, respectively. However, the peroxisomal *APX* usually consist of alanine and valine rich hydrophobic trans-membrane region followed by a positively charged domain [18, 19]. The

peroxisomal localization of APX proteins usually depends upon the overall basic AAs composition rather than the presence of a specific sequence or a single positively charged AA residue [18].

The presence of development related and stress-responsive *cis*-acting elements in the promoter region of *TaAPXs* and *TaAPX-R* genes suggested their role in various biological processes and defense responses. The occurrence of related or similar *cis*-acting elements has been described in the promoter region of *TaSODs*, *TaGPXs* and *GhAPX* genes etc. [22, 26, 27]. These *cis*-acting elements suggest the co-activation of genes involved in ROS detoxification and other antioxidant mediated biological pathways. The specifically high expression of certain *TaAPX* genes in particular tissues suggested their precise role in its growth and development such as involvement of *TaAPX6* during grain maturity, and *TaAPX1* and *TaAPX2* in the flowering, reproduction and grain filling. The function of *AtAPX1* in plant growth and flowering, and *AtAPX6* in seed development has been described in *A. thaliana* [12, 14]. *APXs* are also known to regulate leaf development and senescence, root development, pre and post-reproduction processes, cell elongation etc. through signaling mechanism [28].

Besides this, the importance of *APXs* in defense response during biotic and abiotic stresses is well known in numerous plants [8, 29]. In case of *O. sativa*, *OsAPX8* is involved in providing tolerance against bacterial blight, while *OsAPX1* and *OsAPX2* have been reported in providing tolerance against pathogen attack, oxidative and salinity stress [29]. Further, the enhanced expression of *cAPX* in response to wounding has been shown in sweet potato [30]. The pathogen-specific expression of *TaAPX* genes suggested distinct responses of these genes during biotic stress. The expression analyses of *TaAPX* and *TaAPX-R* genes during HS, DS, HD and salt stress suggested their role in abiotic stress tolerance. The function of *APX* genes in providing salinity tolerance has been earlier described in numerous plant species such as *A. thaliana*, *O. sativa*, *S. lycopersicum* etc. [29]. Similarly, the upregulation of *APX* under heat, drought and salt stresses has been reported in *Brassica campestris*, *H. vulgare* and *O. sativa* [29]. The over expression of *B. campestris* and *H. vulgare* *APX* genes showed enhanced thermal tolerance in *A. thaliana* [31]. The activity of *APX* also gets affected under different stress conditions in *T. aestivum*, as reported in case of other plants. For instance, in rice and soybean the activity of *APX* was higher in salt tolerant genotypes compared to susceptible genotype [32, 33]. While, in some cases such as rice the activity of *APX* was higher in sensitive genotype [34]. Under drought stress condition the higher *APX* activity was observed in soybean [35]. Similarly, the enhanced *APX* activity was observed in various plant species under different stress conditions like *A. thaliana*, *Ipomoea batatas*, *Medicago sativa*, *S. lycopersicum*, *S. tuberosum*, *O. sativa*, *G. hirsutum* etc. [29]. Similarly, we observed increased *APX* activity during most of the analyzed abiotic stress conditions in shoot and root tissues of *T. aestivum*.

The co-expression analysis suggested the interaction of *TaAPX* and *TaAPX-R* genes with the other genes involved in diverse functions. Further, a few co-expressed genes also showed interaction with the other antioxidant enzymes encoding genes such as *TaGPX* [27], indicated their role in signalling during ROS burst. The regulation of *TaAPX* through miRNAs also strengthened their involvement in various biological pathways. These miRNAs were reported to target various plant development and stress-responsive genes in earlier studies. For instance, tae-miR1211a targets superoxide dismutase [Cu-Zn] 4AP, serine/threonine-protein kinase WNK2, Fumarate hydratase 2, SNF1-related protein kinase regulatory subunit beta-1-like etc.,

tae-miR444b targets MADS-box transcription factor, tae-miR390a and tae-miR390b targets ARF (auxin-mediated transcriptional activation/suppression), tae-miR396 targets growth-regulating factor 9, tae-miR319b targets transcription factor PCF etc. [36]. Further, the interactions of TaAPXs with various chemical compounds suggested that they might function as co-factors of either APX or interacting enzymes/proteins. However, the functional validation of each interaction needs to be performed in future studies.

Conclusions

In conclusion, structure analysis suggested conserved nature of *TaAPX* and *TaAPX-R* genes and proteins. Despite of structural similarities, the paralogous *TaAPX* genes were evolved at an unequal evolutionary rate and exhibited functional divergence. Differential expression profiling in developmental stages and stress conditions revealed their diverse biological functions, which were also supported by their co-expression with the other development related and stress responsive genes. These genes were also found to be regulated by miRNAs and various other factors. The stress responsive behavior of these genes might be explored for the development of stress resistant transgenic crop plants in future studies. Further, the interaction of *TaAPX* and *TaAPX-R* genes with each other as well as with the other genes can also be established.

Methods

Identification of TaAPX and TaAPX-R genes and their chromosomal positions

The *TaAPX* and *TaAPX-R* genes were identified by BLASTp search using known APX sequences from *A. thaliana* and *O. sativa* as a query against *T. aestivum* gene and protein model sequences (IWGSC RefSeq assembly v1.0.) downloaded from Ensembl Plants (<http://plants.ensembl.org/index.html>). The identified sequences were used for Hidden Markov Model (HMM) search for the peroxidase domain (PF00141) using Pfam BLAST at e-value 10^{-10} [37]. The non-redundant set of genes was then confirmed through PeroxiScan search [38]. NCBI Conserved Domain Database search was used to reconfirm the legitimacy of APX domain in each TaAPX and TaAPX-R sequence [39]. The existence of identified genes at transcript level was confirmed using BLASTn search against transcriptome shotgun assembly (TSA) sequences of *T. aestivum* at NCBI server (<https://www.ncbi.nlm.nih.gov/genbank/tsa/>). The nomenclature of identified *TaAPX* and *TaAPX-R* genes was carried out following the guidelines of international rules of genetic nomenclature for gene symbolization in wheat (<https://wheat.pw.usda.gov/ggpages/wgc/98/Intro.htm>). The plausible homeologous genes were identified through the standard procedures including bidirectional BLAST search (10^{-10} e-value and $\geq 90\%$ identity) and chromosomal similarity, as reported earlier [40, 41]. However, the similarity of $\geq 80\%$ among intra sub-genomic genes was used for the identification of paralogous genes. The localization of these genes on various chromosomes was retrieved from Ensembl Plants [42] and represented using MapInspect (<https://mapinspect.software.informer.com/>).

Evolutionary and syntenic relationships

The orthologous genes were identified through bi-directional BLAST search (e value 10^{-10}) in *A. thaliana*, *B. distachyon*, *H. vulgare*, *O. sativa*, and *Z. mays*. The non-redundant full-length protein sequences were aligned using MUSCLE [43] and a phylogenetic tree was constructed at 1000 bootstrap value by Neighbor-Joining

(NJ) method using MEGA7 [44]. The syntenic relationship among orthologous was depicted using the Circos-0.69-6 software [45].

Calculation of Ka/Ks, divergence time and Tajima's relative rate test

The nucleotide and peptide sequences of duplicated gene pairs were aligned using the ParaAT2.0 [46]. The alignment was used for the estimation of the number of non-synonymous substitutions per non-synonymous site (Ka), synonymous substitutions per synonymous site (Ks) and the ratio of Ka/Ks using the KaKs Calculator [47]. The formula, $T = Ks/2r$ was used for the calculation of divergence time between the duplicated genes, where T and r represent the divergence time and divergence rate, respectively. For monocotyledonous plants, the divergence rate was presumed to be 6.5×10^{-9} [48]. Additionally, the Tajima's relative rate test was performed to analyze the relative evolutionary rate after gene duplication using MEGA 7 [44].

Analysis of *cis*-regulatory elements, intron/exon, and splice variants

The promoter sequences were retrieved from the genomic sequences as a 1.5 kb upstream region from the CDS start site of each gene. The (+) and (-) strands of these sequences were analysed using the Plant CARE database for the identification of various *cis*-regulatory elements [49]. The organization of intron/exon, their numeral, exon length and intron phases in each gene was analysed by aligning the CDS sequence with the respective genomic sequence. The depiction of arrangement was made using the GSDS 2.0 server [50]. The splice variants were analyzed for different sorts of known splicing mechanisms by comparing the variants with their genomic sequence. The existence of these splice variants at transcript level was confirmed by TSA BLAST search.

Physicochemical and structural properties of proteins

The sequence-dependent properties of TaAPX and TaAPX-R proteins were determined using different servers. The molecular weight, isoelectric point (pI), length, GRAVY value of proteins were determined using ExPasy server and reconfirmed through Ensembl Plants and Sequence Manipulation Suite [42, 51, 52]. The subcellular localization was analyzed using the CELLO v.2.5 [53]. The protein sequences were further screened for the presence of chloroplast transit peptide (cTP) and mitochondrial targeting peptide (mTP) using the ChloroP 1.1 and the TargetP 1.1, respectively [54]. The presence of transmembrane helices was analyzed using the TMHMM v2.0 server [55]. The conserved motifs and domains were analyzed by the MEME Suite [56] and the NCBI conserved domain search [39], respectively.

The multiple sequence alignments were made using the Multalin [57] to examine the primary structure, conserved amino-acid residues, catalytic site, active site, metal-binding sites, and signature motifs. The tertiary structure of proteins was determined by homology modeling through the I-TASSER [58]. The possible ligand-binding site residues were further confirmed using the RaptorX binding site prediction server [59]. The association of organic and inorganic chemicals was analyzed using the STITCH software (<http://stitch.embl.de>) at high confidence (0.7) with not more than 50 interactions in the first shell. The orthologous proteins of *H. vulgare* with more than 85% similarity were considered for the analysis.

miRNA target prediction

The targets of known *T. aestivum* miRNAs [36] in *TaAPX* and *TaAPX-R* genes were identified using the psRNATarget server at default parameters [60]. The interactions between the miRNAs and target genes were represented through the Gephi 0.9.1 [61].

Transcriptional profiling and co-expression analyses

The transcriptional profiling and co-expression analyses of *TaAPX* and *TaAPX-R* genes were performed using high throughput RNA-seq data generated from various plant tissues and stress conditions. The fragments per kilobase of transcripts per million mapped reads (FPKM) value was determined through RNA-Seq by Expectation Maximization (RSEM) using Trinity pipeline [62]. The RNA seq data of *T. aestivum* available at wheat-urgi.versailles.inra.fr/files/RNASeqWheat/, developed in two biological replicates from three developmental stages of root, leaf, stem, spike, and grain [63, 64] were analyzed for the expression of these genes during development.

The RNA-seq data available in replicates (accession no.- PRJNA243835) after 24 h, 48 h and 72 h infestation of two major fungal pathogens; *Puccinia striiformis* (Pst) and *Blumeria graminis* (Bgt) of *T. aestivum* [65] were used for expression analysis under biotic stress, separately. The impact of abiotic stresses, heat (HS), drought (DS) and their combination (HD), were studied using RNAseq data (accession no. - SRP045409) available from the leaves in two biological replicates, after one and six hours of treatments, separately [66]. However, the effect of salinity (150 mM NaCl) stress was analyzed using the RNAseq data (accession no. - SRP062745) generated from the root after 6, 12, 24 and 48 h of treatments, separately [67]. The differentially expressed genes (≥ 2 folds) in various treatments were identified using inbuilt edge R software of Trinity pipeline [68]. Heatmaps for differentially expressed genes were constructed using the HCE (Hierarchical Clustering Explorer 3.5) with the Euclidean distance method of the hierarchical clustering of genes [69]. The gene expression was further confirmed using the WheatExp Server (<https://wheat.pw.usda.gov/WheatExp/>).

The co-expression analysis of *TaAPXs* and *TaAPX-Rs* with the entire gene pool of *T. aestivum* during developmental stages and in each stress condition was accomplished using the CoExpressv.1.5 tool. The genes having maximum expression value less than one FPKM were filtered out, and analysis was performed using the Pearson correlation coefficient with correlation power 1, filtering threshold ≥ 0.9 , bootstraps 100 and false discovery rate (FDR) 10 [70]. The co-expressed genes were annotated and mapped for gene ontology using the Blast2GO software [71]. The interactome was generated for each condition using the Gephi 0.9.1 [61].

Plant samples and growth conditions

The seeds of *T. aestivum* (cv. Chinese Spring) available in our laboratory [40] were sterilized with 1.2% sodium hypochlorite and 10% ethanol. The sterilized seeds were washed with double autoclave water and kept overnight for stratification at 4°C on moist Whattmann filter papers. The seeds were kept for germination at room temperature. The seedlings were grown in autoclaved water after transferring in fresh phytaboxes and kept in the plant growth chamber. Seven days old seedlings were subjected to different abiotic stresses

such as heat (40°C), drought (20% PEG) and their combination for 1 and 6 h, while salinity stress (150 mM NaCl) for 6, 12, 24 and 48 h, separately. The root and shoot samples of normally grown and stressed plants were immediately frozen in liquid nitrogen, and stored at -80°C for further experimentations.

Expression validation by qRT-PCR

The stored samples of root and shoot were ground in liquid nitrogen and total RNA was extracted using the Spectrum™ Plant Total RNA kit (Sigma, USA). The DNA contamination was removed using the TURBO DNA-free™ Kit (Invitrogen, USA). The integrity of isolated RNA samples was checked by gel electrophoresis and quantity was measured using the Nanodrop spectrophotometer. One microgram of RNA was used to synthesize the first-strand cDNA using the Superscript III First Strand Synthesis Supermix (Invitrogen, USA) following the manufacturer's instructions. The gene specific primers were designed for randomly selected *TaAPX* genes from each phylogenetic group using the Oligo Calc software for qRT-PCR analysis [72]. The qRT-PCR was performed using QuantiFast® SYBR® Green PCR Kit (Qiagen, Germany) on Bio-Rad CFX96 Real-Time PCR detection system following the method established [40]. ADP-ribosylation factor (TaARF1, AB050957.1) was used as an internal control [73], and fold change in gene expression was calculated using delta-delta CT-method ($2^{-\Delta\Delta CT}$) [74].

Determination of ascorbate peroxidase enzyme activity

For estimation of APX enzyme activity, 0.5 g of stored plant tissues were ground in liquid nitrogen and homogenized in 50 mM ice cold phosphate buffer (pH 7.0) containing 1.0 mM EDTA, followed by centrifugation at 10000 rpm, 4°C for 15 minutes following the previously described methods [75, 76] with little modifications. The supernatant was collected and immediately used for the enzyme activity assay. The total protein extracts were quantified according to the method established by Bradford [77] and bovine serum albumin (BSA) was used as standard. The APX activity was determined by analyzing the decrease in absorbance of ascorbate at 290 nm with an absorbance coefficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ [78] using Eppendorf BioSpectrometer® kinetic. The assay was performed in 1 ml reaction volume consisting of 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA, and 0.1 mM hydrogen peroxide. The reaction was started by adding 10 µl of crude protein extract and the absorbance decrease was recorded for 3 minutes [78]. All the experiments were performed in triplicates and expressed as mean $\pm$ SD. The APX activity data for heat, drought and their combination was statistically analyzed by analysis of variance (ANOVA) at 5% probability level using SPSS software. The post hoc Tukey's test was used to examine the significant difference among the treatments ($P < 0.05$). In case of salt stress, the fold change in APX activity was analyzed and expressed as mean $\pm$ SD.

Abbreviations

APX: Ascorbate peroxidases; Bgt: *Blumeria graminis*; DS: Drought stress; HD: Heat-drought combination stress; HS: Heat stress; Pst: *Puccinia striiformis*; ROS: reactive oxygen species

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All the data have been included in the manuscript. The publically available transcriptome data were used from URGI and NCBI server.

Competing interests

All the authors declare that there is no competing interest.

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Authors' contributions

SKU conceived the idea. SKU and ST designed the experiments. ST and S performed the experiments. ST, PCV and SKU analyzed the data. ST and SKU wrote the manuscript, and ST, KS and SKU finalized the manuscript. All authors have read and approved the manuscript.

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Tables

Table 1 The ratio of Ka/Ks between duplicated *TaAPX* gene pairs and their divergence time.

Gene A	Gene B	Ka	Ks	Ka/Ks	T = Ks/2r	Selection pressure
<i>TaAPX2-A</i>	<i>TaAPX5-A</i>	0.097633	1.03639	0.0942044	7.972230769	Purifying selection
<i>TaAPX2-B</i>	<i>TaAPX5-B</i>	0.095283	1.20929	0.0787927	9.302230769	Purifying selection
<i>TaAPX2-D</i>	<i>TaAPX5-D</i>	0.101729	1.17689	0.0864383	9.053	Purifying selection

Ka- non-synonymous substitutions per non-synonymous site, Ks- synonymous substitutions per synonymous site, T- Divergence time

Table 2 Tajima’s relative rate test for duplicated *TaAPX* gene pairs.

Gene A	Gene B	Out-group	Nt	Na	Nb	χ^2	<i>P</i>
<i>TaAPX2-A</i>	<i>TaAPX5-A.1</i>	<i>OsAPX1</i>	527	87	28	30.27	< 0.05
<i>TaAPX2-B</i>	<i>TaAPX5-B.1</i>	<i>OsAPX1</i>	578	103	27	44.43	< 0.05
<i>TaAPX2-D</i>	<i>TaAPX5-D</i>	<i>OsAPX1</i>	575	104	29	42.29	< 0.05

Nt Identical sites in all three sequences
Na Unique differences in Sequence A
Nb Unique differences in Sequence B
If *P* < 0.05 χ^2 value is significant and the test rejects the hypothesis of equal rates of substitution between the duplicated genes suggesting that they have different evolutionary rates after divergence

Table 3 Molecular characterization of *TaAPX* genes and proteins.

Gene	Chromosome	ORF (bp)	Protein length	Isoelectric Point	Molecular Weight (kDa)
<i>TaAPX1-A</i>	2A	888	295	8.31	32.36
<i>TaAPX1-B</i>	2B	894	297	8.30	32.38
<i>TaAPX1-D</i>	2D	888	295	8.30	32.35
<i>TaAPX2-A</i>	2A	1227	408	6.95	44.33
<i>TaAPX2-B</i>	2B	765	254	5.00	27.43
<i>TaAPX2-D</i>	2D	771	256	4.89	27.66
<i>TaAPX3-A.1</i>	2A	1038	345	8.72	37.19
<i>TaAPX3-A.2</i>	2A	1041	346	8.42	37.32
<i>TaAPX3-B</i>	2B	1047	348	8.72	37.51
<i>TaAPX3-D</i>	2D	1047	348	8.72	37.54
<i>TaAPX4-A</i>	2A	1059	352	7.76	37.77
<i>TaAPX4-B</i>	2B	1134	377	9.25	40.63
<i>TaAPX4-D</i>	2D	1140	379	9.07	40.74
<i>TaAPX5-A.1</i>	4A	684	227	7.15	24.77
<i>TaAPX5-A.2</i>	4A	753	250	6.17	27.49
<i>TaAPX5-B.1</i>	4B	753	250	6.17	27.47
<i>TaAPX5-B.2</i>	4B	771	256	7.60	27.90
<i>TaAPX5-D</i>	4D	753	250	6.32	27.49
<i>TaAPX6-A.1</i>	5A	939	312	7.02	34.12
<i>TaAPX6-A.2</i>	5A	936	311	7.30	33.99
<i>TaAPX6-A.3</i>	5A	921	306	6.80	33.38
<i>TaAPX6-B.1</i>	5B	924	307	7.06	33.57
<i>TaAPX6-B.2</i>	5B	939	312	7.66	34.18
<i>TaAPX6-B.3</i>	5B	942	313	7.32	34.31
<i>TaAPX6-D.1</i>	5D	924	307	6.61	33.44
<i>TaAPX6-D.2</i>	5D	942	313	6.61	34.21
<i>TaAPX6-D.3</i>	5D	936	311	6.61	34.05
<i>TaAPX7-A</i>	6A	1296	431	6.80	46.86
<i>TaAPX7-B.1</i>	6B	1296	431	7.58	46.75
<i>TaAPX7-B.2</i>	6B	1320	439	8.04	47.65
<i>TaAPX7-B.3</i>	6B	1410	469	8.61	50.90
<i>TaAPX7-D.1</i>	6D	1296	431	7.13	46.80
<i>TaAPX7-D.2</i>	6D	1266	421	7.58	45.57
<i>TaAPX8-A.1</i>	7A	696	231	8.21	24.60
<i>TaAPX8-A.2</i>	7A	990	329	7.00	35.58
<i>TaAPX8-B.1</i>	7B	1026	341	7.38	36.52
<i>TaAPX8-B.2</i>	7B	1002	333	6.99	35.67
<i>TaAPX8-D.1</i>	7D	987	328	6.05	35.12
<i>TaAPX8-D.2</i>	7D	744	247	4.98	26.48
<i>TaAPX9-A</i>	7A	876	291	8.19	31.67
<i>TaAPX9-B</i>	7B	876	291	8.19	31.72
<i>TaAPX9-D.1</i>	7D	876	291	8.19	31.68
<i>TaAPX9-D.2</i>	7D	777	258	8.43	28.55

Table 4 Details of predicted tertiary structures and ligand binding sites in different APX proteins.

Protein details		Structural parameters			Ligand and binding site residues			
Protein	Sub-cellular localization	C-score	TM-score	RMSD	Ligand	P-value	Multiplicity	Binding residues
TaAPX1-A	Peroxisome	-1.55	0.52±0.15	9.7±4.6Å	HEM	4.25e-09	131	P35 I36 L38 R39 W42 P133 D134 A135 L142 F146 L159 S160 G162 H163 L165 G166 K167 A168 R169 R172 S173 W179 L205 T207
TaAPX2-B	Cytoplasm	1.60	0.94±0.05	2.7±2.0Å	HEM	4.69e-10	121	P35 L36 L38 R39 W42 P133 D134 A135 L142 F146 L160 S161 G163 H164 L166 G167 R168 C169 H170 R173 S174 W180 L206 T208
TaAPX3-A.1	Chloroplast	-0.27	0.68±0.12	7.1±4.1Å	HEM	4.24e-12	125	P97 V100 R101 W104 P202 D203 A204 L213 F217

								L230
								S231
								A233
								H234
								L236
								G237
								R238
								S239
								R240
								R243
								S244
								W246
								W266
								L291
								T293
TaAPX8- B.1	Chloroplast	0.38	0.76±0.10	5.7±3.6Å	HEM	3.31e- 08	155	A121
								G122
								L124
(APX- related)								R125
								F128
								P220
								E221
								E222
								L229
								F233
								R247
								Q249
								L254
								S255
								A257
								H258
								I260
								G261
								G262
								K263
								I292
								L294
								T296
								D297

C-score - Confidence score of predicted models and ranged between -5 and 2, where higher value signifies a model with more confidence.

TM-score and RMSD - For quality structure prediction based on the correlation with C-score, where >0.5 TM-score directs a model of precise topology.

Multiplicity - For determining the quality of predicted pocket for ligand binding, where value >40 indicates the higher confidence.

Additional File Legends

Additional file 1 Homeologous gene clustering based on ≥ 90% sequence identity and localized on same chromosome of each A, B and D sub-genomes. Nomenclature assigned in accordance with the guidelines of international rules of genetic nomenclature for gene symbolization in wheat.

Additional file 2 Molecular characterization of TaAPX genes and proteins of *Triticum aestivum* and their various physico-chemical properties.

Additional file 3 Existence of transcripts confirmed using BLASTn search against transcriptome shotgun assembly (TSA) sequences of *T. aestivum* at NCBI server.

Additional file 4 Co-expression analysis, and gene ontology (GO) mapping and annotation of co-expressed genes with *TaAPXs* in tissue developmental stages and during biotic, salt, and heat, drought and their combination stresses.

Additional file 5 Identified target TaAPXs of known miRNAs in *T. aestivum* using psRNATarget.

Additional file 6 Annotation of chemical compounds interact with TaAPXs.

Additional table 1 *Cis*-regulatory elements predicted in promoter region of *TaAPX* genes.

Additional table 2 List of qRT PCR gene specific primers.

Additional fig. 1 Sequences of ten conserved motifs indicating the motif number, e-value, sites, motif width.

Additional fig. 2 Expression analysis using qRT PCR of few randomly selected *TaAPX* genes. qRT PCR was performed using gene specific primers. The bars represents the log₂ fold change expression value under different abiotic stress conditions and vertical lines on bars represent standard deviation. The fold change value of each gene under heat (HS), drought (DS) and their combination (HD) are shown in graphs (A-D), whereas (E-H) represents fold change value under salt stress. The result shows the expression of each *TaAPX* gene was comparable to the expression obtained from RNA seq data.

Figures

Figure 2

Synteny analysis. Syntenic relationship of TaAPXs with the APXs of *Arabidopsis thaliana* (a), *Brachypodium distachyon*(b), *Hordeum vulgare* (c), *Oryza sativa* (d) and *Zea mays* (e). The figure was generated using circos-0.69-6 software.

Figure 4

Exon/Intron organization and motif analysis. Exon/Intron organization in TaAPX genes and their splice variants. The exons are shown as blue boxes interspaced by introns shown as thin black line. Splice variants are marked with black vertical line at 3' end of the transcript. The length of each exon is represented above the respective exon. 0, 1 and 2 indicates the different intron phases (a). Motifs analysis showing the organization of ten conserved motifs represented by colored boxes (b).

Figure 5

Protein sequence and structure analysis of TaAPXs. Multiple sequence alignments of cytosolic TaAPX protein sequences with cAPX of *Pisum sativum* (a), peroxisomal TaAPX proteins with AtAPX5 (b), chloroplastic TaAPX proteins with AtAPXT and AtAPXS (c), TaAPX-related (TaAPX-R) protein sequences with AtAPX4 of *Arabidopsis thaliana* (d). Green, blue and purple shaded boxes represents the active site domain (ASD), heme-binding domain (HBD) and cation binding domain (CBD) present in all the groups, but substituted with new sets of residues in TaAPX-R group proteins. Two additional regions chloroplastic domain 1 and 2 are highlighted with yellow shaded box and present only in chloroplastic APX proteins. Red asterisks and Green marks represent the amino-acid residues involved in heme-binding and ascorbate binding (Lys, Cys and Arg), respectively. The residues important for cytoplasmic and peroxisomal localization

are underlined with thick green and black line, respectively. The tertiary structure of TaAPX2-B (cAPX) (e), TaAPX1-A (mAPX) (f), TaAPX3-A.1 (ChIAPX) (g) and TaAPX8-B.1 (APX-R) (h), indicating the structural differences in proteins of all the groups.

Figure 6

Correlation between the replicates of expression data in various tissue developmental stages (a) and biotic and abiotic stress conditions (b). Expression profiling of TaAPX genes in tissue developmental stages and stress conditions. Heat-map shows the expression profiles of TaAPX genes in three developmental stages

combination stresses (d). Interaction of miRNA targets (e) and chemical associations (f) are also shown. The pictogram was generated using Gephi 0.9.1 software.

Figure 8

Ascorbate peroxidase activity during heat (HS), drought (DS) and their combination stress (HD) in shoot (a) and root (b) at 1 h and 6 h of treatments. Bars represents the mean $\pm$ standard error of enzyme activity (mMug⁻¹min⁻¹). The different letters shows the significant difference (P < 0.05) among treatments. Fold change $\pm$ SD in ascorbate peroxidase activity at 6, 12, 24, and 48 h of salt stress in shoot (c) and root (d).

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