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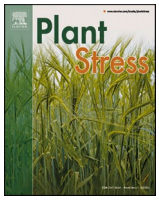

Genome-wide analysis of the class III peroxidase gene family in sesame and *SiPRXs* gene validation by expression analysis under drought stress

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ABSTRACT

Sesame (*Sesamum indicum*) is an important indigenous oilseed crop but its growth and productivity are severely affected by abiotic stresses. Class III peroxidases are key stress related enzymes that exclusively occur in plant kingdom, however, their specific involvement in sesame remains largely unexplored. The present study aimed to identify the PRX gene family in sesame and elucidate their role in conferring drought stress tolerance in contrasting sesame accessions. Through genome-wide analysis of the *PRX* gene family, 45 non-redundant members (designated *SiPRXs*) were identified which were unequally distributed on 13 sesame chromosomes. Motif analysis revealed highly conserved peroxidase domains in all *SiPRX* proteins. To validate the function of identified *SiPRX* family members, sesame accessions were phenotyped under drought stress and irrigated conditions. The contrasting drought-tolerant and drought-sensitive accessions were used to study the relative transcript abundance of the selected 15 *SiPRX* genes by quantitative real-time PCR.Expression analysis revealed differential expression of *SiPRX* genes between drought-tolerant and drought-sensitive accessions, which was consistent with their physiological responses to drought stress. Our findings provide comprehensive insights into the genomic characterization of the *SiPRX* gene family in sesame with special reference to drought stress tolerance. These results emphasize the potential utility of *SiPRX* genes in enhancing drought resilience in sesame with implications for crop improvement strategies.

1. Introduction

Peroxidase (PRX) enzymes are essential for plant growth, development, and defense mechanisms, particularly in response to biotic and abiotic stress conditions (Bindschedler et al., 2006). These enzymes are broadly classified into two major groups based on the presence (haemoglobin peroxidases) and absence (non-haemoglobin peroxidases) of hemoglobin pigment. Among hemoglobin peroxidases, class III peroxidases (EC 1.11.1.7) are oxidoreductases exclusive to the plant kingdom (Passardi et al., 2004). The stability of PRX enzymes in plants is

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Table 1
List of primers used in the study for validation of *SiPRX* genes using qRT-PCR.

Primer's name	Primer sequence (5′ to 3′)	Length	Tm	GC Percent
qPRX5_2023_F	CCAAACATGGCCTCAACATAAC	22	62.07	45.45
qPRX5_2023_R	TGTAGATGCGCTTGGAGAAC	20	62.08	50
qPRX2_2023_F	TCTTGTCGGTGGACCTTATTG	21	61.83	47.61
qPRX2_2023_R	TCATCTGCTGTGGGAAGATTAG	22	61.75	45.45
qPRX11_2023_F	CCACTCTCGATGGCCTTATTT	21	62.01	47.62
qPRX11_2023_R	GCTGTATATCCTAGCACGGAAC	22	62.05	50
qPRX10_2023_F	GTGGATCAGAATCTCCCACTTC	22	62.05	50
qPRX10_2023_R	GAGCACAATCATCTCCTCGATAG	23	62.07	47.83
qPRX7_2023_F	GAGTGTTATGCGGGAGATATGG	22	62.13	50
qPRX7_2023_R	CAGTTGATTGACAGCCCTACA	21	62.12	47.62
qPRX12_2023_F	ATGAGCTTCCTAGTCCCTTAGA	22	61.94	45.45
qPRX12_2023_R	CTGTGCTGTGAGCACCTAATA	21	61.86	47.62
qPRX20_2023_F	TCTCTCTCTCTCCCTCTCTCT	21	62.04	52.38
qPRX20_2023_R	ACCTGCATCAAGCCCATATC	20	62.15	50
qPRX16_2023_F	CATTGCACAACTCTACAGCAATAA	24	62.03	37.5
qPRX16_2023_R	CTCTCCATTCGATCCGGTTAAG	22	62.01	50
qPRX18_2023_F	TGTAGGAGGGCCAACTAAGA	20	62.11	50
qPRX18_2023_R	CAAGAACAATGAAAGCCGACAG	22	62.14	45.45
qPRX24_2023_F	GACGGGAAGTGATGGTGAAA	20	62.08	50
qPRX24_2023_R	AGGATCCATCTACGAGACTACAA	23	62.14	43.48
qPRX31_2023_F	GTCGTCGTGGACTCGTATTTC	21	62.22	52.38
qPRX31_2023_R	ACCCTGTATGCACACCAATC	20	62.25	50
qPRX40_2023_F	AGGAGTTTGCTAGAGCCATTAC	22	61.99	45.45
qPRX40_2023_R	CTGCTTTCTGATCTCACCCTT	21	61.92	47.62
qPRX45_2023_F	CTGGAGACTGGACGTTGAATAG	22	61.89	50
qPRX45_2023_R	ATCTACAAGCAGATGCCCATAA	22	61.81	40.91
qPRX43_2023_F	GGACCTGTCGGGAATTTCTATC	22	62.07	50
qPRX43_2023_R	AATCTGCGGTGGAGAAGAAG	20	61.86	50
qPRX34_2023_F	ACAGGGAATGAAGGTGTGATTAG	23	62.27	43.48
qPRX34_2023_R	TGGATCGGAGTTGCTTCAATTA	22	62.13	40.91
qSiActin_2023_F	CTCCCTTTATGCCAGTGGTCGT	22	61.5	55
qSiActin_2023_R	GCTCAGCTGTTGTAGTGAAGGA	22	58.2	50
qSiUB6_2023_F	CACCAAGCCGAAGAAGATCAAG	22	60	50
qSiUB6_2023_R	CCTCAGCCTCTGCACCTTTC	20	59	60

maintained through glycosylation, which protects them from protease degradation (Zheng and Van Huystee, 1991). Peroxidases play diverse roles in plant species across various developmental stages, particularly in safeguarding plants against abiotic stresses such as high temperature, cold, and drought (Ritonga et al., 2020; Cheng et al., 2022).

PRXs are basically classified in three superfamilies such as animal PRX, catalase, and plant PRX. Plant PRX are classified in three subclasses: Class 1 PRXs, found in bacteria, yeast, and plants such as ascorbate peroxidase, cytochrome c oxidase, bacterial catalase peroxidase; Class II PRXs, present as extracellular enzymes in fungi such as lignin peroxidase and Mn^{+2} dependent peroxidase; Class III PRX enzymes include horseradish peroxidase, which are specific plant enzymes present in vacuoles or extracellular. Glutathione peroxidase has selenium as cofactor instead of heme and is found in both animals and plants (Hiraga et al., 2001). Many plant species harbor numerous class III peroxidases within the PRX family. For instance, *Arabidopsis, Oryza sativa* (rice), *Panicum virgatum, Populus* (poplar)*, Manihot esculenta*, and *Nicotiana tabacum* possesses 73, 138, 200, 93, 91, and 201 the class III peroxidases respectively (Tognolli et al., 2002; Passardi et al., 2004; Ren et al., 2014; Moural et al., 2017; Wu et al., 2019; Cheng et al., 2022). Studies have indicated that peroxidases may serve multiple functions in plant species. Microarray analyses have indicated the involvement of *PRXs* in abiotic stress tolerance in *Zea mays* (Wang et al., 2015) while *GhPOX1* is involved in fiber elongation in cotton (*Gossypium hirsutum*) (Mei et al., 2009; Duan et al., 2019). *PRX* isoforms, *PRX33* and *PRX34,* from *Arabidopsis thaliana*, are involved in root elongation, while *AtPRX72* plays an important role in cell wall lignification (Passardi et al., 2006; Herrero et al., 2013). Lignification is also considered a critical defense mechanism during biotic stresses, such as pathogen attacks. Earlier evidences suggest that overexpression of *PRX* genes in transgenic plants provided tolerance against different abiotic stresses. For example, *TaPRX-2A* overexpressed in wheat protected the plants against salt stress (Su et al., 2020). Similarly, *AtPrx64* gene from Arabidopsis overexpressed in tobacco plants exhibited enhanced tolerance to heavy metal stress such as aluminum (Wu et al., 2017). Although *PRXs* play an essential role in overcoming both abiotic and biotic stresses in plants but comprehensive analyses of the class III peroxidase family is limited to a few model plant / crop species, including *Arabidopsis thaliana*, maize, rice, and potato (Tognolli et al., 2002; Passardi et al., 2004; Wang et al., 2015; Yang et al., 2020).

Sesamum indicum L., commonly called sesame, belongs to the Pedaliaceae family and ranks among the most significant oilseed crops. Sesame is widely grown in tropical and subtropical regions and its seeds are a valuable source of oil (~50 % of its content, with balanced oleic acid and linolenic acids), proteins (23 %), and carbohydrates (13.5 %) with numerous medicinal properties (Were et al., 2006). Sesame is being traded in the national as well as international markets and its consumption is steadily increasing among the public (Teklu et al., 2022). Sesame frequently encounters biotic and abiotic stresses throughout its growth and development, especially during fruit (capsule) formation (Yadav et al., 2022). Despite the acknowledged importance of the *PRX* gene family in growth and development, the role of class III PRX has not been explored in sesame. Consequently, understanding the role of *PRX* gene family in sesame represents a crucial initial step in unraveling their characteristics and physiological significance.

In this study, we investigated the *PRX* gene family in sesame using bioinformatics approach and validated our findings by expression analysis of contrasting sesame accessions in response to drought stress. The *in-silico* study includes identifying sesame class III *PRX* genes, structure and domain prediction, phylogenetic relationships, chromosomal localization, conserved motifs, and substitution rates. We retrieved sequences of *SiPRX* gene family and expression analysis data of *SiPRX* genes under drought stress from the National Centre for Biological Information (NCBI) database. Further, a qRT-PCR-based validation was

Table 2
Characteristics of 45 *PRX* genes under Class III peroxidase family in *Sesamum indicum*.

Gene name	Gene ID	Start position	End position	strand	Chr	Amino acids	MW (kDa)	pI
*SiPRX*1	Sesame00022	237153	244471	+	chr1	319	34.35	9.13
*SiPRX*2	Sesame00842	14753929	14757375	–	chr1	339	37.32	5.67
*SiPRX*3	Sesame02403	4236784	4238477	–	chr2	353	38.21	7.99
*SiPRX*4	Sesame02533	5242071	5246523	+	chr2	412	45.51	9.21
*SiPRX*5	Sesame02840	14722929	14724592	–	chr2	336	36.78	9.59
*SiPRX*6	Sesame02881	15171027	15172794	+	chr2	331	35.78	9.36
*SiPRX*7	Sesame03764	344345	345748	–	chr3	358	38.41	4.48
*SiPRX*8	Sesame05821	29863474	29864829	–	chr3	340	36.93	8.48
*SiPRX*9	Sesame05975	30825230	30829038	+	chr3	354	38.8	7.54
*SiPRX*10	Sesame06661	11451446	11452919	+	chr4	433	48.69	6.15
*SiPRX*11	Sesame07126	15022674	15024588	–	chr4	322	34.57	7.64
*SiPRX*12	Sesame07420	19003672	19006705	+	chr4	377	42.08	5.04
*SiPRX*13	Sesame07947	3737646	3739294	+	chr5	332	35.54	5.25
*SiPRX*14	Sesame08173	14957211	14958668	–	chr5	324	34.96	7.72
*SiPRX*15	Sesame08875	310368	311584	–	chr6	321	35.39	6.13
*SiPRX*16	Sesame08934	1031208	1032756	+	chr6	325	34.63	5.69
*SiPRX*17	Sesame09486	5878569	5880611	–	chr6	340	37.12	4.94
*SiPRX*18	Sesame10415	21173104	21180409	+	chr6	322	35.31	8.88
*SiPRX*19	Sesame10416	21186847	21189881	+	chr6	328	35.58	8.63
*SiPRX*20	Sesame11387	5084838	5086614	+	chr7	319	34.65	9.56
*SiPRX*21	Sesame12317	14062732	14064367	+	chr7	322	35.06	8.32
*SiPRX*22	Sesame12318	14065928	14067407	+	chr7	316	35.08	7.06
*SiPRX*23	Sesame13385	18221339	18222668	–	chr8	330	37.55	8.7
*SiPRX*24	Sesame14508	28416585	28417701	–	chr8	329	35.55	6.98
*SiPRX*25	Sesame14770	30292078	30293027	–	chr8	337	36.85	7.24
*SiPRX*26	Sesame14947	31431896	31435735	–	chr8	288	31.75	7.59
*SiPRX*27	Sesame15207	1073696	1075231	–	chr9	335	35.68	7.93
*SiPRX*28	Sesame17216	23293638	23295859	+	chr9	327	35.51	4.89
*SiPRX*29	Sesame17491	25185567	25186634	–	chr9	327	35.46	7.4
*SiPRX*30	Sesame17498	25218480	25227352	–	chr9	332	36.72	7.26
*SiPRX*31	Sesame18044	2233617	2236489	–	chr10	329	35.88	8.09
*SiPRX*32	Sesame18594	15396734	15400537	+	chr10	318	34.09	4.88
*SiPRX*33	Sesame19093	20625149	20626417	+	chr10	323	36.08	6.28
*SiPRX*34	Sesame19237	21586722	21588392	+	chr10	250	27.98	6.18
*SiPRX*35	Sesame20359	15180814	15182355	+	chr11	321	34.27	9.18
*SiPRX*36	Sesame20377	15274282	15277059	–	chr11	324	35.19	4.48
*SiPRX*37	Sesame20635	16942739	16943910	+	chr11	324	35.08	10.24
*SiPRX*38	Sesame21155	8971778	8974098	+	chr12	324	35.02	9.15
*SiPRX*39	Sesame21156	8984765	8987788	+	chr12	330	37.46	8.54
*SiPRX*40	Sesame22000	15549089	15550675	+	chr12	342	37.58	9.16
*SiPRX*41	Sesame22224	16847345	16852106	–	chr12	320	34.72	7.99
*SiPRX*42	Sesame22558	19003749	19004794	+	chr12	326	36.71	7.35
*SiPRX*43	Sesame22738	1476352	1479399	–	chr13	332	36.41	6.5
*SiPRX*44	Sesame23663	16879262	16880724	–	chr13	326	35.43	4.54
*SiPRX*45	Sesame23811	18197552	18199641	–	chr13	332	36.61	5.31

Table 3
Genes in class III peroxidase family from reported plant species.

S. no.	Species	No. of genes in class III peroxidase family	Genome size (Mbp)
1	*Arabidopsis thaliana*	81	135
2	*Vitis vinifera*	85	500
3	*Oryza sativa*	138	430
4	*Triticum aestivum*	374	17,000
5	*Zea mays*	156	2500
6	*Brachypodium distachyon*	149	335
7	*Chlamydomonas reinhardtii*	6	110
8	*Solanum tuberosum*	102	844
9	*Betula pendula*	90	440
10	*Citrus sinensis*	72	380
11	*Triticum urartu*	159	4790
12	*Aegilops tauschii*	169	4792

conducted for 15 *SiPRX* genes in four contrasting sesame accessions (drought-tolerant and drought-sensitive).

2. Materials and methods

2.1. Screening of sesame peroxidase proteins

Protein sequences corresponding to class III peroxidase were retrieved from the TAIR (The Arabidopsis Information Resource) database (https://www.arabidopsis.org/browse/genefamily/peroxidase.jsp). These sequences were used as 'query sequence' to find corresponding orthologs in sesame using the NCBI BLASTp tool (with *Sesamum indicum* (tax id: 4182) as the specified organism). The selected protein sequences met the criteria of >30 % similarity and an e-value $<e^{-10}$. In addition, the Hidden Markov model (HMM) of the peroxidase protein family was used to search for amino acid sequences. The retrieved sequences were then analyzed to confirm the presence of conserved peroxidase domains using the SMART software tool (Simple Modular Architecture Research Tool; (http://smart.embl-heidelberg.de/). Information related to *SiPRX* genes, CDS, gene IDs, and physical positions was obtained from the NCBI.

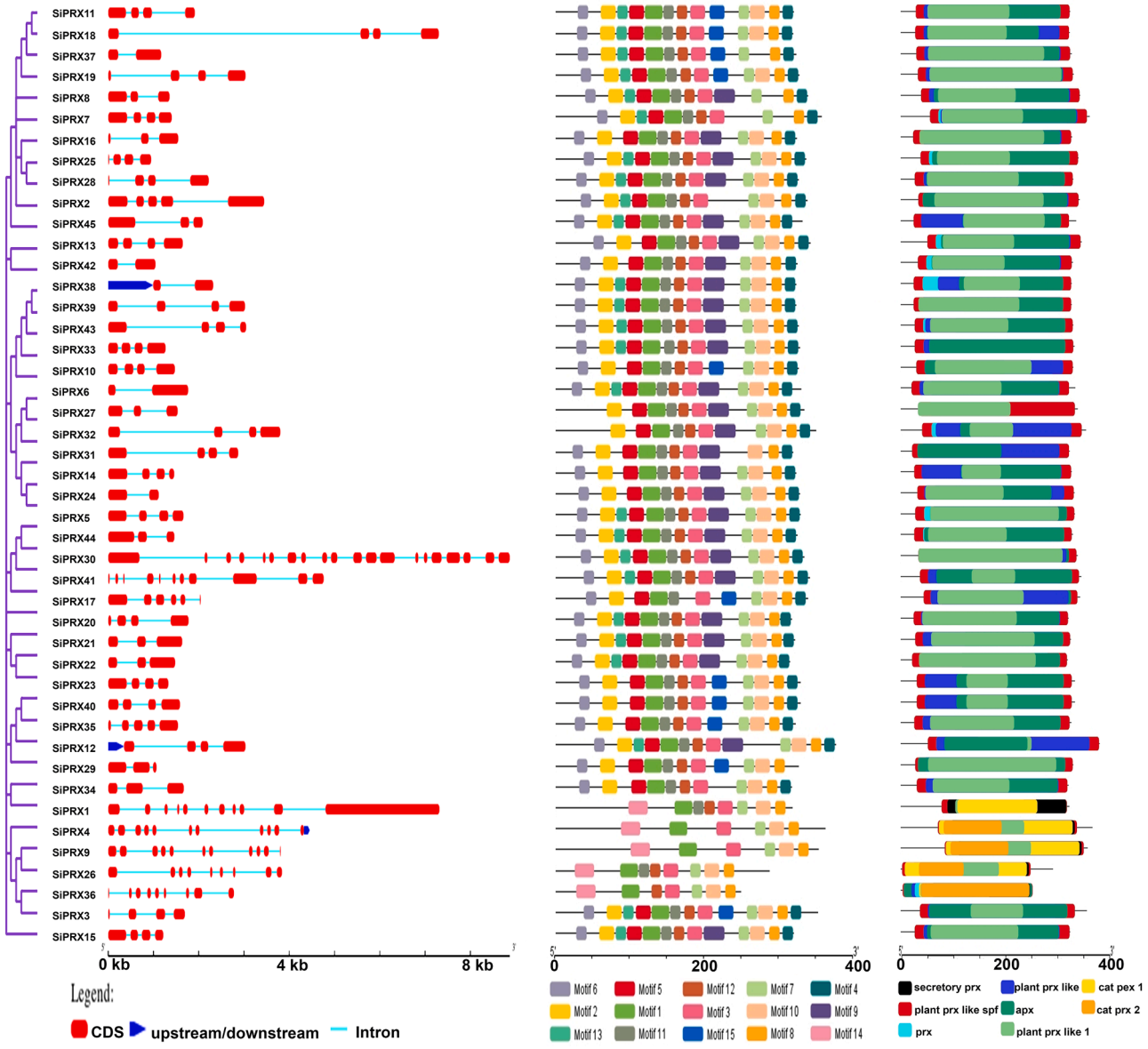

Fig. 1. Gene structure and motif prediction in identified members of *SiPRX*. The exon-intron diagrammatic view of *SiPRX* genes generated using GSDS software. The thick red lines indicate exons, and the turquoise lines indicate introns. Blue arrows represent upstream or downstream regions. Scale shows gene length. For motif prediction, different colored boxes represent different motifs. Each protein member has the conserved PRX domain, represented by other peroxidase-related domains.

2.2. Physicochemical properties of SiPRXs and subcellular location prediction

Various physicochemical properties of *SiPRX* proteins, such as molecular weight, isoelectric point (pI), amino acid composition, GRAVY (grand average of hydropathy), and aliphatic index, were predicted using the ExPASy ProtParam tool (https://web.expasy.org/protparam/). Subcellular localization predictions of *SiPRX* proteins were made using the CELLO2GO web server (http://cello.life.nctu.edu.tw/cello2go/).

2.3. Gene structure and conserved domain analysis

The *SiPRX* gene structure was identified using GSDS (Gene Structure Display Server) software (http://gsds.gao-lab.org/). Conserved motif present in *SiPRX* proteins were identified using MEME (Motif EM for Motif Elicitation) server version 5.3.3 (https://meme-suite.org/meme/tools/meme) with the following parameters: optimum motifs (6–50 amino acids) and number of motifs (15). Further, the annotation of conserved motifs was carried out using CDD (Conserved Domains Database) (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi).

2.4. Phylogenetic analysis

Each *SiPRX* sequence from sesame was searched using the BLAST tool against all Arabidopsis and *Brassica rapa* sequences to identify putative orthologs between species. Hits meeting the >30 % similarity and alignment >300 bp criteria were considered orthologs (Yang et al., 2020). We utilized PRX protein sequences from *Arabidopsis thaliana, Oryza sativa* (model plant) and *Zea mays* (reservoir of abiotic stress tolerant genes), for phylogenetic tree construction. The MUSCLE program aligned all PRX proteins, with the neighbor-joining tree constructed using MEGA-X software (https://www.megasoftware.net/) and 1000 bootstrap replicates. For better view, the phylogenetic tree was visualized using iTOL v6 (https://itol.embl.de/).

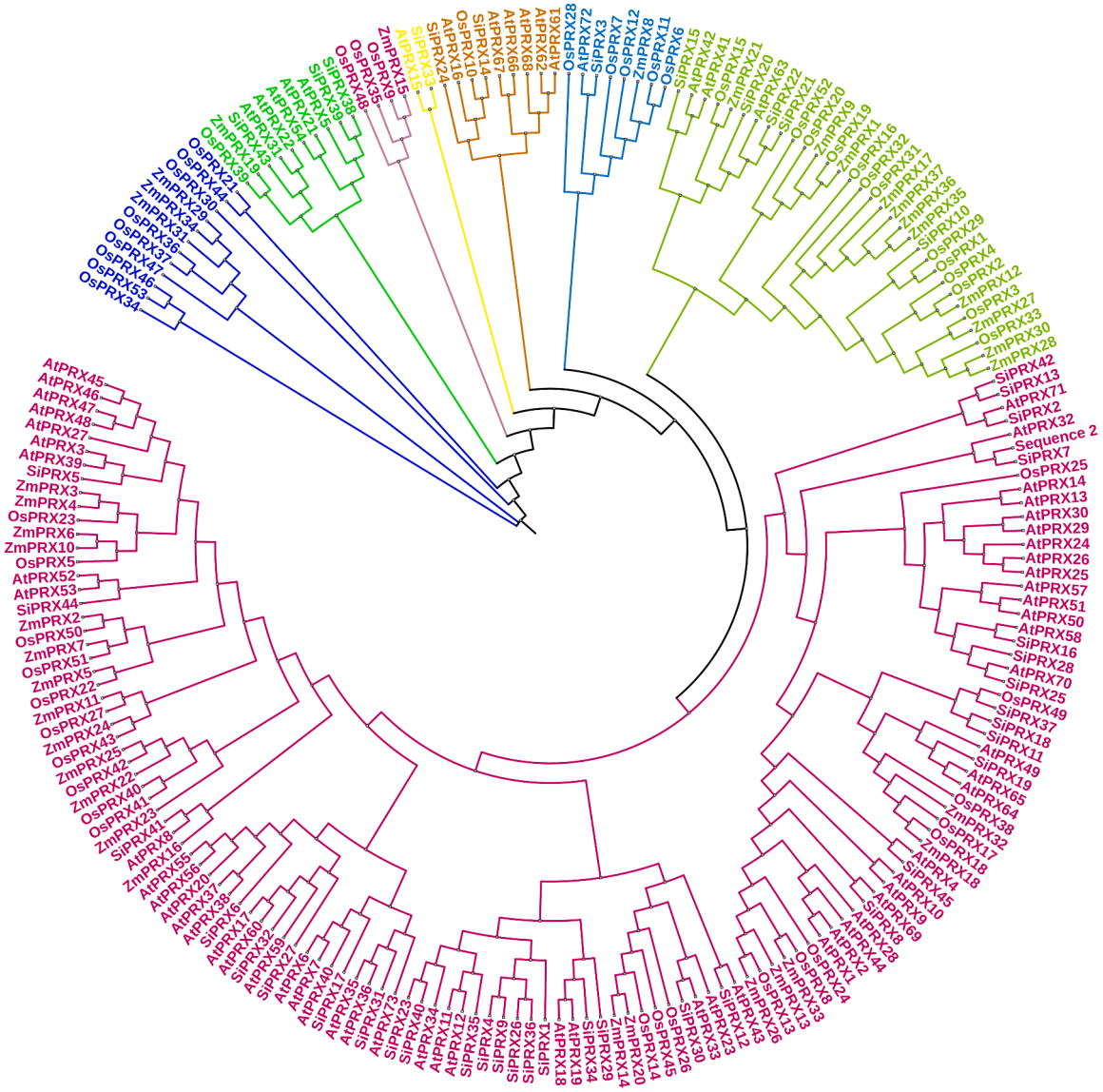

Fig. 2. Diversity of PRX proteins from sesame, Arabidopsis, rice, and maize. The tree was constructed using neighbor-joining method with a bootstrap value of 1000 and full-length amino acid sequences from different plant species. Major clusters are shown in different colors.

2.5. Chromosomal localization, synteny analysis, and gene duplication in sesame PRX genes

The chromosomal locations of *SiPRX* genes were obtained from the sesame genome annotation file available at NCBI (https://www.ncbi.nlm.nih.gov/genome/?term=sesamum%20indicum%20). These genes were renamed based on their physical location within the *S. indicum* genome (Wang et al., 2022). An input file (.txt) containing the start and end positions of sesame chromosomes and *SiPRX* gene positions was prepared and then visualized using Mapchart (https://www.wur.nl/en/show/mapchart.htm). Gene duplication events were analyzed using MCScanX (Wang et al., 2012) with the results plotted using TBtools (Chen et al., 2020). Genes were considered duplicated if the corresponding coding protein pairs exhibited a query coverage of >90 % and shared >50 % identity (Wang et al., 2017). Furthermore, we examined tandem and segmental duplication events based on gene pair distance: tandem duplications have ≤5 genes separating paralog gene pairs within a 100 kb region, while segmental duplications have >5 genes separating paralog gene pairs (Zhang and Li, 2018).

2.6. Determination of synonymous and non-synonymous substitution rates

Sesame orthologous PRX protein sequences were compared to those in Arabidopsis to calculate synonymous (Ks) and non-synonymous (Ka) substitution rates. *SiPRX* protein sequences (as a query) were BLAST-searched against the *Arabidopsis thaliana* TAIR 10 (target) proteome using Phytozome12 software (https://phytozome.jgi.doe.gov/pz/portal.html#). The selected parameters were Target type (proteome), expected threshold (−1), and comparison matrix (BLOSUM62). The gene ID and chromosome number were recorded for the most similar sequence, and the 'sequence' tab was selected to get the CDS and protein sequence of the respective ID. Protein sequences of *SiPRX* (query) and AtPRX (target) were aligned using the Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/), with these results submitted into PAL2NAL tool (http://www.bork.embl.de/pal2nal/) as 'Input File 1.' The corresponding CDS sequence for both species was added to 'Input File 2′ to calculate the Ks and Ka substitution rates. Duplication event times (T, in millions of years ago (Mya)) were estimated as follows: $T = Ks / 2\lambda \times 10^{-6}$, where $\lambda = 6.5 \times 10^{-9}$ (Quraishi et al., 2011).

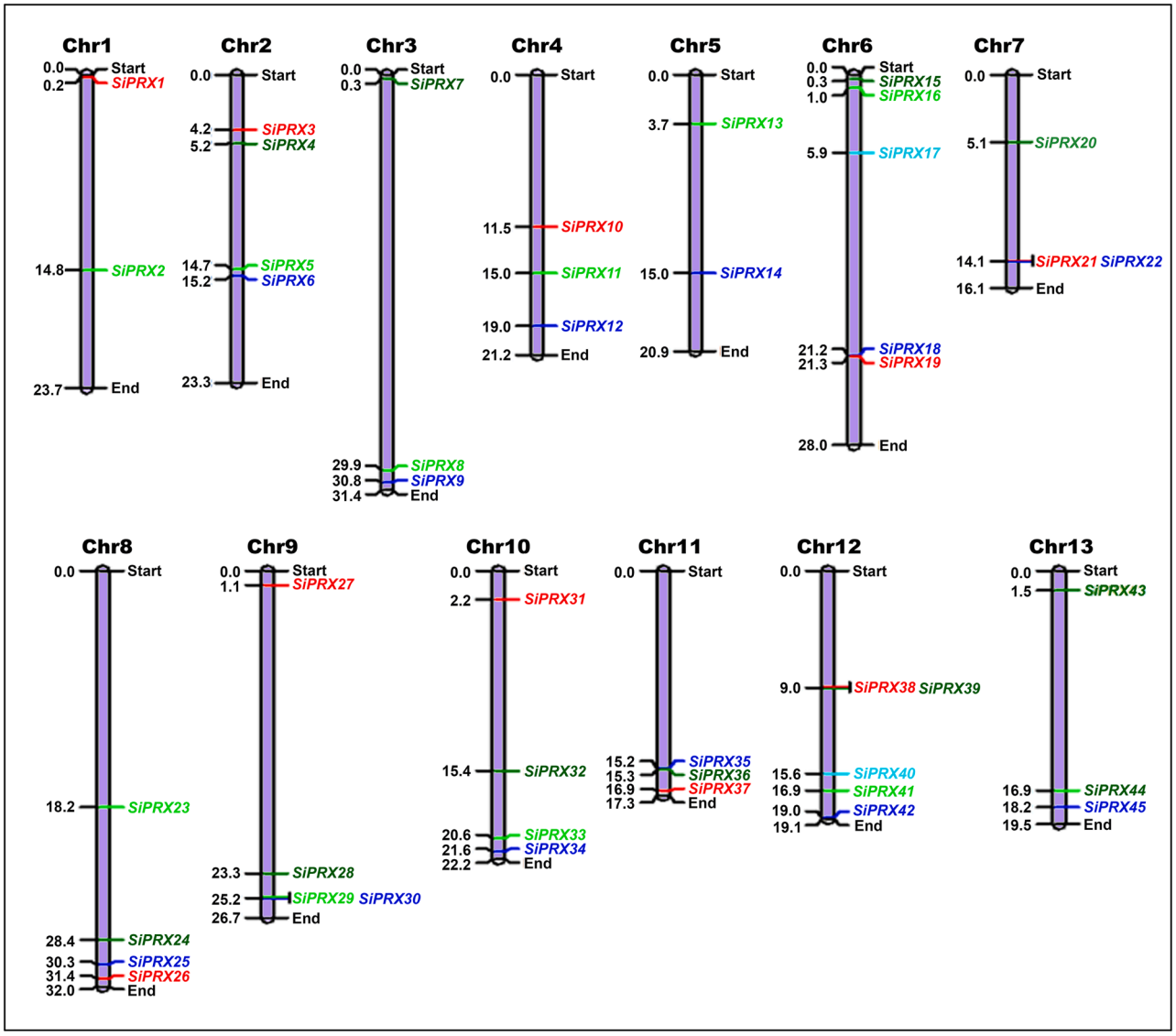

Fig. 3. Chromosomal locations of sesame *PRX* genes on 13 sesame chromosomes. The start and end points represent the whole length of the chromosome (in Mb), while the number denotes gene position in Mb from the starting point. The *SiPRX* gene name on right side of the bars corresponds to predicted locations of each *PRX* gene. The chromosome number is on top of each bar.

2.7. Post-translational modifications and protein–protein interactions

Predictions of post-translational modifications (PTMs), including *N*-linked glycosylation, *O*-linked glycosylation, and phosphorylation sites, were conducted using various bioinformatics tools. *N*-linked and *O*-linked glycosylation sites in *SiPRX* proteins were predicted using NetNGly 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and YinOYang 1.2 (http://www.cbs.dtu.dk/services/YinOYang/) servers, respectively, while phosphorylation sites in *SiPRX* proteins were predicted using MusiteDeep software (https://www.musite.net/). The presence of transmembrane helices (membranes spanning helical domains) was predicted using the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/). The protein–protein interactions were analyzed using STRING v 11.5 software (https://string-db.org/) by uploading all *SiPRX* proteins and selecting '*Arabidopsis thaliana*' from the organism list. The obtained results were saved as a .jpeg image files.

2.8. Gene ontology and in-silico expression analysis of PRX genes in sesame under drought stress and putative promoter prediction

The gene ontology (GO) analysis for all *SiPRXs* was performed using Omicsbox software (https://www.blast2go.com/). The results were categorized as cellular components, molecular functions, and biological processes. The involvement of *SiPRX* genes in different plant metabolic pathways was predicted using KEGG pathway analysis. The expression of *SiPRX* genes under drought stress in two contrasting sesame genotypes was obtained from the NCBI BioProject Database (PRJNA623932). The gene IDs of *SiPRX* family members were searched in the database to retrieve FPKM (fragments per kilobase of transcript per million mapped reads) values under control and stress conditions. Log-transformed FPKM values were used to calculate fold-change expression and a heat map was generated using TBtools software (Chen et al., 2020).

The *SiPRX* gene sequences were BLAST-searched against the sesame genome, with a 2 kb upstream region (upstream to start codon) and retrieved for all identified *SiPRX* genes to identify drought-stress-responsive elements in the putative promoter region. Upstream sequences were analyzed further for *cis*-acting regulatory elements using

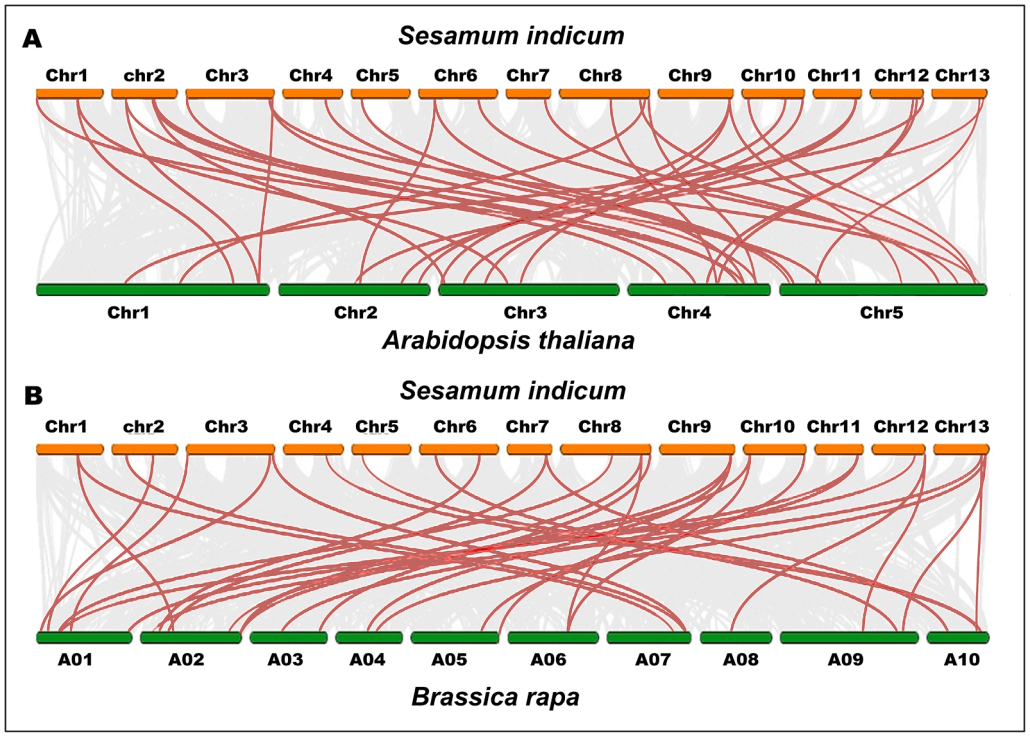

Fig. 4. Synteny analysis of *PRX* genes of *S. indicum* with *A. thaliana* and *B. rapa.* (A) Red lines highlight the syntenic *PRX* gene pairs, while gray lines in the background indicate the collinear blocks within the *S. indicum* and *A. thaliana* genomes, (B) red lines show syntenic *PRX* gene pairs between *S. indicum* and *B. rapa*, while gray lines in the background indicate the collinear blocks within *S. indicum* and *B. rapa* genomes.

PlantCARE software (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.9. Identification of sesame accession with contrasting response to drought stress

Gene expression under drought and control conditions was assessed in contrasting sesame accessions to validate the identified genes. Drought-tolerant accessions were identified through a large-scale preliminary screening of ~5500 diverse sesame germplasm under field conditions for two years at two locations (Sardarkrushinagar Dantiwada Agricultural University, Gujarat, India, and Rani Lakshmi Bai Central Agricultural University, Jhansi, India) (data unpublished). The selected accessions scored well for morpho-physiological and yield traits under drought stress. Based on the results of field screening, nine contrasting accessions (drought tolerant: IC557243, EC370700, PB Til-2, EC350648, IC129772; drought sensitive: Thilak, GT-10, EC344951, IC129677) were further evaluated in a pot experiment under controlled conditions (day/night 28/32 °C, relative humidity >75 %) at the National Phytotron Facility, Indian Agriculture Research Institute, New Delhi, India. The experiment was laid out in a completely randomized design (CRD) with two factors, genotypes and treatments, and 8 replicates for each treatment. Soil was collected from the field (0–30 cm depth), air dried, and well sieved. Pots (6″ dia) were filled with 3.5 kg of a soil: sand mixture (1: 4), with recommended fertilizer dose (80 kg N, 40 kg P_2O_5, and 40 kg K_2O per hectare) applied as urea, single super phosphate, and muriate of potash. Plants were exposed to drought stress by withholding irrigation at flower bud initiation stage until soil moisture levels fell below 10 % (measured daily by a moisture probe using LI-COR 6800 (LI-COR Biosciences, Lincoln, NE, USA). When the plants exhibited drought symptoms (leaf wilting, lower leaf yellowing), physiological traits such as membrane stability index (Meena et al., 2021), relative water content (Barrs and Weatherley, 1962), canopy temperature (by IRT gun), leaf chlorophyll (MC-100, Apogee Instruments, Logan, USA), maximum quantum yield of PSII measured by portable fluorescence spectrometer ($OS30p^+$, Opti-Sciences, Hudson, USA), and shoot dry weight were recorded for drought-stressed and control plants. Hierarchical cluster analysis (Ward's method) was used to identify four contrasting sesame accessions (two drought-tolerant and two drought-sensitive) for gene expression analysis. The procedure for basic statistical analysis, analysis of variance (ANOVA), and hierarchical cluster analysis were carried out using the statistical software R version 3.6.1 (R Core Team, 2019).

2.10. Validation of expression of selected SiPRX genes by qRT-PCR

Total RNA was extracted from leaf tissue of control and drought-stressed plants using a PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, USA). After treating 10 µg RNA with DNase I (Turbo DNase, Invitrogen, Thermo Fisher Scientific, Waltham, USA) to remove genomic DNA contamination, cDNA was prepared using a single-step RT-PCR kit (High-Capacity cDNA Reverse Transcription Kit, Thermo Fisher Scientific, Waltham, USA) with oligo *dT* primer. Real-time PCR was performed in triplicate using 2 × Brillant SYBR Green QPCR (Agilent Technologies, CA, USA) on an Aria Real-Time PCR system (MX20515245, Agilent Technologies, CA, USA). Reaction set-up and cycling parameters for qRT-PCR were in accordance with Sharma et al. (2021). Actin (*SiActin*) and ubiquitin (*SiUB6*) were used as reference genes for normalization and both genes showed very stable expression under drought stress conditions. The average C_T (cycle threshold) value of SiActin (C_T range between 16.38–17.05) and SiUB6 (C_T range between 15.67–16.50) was used for data normalization. Normalized relative transcript levels under drought and irrigated conditions were calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). Primers were designed using IDT (Integrated DNA Technologies) software (www.idtdna.com) with 20–22 base, average T_m value of 62 °C, and GC content ranging from 45 to 50 %. Table 1 presents details the primers used for expression analysis.

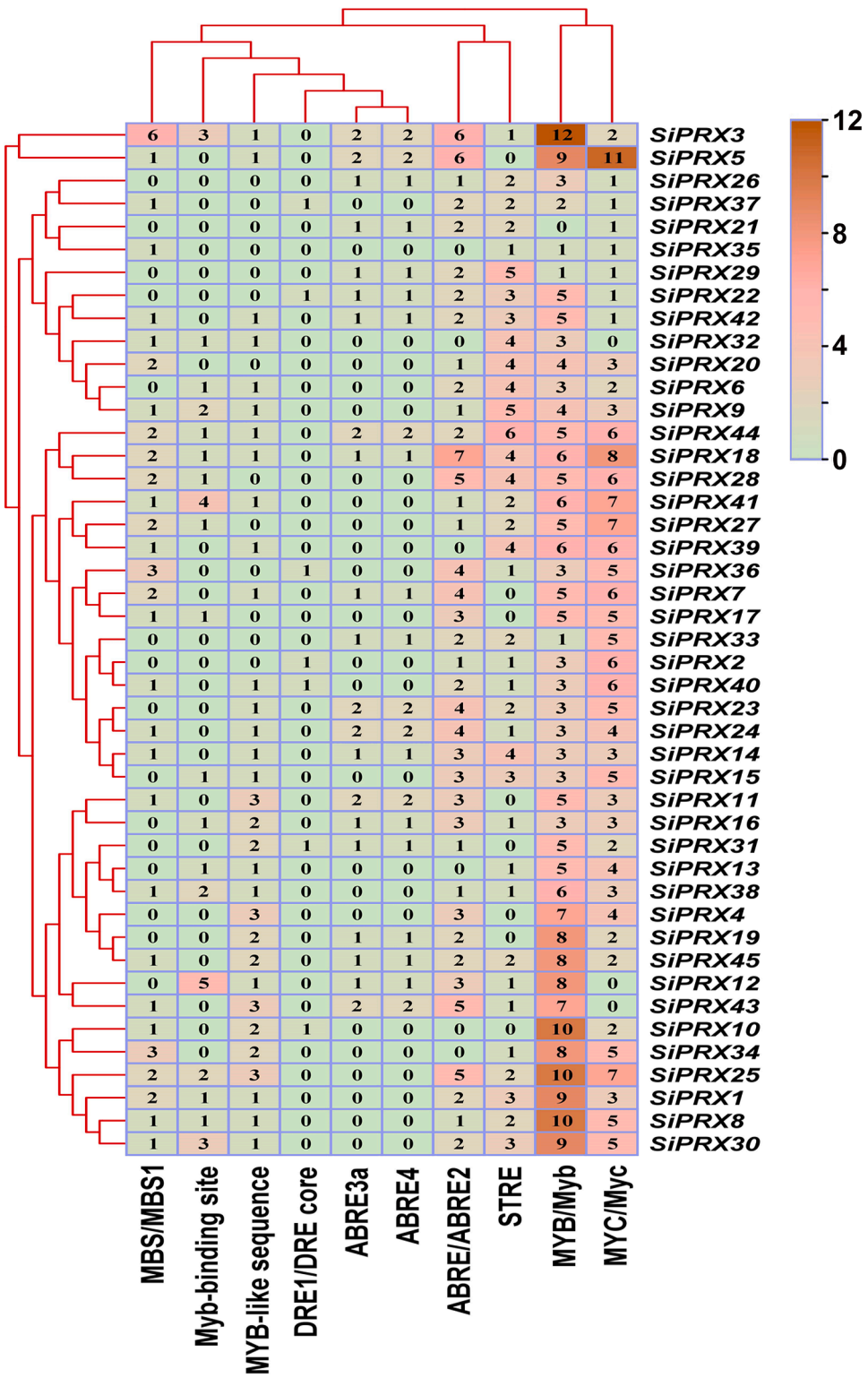

Fig. 5. Analysis of cis-acting regulatory elements in putative promoters (2000 bp upstream) of *SiPRX* genes. Distribution of different *cis*-acting elements for *SiPRX* genes putative promoters. Counts of each *cis*-acting motif are in the columns. The colour code key is on the top right-hand side.

3. Results

3.1. Identification and characterization of PRX gene family in sesame

We identified genes encoding class III peroxidase using whole genome sequence information for sesame (Wang et al., 2022). Based on 73 *PRXs* (AtPER 1 to AtPER 73) present in *Arabidopsis*, we identified 45 *PRX* genes in the sesame genome based on CDD. The identified genes (*SiPRXs*) were assigned *SiPRX1* to *SiPRX45* based on their locus on sesame chromosomes (Table 2). Among the 45 *SiPRX* genes, 23 were in the forward direction (+ve), and 22 were in the reverse direction (–ve). The average molecular weight of the *SiPRX* proteins was 36.31 kDa, ranging from 27.98 kDa (*SiPRX*34) to 48.69 kDa (*SiPRX*10). The isoelectric point (pI) ranged from 4.48 (*SiPRX*7, *SiPRX*36) to 10.24 (*SiPRX*37), indicating

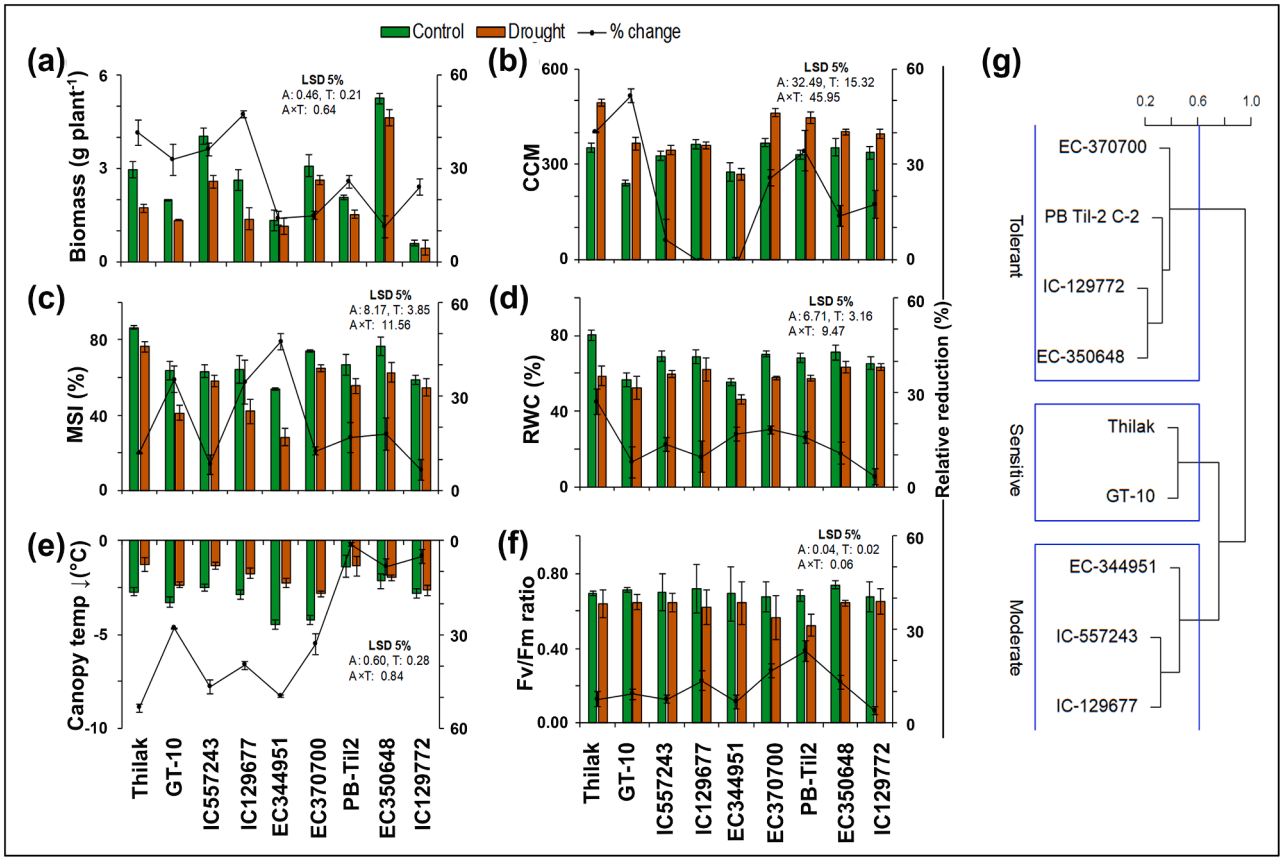

Fig. 6. Effect of drought stress on (a) shoot biomass, (b) chlorophyll concentrations, (c) membrane stability index, (d) relative water index, (e) canopy temperature depression, and (f) F_v/F_m ratio in nine sesame accessions. Bars represent the mean of eight replications ± SE, while the line graph represents the relative change with respect to drought. (g) Ward's method utilized the relative value of all traits for clustering sesame accessions. A, accession; T, treatment.

highly acidic to highly basic properties within the same *PRX* gene family (Table 2). The comparison of the number of *PRX* genes in the class III peroxidase family across different plant species for a range of genome content suggests that there is no correlation between genome size and *PRX* gene numbers (Table 3). Subcellular localization analysis revealed that most *SiPRX* proteins (65 %) were present in the extracellular region, followed by chloroplasts and nuclear membranes, while *SiPRX* proteins targeting the nucleus were the least abundant (Supplementary Fig. 1).

3.2. Gene structure and protein motif analysis of SiPRXs

Gene structure analysis revealed that *SiPRX* genes had 2 to 20 exons with an average of ~5 exons per gene (Fig. 1). *SiPRX30* had the most exons (20), followed by *SiPRX1, SiPRX4, SiPRX9*, and *SiPRX41* with 11 exons each, *SiPRX26* and *SiPRX36* with nine exons each, and *SiPRX17* with six exons, *SiPRX2* and *SiPRX35* with five exons each. Twenty-one *SiPRXs* (*SiPRX3, SiPRX5, SiPRX7, SiPRX10, SiPRX11, SiPRX12, SiPRX13, SiPRX14, SiPRX15, SiPRX18, SiPRX19, SiPRX20, SiPRX23, SiPRX25, SiPRX28, SiPRX31, SiPRX32, SiPRX33, SiPRX39, SiPRX40* and *SiPRX43*) had four exons each, nine *SiPRXs* (*SiPRX8, SiPRX16, SiPRX21, SiPRX22, SiPRX27, SiPRX29, SiPRX34, SiPRX44* and *SiPRX45*) had three exons each, and five *SiPRXs* (i.e., *SiPRX6, SiPRX24, SiPRX37, SiPRX38,* and *SiPRX42*) had two exons each (Fig. 1).

We identified 15 motifs in the *SiPRX* proteins (Fig. 1). Supplementary Table S1 presents the details of motifs present in the *SiPRX* protein sequences. At least one typical PRX motif was present in all sesame PRX family members (Fig. 1). However, *SiPRX*27 and *SiPRX*32 lacked the presence of typical motif (motif no. 6). Using the CDD tool, all 15 motifs were functionally annotated and checked for presence of the peroxidase domain (Fig. 1).

3.3. Phylogenetic analysis

Supplementary Fig. S2 shows the aligned *SiPRX* protein sequences. Diversity within the PRX family in sesame were further explored by including PRX proteins from *Arabidopsis thaliana, Oryza sativa,* and *Zea mays* (Fig. 2). The phylogenetic tree grouped the protein sequences of all PRXs into eight clusters. This helped to identify the corresponding orthologs present in sesame when compared with other species.

3.4. Chromosome location, synteny analysis, and duplication of SiPRX genes

The chromosomal localization for 45 *SiPRX* genes was constructed to reveal their distribution pattern across the 13 chromosomes which highlighted the uneven distribution pattern with 2–5 genes per chromosome (Fig. 3). Chromosomes 6 and 12 had the most *SiPRX* genes (five each), while chromosomes 1 and 5 had the least (two each). Some *SiPRX* genes were in tandem to each other, e.g., *SiPRX21* and *SiPRX22* on chromosome 7 and *SiPRX29* and *SiPRX30* on chromosome 9 (Fig. 3).

Gene duplication plays a crucial role in gene family's expansion during evolutionary process. The analysis of Ka/Ks ratios for *SiPRX* genes showed that all genes had Ka/Ks values <1, indicating they had undergone negative selection (Wang et al., 2015; Duan et al., 2019) (Supplementary Table S2). The MCScanX analysis identified 35 and 32 orthologs for sesame with *Arabidopsis* and *Brassica* respectively (Fig. 4).

3.5. Prediction of N-linked and O-linked glycosylation sites, phosphorylation sites, TMHs, and protein–protein interactions

In this study, we predicted up to 14*N*-linked glycosylation sites in

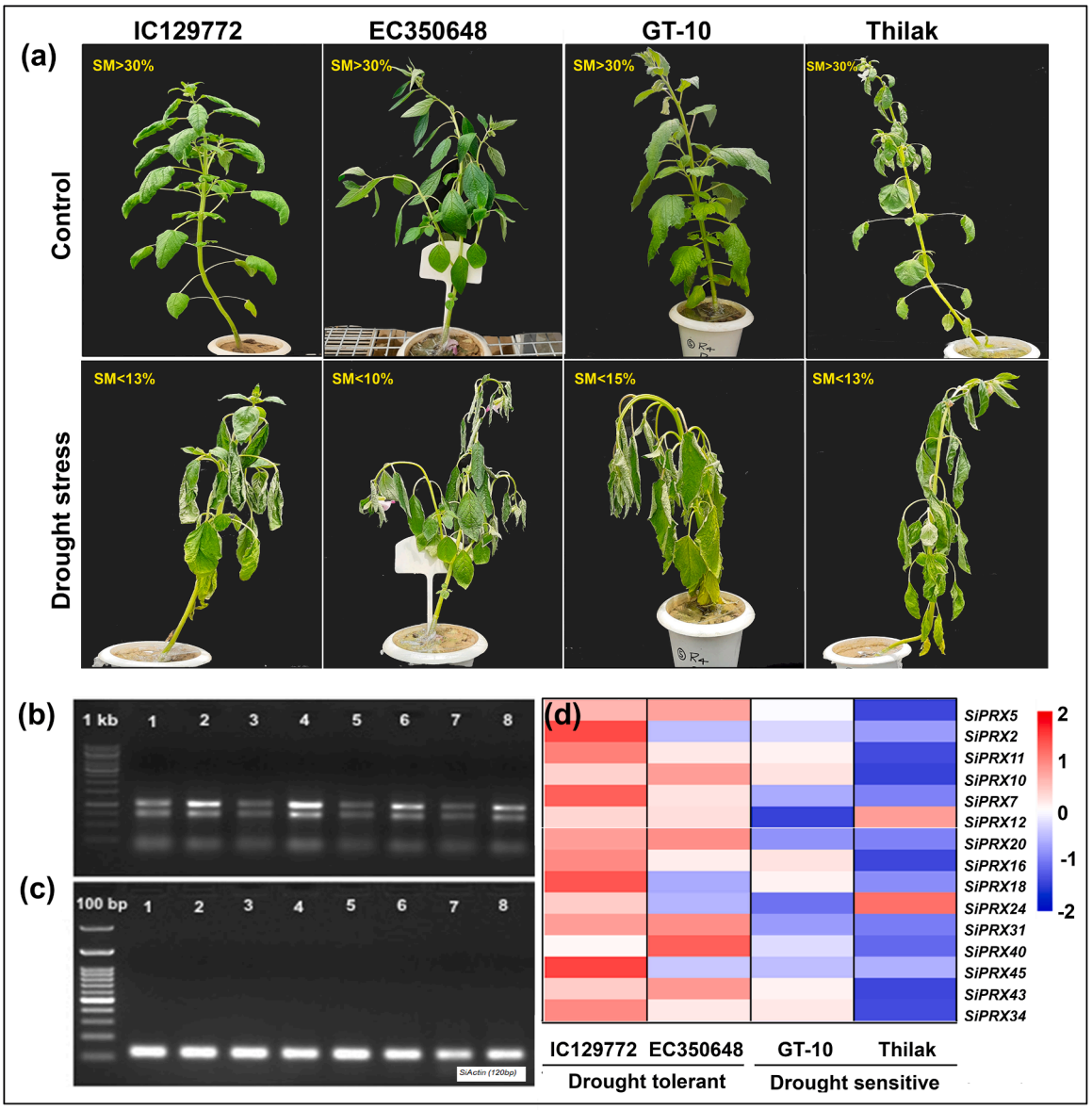

Fig. 7. Real-time validation of *SiPRX* genes in contrasting sesame accessions. (A) Sesame accessions under different soil moisture (SM) contents. (B) Total RNA isolation from two contrasting accessions grown under drought stress and control conditions. Lanes 1 to 4: control IC129772, EC350648, GT10, and Thilak; Lanes 5 to 8: drought-stressed IC129772, EC350648, GT10, and Thilak. (C) semi q-RT-PCR for housekeeping gene (*SiActin*). Lanes 1–4: control IC129772, EC350648, GT10, and Thilak; Lanes 5 to 8 drought-stressed IC129772, EC350648, GT10, and Thilak. (D) qRT-PCR of 15 selected *SiPRX* genes in contrasting sesame accessions. Heat map showing the effect of drought stress on the relative expression of *SiPRX* genes in the leaves of four contrasting sesame accessions. The gene expression levels are presented using fold-change values transformed to Log_2 format compared to control. Color ranges are set from blue to red to visualize low to high gene expression, respectively.

SiPRX proteins, with an average of 6.7*N*-linked sites per *SiPRX* protein (Supplementary Table S3). *SiPRX*10 possessed the most *N*-linked sites (14), while *SiPRX*1 and *SiPRX*36 exhibited the least (one each). Only three Sipra proteins showed the presence of potential *O*-linked sites: *SiPRX*1 (1 site), *SiPRX*4 (1 site), and *SiPRX*11 (2 sites) (Supplementary Table S3). Glycosylation is an essential post-translational modification for membranes and secretory proteins in plants, with one-third of the *SiPRX* proteins in sesame being membrane-bound (with TMHs, transmembrane helixes) (Supplementary Table S3). The TMHs contained 15.13–22.97 amino acids (average 19.11), contributing to TMH formation. Supplementary File S1 presents the phosphorylation (phosphorylated serine and threonine) sites in *SiPRX* proteins.

The protein–protein interaction analysis revealed that *SiPRX* proteins interacted with other proteins, including root hair-specific proteins (RHS12, RHS13, RHS18, RHS19), catalase, cytosolic ascorbate peroxidase, mono-dehydorascorbate reductase (MDHAR), cys-peroxiredoxin, glutathione reductase, and NADPH-dependent thioredoxin reductase (Supplementary Fig. S3).

3.6. Gene ontology analysis, promoter prediction of SiPRX genes, and in-silico expression analysis under drought stress

The GO analysis revealed that the *SiPRX* genes were involved in plant stress defense mechanism (Supplementary Fig. S4). Enriched KEGG pathway analysis highlighted the involvement of *SiPRXs* in four major metabolic pathways: phenylpropanoid biosynthesis, secondary metabolite biosynthesis, ascorbate and aldarate metabolism, and glutathione metabolism (Supplementary Fig. S5). Furthermore, *in-silico* analysis of *SiPRX* gene expression under drought stress identified more upregulated *SiPRX* genes in the drought-tolerant genotype than in the drought-

sensitive genotype (Supplementary Fig. S6). These results suggest that *SiPRX* genes play a major role in providing abiotic stress tolerance to sesame plants, especially under drought stress.

The promoter region of *SiPRX* genes revealed the presence of two *cis*-regulatory elements for drought stress tolerance linking with ABA-dependent and ABA-independent pathways. ABA-dependent *cis*-regulatory elements included DRE1/DRE core, ABRE3a, ABRE4, ABRE/ABRE2, and STRE (Fig. 5), while ABA-independent *cis*-regulatory elements included MBS/MBS1, Myb-binding site, MYB-like sequence, and MYB, MYC. The elements exhibit high variability in the promoter region of *SiPRX* genes and might be associated with differential expression pattern for *SiPRX* genes under drought stress.

3.7. Wet-lab validation of SiPRX genes in contrasting sesame accessions in response to drought stress

Physiological trait analysis revealed significant ($p \leq 0.05$) effects of accession (A), drought (D), and $A \times D$ interaction for all physiological traits. Except for chlorophyll concentration, all other physiological traits including shoot biomass, membrane stability index (MSI), relative water content (RWC), canopy temperature depression (CTD), and maximum quantum yield of PSII (F_v/F_m ratio) exhibited a significant decrease under drought, compared to the control (Fig. 6a–f). All nine accessions were grouped by Ward's clustering using relative values (ratio of drought to control) of all traits (shoot biomass, chlorophyll concentrations, CTD, RWC, MSI, and F_v/F_m ratio) (Fig. 6g) to identify contrasting sesame accessions for gene expression analysis. Three clusters were identified: tolerant (EC370700, PB Til-2, IC129772, EC350648), moderate (EC344951, IC557243, IC129677), and sensitive (Thilak, GT-10). The accessions in the tolerant group typically exhibited minimal reductions in most traits under drought stress compared to the control. Based on the performance of accessions under drought stress, two drought-tolerant (IC129772 and EC350648) and two drought-sensitive accessions (Thilak and GT-10) were selected for gene expression studies.

To validate the *in-silico* identified *PRX* genes at the transcription level, 15 genes were selected from the pool of 45 *SiPRX* genes. Total RNA isolated from drought-tolerant and drought-sensitive accessions that were subjected to drought stress for validation through qRT-PCR (Fig. 7a–c). Ten genes in the drought-tolerant accession IC129772 exhibited >2-fold higher expression under drought stress, while those in drought-sensitive accessions were either downregulated or showed no significant difference (e.g., all genes except *SiPRX12* and *SiPRX24* in Thilak) as compared to the control (Fig. 7d). However, genes *SiPRX5, SiPRX7, SiPRX20,* and *SiPRX31* exhibited significant contrasting expression in drought-tolerant and drought-sensitive accessions.

4. Discussion

The present study on the class III *PRX* gene family in sesame identified 45 *SiPRXs* through genome-wide analysis similar to those conducted in model plant species such as Arabidopsis, rice, and maize (Tognolli et al., 2002; Passardi et al., 2004; Wang et al., 2015). However, this number in sesame is less than those reported in Arabidopsis (*Arabidopsis thaliana*) (73), rice (*Oryza sativa*) (138), maize (*Zea mays*) (119), wheat (*Triticum aestivum*) (374), carrot (*Daucus carota*) (102), and soybean (*Glycine* max) (124) (Su et al., 2020; Meng et al., 2021; Aleem et al., 2022). The class III peroxidase gene family exhibited its conserved nature irrespective of whether the plant species is dicot or monocot. Further, the number of peroxidases varied in different plant species, and the peroxidase genes present in the family is not related to the genome size (Kidwai et al., 2020). In terms of subcellular localization, PRX proteins are typically characterized as secretory proteins. Our findings align with this characteristic, as nearly two-thirds of the *SiPRX* proteins were extracellular in localization. Meng et al. (2021) reported a similar trend for DcPRX proteins in carrot, which revealed that PRX proteins in other plant species are also mostly extracellular.

The MEME prediction analysis revealed that closely related *SiPRX* proteins were grouped in the same subfamily (Fig. 1). This observation aligns with earlier reports on PRX gene families in Arabidopsis, rice, maize, and potato (*Solanum tuberosum*) (Tognolli et al., 2002; Passardi et al., 2004; Wang et al., 2015; Yang et al., 2020). We found approximately 42 % of the *SiPRX* genes exhibiting a 3-intron/4-exon pattern which is consistent with observations in Arabidopsis where a 3-intron/4-exon model covered a significant portion of *PRX* genes (Tognolli et al., 2002). Our results also align with those observed in rice and potato (Passardi et al., 2004; Yang et al., 2020) indicating a highly conserved exon/intron model of *PRX* genes.

The present study highlighted that a few of *SiPRX* genes underwent tandem duplication events, a key factor for the *SiPRX* gene family expansion (Van de Peer et al., 2009; Elsheery, 2019). Gene duplication events include segmental and tandem duplications (Cannon et al., 2004). Our results are consistent with previous studies on class III peroxidase gene family in *Populus trichocarpa* and *Betula pendula*, where tandem duplication significantly contributed to gene family expansion for *PRX* genes (Ren et al., 2014; Cai et al., 2021). In addition, segmental duplication has been reported as a driver of *PRX* gene family expansion, for example, in Chinese pear (Cao et al., 2016). Maize is another example exhibiting segmental and tandem duplications in *PRX* gene family expansion (Wang et al., 2015). Thus, the results collectively emphasize that the peroxidase gene in different species may have different patterns of gene family expansion. Notably, many of the tandem duplicated genes in the present study were located proximal to the telomeric regions of chromosomes. Further, we found Ka/Ks <1 in all *SiPRX* genes which is in agreement with previous reports on different plant species such as lettuce (*Lactuca sativa*) (Park et al., 2020), cotton (*Gossypium hirsutum*) (Akram et al., 2020), and many gene families of soybean such as SWEET gene family (Patil et al., 2015), GRAS (Wang et al., 2020), and NOX gene family (Zhang et al., 2019) where majority of *peroxidase* genes had Ka/Ks <1. A recent study on soybean showed that 81 % gene pairs were segmentally duplicated while 19 % were tandemly duplicated (Aleem et al., 2022). The hexaploid wheat (2n = 6x = 42), possessed 374 peroxidase genes (Su et al., 2020) while tobacco (*Nicotiana tabacum*) an allotetraploid (2n = 4x = 48) showed 210 peroxidase genes (Cheng et al., 2022). Regarding evolutionary studies, a recent report in sweet orange (*Citrus* spp.) and tobacco found that out of all peroxidase genes, only a few experienced positive selection (Li et al., 2020; Cheng et al., 2022). So, there is possibility that the segmental duplication in sesame is the major driving force for evolution and expansion of gene families and at the same time, selection pressure is not same on all genes present in a family.

Peroxidases are classified as membrane bound and secretory PRXs, having signal peptide present at N-terminal for targeting plasma membrane, vacuole, thylakoid and endoplasmic reticulum (Lüthje and Martinez-Cortes, 2018). In the present study, 65 % peroxidases were found to be extracellular, followed by plasma membrane, chloroplast, mitochondria, and nuclear which is consistent with those reported in tobacco class III peroxidase family (Cheng et al., 2022).

Understanding gene function is a crucial aspect of plant biology, and gene expression patterns under control and stress conditions shed light on its functionality. The *in-silico* analysis revealed highly variable expression patterns of four *SiPRX* genes (*SiPRX5, SiPRX7, SiPRX20, SiPRX31*) in sesame genotypes contrasting in response to drought stress, akin to that reported in potato *SiPRXs* under drought stress (Yang et al., 2020). This variability suggests functional differences among *PRX* genes within the class III peroxidase gene family. The candidate genes associated with enhanced tolerance to drought stress in the drought-tolerant genotype could serve as valuable genomic resource for improving sesame crop through breeding or transgenic approaches, especially in the face of global climate change. In the drought-tolerant sesame genotype, the tolerance was linked to accumulated osmoprotectants (proline) and antioxidant enzymes, especially PRXs (Dossa et al., 2017). Earlier studies in Arabidopsis, rice, maize, and potato also demonstrated the

upregulation of *PRXs* under abiotic stress conditions (Tognolli et al., 2002; Passardi et al., 2004; Wang et al., 2015; Yang et al., 2020), emphasizing the significant role of *PRXs* in plant abiotic stress tolerance. *SiPRXs* protein-protein interactions are primarily involved in pathways related to H_2O_2 (hydrogen peroxide) removal, lignin metabolism, auxin catabolism, suberization, pathogen attack, and oxidative stress response. These interactions aid in plant cell survival under abiotic stress conditions (Zhang et al., 2021). Jasmonic acid, salicylic acid, polyethylene glycol (PEG), and hydrogen peroxide (H_2O_2) also trigger specific *PRX* genes in maize (Wang et al., 2015). However, a few genes exhibited variability in expression (Supplementary Figs. S6 and 7). This finding aligns with the expression patterns of potato *PRX* genes clustered in group G which are specifically involved in stress responses (Yang et al., 2020). They demonstrated through qRT-PCR-based analysis that the expression patterns of *PRX* genes varied under different abiotic stresses, such as heat, salt, and drought. Likewise, in the present study, qRT-PCR analysis revealed differential expression levels of *SiPRXs* genes under drought stress condition. The identified *SiPRX* genes (*SiPRX5, SiPRX7, SiPRX20, SiPRX31*) can be utilized in crop improvement program against drought stress in sesame and related species.

5. Conclusion

We conducted a genome-wide survey for *SiPRX* genes to assess structural and functional diversity. The wet-lab experiments, which examined the expression patterns of *SiPRX* genes in drought-tolerant and drought-sensitive sesame accessions, strongly suggest their involvement in drought stress tolerance. We found four potential *SiPRX* genes demonstrating role in drought stress tolerance and an in-depth study of *SiPRX*5 protein structure revealed potential active sites. Thus, these findings have strong future application value in developing drought-tolerant sesame varieties foreseeing the future impacts of globally changing climatic condition.

Ethics approval

N/A.

Consent to participate

N/A.

Consent for publication

N/A.

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CRediT authorship contribution statement

Harinder Vishwakarma: Conceptualization, Visualization, Data curation, Formal analysis, Software, Validation, Writing – original draft, Writing – review & editing. **Sandeep Sharma:** Conceptualization, Visualization, Data curation, Formal analysis, Methodology, Writing – review & editing. **Kishor Prabhakar Panzade:** Conceptualization, Visualization, Data curation, Formal analysis, Software. **Pawankumar S. Kharate:** Conceptualization, Visualization, Formal analysis. **Ajay Kumar:** Conceptualization, Visualization, Data curation, Formal analysis, Methodology, Software. **Nisha Singh:** Conceptualization, Visualization, Data curation, Formal analysis, Methodology. **Himanshu Avashthi:** Conceptualization, Methodology, Visualization. **Parimalan Rangan:** Conceptualization, Visualization, Data curation, Project administration, Supervision, Writing – review & editing. **Anuj Kumar Singh:** Conceptualization, Visualization, Data curation, Investigation, Methodology. **Artika Singh:** Conceptualization, Visualization, Data curation, Formal analysis, Methodology. **Ulavappa Basavanneppa Angadi:** Conceptualization, Project administration, Visualization, Data curation, Formal analysis, Writing – review & editing, Methodology, Software. **Kadambot H.M. Siddique:** Conceptualization, Visualization, Supervision, Writing – review & editing, Project administration, Methodology. **Kuldeep Singh:** Conceptualization, Visualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing. **Gyanendra Pratap Singh:** Conceptualization, Visualization, Writing – review & editing, Project administration, Resources, Supervision. **Renu Pandey:** Conceptualization, Visualization, Data curation, Formal analysis, Project administration, Methodology, Writing – review & editing. **Rashmi Yadav:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2024.100367.

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