

Article

Genome-Wide Analysis of the Chickpea Peroxiredoxin Gene Family Reveals Subfamily-Specific Expression Partitioning

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Abstract

Peroxiredoxins (Prxs) are thiol-dependent peroxidases that couple hydrogen peroxide (H₂O₂) detoxification with redox signalling, but the family has not been characterized in chickpea (*Cicer arietinum* L.). Here, we performed a genome-wide analysis of the *CaPrx* family integrating phylogeny, gene structure, conserved motifs, duplication history, promoter architecture, and public RNA-seq data. Eight *CaPrx* genes were identified and classified into the four canonical Prx subfamilies. The duplicated pair *Ca2CPrxA/Ca2CPrxB* showed significant purifying selection ($K_a/K_s = 0.058$; $p < 10^{-20}$), whereas *Ca1CPrxA* and *Ca1CPrxB* differed at only three nucleotide positions, which is consistent with a very recent duplication. In a 32-tissue expression atlas, expression was partitioned by subfamily: *Ca2CPrxA*, *Ca2CPrxB*, and *CaPrxQ* were most abundant in photosynthetic tissues, the 1-Cys Prx transcript accumulated strongly during late seed maturation, and *CaPrxIIA* was highly and constitutively expressed. In the roots of a drought-sensitive and a drought-tolerant genotype, no *CaPrx* gene changed by more than two-fold under drought. These results provide a curated genomic and expression reference for the chickpea Prx family and point to subfamily-specific expression domains, particularly the seed-associated 1-Cys Prx, as priorities for functional studies.

Keywords: peroxiredoxin; oxidative stress; gene duplication; purifying selection; redox regulation; expression atlas; seed maturation

1. Introduction

Reactive oxygen species (ROS) are continuously generated as inevitable by-products of aerobic metabolism in plants [1,2]. Under optimal conditions, ROS act as essential signaling molecules, regulating cell proliferation, growth, development, and responses to environmental stimuli. However, environmental stresses such as drought, salinity, extreme temperatures, and high irradiance disrupt cellular redox homeostasis, leading to excessive ROS accumulation that damages proteins, lipids, nucleic acids, and biological membranes [2]. Consequently, plant survival depends on a highly coordinated antioxidant network capable of simultaneously limiting oxidative damage while preserving ROS-mediated signaling required for physiological acclimation [1,3].

Among the enzymatic components of this antioxidant system, peroxiredoxins (Prxs; EC 1.11.1.15) constitute a highly conserved family of thiol-dependent peroxidases that

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catalyze the reduction of hydrogen peroxide (H_2O_2), alkyl hydroperoxides, and peroxy-nitrite. Unlike several antioxidant enzymes whose primary function is ROS detoxification, Prxs also participate directly in redox signaling by coupling peroxide scavenging with the regulation of cellular metabolism, photosynthesis, growth, development, and stress acclimation [3,4].

Based on their catalytic cysteine composition and structural characteristics, plant Prxs are classified into four major subfamilies: 1-Cys Prx, 2-Cys Prx, Type-II Prx, and PrxQ [5,6]. Members of these subfamilies are distributed across multiple subcellular compartments, including chloroplasts, mitochondria, cytosol, nuclei, and the extracellular space, reflecting their broad participation in redox-regulated cellular processes [3,7]. The subfamilies also differ in their typical sites of action: 2-Cys Prx and PrxQ are chloroplast enzymes closely linked to photosynthetic metabolism [8,9], type II Prxs occur in the cytosol, mitochondria, and plastids [8,10], and 1-Cys Prx is expressed mainly in seeds, where it has been associated with desiccation tolerance and dormancy [11].

Comparative genomic analyses have shown that the Prx family is compact and conserved across land plants [5,12]. Gene duplication has nevertheless contributed to its diversification. After duplication, paralogous genes often retain conserved coding sequences (CDS), and their fate depends largely on how their expression evolves: duplicates may partition ancestral expression domains (subfunctionalization), acquire new ones (neofunctionalization), or be retained with largely redundant expression, for example, because of dosage requirements [13–16]. Distinguishing among these outcomes requires combining analyses of sequence divergence and selection with expression data across tissues and conditions [17–19].

Chickpea (*Cicer arietinum* L.) is one of the world's most important grain legumes and a major source of dietary protein for millions of people, particularly in semi-arid and drought-prone regions, where the crop is often grown on residual soil moisture and exposed to terminal drought [20]. As a self-pollinated diploid species ($2n = 2x = 16$), chickpea possesses a relatively well-characterized genome and has become an important model for genomic studies aimed at improving stress tolerance and crop productivity [21,22]. Despite the biological importance of peroxiredoxins in maintaining cellular redox homeostasis, the evolutionary relationships, duplication history, and expression patterns of the *Prx* gene family remain poorly understood in chickpea.

Therefore, this study characterized the *Prx* gene family in *C. arietinum* through an integrated genome-wide approach combining phylogenetic inference, chromosomal organization, gene duplication, structural conservation, conserved motifs, and promoter architecture with publicly available transcriptomes covering 32 tissues and developmental stages and a drought experiment in contrasting genotypes. We specifically asked how *CaPrx* expression is distributed among organs and subfamilies, and whether duplicated *CaPrx* genes have retained conserved protein structures and diverged in expression across tissues and under drought. These data provide a genomic and expression reference for future functional studies of redox regulation in chickpea.

2. Results

2.1. Genome-Wide Identification and Molecular Characterization of the *CaPrx* Gene Family

A genome-wide search of the *C. arietinum* L. genome identified eight genes encoding putative peroxiredoxins (*CaPrxs*) (Table 1). Based on their chromosomal distribution and phylogenetic relationships, the identified genes were designated *CaPrxIIA*, *CaPrxIIB*, *CaPrxIIC*, *Ca1CPrxA*, *Ca1CPrxB*, *Ca2CPrxA*, *Ca2CPrxB*, and *CaPrxQ*. The CDS ranged from 489 to 798 bp, encoding proteins comprising 162–265 aa, with predicted molecular weights varying from 17.23 to 29.05 kDa.

The predicted physicochemical properties showed moderate variation among CaPrx proteins. The theoretical pI ranged from 4.95 to 9.52. Six proteins presented negative GRAVY values, whereas CaPrxIIA and CaPrxIIB exhibited slightly positive values. Subcellular localization predictions indicated that CaPrx proteins are distributed among the chloroplast, cytoplasm, nucleus, and mitochondrion, with most family members predicted to localize in the nucleus or chloroplast (Table 1).

Table 1. Genomic and physicochemical characteristics of the CaPrx proteins identified in *C. arietinum* L.

Gene	ID	Chromosome Position	Length (aa)	CDS (bp)	MW (kDa)	pI	GRAVY	Subcellular Localization
CaPrxIIC	Ca_00604 (PAC:40149207)	Ca1:5150502..5153208 (–)	196	591	21.17	8.74	–0.116	Mitochondrion
CaPrxIIB	Ca_00760 (PAC:40156755)	Ca3:34829559..34830213 (–)	217	654	23.29	7.67	0.025	Nucleus
Ca2CPrxA	Ca_00840 (PAC:40156866)	Ca3:35564193..35566353 (+)	201	606	22.54	4.95	–0.153	Chloroplast/Cytoplasm
Ca2CPrxB	Ca_14411 (PAC:40147111)	Ca4:24016241..24018687 (–)	265	798	29.05	6.31	–0.078	Chloroplast/Cytoplasm
Ca1CPrxA	Ca_05125 (PAC:40137920)	Ca6:13543775..13544715 (+)	218	657	24.36	6.08	–0.453	Nucleus
Ca1CPrxB	Ca_21594 (PAC:40137018)	Ca6:40720281..40721221 (–)	218	657	24.28	6.07	–0.441	Nucleus
CaPrxQ	Ca_03122 (PAC:40142235)	Ca7:3426338..3427573 (–)	228	687	23.21	9.52	–0.375	Nucleus
CaPrxIIA	Ca_17900 (PAC:40153576)	Ca8:15411496..15413198 (–)	162	489	17.23	5.01	0.130	Cytoplasm/Nucleus

ID, gene identifier (Phytozome v13, *C. arietinum* v1.0); chromosome position, start–end coordinates and strand; length, number of amino acids excluding the stop codon; CDS, coding sequence; MW, molecular weight; pI, theoretical isoelectric point; GRAVY, grand average of hydropathy.

2.2. Chromosomal Distribution, Gene Duplication and Evolutionary Analysis

The eight CaPrx genes were unevenly distributed across six of the eight chickpea chromosomes (Figure 1A). Chromosomes 3 and 6 harbored two family members each, whereas chromosomes 1, 4, 7, and 8 contained a single CaPrx gene. No CaPrx genes were identified on chromosomes 2 or 5.

Two putative segmentally duplicated gene pairs, Ca1CPrxA/Ca1CPrxB and Ca2CPrxA/Ca2CPrxB, were identified (Figure 1B).

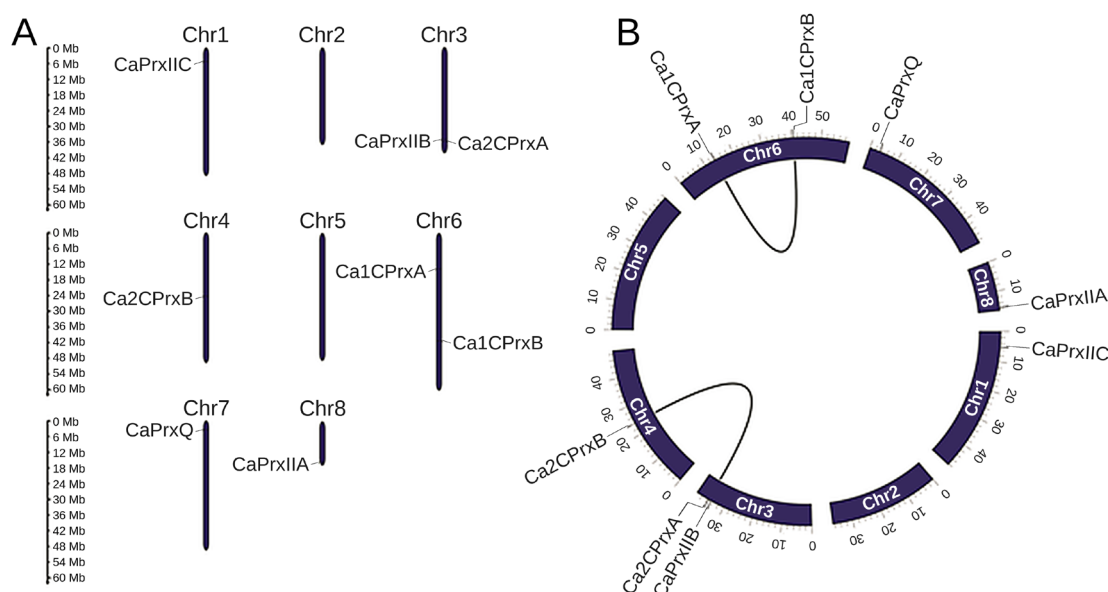


Figure 1. (A) Localization of CaPrxs genes across the eight chromosomes, with chromosome numbers and sizes (Mb) indicated. (B) Duplication events, with black lines connecting putative segmentally duplicated gene pairs.

The two pairs differed markedly in the numbers of synonymous (Ks) and non-synonymous (Ka) substitutions per site (Table 2). *Ca2CPrxA/Ca2CPrxB* (Ks = 0.451) showed Ka/Ks = 0.058 (95% bootstrap CI 0.026–0.104), significantly below 1 (one-sided Fisher's exact test, $p = 5.1 \times 10^{-21}$), indicating purifying selection, with an estimated duplication time of 36.95 million years ago (MYA). In contrast, the CDS of *Ca1CPrxA* and *Ca1CPrxB* differed at only three of 657 positions (one synonymous and two non-synonymous differences, all within an 18 bp stretch; Ks = 0.007). With so few substitutions, Ka/Ks (0.59) could not be distinguished from 1 ($p = 0.54$), and the bootstrap interval was not estimable because Ks was zero in 35% of resamples.

Table 2. Divergence and selection analysis of the duplicated *CaPrx* gene pairs.

Pair	Chr.	Codons	Sd	Nd	Ka	Ks	Ka/Ks	95% CI	<i>p</i>	T (MYA)
<i>Ca1CPrxA/B</i>	6/6	218	1.0	2.0	0.0041	0.0070	0.59	n.e.	0.54	0.57
<i>Ca2CPrxA/B</i>	3/4	201	44.5	11.5	0.0263	0.4508	0.058	0.026–0.104	5.1×10^{-21}	36.95

Chr., chromosomes; Sd and Nd, numbers of synonymous and non-synonymous differences (Nei–Gojobori); Ka and Ks, Jukes–Cantor-corrected non-synonymous and synonymous substitutions per site; 95% CI, bootstrap confidence interval of Ka/Ks (5000 resamples over codons); n.e., not estimable (Ks = 0 in 35% of resamples); *p*, one-sided Fisher's exact test of Ka < Ks; T = Ks/2λ with λ = 6.1×10^{-9} substitutions site^{−1} year^{−1} [23]; MYA, million years ago.

2.3. Phylogenetic Relationships, Gene Structure and Conserved Motifs

Phylogenetic analysis was performed using the full-length protein sequences of eight *CaPrxs* together with Prx proteins from *A. thaliana* and *O. sativa* L. All proteins were grouped into the four canonical plant Prx subfamilies: Type II Prx, 1-Cys Prx, 2-Cys Prx, and PrxQ (Figure 2). Type II Prx was the largest group, comprising 14 members, followed by 2-Cys Prx with six members, 1-Cys Prx with five, and PrxQ with three. Each group contained representatives from the three species.

Analysis restricted to the chickpea proteins also separated *CaPrxs* according to their respective subfamilies (Figure 3A). Members belonging to the same phylogenetic group generally shared similar exon–intron organization and motif composition (Figure 3B,C). In particular, the duplicated pairs *Ca1CPrxA/Ca1CPrxB* and *Ca2CPrxA/Ca2CPrxB* showed highly similar gene structures.

Ten conserved motifs were identified, with most *CaPrx* proteins containing between five and nine motifs (Figure 3B). Motif 1, containing the conserved catalytic PxxxTxxC signature, was present in all *CaPrx* proteins. Other motifs showed a more restricted distribution. Motifs 3 and 6 were restricted to members of the 1-Cys Prx and 2-Cys Prx groups, whereas motif 8 was detected only in *CaPrxIIB* and *CaPrxIIC*.

Analysis of exon–intron organization revealed substantial structural variation among *CaPrx* genes (Figure 3C), with the number of exons ranging from one to seven. *CaPrxIIB* exhibited the simplest structure, consisting of a single exon, whereas *Ca2CPrxB* contained the highest number, with seven exons. The duplicated pair *Ca1CPrxA/Ca1CPrxB* showed identical exon–intron organization, with three exons and two introns in each gene. In contrast, *Ca2CPrxA* and *Ca2CPrxB* contained six and seven exons, respectively. *CaPrxQ* and *CaPrxIIC* each comprised five exons, whereas *CaPrxIIA* contained three exons.

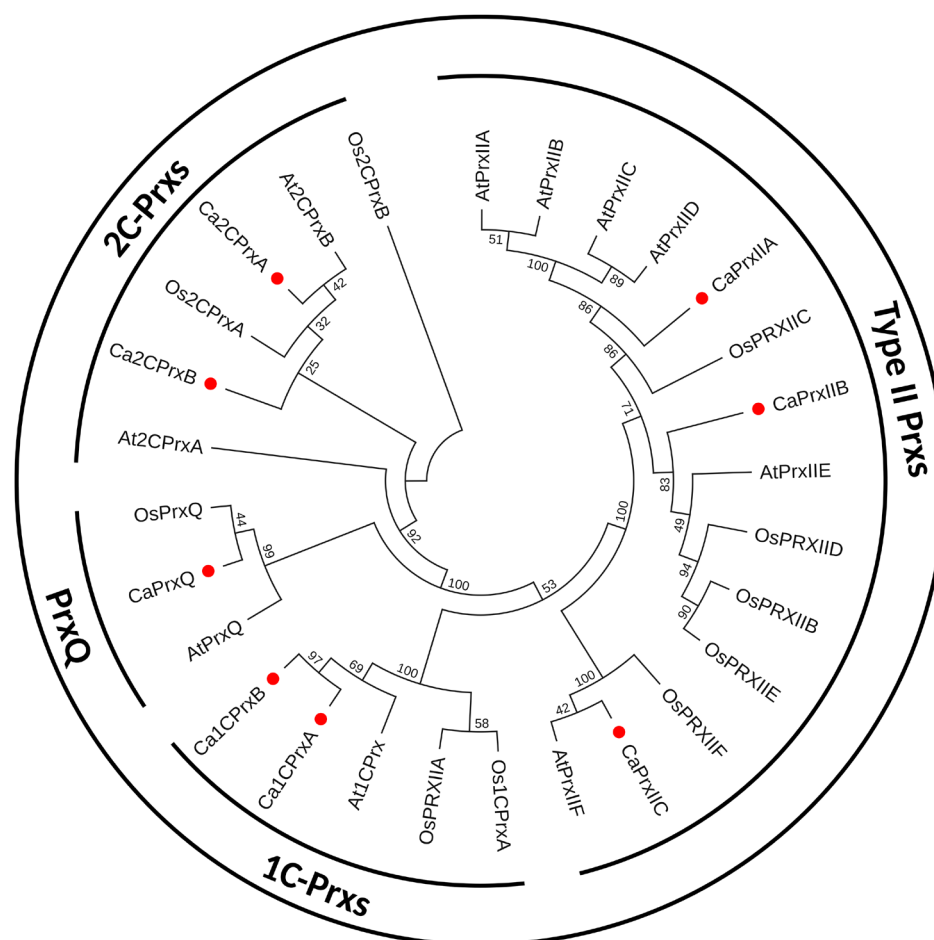


Figure 2. Phylogenetic analysis of Prx proteins from *Arabidopsis thaliana* (At), *Oryza sativa* L. (Os), and *Cicer arietinum* L. (Ca) was constructed using the ML method. *CaPrxs* were highlighted in red circles. The name of each Prx group is indicated next to the corresponding group.

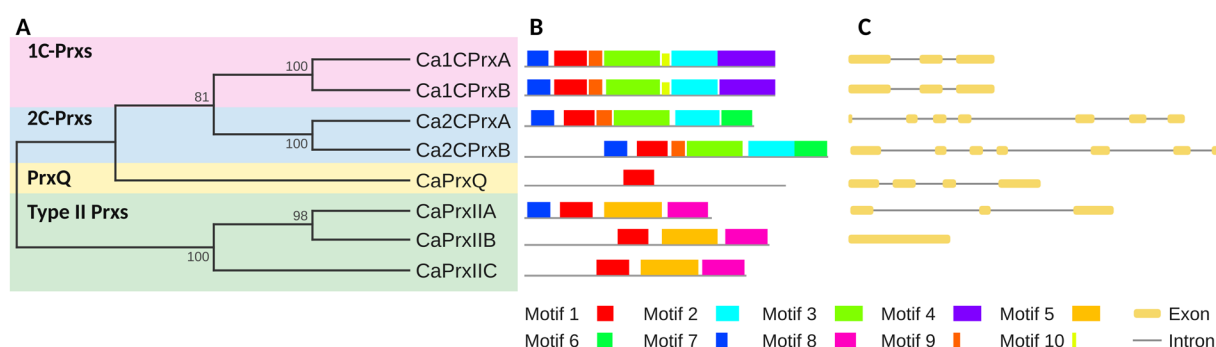


Figure 3. Conserved gene structure and protein architecture of the CaPrx family. **(A)** Phylogenetic relationships among CaPrx proteins. Colored backgrounds indicate the four Prx subfamilies: 1C-Prx (pink), 2C-Prx (blue), PrxQ (yellow) and Type II Prx (green). **(B)** Conserved motifs in CaPrx proteins obtained by MEME suite program. Each colored box represents a motif, and grey lines indicate protein length drawn to scale. **(C)** Gene structure of *Prxs* in *C. arietinum* L. as revealed by the GSDS2.0 software. Yellow boxes represent exons and grey lines represent introns.

2.4. Cis-Regulatory Elements in CaPrx Promoters

Analysis of the 1500 pb upstream regions revealed a diverse set of *cis*-regulatory elements associated with phytohormone signalling, abiotic stress responses, light respon-

siveness, defense, and plant development (Figure 4). Light-responsive elements were the most abundant category and were detected in all CaPrx promoters.

Cis-elements associated with abscisic acid (ABA), auxin, gibberellin (GA), and salicylic acid (SA) responses were also identified, together with elements related to drought and low-temperature responses. Their abundance and distribution differed among *CaPrx* genes. *Ca2CPrxB*, *CaPrxIIA*, and *CaPrxIIB* contained a higher number of abiotic stress-related elements. Motifs associated with cell cycle regulation and meristem expression were detected only in *CaPrxIIB* and *Ca2CPrxB*, whereas circadian-responsive elements were restricted to *Ca2CPrxA* and *CaPrxQ*.

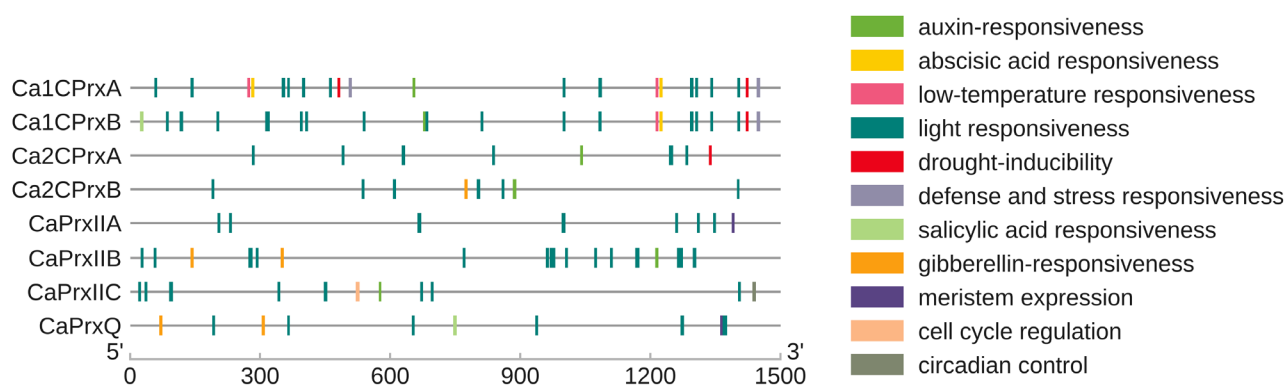


Figure 4. Distribution of *cis*-regulatory elements in the promoter regions of *CaPrx* genes in *C. arvense* L. Colored markers represent elements associated with hormonal responses (auxin, ABA, GA, and SA), abiotic stresses (light, temperature, and drought), and developmental or defence-related processes (circadian control, meristem expression, cell cycle regulation).

2.5. Expression of *CaPrx* Genes Across Tissues and Developmental Stages

The *CaPrx* CDS were matched to transcripts of the chickpea expression atlas by sequence alignment (100% identity over $\geq 95\%$ of the CDS; Table S1a), except that *Ca1CPrxA* and *Ca1CPrxB* matched the same transcript (100% and 99.5% identity, respectively), and could not be quantified separately; they are therefore reported together as *Ca1CPrxA/B*.

Expression was partitioned largely by subfamily (Figure 5A; Table S1b). The two 2-Cys Prx genes and *CaPrxQ* reached their highest abundance in young leaves (875–1315 transcripts per million, TPM) and were 13-fold (*Ca2CPrxA*, *Ca2CPrxB*) to 240-fold (*CaPrxQ*) more abundant in photosynthetic tissues (leaves, shoot, shoot apical meristem [SAM], and bracteole) than in roots, root hairs, and nodules. *Ca1CPrxA/B* was below 10 TPM in 23 of the 32 samples but accumulated strongly during seed maturation, reaching 649 TPM at 20 DAP and 5716 TPM at 30 DAP; it was also expressed in root hairs (321 TPM). The type II genes were broadly expressed: *CaPrxIIA* was the most abundant family member (median 611 TPM; range 112–1534 TPM) and accounted for a median of 43% of total *CaPrx* transcripts per sample, whereas *CaPrxIIB* and *CaPrxIIC* showed intermediate, relatively uniform levels.

Consistent with these patterns, only *Ca1CPrxA/B* approached the threshold for tissue-specific expression ($\tau = 0.77$), whereas all other genes showed broad expression ($\tau = 0.13 - 0.34$; Figure 5B; Table S2). The expression profiles of *Ca2CPrxA* and *Ca2CPrxB* were strongly positively correlated across the 32 samples (Spearman's $\rho = 0.95$, $p = 3.1 \times 10^{-16}$; Figure 5C; Table S3), indicating that this duplicated pair has retained a largely shared expression pattern.

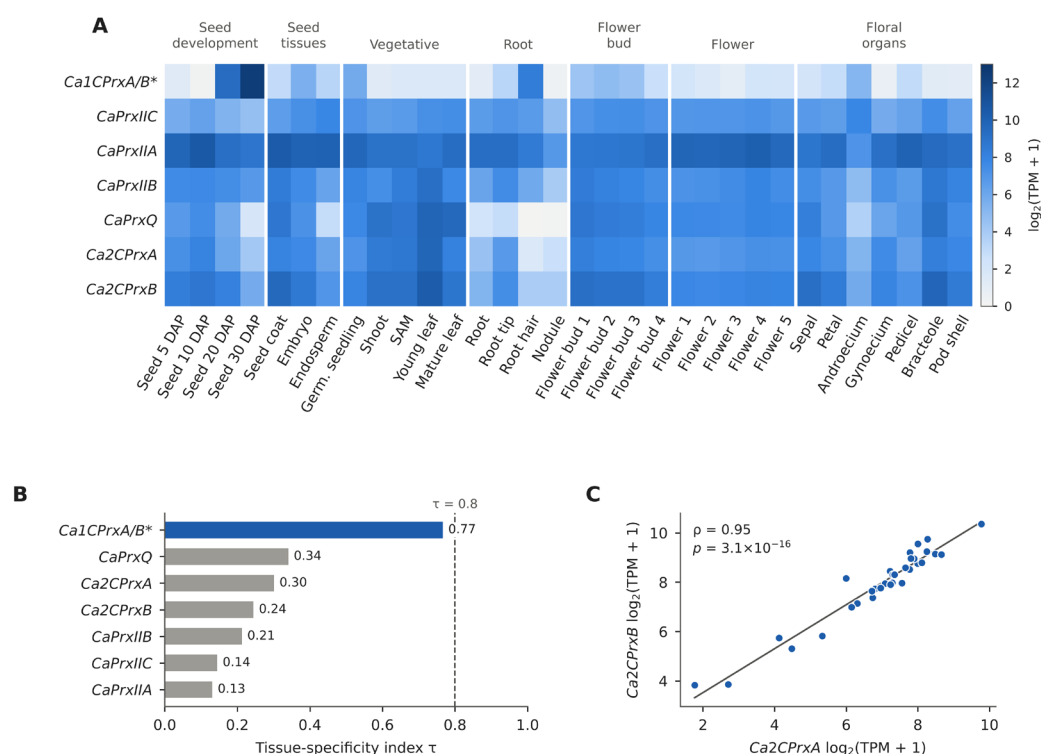


Figure 5. Expression of *CaPrx* genes across 32 chickpea tissues and developmental stages (GSE147831). **(A)** Heatmap of $\log_2(\text{TPM} + 1)$ values; tissues are grouped by organ and developmental stage, and genes are clustered by Pearson correlation distance and average linkage. *Ca1CPrxA* and *Ca1CPrxB* match the same atlas transcript and are shown together (*Ca1CPrxA/B**). **(B)** Tissue-specificity index (τ) calculated from $\log_2(\text{TPM} + 1)$ values; the dashed line indicates $\tau = 0.8$, the threshold commonly used to define tissue-specific expression [24]. The blue bar highlights the gene with the highest τ value; grey bars indicate the remaining genes. **(C)** Relationship between the expression profiles of the duplicated pair *Ca2CPrxA*/*Ca2CPrxB* across the 32 samples; each dot represents one tissue/developmental stage, the line is a least-squares fit, and ρ is Spearman's correlation coefficient. DAP, days after pollination; SAM, shoot apical meristem.

2.6. Expression of *CaPrx* Genes in Roots Under Drought Stress

In roots of the drought-sensitive ICC1882 and drought-tolerant ICC4958 genotypes, *CaPrxIIA* was the most abundant family member (514–953 TPM), followed by *CaPrxIIC*, *CaPrxIIB*, and *Ca2CPrxB* (61–155 TPM) and *Ca2CPrxA* (16–21 TPM), whereas *CaPrxQ* (1.9–2.9 TPM) and *Ca1CPrxA* (≤ 0.9 TPM) were expressed at low levels, and no reads were assigned to *Ca1CPrxB* (Figure 6A), most likely because its CDS is 99.5% identical to that of *Ca1CPrxA*, so reads cannot be assigned uniquely to either copy. Drought caused only modest changes in transcript abundance (Figure 6B). No gene exceeded a two-fold change ($|\log_2\text{FC}| \geq 1$) in either genotype: $\log_2\text{FC}$ ranged from -0.40 to $+0.10$ in ICC1882 and from -0.30 (*CaPrxQ*) to $+0.73$ (*Ca1CPrxA*, from 0.14 to 0.89 TPM) in ICC4958.

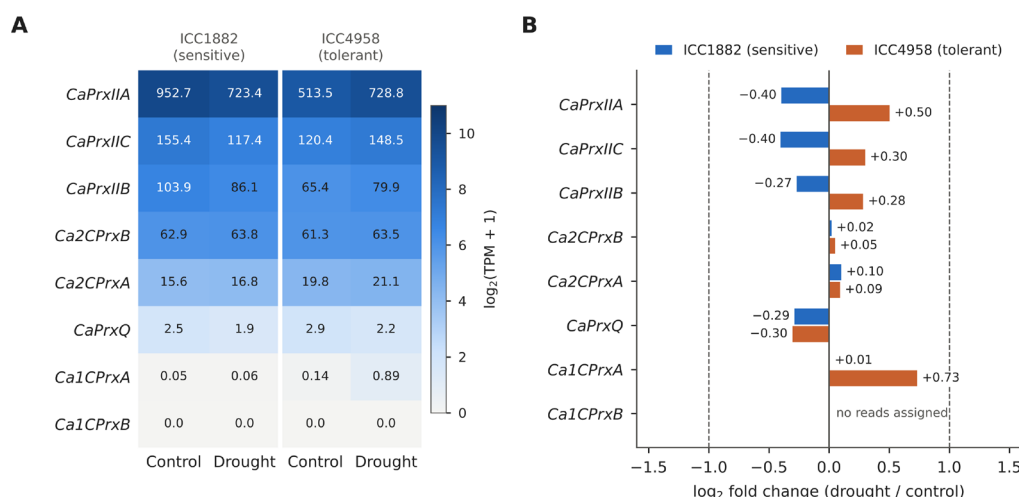


Figure 6. Expression of *CaPrx* genes in roots of the drought-sensitive ICC1882 and drought-tolerant ICC4958 genotypes under control and drought conditions (GSE193077). **(A)** Heatmap of $\log_2(\text{TPM} + 1)$ values; numbers in cells are TPM. **(B)** $\log_2$ fold change (drought/control) for each gene and genotype; dashed lines indicate a two-fold change ($|\log_2\text{FC}| = 1$). *Ca1CPrxB* had no assigned reads in any condition.

3. Discussion

3.1. Structural Conservation and Evolutionary History of the *CaPrx* Gene Family

The identification of eight *CaPrx* genes indicates that the peroxiredoxin family is relatively compact in chickpea. A similar number of *Prx* genes has been reported in other legumes, including *Phaseolus vulgaris* L., suggesting limited expansion of this antioxidant gene family during legume evolution [12]. Despite variation in protein size, physicochemical properties, and predicted subcellular localization, the overall characteristics of *CaPrxs* are consistent with the structural diversity described for plant peroxiredoxins [3,8,25].

The phylogenetic analysis placed *CaPrxs* within the four plant *Prx* subfamilies and showed representatives of chickpea, *Arabidopsis*, and rice in each major group. This pattern is consistent with the main *Prx* lineages having been established before the divergence of monocots and dicots, as indicated by broader phylogenetic surveys [5], although sampling three species does not by itself resolve deeper evolutionary relationships. The close correspondence between phylogenetic relationships, exon–intron organization, and conserved motif composition further supports the evolutionary conservation of this family. Similar patterns have been reported for *Prx* families in common bean, cotton, and *Brachypodium distachyon* [12,25,26].

The two paralogous pairs reveal contrasting patterns of sequence divergence within the *CaPrx* family. *Ca2CPrxA/Ca2CPrxB* diverged approximately 37 MYA, and its low Ka/Ks ratio supports long-term conservation under strong purifying selection, consistent with the evolutionary maintenance of proteins involved in conserved cellular functions [15,27]. In contrast, *Ca1CPrxA* and *Ca1CPrxB* display remarkable sequence conservation, differing at only three nucleotide positions clustered within an 18 bp region. Their close similarity is further reflected in the full-length transcriptome of genotype ICC 4958, in which a single assembled transcript matches both annotated copies. Whereas *Arabidopsis* carries a single 1-Cys *Prx* gene [28], the chickpea reference contains two near-identical *Ca1CPrx* loci, suggesting a recent duplication. Together, these findings distinguish the long-term retention of conserved 2-Cys *Prx* paralogues from the minimal divergence of

the annotated 1-Cys Prx pair, revealing distinct evolutionary patterns within the chickpea Prx repertoire.

3.2. Subfamily-Specific Expression Partitioning of *CaPrx* Genes

A clear partitioning of *CaPrx* expression that largely followed subfamily boundaries. The 2-Cys Prx genes and *CaPrxQ* were most abundant in photosynthetic tissues. This is consistent with the role of these subfamilies in the chloroplast, where 2-Cys Prx and PrxQ reduce peroxides generated by photosynthetic electron transport and participate in redox regulation of plastid metabolism [3,8,9].

The 1-Cys Prx transcript showed the most restricted pattern, with strong accumulation during late seed maturation. This closely mirrors the seed-specific, dormancy-related expression of *AtPER1* in *Arabidopsis* [11] and the seed-associated expression of *Prx* genes in rice [29]. These observations suggest that the seed-associated function of 1-Cys Prx is conserved in chickpea, where it may protect embryonic tissues against oxidative damage during desiccation. In contrast, *CaPrxIIA* was highly expressed in all tissues and represented nearly half of the *CaPrx* transcript pool, suggesting a housekeeping role in cytosolic peroxide homeostasis, while *CaPrxIIC*, predicted to be mitochondrial (Table 1), showed intermediate and uniform expression, consistent with the general antioxidant function of mitochondrial type II Prxs [10].

The duplicated pair *Ca2CPrxA/Ca2CPrxB* showed nearly identical expression profiles across tissues despite approximately 37 MYA of divergence. Thus, in contrast to what is often reported for duplicated genes [15,16,18,30,31], we found no evidence of expression partitioning between the two 2-Cys Prx copies. Their retention may instead reflect dosage requirements for one of the most abundant chloroplast peroxidases. Differences in promoter *cis*-element composition between the two genes (Figure 4) did not translate into divergent tissue expression in this dataset, although they may affect responses to conditions not represented in the atlas. Because *cis*-element predictions are based on sequence motifs alone, they indicate potential regulatory inputs [32].

3.3. Expression of *CaPrx* Genes in Roots Under Drought

In the drought transcriptome, *CaPrx* expression in roots was largely stable, with no gene changing by more than two-fold in either genotype. The expression hierarchy (*CaPrxIIA* » other type II and 2-Cys genes » *CaPrxQ* and *Ca1CPrxA*) was consistent with the root samples of the developmental atlas, which supports the reliability of both datasets. The small increase in *Ca1CPrxA* in ICC4958 (+0.73 log₂FC) occurred at very low absolute abundance (0.14 to 0.89 TPM) and should not be interpreted as drought induction. Stable transcript levels do not exclude a role for *Prxs* in drought responses, because Prx activity is strongly regulated post-translationally, through cysteine hyperoxidation, oligomerization, and interaction with redox partners [33]. However, this analysis is limited to root tissue, and a single time point. Drought responses of *CaPrx* genes in leaves, where the photosynthetic 2-Cys Prx and PrxQ are most abundant, remain to be determined.

3.4. An Integrated View of the *CaPrx* Family

Taken together, the chickpea *Prx* family combines a compact gene set and conserved protein architecture with clear expression partitioning among subfamilies: plastid-associated 2-Cys Prx and PrxQ in photosynthetic tissues, a seed-maturation-associated 1-Cys Prx, and broadly expressed type II Prxs, with *CaPrxIIA* as the dominant transcript. Among duplicated genes, the older pair has been retained under purifying selection with conserved expression, whereas the near-identical *Ca1CPrx* copies require confirmation in an improved genome assembly. The strong and specific accumulation of

1-Cys Prx transcripts during seed maturation makes this gene a priority candidate for functional studies of seed redox protection and longevity in chickpea.

4. Materials and Methods

4.1. Identification of Prx Genes in the Chickpea Genome

The protein sequences of *Arabidopsis thaliana* Prx genes [28] were used as queries in BLASTp v2.12.0+ searches, with default parameters, against the predicted proteome of the *C. arietinum* L. genome (version 1.0) using the BLAST tool of Phytozome v13 (<https://phytozome-next.jgi.doe.gov/>). Predicted amino acid (aa), genomic DNA, and coding sequence (CDS) were retrieved for all candidate genes. Candidate proteins were retained only when a peroxiredoxin domain (AhpC-TSA, PF00578, or Redoxin, PF08534) was confirmed in the Pfam database (<http://pfam.xfam.org>). Redundant entries, alternative splice variants, incomplete protein sequences, and sequences lacking the characteristic Prx domain were excluded. *CaPrxQ* was retained despite an internal stretch of undetermined nucleotides in the v1.0 assembly because its gene model has complete start and stop codons and conserves the catalytic peroxidatic cysteine motif. Gene nomenclature was assigned using the prefix Ca, followed by Prx and an identifier according to chromosomal position.

4.2. Physicochemical Characterization and Subcellular Localization

The physicochemical properties of the identified CaPrx proteins, including protein length (aa), molecular weight (kDa), theoretical isoelectric point (pI), and grand average of hydropathy (GRAVY), were determined using the ProtParam tool available in the Expert Protein Analysis System (ExPASy). The predicted subcellular localization of each CaPrx protein was determined using the Plant-mPLoc server [34].

4.3. Phylogenetic Analysis

Multiple sequence alignment of Prx proteins was performed using T-Coffee [35] with default parameters. Protein sequences from *A. thaliana* and *Oryza sativa* L. were included for comparative analysis. Phylogenetic relationships were inferred using MEGA12 (Molecular Evolutionary Genetics Analysis) [36]. The best-fitting aa substitution model was selected with the Find Best Model (ML) tool of MEGA12 based on the lowest Bayesian Information Criterion (BIC) score, which identified the Whelan and Goldman model with gamma-distributed rate variation among sites (WAG + G; Γ shape parameter = 2.15) [37]. The ML tree was then inferred under the WAG + G model with 1000 bootstrap replicates.

4.4. Gene Structure, Conserved Motifs and Chromosomal Organization

Gene structure, including exon/intron organization, was analyzed using the Gene Structure Display Server (GSDS) [38] based on genomic and CDS. Conserved protein motifs were identified using the Multiple Em for Motif Elicitation (MEME) Suite [39], with zero or one occurrence of each motif per sequence, a motif width of 6–50 residues, and a maximum of 10 motifs, keeping all other parameters at their default values. Chromosomal positions of *CaPrx* genes were obtained from the *C. arietinum* L. genome database, and chromosome maps were generated using the MG2C web server [40].

4.5. Gene Duplication and Evolutionary Analyses

Reciprocal BLASTn searches were performed to identify putative paralogous gene pairs using an E-value threshold $<1 \times 10^{-50}$, alignment score >200 , minimum sequence coverage of 80%, and sequence identity $\geq 70\%$ [41]. The CDS of each pair was aligned co-

don by codon using the protein alignment as a guide, and codons with gaps were removed. Numbers of synonymous and non-synonymous sites and differences were estimated using the Nei–Gojobori method [42], and Ka and Ks were obtained with the Jukes–Cantor correction. Departure of Ka/Ks from neutrality was tested with a one-sided Fisher’s exact test comparing the proportions of non-synonymous and synonymous differences per site, and 95% confidence intervals for Ka/Ks were obtained from 5000 bootstrap resamples over codons. Values of Ka/Ks <1 indicate purifying selection, ≈ 1 neutral evolution, and >1 positive selection. The approximate duplication time was estimated as $T = Ks/2\lambda$, where $\lambda = 6.1 \times 10^{-9}$ synonymous substitutions site⁻¹ year⁻¹ is the rate estimated for legumes [23]. Analyses were performed in Python (v3.11) with Biopython and SciPy.

4.6. Cis-Regulatory Element Analysis

Putative *cis*-regulatory elements were identified within the 1500 base pairs (bp) upstream promoter regions of each *CaPrx* gene using the PlantCARE database with default settings [43]. The identified *cis*-acting regulatory elements were subsequently classified according to their predicted biological functions and graphically visualized using TBtools-II [44].

4.7. Tissue- and Developmental-Stage Expression Profiling

Expression of *CaPrx* genes across tissues and developmental stages was examined using the chickpea expression atlas deposited in the curated by the National Center for Biotechnology Information (NCBI Gene Expression Omnibus (GEO)) under accession number GSE147831 [45]. This dataset comprises 94 RNA-seq libraries from 32 tissues/developmental stages of genotype ICC 4958: germinating seedling, shoot, root, root tip, root hair, nodule, shoot apical meristem (SAM), young and mature leaves, bracteole, pedicel, pod shell, sepal, petal, androecium, gynoecium, flower buds (stages 1–4), flowers (stages 1–5), seed coat, embryo, endosperm, and seeds at 5, 10, 20, and 30 days after pollination (DAP). Each tissue was represented by three biological replicates, except seeds at 20 and 30 DAP (two replicates), and the deposited files provide replicate-averaged FPKM (fragments per kilobase of transcript per million mapped reads) values for transcripts of a full-length transcriptome assembly [45]. To place all samples on a common scale, FPKM values were converted to transcripts per million (TPM) by dividing each value by the sum of FPKM values of the sample and multiplying by 10^6 .

For the heatmap, TPM values were $\log_2(\text{TPM} + 1)$ -transformed, and genes were clustered hierarchically using Pearson correlation distance and average linkage; tissues were ordered by organ and developmental stage. Tissue specificity was quantified with the index τ [24], calculated for each gene as $\tau = \sum_{i=1}^N (1 - \hat{x}_i)/(N - 1)$, where $\hat{x}_i = x_i/\max(x)$, x_i is the $\log_2(\text{TPM} + 1)$ value in tissue i , and $N = 32$. τ ranges from 0 (uniform expression) to 1 (expression restricted to a single tissue), and $\tau \geq 0.8$ was taken to indicate tissue-specific expression [46]. Expression similarity between duplicated genes was assessed by Spearman’s rank correlation (ρ) across the 32 tissues. Analyses were performed in Python (v3.11) with pandas, SciPy, and matplotlib.

4.8. Expression Under Drought Stress

Drought-responsive expression was examined using the processed RNA-seq data deposited in GEO under accession number GSE193077 [47]. In that experiment, the drought-sensitive genotype ICC1882 and the drought-tolerant genotype ICC4958 were grown in a growth chamber, and drought was imposed on 11-day-old plants by withholding water for 15 days, while control plants were irrigated regularly; roots were then harvested for RNA-seq [47]. Reads (49 nucleotides (nt, single-end)) were mapped to the

kabuli chickpea genome (v1.0) with TopHat v2.1.1 and quantified with Cufflinks v2.1.1, and the deposited file provides one FPKM value per gene for each genotype × treatment combination (two biological replicates per condition are listed in GEO). Because the total FPKM differed among the four samples (1.20–1.66 million), values were converted to TPM as described in Section 4.7. For each genotype, the drought response was expressed as $\log_2FC = \log_2[(TPM_{\text{drought}} + 1)/(TPM_{\text{control}} + 1)]$, where the pseudocount of 1 avoids undefined values when a gene has zero abundance in one condition and dampens spurious large fold changes for genes expressed at very low levels. All eight *CaPrx* genes are reported; *Ca1CPrxB* had no assigned reads in any sample. $\log_2FC$ values are presented descriptively, and $|\log_2FC| \geq 1$ (two-fold change) was used only as a reference threshold.

5. Conclusions

This study provides a curated genome-wide characterization of the *CaPrx* gene family in chickpea, linking evolutionary conservation to distinct expression patterns. The eight *CaPrx* genes encode structurally conserved proteins belonging to the four canonical Prx subfamilies. The older duplicate pair, *Ca2CPrxA/Ca2CPrxB*, has been retained under purifying selection and exhibits highly correlated expression, whereas the near-identical *Ca1CPrxA/Ca1CPrxB* copies suggest a more recent duplication. Expression profiles across 32 tissues revealed clear subfamily-associated patterns: 2-Cys Prx and PrxQ predominated in photosynthetic tissues, 1-Cys Prx transcripts accumulated during seed maturation, and *CaPrxIIA* showed consistently high expression. In roots, transcript levels remained largely stable under drought. Together, these findings establish a foundation for investigating the functional diversification of chickpea peroxiredoxins and highlight 1-Cys Prx as a promising target for elucidating redox protection during seed development.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/stresses6040082/s1>, Table S1. (a) Assignment of *CaPrx* genes to transcripts of the chickpea expression atlas (GSE147831) by alignment of coding sequences (minimap2). When two transcripts matched one gene, their values were summed. (b) Expression (TPM) of *CaPrx* genes across 32 chickpea tissues/developmental stages (GSE147831; genotype ICC 4958). FPKM values were converted to TPM within each sample. *Ca1CPrxA* and *Ca1CPrxB* match the same atlas transcript and are reported together (*Ca1CPrxA/B*). Table S2. Tissue-specificity index (τ) of *CaPrx* genes, calculated from $\log_2(TPM + 1)$ values across 32 tissues/developmental stages. Table S3. Spearman correlation between the expression profiles of the duplicated *CaPrx* gene pairs across 32 tissues/developmental stages.

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Abbreviations

The following abbreviations are used in this manuscript:

ROS	Reactive oxygen species
pI	Isoelectric point
kDa	molecular weight
GRAVY	Grand Average Hydropathicity
ABA	Absciscic Acid
CDS	coding sequences
NCBI	National Center for Biotechnology Information
MEME	Multiple Em for Motif Elicitation
RNA-seq	RNA sequencing
FPKM	Fragments Per Kilobase of Transcript Per Million Mapped Reads
TPM	Transcripts Per Million
DAP	Days after pollination
Ka	Non-synonymous substitutions per non-synonymous site
Ks	Synonymous substitutions per synonymous site
MYA	Million years ago
SAM	Shoot apical meristem
GEO	Gene Expression Omnibus

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