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Structural and Functional Characterization of the Peroxidase Gene in Chickpea under Aluminum Stress

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Abstract

Aluminum (Al^{3+}) toxicity is a significant limitation to chickpea (*Cicer arietinum* L.) productivity in acidic soils by impairing root growth and increasing reactive oxygen species (ROS). Class III peroxidases are major antioxidant enzymes important for ROS scavenging; however, the structural and functional role of peroxidases in chickpea under Al^{3+} stress remains unclear. This study conducted comprehensive bioinformatics and molecular docking of the chickpea peroxidase 43 isoform X2 gene. BLASTn and BLASTx analyses confirmed that the gene encodes a peroxidase-like protein. Further comparative analysis of peroxidase 43 from nine legumes revealed a high degree of sequence conservation, especially with *Medicago truncatula*, *Pisum sativum*, and *Vigna radiata*. Multiple sequence alignment with phylogenetic analysis highlighted conserved motifs for the catalytic mechanism and evolutionary divergence, with *Cajanus cajan* having the lowest conservation. The analysis of conserved domains confirmed that peroxidases have specific motifs, while 3D structure prediction confirmed the integrity of the protein fold. Protein-protein interaction analysis revealed a strong connection to enzymes involved in oxidative stress. In addition, molecular docking results revealed that the Al^{3+} ion has a strong binding affinity to the active site, which could destabilize enzymatic activity and function. In contrast, Fe^{2+} supports activity and function, while Cu^{2+} strongly competitively binds to peroxidase. Overall, these results give insight into peroxidase-mediated antioxidant defense mechanisms as well as their potential use in breeding strategies for Al^{3+}-tolerant chickpea varieties

Keywords: Chickpea; Aluminum stress; Antioxidant defense; Molecular docking; Reactive oxygen species (ROS); Phylogenetic analysis

Introduction

Class III plant peroxidases (PRXs) are heme-containing glycoproteins that are critical for plant defense and plant developmental processes. PRXs scavenge reactive oxygen species (ROS), contribute to modifications of the cell wall, and play a role in lignin polymerization, suberization, auxin catabolism, and pathogen defense (Cosio and Dunand, 2009; Pandey et al., 2017; Yadav and Chattopadhyay, 2023). PRXs are typically localized in the apoplast of plants, and they reduce H_2O_2 while oxidizing phenolic substrates, playing a role in maintaining redox balance during periods of environmental stress (Kidwai et al., 2020; Li et al., 2024).

Al^{3+}stress is a major area of concern to crop productivity on acidic soils, constituting approximately 40% of the world's agricultural land. Chickpea (*Cicer arietinum* L.) exposed to Al^{3+} toxicity responds with enhanced accumulation of ROS, lipid peroxidation, although this response occurs at the cost of inhibited root growth and disrupted metabolism, (Singh et al., 2012; Choudhury and Sharma, 2014; Hossain et al., 2023). Evidence of enhanced activity of antioxidants has been provided, including peroxidases, as adaptive mechanisms to Al^{3+} stress (Samad et al., 2020). Furthermore, transcriptome studies conducted with legumes have shown that peroxidase genes are upregulated

to abiotic stresses like heat, salinity, drought, and metal toxicity (Singh et al., 2019; Sharma et al., 2021; Matamoros and Becana, 2021). Nevertheless, there is still a limited understanding of the structural and functional properties of individual peroxidase isoforms within chickpea under Al^{3+}stress conditions. Other plant studies have shown that Al^{3+} binding to antioxidant enzymes may create steric hindrance at the catalytic sites needed for the detoxification of ROS (Jouili et al., 2011). Computational docking can be a useful method for predicting binding affinities and developing a mechanistic understanding of the inhibition of enzymes due to stress conditions (Liu et al., 2023). Therefore, this study utilized an integrated structural and functional characterization of chickpea peroxidase 43 isoform X2 to identify its role in Al^{3+} stress tolerance. Specifically, this study sought to identify conserved domains and heme-binding residues, predict and assess their three-dimensional structure, assess the interaction network, and binding affinities with Al^{3+}, Fe^{2+}, Cu^{2+}, and Zn^{2+}. Overall, this study leads to new avenues of research focused on peroxidase-mediated antioxidant defense that includes the potential in the development of Al^{3+}-tolerant chickpea cultivars.

Materials and Methods

Sequence confirmation and retrieval

To validate the identity of the aluminium (Al^{3+}) responsive peroxidase gene in chickpeas (*Cicer arietinum*), nucleotide and corresponding protein sequences were analyzed using the NCBI BLASTn and BLASTx tools (https://blast.ncbi.nlm.nih.gov/Blast.cgi). BLASTn was used to assess nucleotide-level homology, whereas BLASTx was used to confirm the translated protein identity by comparison with annotated peroxidase proteins. Following sequence confirmation, full-length peroxidase protein sequences were retrieved from the NCBI Protein Database for chickpeas and eight other legumes: *Glycine max*, *Medicago truncatula, Phaseolus vulgaris, Vigna radiata, Vigna unguiculata, Cajanus cajan, Lens culinaris, and Pisum sativum*. These were used for comparative and evolutionary analysis.

Multiple sequence alignment and phylogenetic analysis

The protein sequences of peroxidase from selected legumes were aligned using the COBALT (Constraint-Based Multiple Alignment Tool) from NCBI (https://www.ncbi.nlm.nih.gov/tools/cobalt/). Conserved and variable regions were determined across all species. A phylogenetic tree was constructed using elements from the COBALT interface, Phylogenetic Tree, to investigate evolutionary relationships and divergence of peroxidase proteins across legumes.

Global protein alignments and similarity analysis

Pairwise global alignments of chickpea peroxidase and legume orthologs were done using the Needleman–Wunsch algorithm through the EMBOSS Needle tool (https://www.ebi.ac.uk/Tools/psa/emboss_needle/). From the alignments; sequence identity, similarity scores, and gap percentages were obtained. Dot plots were constructed to visually examine the structural alignment and conserved blocks.

Conserved domain identification

The sequences were analysed for annotation of conserved and functionally-significant motifs for Peroxidase proteins through the Conserved Domain Database (CDD) using the NCBI CD-Search tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Functional domains were identified according to similarities to well-characterized members of the peroxidase family, focusing on residues involved in catalysis and the heme-binding motifs.

3D *structure prediction*

The three-dimensional shape of peroxidase was modelled using the SWISS-MODEL server (https://swissmodel.expasy.org/) based on other plant peroxidases as homologous templates. The predicted models were validated using QMEAN scores and Ramachandran plots. When possible, where available, AlphaFold Predicted Structure were consulted for refinement.

Protein-protein interaction analysis

Protein–protein interaction (PPI) of chickpea Peroxidase was analysed using the STRING v11.5 database (https://string-db.org/) with a minimum confidence score of 0.7 (high confidence). Interactions were derived from co-expression, experimental evidence, and computational predictions. Focusing on partners involved in oxidative stress and reactive oxygen species (ROS) detoxification.

Molecular docking studies

For investigating the interactions with metal ions under aluminum stress, molecular docking studies were performed with Peroxidase from *Cicer arietinum* with Al^{3+} and Fe^{2+}, Cu^{2+}, and Zn^{2+} ions using PyRx v0.8 as the backend (AutoDock Vina). The 3D structure of peroxidase was modeled using SWISS-MODEL, while the ligand structures were obtained from PubChem. Ligands were retrieved in .sdf format and converted to. pdbqt after energy minimization. The proteins were also energy minimized and prepared for docking. The binding affinity scores (kcal/mol^{-1}) and interaction residues were evaluated to assess how metal ions can affect the peroxidase function. The results were visualized using BIOVIA Discovery Studio Visualizer for determining the types of interactions, the interaction sites of the binding molecules, and potential interference with the catalytic domains.

Results

Validation of sequence using BLASTn and BLASTx

To verify the identity of the Al^{3+} responsive gene suspected to encode a peroxidase, both nucleotide and protein sequence homology analyses were performed using NCBI's BLASTn and BLASTx tools. The BLASTn results illustrated nucleotide-level similarities between the cloned peroxidase genes of several leguminous species. The best hits included sequences of several leguminous species, such as *Cicer arietinum, Glycine max,* and *Medicago truncatula*, with percent identity scores over 90% and E-values close to zero. These results demonstrate that this gene is evolutionarily conserved at the nucleotide level in legumes.

BLASTx analysis of the translated protein sequence confirmed the identity of the cloned gene as a peroxidase (**Fig. 1**). The predicted amino acid sequence showed significant similarity to known plant peroxidase proteins, especially class III peroxidases. Conserved domains, including the heme-binding region and catalytic residues such as His and Arg, which are essential for hydrogen peroxide detoxification, were located and aligned in different species. This demonstrates that the gene is functional in reactive oxygen species (ROS) scavenging. In conclusion, the cloned peroxidase gene is likely involved in mitigating oxidative stress in chickpeas under aluminum toxicity.

Sequence of Clone 3 (*Peroxidase*)

>ACGTGGTTGTAGACACAACCACTGCTCAACTCCAGAAAAAATCAAAGACTTCGTCACCAACTGTCCCACTCTCCGCGCTCCTTACT
ACGATTGTTCGAATGTATGGTGCTGTCGTCGTTCATCAACGCCTTGCAACAAAGCCGTACGGACTCGCCCAGTTGCAAAAACAAACT
CTTGTGCTGCGATATTCTTGTTTTCGCCACCCGTGGCTGTGTCGTATTGTTGTTGTCGCCGACTACGGTCCGACCATGCGTGGGTGCT
CAACAAAGTTACCATACAAAAACAAACTTACCGACATGCGCTGCCCGCTGTTCTACAACTTCAACCTCACACACGCCGTCCCAACTCT
ACACAAGCCTACGCTACAGAACTTCAGTTGGCTTGTCCCAAGACAGTTGACCCAAGAACGCCATCACATGGACCCAACCACTCCAA
GACAATTCGACAACATTACGGCAAAGGACTCTTCACTTCCACTITCACGATGGTCGCTCAAACCCACCGTTACOGATTGGGCCAAGA
ATTCGTTGCTTTCAACAAGGCTTTCGTACCCGCTATGCCCAAACTCGGCCGCGTTGGGATGTTGTCATGCGTGTCTGTGGTGCCTTTT
ACTGICAICTTGTTTTCGAGATTIGTTCTTTGTCATGITATGGGTTTTTTGCTICIGITTGTTTCTGCTTCTTGTATTTCTATIACACTTGTTT
ACTCAATCC

Fig.1. BLASTn alignment showing high nucleotide similarity of the cloned chickpea peroxidase gene with legume peroxidase genes.

Retrieval of peroxidase protein sequences from legumes

For structural, evolutionary, and functional characterization, peroxidase homolog protein sequences were retrieved from the NCBI protein database. The sequences were obtained from nine legume species: *Cicer arietinum, Medicago truncatula*, *Glycine max, Pisum sativum, Arachis hypogaea, Vigna unguiculata, Vigna radiata, Phaseolus vulgaris*, and *Cajanus cajan* **(Table 1)**. The sequences provided the basis for further comparative work, including multiple sequence alignment, conserved domain analysis, phylogenetic tree construction and docking studies. The emphasis was primarily on peroxidase 43 isoforms, which are known to be involved in the scavenging of reactive oxygen species (ROS) during abiotic stress (e.g., aluminum). The pertinent information for the selected protein sequences, including accession numbers, is provided below.

Table 1. List of peroxidase protein sequences retrieved from legumes for comparative analysis.

S. No.	Species	Protein Description	Accession No.
1.	*Cicer arietinum*	Peroxidase 43 isoform X2	XP_027193363.1
2.	*Medicago truncatula*	Peroxidase 43 isoform X1	XP_003593134.1
3.	*Glycine max*	Peroxidase 43 isoform X1	XP_040874190.1
4.	*Pisum sativum*	Peroxidase 43	XP_050881506.1
5.	*Arachis hypogaea*	Peroxidase 43	XP_072087021.1
6.	*Vigna unguiculata*	Peroxidase 43	XP_027930397.1
7.	*Phaseolus vulgaris*	Peroxidase 43 isoform X1	XP_068497273.1
8.	*Vigna radiata* var. *radiata*	Peroxidase 28-like	XP_014519734.2
9.	*Cajanus cajan*	Peroxidase 43	XP_020221464.1

Multiple sequence alignment (MSA)

We examined the conservation of peroxidase protein sequences across legumes by conducting a multiple sequence alignment (MSA) using the NCBI COBALT tool. We aligned the peroxidase protein sequences from *Cicer arietinum* and eight other leguminous species, including *Pisum sativum, Vigna unguiculata, Phaseolus vulgaris, Arachis hypogaea, Medicago truncatula, Glycine max, Vigna radiata, and Cajanus cajan*. The MSAs revealed a highly conserved domain spanning amino acids 250-630 (**Fig. 2**). The areas of conservation (marked in red blocks in the MSA) suggest potentially important functional motifs, such as heme-binding sites, catalytic histidine, and reactive tyrosine in Class III peroxidases. We observed less variability in the areas known to be functional than in the areas outside the conserved domain of the protein, likely representing phylogenetically distinct isoforms or species with functionally divergent isoforms that possess species-specific localization signals.

Fig.2. Multiple sequence alignment of peroxidase proteins from *Cicer arietinum* and related legumes. Red blocks denote highly conserved amino acid regions, and grey gaps represent insertions or deletions indicative of evolutionary divergence.

The percent identity values between *Cicer arietinum* and the other legume peroxidases were all ~81–89%, with the highest percent identity held by *Medicago truncatula* (89.02%) and *Cajanus cajan* (85.71%), indicative of close evolutionary relationships **(Table 2**). Our discovery of high levels of protein identity among legume peroxidase proteins supports the premise that peroxidase genes have maintained an evolutionary conservation pattern across legumes with a conserved role in ROS scavenging and response to aluminum stress.

Table 2. Details of Protein Sequences Used for Multiple Sequence Alignment

Accession	Species Name	% identity
XP_027193363.1	*Cicer arietinum*	100.00%
XP_050881506.1	*Pisum sativum*	82.96%
XP_027930397.1	*Vigna unguiculata*	81.51%
XP_068497273.1	*Phaseolus vulgaris*	85.15%
XP_072087021.1	*Arachis hypogaea*	83.22%
XP_003593134.1	*Medicago truncatula*	89.02%
XP_040874190.1	*Glycine max*	81.50%
XP_014519734.2	*Vigna radiata*	81.16%
XP_020221464.1	*Cajanus Cajan*	85.71%

Phylogenetic analysis

We constructed a phylogenetic tree to examine the evolutionary relationships of the peroxidase protein in *Cicer arietinum* with other legume species based on the aligned peroxidase sequences from NCBI. The analysis revealed distinct evolutionary groupings, where sequences reflected the conservation and divergence of peroxidase isoforms in legume species (**Fig.3**). *Vigna radiata* diverged from the other species on the tree and had the greatest evolutionary distance from the other species, suggesting that this species had unique evolutionary adaptations for peroxidase function. *Cicer arietinum* showed the greatest homology to *Cajanus cajan* in the last clade shown on the tree. Sequence homology suggests that *peroxidases in Cicer arietinum* and *Cajanus cajan* have similar functional roles in response to oxidative stress and aluminum toxicity.

The next clade, including *Glycine max*, *Vigna unguiculata*, and *Phaseolus vulgaris*, is indicated to have close evolutionary relationships, an ancestral lineage, and conserved functions within this group. The identified branches, showing some well-supported branches, emphasized that there is conservation of important catalytic motifs; however, there is diversification that introduces species-specific regulatory functions. The evolutionary information from this phylogenetic analysis, in conjunction with the high sequence identity observed in the MSA and conserved catalytic regions

(**Fig. 2**), supports the idea that peroxidase genes across legumes are under strong selective pressure for functional conservation. However, the observed phylogenetic divergence also suggests potential specialization in response to different environmental stressors or physiological contexts.

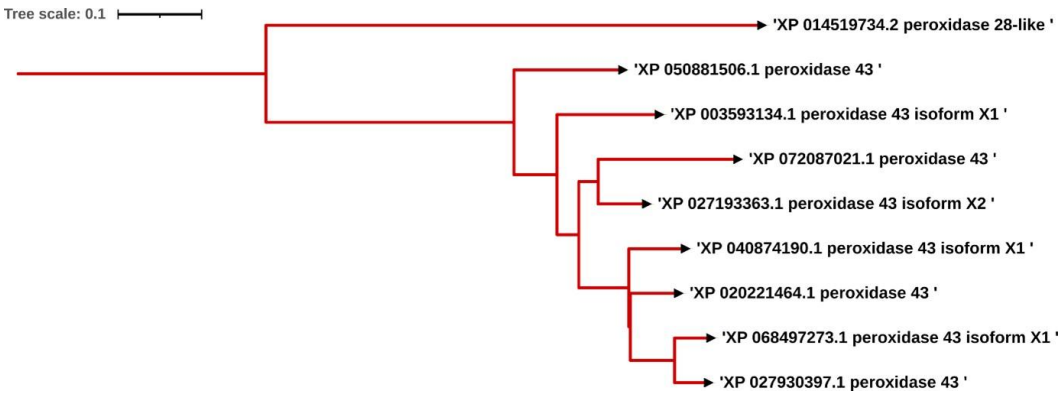

Fig. 3. Phylogenetic tree of peroxidase proteins from *Cicer arietinum* and eight other legume species, illustrating their evolutionary relationships and sequence divergence. Closely clustered branches represent conserved evolutionary paths, whereas distant nodes reflect divergent evolutionary adaptations.

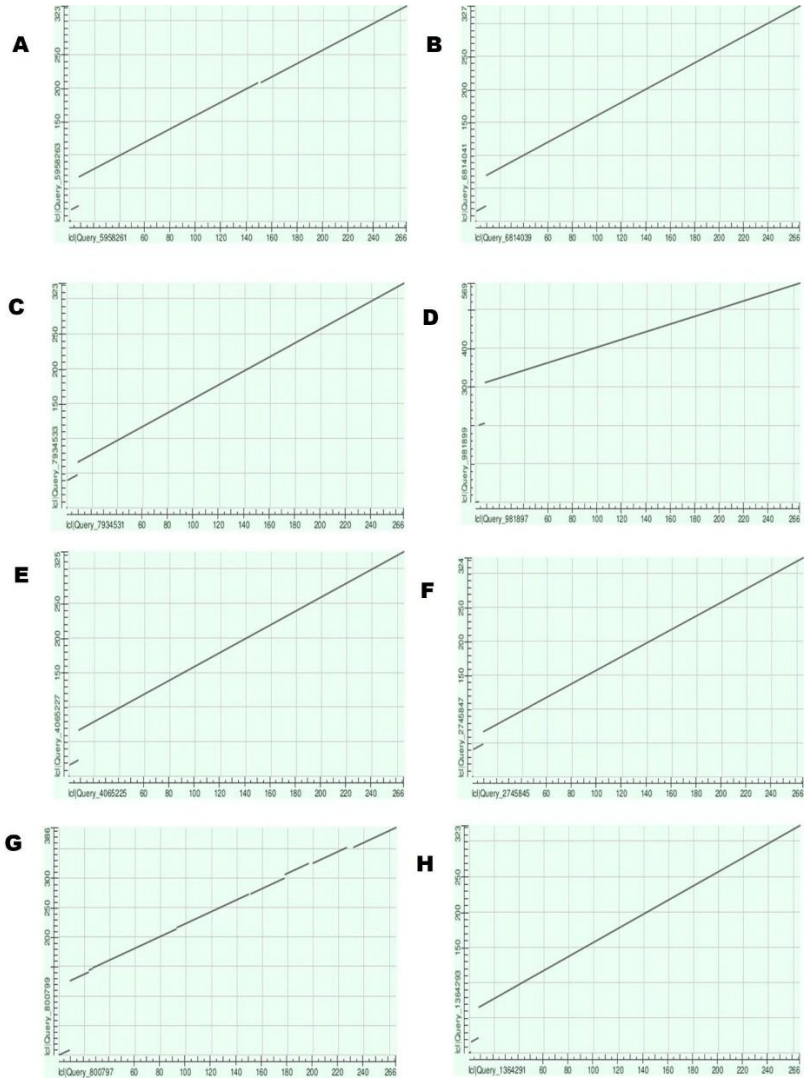

Fig. 4. Dot plot alignments of chickpea peroxidase 43 isoform X2 with orthologs from eight legume species.(A) *Medicago truncatula* (XP_003593134.1), (B) *Glycine max* (XP_040874190.1), (C) *Pisum sativum* (XP_050881506.1), (D) *Arachis hypogaea* (XP_072087021.1), (E) *Vigna unguiculata* (XP_027930397.1), (F) *Phaseolus vulgaris* (XP_068497273.1), (G) *Vigna radiata* var. *radiata* (XP_014519734.2), and (H) *Cajanus cajan* (XP_020221464.1). Continuous diagonal traces indicate strong sequence conservation across species, whereas minor gaps or discontinuities suggest regions of divergence.

Global protein alignment and similarity

Global protein alignment of *Cicer arietinum* peroxidase was performed using homologous sequences from eight legume species to assess evolutionary divergence and sequence conservation. Overall, the sequence alignment results exhibited considerable conservation across most of the legume species based on the dot plots, as uninterrupted diagonal traces were shown, and their similarity scores were indicated (**Table 3**). Dot plot analysis revealed uninterrupted alignment traces from some species, such as *Medicago truncatula* (**Fig. 4A**), *Glycine max* (**Fig. 4B**), *Pisum sativum* (**Fig. 4C**), *Vigna unguiculata* (**Fig. 4E**), *Phaseolus vulgaris* (**Fig. 4F**), *Vigna radiata var. radiata* (**Fig. 4G**), and *Cajanus cajan* (**Fig. 4H**). The uninterrupted traces indicated strong homology among the plant species, which may reflect the evolutionary conservation of functional regions within the peroxidase protein. The *Arachis hypogaea* plot showed a peroxidase protein sequence that was much longer (570 amino acids) than any of the legume species, which ranged from 323 to 386 amino acids. The dot plot (**Fig. 4D**) shows a conserved portion of the *Arachis hypogaea* sequence within its larger protein structure. The unique segments of peroxidases probably provide some kind of regulatory or accessory domain that contributes to species-specific functional adaptations. Species such as *Cajanus cajan* and *Pisum sativum* have only a shorter (~323 amino acids) peroxidase structure. Still, they had dot plots (**Fig. 4H** and **4C, respectively**) with dense continuous alignment traces, which suggested that they were the cornerstone peroxidase domains required to carry out enzymatic functions.

Overall, the high degree of sequence conservation present in the aligned leguminous peroxidase proteins strongly supports the idea that this gene family is functionally and evolutionarily important in plant defense and critical under oxidative and aluminum stress. Some changes in sequence length or domain structure may have evolved in lineage-specific diversifications of function.

Table 3. Comparative global alignment of Peroxidase proteins across leguminous species

Figure	Species	Accession No.	Protein Name	Query Length (aa)	No. of Matches	Alignment Quality
5.4A	*Medicago truncatula*	XP_003593134.1	Peroxidase 43 isoform X1	323	1	High (linear with minor gap)
5.4B	*Glycine max*	XP_040874190.1	Peroxidase 43 isoform X1	327	1	High (continuous diagonal)
5.4C	*Pisum sativum*	XP_050881506.1	Peroxidase 43	323	1	High (short but conserved)
5.4D	*Arachis hypogaea*	XP_072087021.1	Peroxidase 43	570	1	Moderate–High (conserved core region)
5.4E	*Vigna unguiculata*	XP_027930397.1	Peroxidase 43	325	1	High (strong diagonal)
5.4F	*Phaseolus vulgaris*	XP_068497273.1	Peroxidase 43 isoform X1	324	1	High (conserved over full length)
5.4G	*Vigna radiata var. radiata*	XP_014519734.2	Peroxidase 28-like	386	1	High (core conserved with extension)
5.4H	*Cajanus cajan*	XP_020221464.1	Peroxidase 43	323	1	High (short sequence, well-aligned)

BLAST alignment summary of peroxidase 43 isoform X2

To continue investigating the associated sequence homology and functional conservation, BLAST alignments were generated for *Cicer arietinum* peroxidase 43 Isoform X2 (XP_027193363.1) to available homologous sequences from eight legume species (**Table 4**). Many of the sequences displayed a high level of similarity, indicating that the peroxidase isoform in the eight leguminous species is conserved from an evolutionary perspective, in stress-related processes. *Medicago truncatula* yielded the best alignment (**Fig. 5A**), revealing more than one broadly distributed, conserved region throughout the protein sequence. This high level of sequence similarity suggests a close evolutionary relationship in the first and strongest facet, likely paralleling functional equivalence in the oxidative stress responses related to hypoxia or low oxygen. *Glycine max* and *Pisum sativum* also showed high sequence conservation (**Fig. 5B and 5C, respectively**). In particular, *Pisum sativum* demonstrated strongly conserved domains at both the N- and C-terminal ends,

whereas, in many cases, other terminal domains are often important for the catalytic activity and substrate specificity of peroxidases.

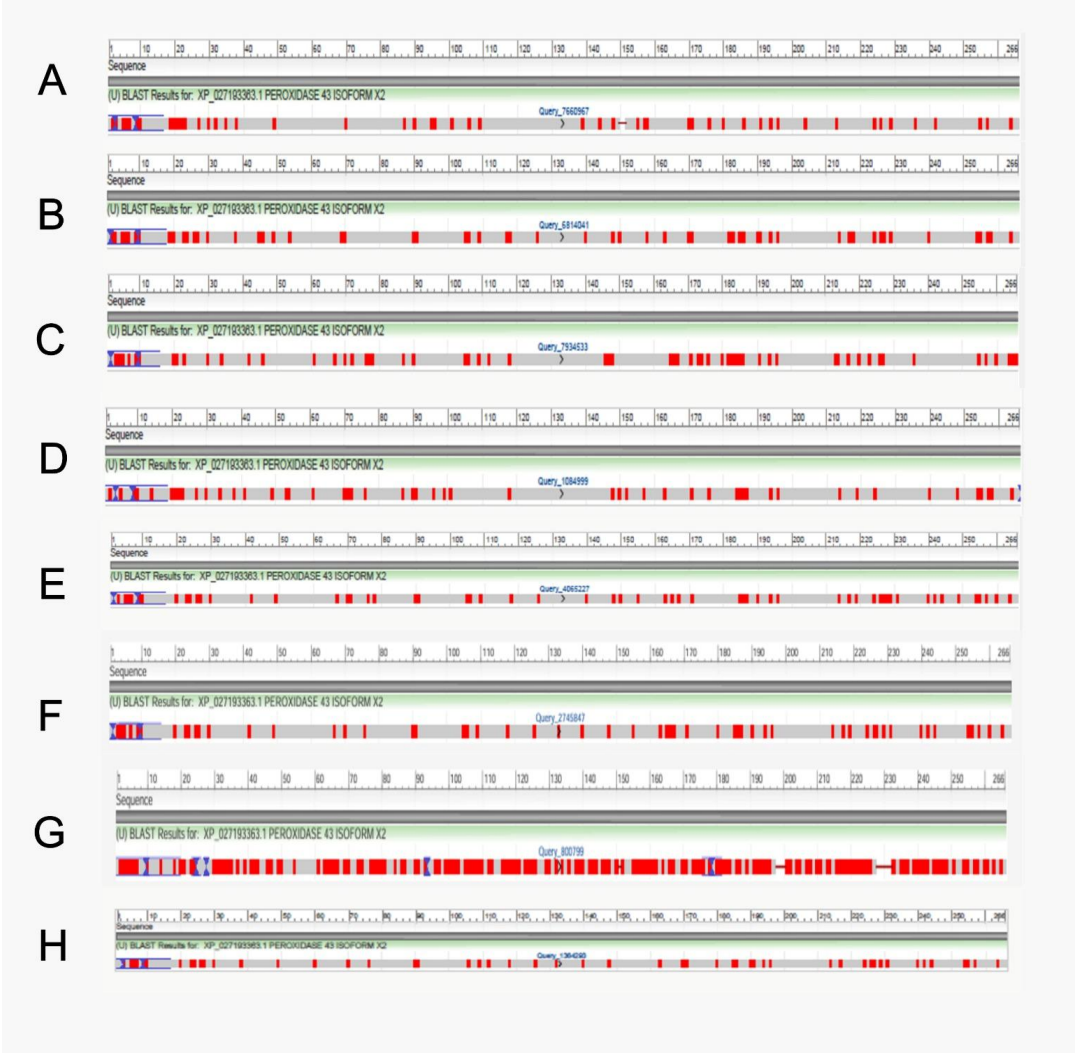

Fig. 5. BLAST alignments of chickpea peroxidase 43 isoform X2 (XP_027193363.1) with orthologs from eight legumes. (**A**) *Medicago truncatula*, (**B**) *Glycine max*, (**C**) *Pisum sativum*, (**D**) *Arachis hypogaea*, (**E**) *Vigna unguiculata*, (**F**) *Phaseolus vulgaris*, (**G**) *Vigna radiata var. radiata*, and (**H**) *Cajanus cajan*. Conserved regions (red boxes) are distributed across the protein length, with most species showing strong sequence similarity and functional conservation. *Cajanus cajan* (**H**) exhibited fewer conserved blocks and more gaps, suggesting possible functional divergence compared to other legumes.

In the alignment with *Arachis hypogaea*, (**Fig. 5.D)** identifies a continuous strong alignment match with the length of the protein, while the red boxes identify conserved regions that are uniformly dispersed along the sequence. Together, these BLAST results further validate the case for peroxidase gene functional conservation, supporting the hypothesis that there is functional conservation in stress-responsive pathways. *Vigna unguiculata* and *Phaseolus vulgaris* (**Fig. 5.E and 5.F**) were closely aligned. *V. unguiculata* resulted in aligned conserved segments, density variables, while *P. vulgaris* had comparatively less conservation overall, indicating some sequence divergence in non-essential regions. *Vigna radiata var. radiata* provided the most closely aligned conserved segments (**Fig. 5.G**) with no gaps and essentially unified homology throughout the length of the protein. This probably means that the ortholog would be extremely similar, sharing possibly identical biological functions related to peroxidase-mediated oxidative defenses or cell wall lignification. In comparison, *Cajanus cajan* resulted in the worst conservation alignment (**Fig. 5.H**), resulting in many gaps or dashed semi-conserved blocks. Clearly, evolutionary divergence into separate lineages appears likely, and perhaps some additional functional specialization or divergence of the peroxidase gene block in this species.

In conclusion, BLAST analysis strongly supports that *Cicer arietinum* peroxidase 43 Isoform X2 is a well-conserved protein across most legume species, especially those in the most common branch of environmental stress responses. Although *Cajanus cajan* and, to a lesser extent, other species

reflected some variability and sequence divergence, possibly implying functional adaptation of adaptation shifts at specific instants of evolutionary distances.

Table 4. Global BLAST alignment summary of Peroxidase 43 isoform x2 (XP_027193363.1) with leguminous species

Query Species	Figure	Alignment Strength	Homology Interpretation
Medicago truncatula	5.5A	Very High	Strong alignment with widespread conserved regions; indicates functional and evolutionary similarity.
Glycine max	5.5B	High	Continuous alignment and conserved domains suggest functional equivalence.
Pisum sativum	5.5C	Very High	Strong conservation at both terminal ends; implies retention of peroxidase function.
Arachis hypogaea	5.5D	High	Broadly conserved regions throughout the sequence suggest oxidative stress-related roles.
Vigna unguiculata	5.5E	High	Conserved regions with moderate variation; likely functional conservation.
Phaseolus vulgaris	5.5F	Moderate to High	Strong alignment overall; sparse regions may indicate minor divergence.
Vigna radiata var. radiata	5.5G	Very High	Densely aligned blocks with minimal gaps; suggests a very close ortholog.
Cajanus cajan	5.5H	Low to Moderate	Scattered conservation and frequent gaps indicate divergence and possible specialization.

Conserved domain identification in peroxidase 43 isoform X2

Domain analysis of *Cicer arietinum* Peroxidase 43 Isoform X2 (XP_027193363.1) resulted in a conserved secretory peroxidase domain as indicated in (**Fig. 6**). This domain spans the length of the protein and is associated with functional roles in a diverse range of biological processes, including the detoxification of hydrogen peroxide (H_2O_2), degradation and biosynthesis of lignin, modification of the cell wall, and response to environmental stressors, including pathogen attack, wounding, and oxidative stress. In addition to the primary domain hit, there were also non-specific hits to the PLN03030 domain family, which accounts for plant peroxidase-related proteins. Perhaps even more importantly, the protein was also assigned to two superfamilies: the plant_peroxidase_like superfamily, as well as the PLN03030 superfamily, which further supports the functional classification as a Class III peroxidase.

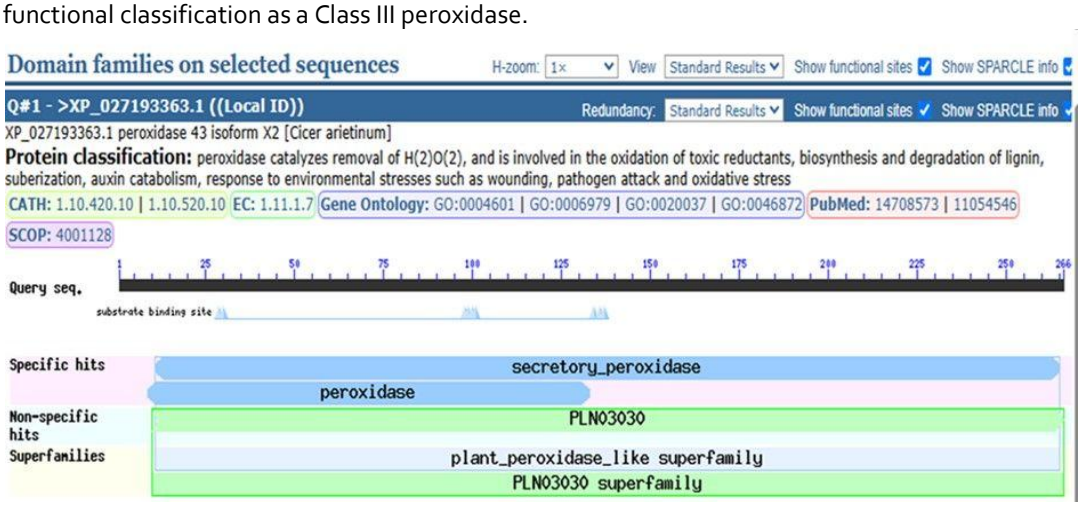

Fig. 6. Conserved domain analysis of *Cicer arietinum* Peroxidase 43 Isoform X2 (XP_027193363.1). Specific hits included a full-length secretory peroxidase domain and a peroxidase family domain (PLN03030). Non-specific matches aligned with the plant_peroxidase_like and PLN03030 superfamilies, confirming its structural and functional classification as a Class III plant peroxidase involved in oxidative stress defense and secondary metabolism.

These conserved structural features show that this peroxidase isoform is biophysically equipped for the catalytic degradation of oxidative compounds such as H_2O_2 and reactive oxygen species (ROS). This function is critical for establishing redox regulation and adaptive stress mechanisms in legumes. The conserved domains also correlated with its upregulation under aluminum stress, a possible mechanism for maintaining oxidative balance in *C. arietinum* roots, with implications for studying redox reactions.

The 3D structure of cicer arietinum structural modeling and validation

Peroxidase 43 Isoform X2 was predicted using the SWISS-MODEL server. The structure contains a well-defined array of α-helices, β-strands, and flexible loop areas, which are characteristic of members of the Class III peroxidase family in plants. The predicted structure subsequently aligned with the selected template of a homologous peroxidase (**Fig.7A**), which provided evidence for structural validity, especially regarding homologous structural conservation. The predicted model retains the key features of the structure that are important for the catalytic activity of the enzyme. The model maintains conserved motifs for substrate binding and potential catalytic residues required for the destabilization of hydrogen peroxide, supporting the proposed functional role of peroxidase in *C. arietinum*, particularly in redox regulation, modification of lignin, and signaling defense pathways, such as ROS signaling.

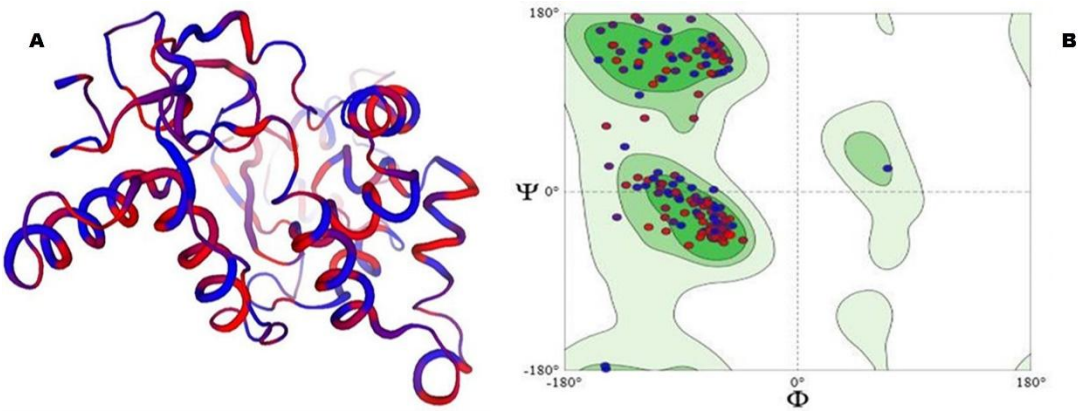

Fig. 7. Predicted 3D structure and stereochemical validation of chickpea peroxidase 43 isoform X2. (A) Ribbon model generated using SWISS-MODEL, showing α-helices and loops characteristic of Class III peroxidases. Superimposed query (blue) and template (red) structures demonstrate high structural similarity. (B) Ramachandran plot of the predicted model, confirming stereochemical quality, with most residues located in favoured regions, indicating a stable and well-folded protein.

The predicted quality of the model structure was further assessed using Ramachandran plots, which showed that most of the observed amino acid residues fell into the favored and allowed regions, indicating excellent stereochemistry and conformational integrity of the protein structure (**Fig.7B**). The relatively few residues in the disallowed regions indicated little structural strain in the predicted model and some reliability of the model structure. These findings provide evidence for the functional description of Peroxidase 43 Isoform X2 as a structurally conserved member of the Class III peroxidase family. The validated 3D structure provides a solid foundation for further studies, including docking, substrate interactions, and exploration of the structure-function relationship.

Protein-protein interaction network of peroxidase (LOC101506026)

To investigate the functional context of the *Cicer arietinum* peroxidase protein (LOC101506026), a protein–protein interaction (PPI) network was constructed using the STRING database. The PPI network showed that LOC101506026 is a central (hub) protein that directly connects multiple proteins and has a subnetwork of strongly connected proteins (**Fig. 8**). It is interesting to note that LOC101499511, LOC101504377, LOC101506453, and LOC101502042 were common interacting partners and well-connected with one another, suggesting they could potentially be co-expressed or co-regulated. These proteins may be related through their involvement in oxidative stress responses, cell wall modification, or both secondary metabolic pathways, and peroxidases play a prominent role in all of these processes.

The topology of the PPI network appears to be a hub-and-spoke model, with LOC101506026 as the core node of the network, with both tightly connected clusters and loosely connected proteins. Loosely connected proteins, such as LOC101492546, LOC101490320, and LOC101492141, were only connected to the peroxidase hub, suggesting that they interact only in situ or conditionally, such as during pathogen challenge or under environmental stress. Most importantly, the interaction evidence was derived from various sources, including experimental datasets (purple edges), gene neighborhood and co-occurrence (green and blue), text mining (yellow), and gene co-expression (black). Multiple lines of evidence increase confidence in the predicted functional associations. These results support that Peroxidase 43 is multifunctional in *C. arietinum* and is an important regulatory protein in legume stress physiology. Within the interaction network, it is the most central

protein and suggests coordination with various proteins involved in redox balance, signaling, and defense.

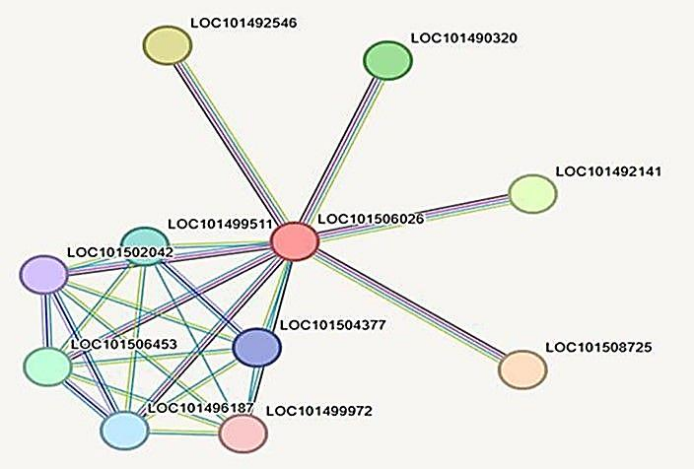

Fig. 8. Protein–protein interaction (PPI) network of *Cicer arietinum* peroxidase (LOC101506026) generated using the STRING database. LOC101506026 (central red node) directly interacts with multiple proteins, forming a tightly connected cluster with proteins such as LOC101499511, LOC101504377, and LOC101506453. The edge colors represent different sources of interaction evidence: purple (experimental), green (gene neighborhood), blue (co-occurrence), yellow (text mining), and black (co-expression). The network analysis indicated that peroxidase 43 acts as a central regulator of oxidative stress and metabolic signaling pathways.

Molecular docking analysis of peroxidase

Molecular docking studies were performed to obtain insights into the interaction of *Cicer arietinum* peroxidase with statistically significant concentrations of three metals (Al^{3+}, Fe^{2+}, Cu^{2+} and Zn^{2+}, which allowed for a modern understanding of their influence on the peroxidase structure and function during abiotic stressors. Aluminum (Al^{3+}) showed the highest binding affinity to the peroxidase active site, with a binding energy of -8.2 kcal/mol. The docking results suggest polar interactions with key residues Lys46, Glu44, Glu228, and Asp231, which are proximal to the substrate-binding region (**Fig. 9A**). Strong polar interactions indicate that the binding of Al^{3+} would produce conformational changes or distortion of the active site, potentially inhibiting peroxidase activity and ROS detoxification during Al^3 stress.

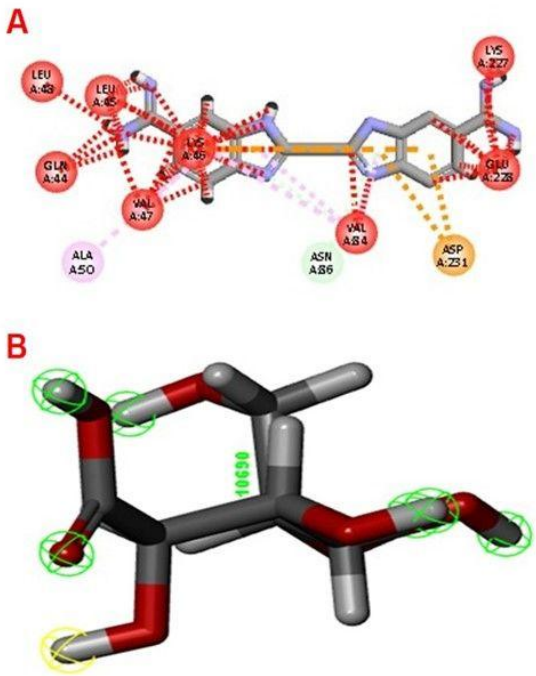

Fig. 9A. Docking model showing aluminum ion (Al^{3+}) interaction with peroxidase residues Lys46, Glu44, Glu228, and Asp231. The red dashed lines represent strong polar contacts, suggesting significant structural disruption. **(B)** Docking of iron ions (Fe^{2+}) with peroxidase, showing weak dispersed interactions away from the active site, indicating minimal functional impact.

Iron (Fe^{2+}) had a weaker affinity (-6.9 kcal/mol), and interactions were situated away from the catalytic core and showed little contact with functional site residues (**Fig. 9B**). Evidence suggests that Fe^{2+} is less disruptive and may stabilize the structure, supporting the idea that the peroxidase is in the reduced form and is an important antioxidant. Cu^{2+}showed a binding energy of -8.3 kcal/mol, thus binding somewhat stronger than Al^{+}, but formed stable interactions towards the less relevant end of the binding site. As to Al^{3+}, this suggests that Cu^{2+} could potentially exert beneficial or adverse effects on peroxidase activity, depending on its concentration. Zinc (Zn^{2+}) displayed the weakest binding affinity (-6.0 kcal/mol) with weak interactions, indicating little to no structural or functional interference with the peroxidase protein.

Based on the comparative docking results (**Table 5**), it is likely that Fe^{2+} and Zn^{2+} can activate or maintain peroxidase activity, while Al^{3+} and Cu^{2+} can, under conditions of great stress, even alter the structural integrity or functional metabolism of the enzyme. These results further support the susceptibility of peroxidase-mediated detoxification of ROS to aluminum toxicity, as well as the possibility that metalloid competition could mediate stress response in legumes.

Table 5. Comparative molecular docking results of *Cicer arietinum* peroxidase protein with metal ions using PyRx.

Metal Ion	Binding Affinity	RMSD Stability	Functional Impact	Role in Al^{3+} Detoxification
Al^{3+}	-8.2 kcal/mol	Unstable (high RMSD)	Disrupts peroxidase structure/function	Promotes toxicity (inhibits ROS detox pathway)
Fe^{2+}	-6.9 kcal/mol	Stable (low RMSD)	Supports enzymatic function	Helps maintain antioxidant defense
Cu^{2+}	-8.3 kcal/mol	Stable	May aid or impair function depending on levels	May support or compete with Fe^{2+}at active sites
Zn^{2+}	-6.0 kcal/mol	Less stable	Weak impact on function	Minimal role in peroxidase-mediated detoxification

Discussion

This study presents a detailed structural and functional characterization of the peroxidase 43 isoform X2 gene (LOC101506026) in *Cicer arietinum*, with a focus on its involvement in the response to aluminum (Al^{3+}) stress, using sequence verification, phylogenetic analysis, structural modeling, protein–protein interaction networks, and molecular docking. Sequence verification using BLASTn and BLASTx indicated that the cloned sequence was a Class III peroxidase that had catalytic and heme-binding residues associated with detoxification of reactive oxygen species (ROS) (Zámocký and Obinger, 2010). It also exhibited high sequence identity (>90%) with peroxidases in *Medicago truncatula* and *Glycine max*, which further supported our hypothesis that peroxidases are evolutionarily constrained and central in the spikes of oxidative stress (Ivanova et al., 2017; Gualtieri et al., 2020; Zhou et al., 2024).

Using homologous sequences extracted from nine other legumes, multiple sequence alignments (MSAs) and phylogenetic analyses were performed to assess evolutionary relationships and functional conservation. Across the full-length MSA, evolutionary conserved domains were shown, especially in the sequence of amino acids 250–630 with their catalytic histidine and tyrosine motifs that are important in defining the ROS scavenging activity of peroxidases (Lourenço et al., 2018). Phylogenetic analyses from the current study, *Cicer arietinum* more closely clustered with the *Cajanus cajan*, suggesting comparable molecular actions in stress responses with the utilization of the same reactive components to develop solutions. In comparison, a greater evolutionary distance for *Vigna radiata* from *C. arietinum* suggests that organisms adapt to regulatory mechanisms for their particular niches or cultivated environments (Kaila et al., 2016).

Our global alignment and dot plot analyses provided additional support for the vast conservation of peroxidase regions across legumes. In the alignment study, it was interesting to note that both *Arachis hypogaea* and *Vigna radiata* had longer peroxidase proteins than other legumes, perhaps because of the extensions of additional regulatory or accessory domains (Muñoz et al., 2016; Raza et al., 2022). Even though the peroxidase proteins from differing species, such as *Pisum sativum* and *Cajanus cajan*, were much shorter they maintained functional equivalence, which means that the core regions of the enzymes were also conserved across legumes.Our BLASTp alignments against *Vigna radiata*, *Medicago truncatula* and *Pisum sativum* showed high conservation in the regions

surrounding the active sites of the three legumes; *Cajanus cajan* showed lower conservation which could reflect potential sub-functionalization, or separate evolutionary divergence from related crops (Jacob et al., 2024; Kotapati et al., 2015). Our conserved domain search results identified the secretory peroxidase domain as well as the PLN03030 family and LOC101506026 was classified as a Class III plant peroxidase (Wang et al., 2023). These findings central highlight the proteins function to detoxify hydrogen peroxide and keep redox homeostasis for biological activities. The structural modeling results confirmed stable α-helical structural similarity, supported through Ramachandran plots that suggested that most residues were in energetically favorable spaces, thus supporting the stereochemical viability of the predicted model (Kudapa et al., 2024).

Network analysis of protein–protein interactions (PPIs) identified LOC101506026 as a hub gene, because of its strong interactions with orthologous genes (e.g. LOC101499511 and LOC101504377), which indicate potential co-expression of regulatory roles within stress domain responses and ROS detoxification (Kudapa et al., 2024). The peripheral nodes of the network involved regulatory role interactions under specific conditions that in interaction with each other may add additional functional regulation (Poddar et al., 2022).

The results from molecular docking analysis showed that Al^{3+} had a strong binding preference for either binding at the active site, or to the close surrounding residues at Lys46, Glu228 or Asp231, with a binding energy score of -8.2 kcal/mol. The binding of Al^{3+} could, potentially cause conformational changes, and possibly reduce the efficiency of the enzyme, or reduce the tolerance of oxidative stress under toxicity from aluminum (Taunk et al., 2023). Conversely, Fe^{2+}, Cu^{2+}, and Zn^{2+} are predicted to have relatively weak binding or potential distal-well and plate-thickness interactions, therefore do not demonstrably affect the overall structure and conformational state like biological cofactors (Cakmak and Horst, 1991).

From these results it can be concluded that *Cicer arietinum* peroxidase 43 isoform X2 is a conserved and functional enzyme involved in ameliorating oxidative stress. There is evidence of a high-affinity interaction with Al^{3+}indicating potential vulnerability in the antioxidant defense mechanism when subjected to aluminum stress. Evidence from sequence conservation, structure modeling, interaction networks, and docking provides ample evidence fits identity in aluminum tolerance and ROS regulation in legumes.

Conclusion

This research shows that the peroxidase 43 isoform X2 of *Cicer arietinum* is a well-conserved Class III peroxidase with essential functions in both detoxification of ROS and adaptation to stress. The sequence and domain analysis, in addition to the modeling the 3D structures demonstrated that peroxidase 43 isoform X2 had conservation across legumes that was coincidental with its function. Molecular docking suggested that Al^{3+}binds with high affinity in the catalytic area of peroxidase 43 isoform X2 and likely hinders the enzyme activity; however, Fe^{2+} may stabilize the enzyme. Protein-protein interaction analysis further identified peroxidase 43 as a central hub in oxidative stress signaling pathways. Overall, peroxidase 43 isoform X2 represents a conserved, key enzyme in chickpea oxidative stress responses. Their strong interaction with Al^{3+}implies a direct role in aluminum toxicity, as such, targeting this gene with breeding or genetic engineering may provide a mechanism to improve chickpea tolerance under acidic soil.

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Author Contributions

PV: Experiments, Methodology, Data curation, Formal analysis, Manuscript writing, Molecular docking; **BNT:** Conceptualization, Editing of the manuscript, Investigation, Supervision, Funding.

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