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In Silico Molecular Modeling and Structural Analysis of Peroxidase Enzymes from five different plants species.

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ABSTRACT

Upon attacked by pathogens, plants defend themselves by producing array of defense related molecules. Plant peroxidase enzymes are about 300-350 residue long and have multiple isoenzymes that differ in substrate specificity and localization within the plant. In this study, 3D structure of peroxidase enzymes from five different plants was predicted by homology modeling method. The quality of the 3D structure of the model was confirmed by various web based validation programs. When compared secondary and tertiary structure of the model, it showed two peroxidase signature domains (PEROXIDASE_1 and PEROXIDASE_2) are present in the central region. Degree of sequence conserveness showed that sequence size differences do not make any impact on their basic functions.

Keywords: 3D structure, Signature domain, Secondary structure, Tertiary structure

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INTRODUCTION

Pathogenesis related proteins (PRs) are the important class of proteins that play a vital role in plant defense mechanism by inducing defense enzymes in stressful environment. This PR enzyme boosts the plant defense by catalysis of the last step in the biosynthesis of lignin and other oxidative phenols [1, 2] as well as maintains the pathogenic loads. Amongst the important PR proteins, plant peroxidases (EC 1.11.1.7) also known as PR-9 or Ypr9 or Prx are important in diverse cellular functions throughout the plant life cycle, probably due to plenty of enzymatic isoforms (isoenzymes) and to the versatility of their enzyme-catalyzed reactions [3]. Plant peroxidases are involved in auxin metabolism, lignification, suberization, cross-linking of cell wall components, phytoalexin synthesis and detoxification of hydrogen peroxidase [4, 5, 6]. Peroxidase is involved in the production or modulation of active oxygen species which may play important roles behind reducing pathogen viability and spread. Prx plays a central role in triggering the hypersensitive reaction (HR) in the crosslinking and lignification of the cell wall and in transducing signals to adjacent non-challenged cells [2]. Strengthening of cell wall barrier synthesis involves the influence of peroxidase enzyme, which may restrict further entry by the pathogen [7] and it was also found to inhibit the spore germination and mycelial growth of certain fungi [8].

Peroxidase enzymes are heme-containing glycoprotein and usually classified as acidic, basic or neutral according to their isoelectric point [5]. These enzymes are encoded by a large multigene family in plants. More than 100 expressed sequence tags (ESTs) encoding different peroxidase isoenzymes are found in Arabidopsis [4]. This enzyme is composed of a single polypeptide chain of about 300 residues in length [9] and they contain ferriprotoporphyrin IX as a prosthetic group. Each plant has numerous peroxidase isoenzymes that differ in substrate specificity and localization within the plant. In addition, isoperoxidases within some species and those from different species exhibit size heterogeneity. Isozymes have the molecular weight range from 30000 to 50000 daltons [10]. Peroxidase enzymes are generally categorized into three classes based on sequence alignments and biological origin [11]. Class I includes the intracellular peroxidases found in the cytosol or chloroplasts. Class II and III are known as secretory peroxidases from fungus and plant sources respectively. Both of these two classes of peroxidases posses monomeric glycoproteins with four conserved disulfide bridges and two calcium ions, but the position of the disulfides differs from each other [9]. However, sequence or structural differences do not make any impact on their basic functions. Therefore, the present study aims to examine the amino acid sequence analysis of peroxidase enzymes of different model plant species viz. *Arabidopsis thaliana* (Family: Brasicaceae); *Capsicum annuum* (Family: Solanaceae); *Nicotiana tabacum* (Family: Solanaceae); *Oryza sativa* (Family: Poaceae); *Zea mays* (Family: Poaceae). Since the 3D structures of proteins can yield essential information about their functional mechanisms, and hence an initiative was taken to *in silico* structural characterization and analyses of these enzyme using molecular modeling techniques. Degree of sequence conserveness and their evolutionary relationship were also studied to comment on their functional relatedness.

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MATERIALS AND METHODS

Sequence retrieval and analysis

Sequence retrieval

Nucleotide sequences of plant peroxidase enzymes were retrieved in FASTA format from NCBI (National Centre for Biotechnology Information; http://www.ncbi.nlm.nih.gov/) nucleotide data bank (Table 1). Plants chosen for this study are: *Arabidopsis thaliana*, *Capsicum annuum, Nicotiana tabacum, Oryza sativa* and *Zea mays*. These sequences were then converted to their corresponding protein sequences using an online DNA to Protein conversion tool (insilico.ehu.es/translate/) and used in further experimental process.

Fingerprint analysis

Sequences in FASTA format were submitted to the ScanProsite tool (http://prosite.expasy.org/scanprosite/) for searching the fingerprint. Sequence logo was created using WebLogo program (http://weblogo.berkeley.edu/logo.cgi; Crooks et al., 2004).

Secondary structure analysis

The secondary structure of the proteins was analyzed using three different online software viz. GORIV, HNN and SOPMA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html).

Three dimensional structure predictions and analysis

Consensus amino acid sequences of peroxidases of each plant obtained using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and Jalview (Waterhouse et al., 2009) were used for 3D structure prediction. Sequences were submitted to the ModWeb server (https://modbase.compbio.ucsf.edu/scgi/modweb.cgi) for automated structure prediction. Initial models obtained from the server were further analyzed for suitable structure selection. Selected structures were refined by energy minimization using SWISS PDB VIEWER (Version 4.0.1). Structural optimization involved energy minimization (100 steps each of steepest descent and conjugate gradient methods) *in vacuo* with the GROMOS96 43B1 parameter set without reaction field. This process of minimization was repeated until satisfactory conformational parameters were obtained. Each loop was separately regularized applying position constraints to the rest of the atoms of the protein, which were 2 amino acids away from the desired loop by energy minimization followed by evaluation of the structural parameters. The final structure was energy minimized 100 steps each with steepest descent and conjugate gradient methods keeping all the atoms of the protein free.

PROCHECK and ERRAT were used for checking the stereochemical quality of the refined structures in each step of energy minimization. PROSA was used to determine the overall model quality of the final structures [12]. Ribbon structure and electrostatic surface

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potential map were generated using UCSF CHIMERA (Version1.4) software. Structures which passed the quality check were deposited in PMDB database.

Table 1: Signature sequences and their regions

Plant name	Accession number	Protein length	PEROXIDASE_2 Peroxidases active site signature	Sequence region	PEROXIDASE_1 Peroxidases proximal heme-ligand signature	Sequence region
Arabidopsis thaliana	gi|186510609|	352	AAsiLRLhFHDC	62-73	DLVALSGGHTF	191-201
	gi|18874553|	329			DMIALSGAHTL	189-199
	gi|145359134|	312			DMVTLSGGHTI	173-183
	gi|145358744|	317			DLVALSGGHTL	180-190
	gi|30686383|	326	AAplIRMhFHDC	57-68	DLVLLSGAHTI	183-193
	gi|18252200|	326	AAplIRMhFHDC	57-68	DLVLLSGAHTI	183-193
	gi|17065479|	329			DMIALSGAHTL	189-199
	gi|24899820|	349	AAslLRLhFHDC	62-73	DLVALSGGHTF	191-201
	gi|23308362|	328	AAslIRLhFHDC	61-72	DVVALSGAHTF	185-195
	gi|21386964|	329	AAplIRMhFHDC	60-71	DLVLLSGAHTI	186-196
	gi|20466178|	349	AAslLRLhFHDC	62-73	DLVALSGGHTF	191-201
	gi|20260463|	331	AAgtLRLfFHDC	61-72	EMVALSGAHTI	190-200
Capsicum annuum	gi|222159966|	324	GAslLRLfFHDC	60-71	DMVALSGAHTI	188-198
	gi|94962423|	322	AAslIRLhFHDC	60-71	DMVALSGSHSI	188-198
	gi|17066702|	332			DLVTLVGGHTI	188-198
	gi|110348875|	322	AAslIRLhFHDC	60-71	DMVALSGSHSI	188-198
Nicotiana tabacum	gi|64976604|	360	GAslIRLhFHDC	97-108	EMVALAGAHTV	227-237
	gi|63253079|	360	GAslIRLhFHDC	97-108	EMVALAGAHTV	227-237
	gi|14031050|	329	AAslLRLhFHDC	59-70	DLVALSGAHTI	187-197
	gi|14031048|	354	AAglLRLhFHDC	67-78	DVVALSGGHTI	197-207
Oryza sativa	gi|113869754|	335	AAslVRLhFHDC	62-73	DVVALSGGHTI	190-200
	gi|2443458|	122	AAglVRIfFHDC	65-76		
	gi|8901179|	311	GAslLRLhFHDC	54-65	DMVALSGAHTI	177-187
	gi|2429291|	314	GAslVRLhFHDC	56-67	DMVALSGAHTI	179-189
	gi|2429289|	314	GAslLRLhFHDC	55-66	DLVALSGAHTI	178-188
	gi|2429287|	315	GAslLRLhFHDC	52-63	DMVALSGAHTI	180-190
	gi|2429285|	317	GAslLRLhFHDC	58-69	DMVALSGAHTI	181-191
Zea mays	gi|162460927|	320	GAslLRLhFHDC	62-73	DMVALSGAHTI	187-197
	gi|162460927|	308	GAslLRLhFHDC	47-58	DLVALSGAHTI	175-185
	gi|162460661|	324	GAavIRMlFHDC	66-77	DLVVLSGAHTV	197-207
	gi|226532577|	339			DLVVLSGSHTI	197-207
	gi|226530538|	316	GAsiIRLfFHDC	57-68	DMTALSGAHTV	185-195
	gi|226530300|	352	APglIRLhFHDC	61-72	DMVVLSGSHTI	190-200
	gi|226528592|	327	GAslLRLfFHDC	65-76		
	gi|226510116|	327	VGstVRLfFHDC	61-72	DLVALSAAHSV	190-200
	gi|226510060|	333	AAslVRLhFHDC	61-72	DVVALSGGHTI	189-199
	gi|226509889|	307	APaaLRLfFHDC	59-70	EMVALLGAHTL	187-197
	gi|226507319|	328	AGplLRLhFHDC	58-69	DLVVLSGGHTL	184-194
	gi|226506661|	329	GAglIRMhFHDC	52-63	DMVTLSGAHTV	181-191
	gi|226495736|	338	AGplLRLhFHDC	65-76	DLAVLSGAHTL	192-202
	gi|226495500|	360	AAgmLRVfFHDC	70-81	ELVALSGAHTL	199-209
	gi|226493662|	324			DLVWLSGAHTI	182-192
	gi|226493477|	323	GAsiVRLfFHDC	69-80	DMVALSGAHTI	197-207
	gi|226492566|	346			DLAVLSGAHAI	209-219
	gi|226491561|	339	GAalVRLiFHDC	62-73	ELVILTGAHSI	192-202
	gi|226491045|	317	APatLRLfFHDC	60-71	DMIALSGGHTI	188-198
	gi|162464361|	360	AAglIRLhFHDC	59-70	DMVVLSGAHTV	187-197

Multiple Sequence Alignment and Cluster Analysis of peroxidase enzyme

Consensus amino acid sequences of the peroxidase enzyme of five plants (i.e. *Arabidopsis thaliana*, *Capsicum annuum*, *Nicotiana tabacum*, *Oryza sativa* and *Zea mays*) in FASTA format subjected to multiple sequence alignment using CLUSTALW [13]. Phylogenetic analyses were conducted in MEGA4 [14] using the Maximum Parsimony (MP) method [15] with 1000 bootstrap value [16]. The Close-Neighbor-Interchange algorithm [17] with search level 3 [16, 17] was used to obtain the MP tree. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option).

RESULTS

Sequence retrieval and primary sequence analysis

In the present study we have taken peroxidase enzymes of five different species e.g. *A. thaliana*, *C. annuum*, *N. tabacum*, *O. sativa* and *Z. mays*. For each species we found more than one mRNA sequences. Therefore all of them were downloaded from NCBI database in FASTA format and converted to corresponding protein sequences using the mentioned tool. Result shows that the protein length varied from 312 to 352 amino acids for *A. thaliana*, 322-332 amino acids for *C. annum*, 329-360 amino acids for *N. tabacum*, 122-335 amino acids for *O. sativa* and 307-360 amino acids for *Z. mays*. The average length of the peroxidases of all the 5 species under study is 331 (Table 1). ScanProsite result of peroxidase enzymes revealed that the sequences contain PEROXIDASE_2 (Peroxidases active site signature) and PEROXIDASE_1 (Peroxidases proximal heme-ligand signature) (Table 1) along with the other functionally important sites CK2 Phosphorylation, Myristoylation, PKC Phosphorylation, N-glycosylation etc. In this present study, only the peroxidase domains were considered for further structural analysis of these enzymes.

These two peroxidase domains having conserved sequences of twelve and eleven amino acid lengths respectively and are present in all the selected plant species with a few exceptions (Table 1). Both the sequences are found to be variable at several positions. From the WebLogo (Figure 1) it is observed that in PEROXIDASE_1 sequence Lysine at 5^{th} and Histidine at 9^{th} position is conserved for all the five plants. In case of PEROXIDASE_2 Arginine at 6^{th} position and last four positions i.e. Phenylalanine, Histidine, Aspertic acid and Cystine (9^{th} to 12^{th}) are conserved in all the five chosen plant species (Figure 1).

Sequence based secondary structure analysis

Secondary structure of the selected five plant species shows that α helix ranges from 27.91 to 40.84% in *A. thaliana*; 25.28 to 39.91% in *C. annuum*; 34.23 to 42.74% in *N. tabacum*; 33.90% to 43.11% in *O. sativa* and 34.69% to 41.42% in *Z. mays*. Random coils ranges from 44.80% to 53.00% in *A. thaliana*; 44.39% to 59.83% in *C. annuum*; 44.64% to 52.20% in *N. tabacum*; 44.89% to 53.12% in *O. sativa* and 46.10% to 51.56% in *Z. mays* (Table 2). From the percentage of occurrence of the above three secondary structures it was observed that all the peroxidase enzyme from five different plants predominantly have a tendency to have alpha (α) helical structure followed by random coil and then by beta (β) sheet.

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Table 2: Comparative analysis of secondary structures of peroxidase enzymes (values are the average of all the peroxidase enzymes of individual plants with ±SD values)

Name of the Plant	HNN			GOR IV			SOPMA		
	α helix	Ex. Strand	R. Coil	α helix	Ex. Strand	R. Coil	α helix	Ex. Strand	R. Coil
A. thaliana	38.55, ±2.56	12.86, ±2.41	48.63, ±2.23	27.91, ±4.18	19.09, ±1.49	53.00, ±3.39	40.84, ±2.70	14.36, ±0.81	44.80, ±2.60
C. annuum	35.29, ±6.25	14.57, ±4.90	50.15, ±6.02	25.28, ±5.63	14.89, ±2.98	59.83, ±3.72	39.91, ±3.10	15.70, ±2.58	44.39, ±0.93
N. tabacum	40.14, ±8.03	12.49, ±6.03	47.38, ±2.17	34.23, ±3.15	13.58, ±2.12	52.20, ±1.58	42.74, ±2.75	12.64, ±1.78	44.64, ±1.02
O. sativa	43.11, ±4.08	9.54, ±3.61	47.35, ±4.24	33.90, ±5.86	12.97, ±2.38	53.12, ±6.11	40.76, ±2.64	14.34, ±2.16	44.89, ±2.69
Z. mays	41.42, ±5.25	10.37, ±3.52	48.21, ±3.95	34.69, ±6.93	13.75, ±4.25	51.56, ±4.77	39.73, ±2.74	14.17, ±1.46	46.10, ±2.63

3D structure prediction and analysis

Model construction, refinement and stereochemical evaluation

Table 3: Steriochemical evaluations of the predicted models

Organism	Protein size	Model ID (obtained from PMDB database)	Model data			Corresponding template		Model quality	
			Modeled Segment	Size	Sequence ID (%)	PDB Code	PDB Segment	PROSA (Z score) Overall Model quality	ERRAT Overall Quality Factor
A. thaliana	355	PM0077500	30-340	311	57	1GWU	1-306	-9.52	90.099
C. annuum	322	PM0077478	29-322	294	58	1SCH	2-294	-8.23	87.063
N. tabacum	360	PM0077479	57-356	300	39	1BGP	1-308	-7.13	92.784
O. sativa	316	PM0077481	25-315	291	67	1SCH	2-294	-9.28	91.519
Z. mays	363	PM0077499	38-354	317	52	1PA2	1-305	-8.55	90.291

In the present study we have found that the peroxidase enzymes of the five plant species have varied sequence length and secondary elements but their function are same. Since the structure of a protein gives much more insight in the function of protein than its sequence, 3D structure of all the peroxidases under study was predicted using molecular modeling techniques. Consensus sequence of peroxidase from each plant species was used for structure prediction. Sequences were submitted to the ModWeb server for tertiary structure prediction. Two models of each peroxidase were received from the server based on different templates. Best model of each of the plant peroxidase was determined by sequence identity and length coverage (Table 3).

Qualities of the backbone of the modeled structures were checked with PROCHECK. It was observed that although most of the ϕ-ψ pairs were distributed in the most favored and additional allowed regions of the Ramachandran's plot, the backbone conformation of some of the amino acids were in the generously allowed and disallowed regions as shown in figure 2 and values are given in table 4. These were grouped into different segments of the structure and refined by energy minimization until most of the backbone conformations fell in the desired regions (Figure 2).

Table 4: Ramchandran's plot statistics after energy minimization.

Model ID	% Residues in				% Bad back bone bond	% Bad back bone angle
	Most Favored Region	Additional Allowed Region	Generously Allowed Region	Disallowed Region		
PM0077500	90.5	9.5	0.0	0.0	0.00	0.06
PM0077478	89.5	9.3	1.2	0.0	0.00	0.00
PM0077479	87.5	11.0	0.8	0.8	0.00	0.00
PM0077481	91.8	7.4	0.8	0.8	0.00	0.00
PM0077499	87.8	11.4	0.4	0.4	0.00	0.25

The overall model quality (Z score) and overall quality factor of the model were measured using PROSA and ERRAT. These values were compared with the relevant x-ray structures of the model used in homology modelling (Table 3). These structural parameters of the models indicate that the modeled structures are reliable and can be used for further study. The final models of the target protein of each species were deposited in the Protein Model Data Base (Table 3).

Analysis of secondary structure and electrostatic potential surface of the peroxidase enzymes

Most of the members of the peroxidase enzymes exhibit extensive ordered secondary structures of α-helix, random coils and β-sheets. All the modeled structures of the peroxidase enzymes contained two peroxidase motifs namely PEROXIDASE_1 and PEROXIDASE_2 which resides in the helical region of all the five peroxidase enzymes, depicted in red and blue color in the models respectively (Figure 3). These regions of all the 5 plant peroxidase enzymes are superposed. The result highlights the conserve nature of the two domains e.g. PEROXIDASE_1 and PEROXIDASE_2 and points out their functional importance in plant defence. Upon detailed analysis of the peroxidase catalytic domain, it was observed that it consists two helical motif PEROXIDASE_2 and PEROXIDASE_1 containing twelve and eleven amino acid residues respectively. Peroxide binding domain (PEROXIDASE_2) which is mainly responsible for catalysis of H_2O_2, contains neutral (Ala, Ser, Leu, Phe, Cys, Ile etc.), positive (Arg, His) and negative (Asp) amino acid residues.

From ESP analysis it can be concluded that the binding region is present in the core of the structure and mainly acidic in nature and this region is astounded by alternatively placed positive, neutral and negative amino acid residues (Figure 4).

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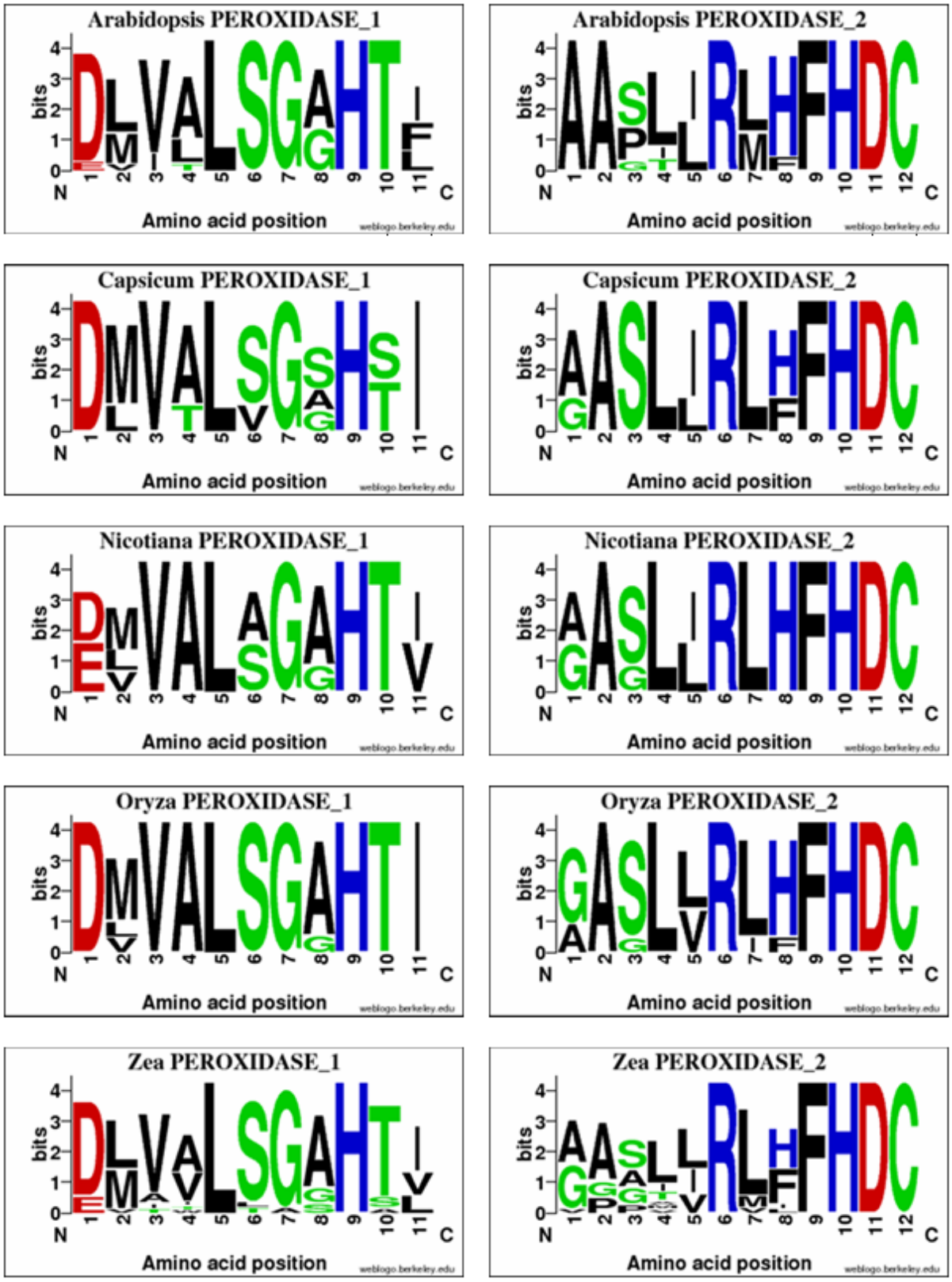

Figure 1: WebLogo of signature domains of peroxydase enzymes (PEROXIDASE_1 and PEROXIDASE_2 domains).

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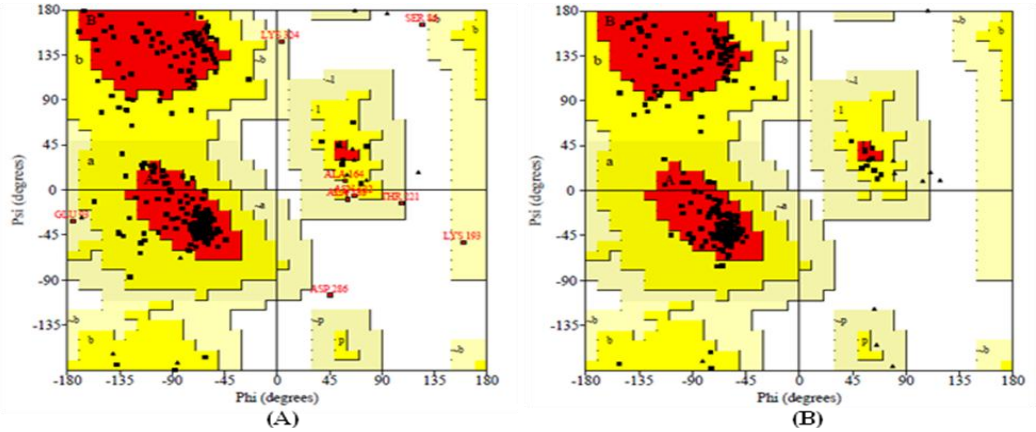

Figure 2: Ramachandran's plot of the predicted *A. thaliana* peroxidase structure (PM0077500). (A) Initial model and (B) Final model. Red region, deep yellow, light yellow and white regions show the core, allowed, generously allowed and disallowed region. Residues of initial model in generously allowed and disallowed region are highlighted in red color.

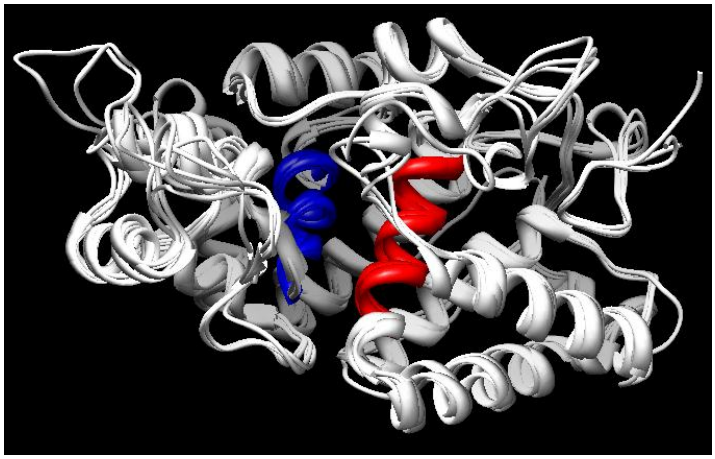

Figure 3: Five peroxidase structures were superposed and two active domains remained in the helical region. Helix coloured in red represents PEROXIDASE_1 (Peroxidases proximal heme-ligand signature) and blue represents PEROXIDASE_2 (Peroxidases active site signature) domain.

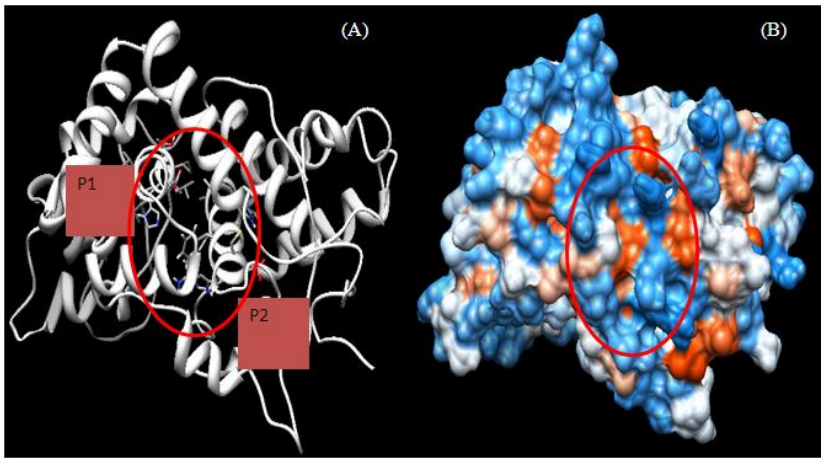

Figure 4: Peroxidase active domain representation in model PM0077500. (A) In Ribbon representation P1: PEROXIDASE_1 domain and P2: PEROXIDASE_2 domain. B. Electrostatic potential surface (ESP) of the model. Active domain region is encircled with red colour. In electrostatic potential surface diagram positive, negative and neutral molecular surface potential were shown in blue, red and white respectively.

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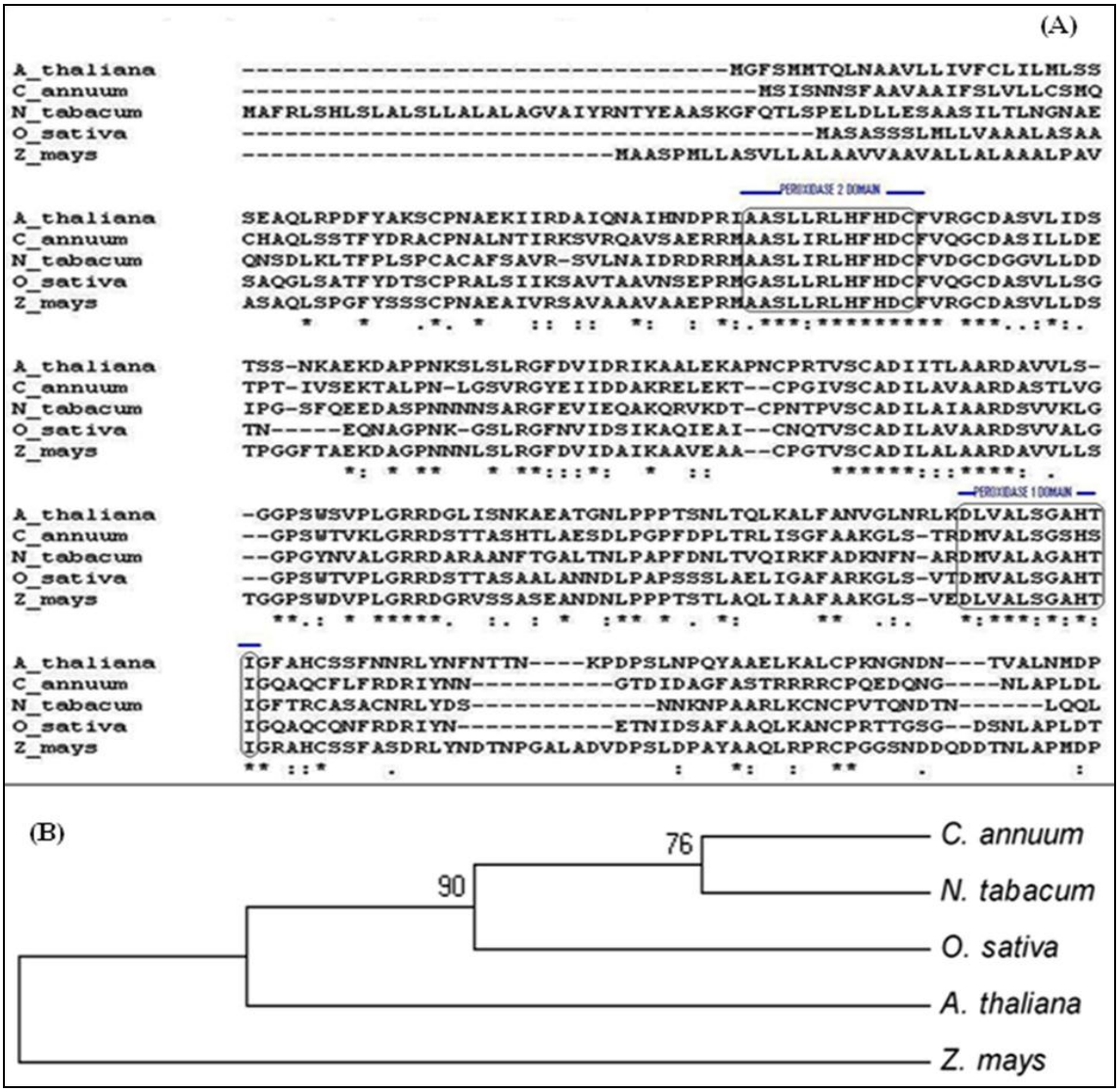

Figure 5: Multiple Sequence Alignment and Phylogenetic Analysis of consensus peroxidase amino acid sequences from five different plant species. (A) Multiple Alignments was done using ClustalW. Boxes depicted PEROXIDASE_1 and PEROXIDASE_2 signature domais. Highly conserved amino acid residues are marked with '*'. (B) The phylogenetic tree of consensus peroxidase sequences from five plants. All the peroxidase sequences were diverged from a common ancestral origin.

Multiple Sequence Alignment and Phylogenetic Analysis

The multiple sequence alignments of the five peroxidases from five different taxa are shown in figure 5. The residues within the PEROXIDASE_1 and PEROXIDASE_2 domains are evolutionary conserved, indicating their structural and functional importance in plant pathogenic response mechanism.

The consensus Phylogenetic tree of all the five plant peroxidase enzymes of our interest was constructed using MEGA4 [14] (Figure 5). A phylogeny, or evolutionary tree, represents the evolutionary relationships among a set of organisms. The root of the tree represents the ancestral lineage, and the tips of the branches represent the descendants of that ancestor. The evolutionary history was inferred using the Maximum Parsimony method [15]. The most parsimonious tree with length = 456 is shown (Figure 5). The consistency

index is (0.772358), the retention index is (0.533333), and the composite index is 0.500585 (0.411924) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [14]. The MP tree was obtained using the Close-Neighbor-Interchange algorithm [17] with search level 3 [16, 17] in which the initial trees were obtained with the random addition of sequences (0 replicates). All positions, containing gaps and missing data, were eliminated from the dataset (Complete Deletion option). There were a total of 302 positions in the final dataset, out of which 60 were parsimony informative. Phylogenetic analyses were conducted in MEGA4 [14].

The Phylogenetic tree states that Peroxidase enzyme of *C. annuum* and *N. tabacum* is evolutionary closely related to each other than either is to *O. sativa* and *A. thaliana* whereas, Peroxidase enzyme of *Z. mays* was found to be most diverged from the common ancestral origin (Figure 5). This finding indicates that though, the core remained relatively conserved, some segments of the peroxidases varied a lot during evolution.

DISCUSSION

Peroxidase (EC 1.11.1.7) oxidizes a vast array of compounds (hydrogen donors) in the presence of H2O2. Plant peroxidases are heme-containing glycoproteins and are usually classified as acidic, neutral, or basic, according to their isoelectric points. Higher plants possess a large number of peroxidase isoenzymes, which are encoded by multigene families [5]. Several physiological functions for peroxidases in plants have been reported, such as removal of reactive oxygen species (ROS), biosynthesis of lignin [2], induction of defense during pathogenesis [18, 19]. The biochemistry and enzymology of the plant peroxidase superfamily has been reviewed extensively [4, 11], but further progress towards the understanding of biological chemistry of plant peroxidases at the structural level is still required. The mechanisms of reaction with peroxide and reducing substrates can only be described in detail with the increasing availability of the suitable *in silico* models generated on the basis of crystallographic, spectroscopic and kinetic data. This will also be helpful in learning about the actual role of specific isoenzymes along with identification of physiological substrates for each peroxidase enzymes. In this work five consensus peroxidase enzyme models from five different plants were used for the study. These five consensus sequences varied in size. But superposed structures of five peroxidase enzyme revealed that the two peroxidase signature domains remained in the same position (Figure 3) making no impact on their basic functions. It can be concluded that further structural analysis of peroxidase and its isoenzymes will offer new insights into the relationship between enzymes and their physiological substrates.

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