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Comparative study of pathogenesis-related protein 5 (PR5) of different *Zingiberaceae* species

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Pathogenesis-related protein 5 (PR5) is a family of proteins that are induced by different phytopathogens in many plants and share significant sequence similarity with thaumatin. In the present study, 8 putative PR5 genes were cloned from different species of *Zingiberaceae*, *viz.*, *Alpinia luteocarpa*, *Curcuma amada*, *C. aromatica*, *C. longa*, *Elettaria cardamomum*, *Hedychium coronarium*, *Zingiber officinale* and *Z. zerumbet.* Deduced sequences encode precursor proteins of 215 to 230 amino acid residues and share high homology with a number of other PR5 genes. The phylogenetic analysis of all the PR5s of *Zingiberaceae* species showed that all of them share significant evolutionary history. The secondary and 3-D structure comparison with 3 known PR5s (*Ca*PR5, *Zo*PR5 & *Zz*PR5) revealed many striking similarities. In addition, we also generated models of their 3-D structure, the docking interactions between PR5 and the ligand β-(1,4)-D-glucan, and the analysis of various protein properties, which has helped in determining the efficiency of PR5 proteins. These analyses further led to the identification of putative antimicrobial domains in these PR5s, which remain conserved.

Keywords: Disease resistance, 3-D modeling, pathogenesis-related proteins (PR5), *Zingiberaceae*

Introduction

The defense strategy of plants against stress factors contains a multitude of tools, including various types of stress proteins with putative protective functions. A group of such proteins induced by different stress stimuli are called 'pathogenesis-related (PR) proteins', which play an important role in plant defense against pathogenic constraints and in general adaptation to stressful environment. They are induced by various biotic factors, such as, pathogens, insects, nematodes, herbivores. Pathogen derived elicitors are potent inducers of PR proteins. Besides being induced by the environmental/external cues, PR protein synthesis can be triggered by internal plant developmental stimuli[1]. These proteins were first detected in tobacco. Now they are found to be ubiquitously distributed in the plant kingdom in both monocotyledonous and dicotyledonous plants across different genera. The PR proteins have different functions. They exhibit chitinase activity and antifungal, antibacterial, insecticidal, nematicidal and antiviral actions. The PR proteins are broadly classified into 16 families, of which PR5 protein is part of our present study. PR5 proteins share significant amino acid sequence homology with the sweet tasting protein in the fruits of the tropical plant *Thaumatococcus daniellii* and have been named as Thaumatin like proteins (TLPs)[2].

To date, various PR5 genes have been cloned from different plants, such as, PR5 from cherry tomato[3] and *Ph*OSM from *Petunia*[4]. Recently, Nair *et al*[5] has reported the significant up regulation of PR5 gene in *Zingiber zerumbet* in response to infection by *Pythium aphanidermatum*. Two putative PR5 genes, *Ca*PR5 and *Zo*PR5, amplified in *Curcuma amada* and ginger, respectively, have been reported to share high homology with a number of other PR5 genes. However, the expression of *Ca*PR5 and *Zo*PR5s under *Ralstonia solanacearum* inoculation revealed that *Ca*PR5 was readily induced by the bacterium in *C. amada*, while *Zo*PR5 induction was very weak and slow in ginger. Promoter analysis indicated the presence of a silencing element binding factor in *Zo*PR5-promoter, but not in case of *Ca*PR5[6]. Other studies have shown that over-expression of PR5 protein enhances the pathogen resistance in plants[7].

The aim of the present study was to clone and characterize the PR5 cDNA in different species of *Zingiberaceae* as well as to study likely function of proteins encoded by the PR5 genes.

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Materials and Methods

Plant Material and Pathogen Inoculation

The plant samples of different species of *Zingiberaceae* were obtained from the Experimental Farm of Indian Institute of Spice Research (IISR) at Peruvannamozhi, Kozhikode (Kerala), India. The different species used in the study were *Alpinia luteocarpa*, *Curcuma amada*, *C. aromatica*, *C. longa*, *Elettaria cardamomum*, *Hedychium coronarium*, *Zingiber officinale* and *Z. zerumbet.* The virulent colonies of *Ralstonia solanacearum* (R4Bv4), cultured on Casamino acid-Peptone-Glucose (CPG) medium, were multiplied in sucrose peptone broth (g L^{1}: sucrose, 20; peptone, 10; K_2HPO_4, 0.5; $MgSO_4$, 0.25; pH 7.2) for 2 d. The resultant bacterial culture was centrifuged at 10,000 g for 20 min at 4°C, pellet resuspended in water and poured around the base of the 45-d-old plants as water suspension at a concentration of 109 cells mL^{-1} of water. The inoculated plants were grown in growth chamber (28±2°C, 12 h light, 65% RH) and were monitored for wilt disease.

RNA Isolation

Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions from different species after inoculation with *R. solanacearum*. All RNA extracts were first treated with DNase I (Promega) and then cleaned up with RNeasy mini kit (Qiagen). For the isolation of PR5 cDNAs, first-strand cDNA synthesis was carried out using 20 µg of total DNase-treated RNA (pooled total RNA obtained from different time intervals) in a 50 µL aliquot.

Isolation of PR5 Sequences and Sequence Analysis

Based on the sequence similarity of three PR5 proteins from *Zingiberaceae* species (JN024682.1, JN024683.1 & FJ550342.1), 3 degenerate (two forward and a common reverse) primers, PR5 F1 (ATCATGGCTACATCAACCACCGC) (for full length), PR5 F2 (GCCCTGCT CTGTTTCTTCC TTTTCC) and PR5 R (CAAGGGCA GAAGGTGA CACTGTAGT) were designed from the conserved regions to amplify the PR5 orthologs from the 8 afore-mentioned species of *Zingiberaceae*. PCR conditions were 1 cycle of 1 min at 95°C, followed by 14 cycles of 40 sec at 58°C, 1 min 15 sec at 72°C and 1 min at 72°C. The isolated fragment was cloned in pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced. The sequences were compared with other PR5 sequences in the database using the BLAST program[8]. Alignments of the predicted protein sequences were performed with ClustalX[9] and GeneDoc[10]. Finally, 8 clones with homology to a PR5 were isolated.

Phylogenetic Analysis

A phylogenetic tree was constructed for all the sequences by using molecular evolutionary genetics analysis 4 (MEGA4)[11]. The tree was constructed for comparing the sequenced TLPs with those from other eudicot and monocot families. The evolutionary history was inferred using the neighbor-joining method[12].

Protein Structure Analysis

Protein structure analysis was done by using Expasy-Protparam tool[13] to analyze the various physical and chemical parameters for a given protein. The computed parameters included the mol wt, theoretical isoelectric point, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity (GRAVY). The GRAVY values indicate the hydrophobicity and hydrophilicity of a protein. The secondary structures of all the samples were identified by using GOR[14].

Molecular Modeling

The protein sequences were used for molecular model generation using Modeller9v8 software[15] and 3-D structures were developed for all of them. The template used for modeling was 1Z3Q protein from *Musa acuminata* (banana). Energy minimization was done using Argus lab[16].

Docking Studies

Molecular docking was conducted by using the Molegro Virtual Docker (MVD)[17]. The entire protein structure was loaded onto the MVD platform for docking process. It performed flexible ligand docking so that the optimal geometry of the ligand was determined. MVD gave multiple poses representing different potential binding modes. MolDock score, Rerank score and hydrogen bond interactions were observed for the interpretation of dock results and interactions.

Antimicrobial Peptide Prediction

The conserved motifs present in all the 11 PR5 sequences were identified by MotifMaker online tool (http://landau.utmb.edu:8080/pcpmer//Tools/SubmitFormMotifMaker.jsp). The antimicrobial activity of the conserved motifs was then detected by antimicrobial peptide database[18]. The evolved residues with

conserved antimicrobial activity were identified from the multiple sequence alignment of the motifs in all the PR5 sequences.

Results and Discussion

The present study describes the isolation and characterization of eight PR5 genes from different species of *Zingiberaceae*.

Isolation of PR5 cDNA and Sequence Analysis

PCR amplifications resulted in the isolation of cDNAs with the expected size from the 8 different *Zingiberaceae* species [*A. luteocarpa* (*Al*PR5; JX458082.1), *C. amada* (*Ca*PR5-1; JX458076.1), *C. aromatica* (*Car*PR5; JX458080.1), *C. longa* (*Cl*PR5; JX458079.1), *E. cardamomum* (*Ec*PR5; JX458081.1), *H. coronarium* (*Hc*PR5; JX458083.1), *Z. officinale* (*Zo*PR5-1; JX458077.1) and *Z. zerumbet* (*Zz*PR5-1; JX458078.1). The different cDNA clones revealed that full-length cDNA was in the range of 652 (*Zz*PR5-1) to 687 bp (*Ec*PR5). The deduced nucleotide sequences exhibited strong similarity to the *Z. zerumbet* PR5 (*Zz*PR5; ACL80664.1), and *C. amada* (*Ca*PR5; AEH41422.1) and *Z. officinale* (*Zo*PR5; AEH41423.1) mRNAs. The comparison of the nucleotide sequences with three other PR5s is given in Supplementary Fig. 1.

The number of amino acids varied from 217-228, with *Z. zerumbet* having the lowest (217) and *C. longa* having the highest (228) number of amino acids (Table 1). The comparison of deduced amino acid sequences with other PR5s revealed expected similarities, confirming them to be PR5 gene belonging to the 'thaumatin-like' sub group (Fig. 1). However, does the addition or deletions of amino acids in certain PR5 have any role for the activity of this protein, remains to be seen. *Ca*PR5-1 was predicted to encode a precursor protein of 224 amino acid residues with an isoelectric point of 7.69, whereas *Zo*PR5-1, which also encoded a precursor protein of 224 amino acids, showed an isoelectric point of 4.56, suggesting significant differences in the non-essential regions of the protein. The precursor proteins of other species, *viz.*, *A. luteocarpa, C. aromatica, C. longa, E. cardamomum,*

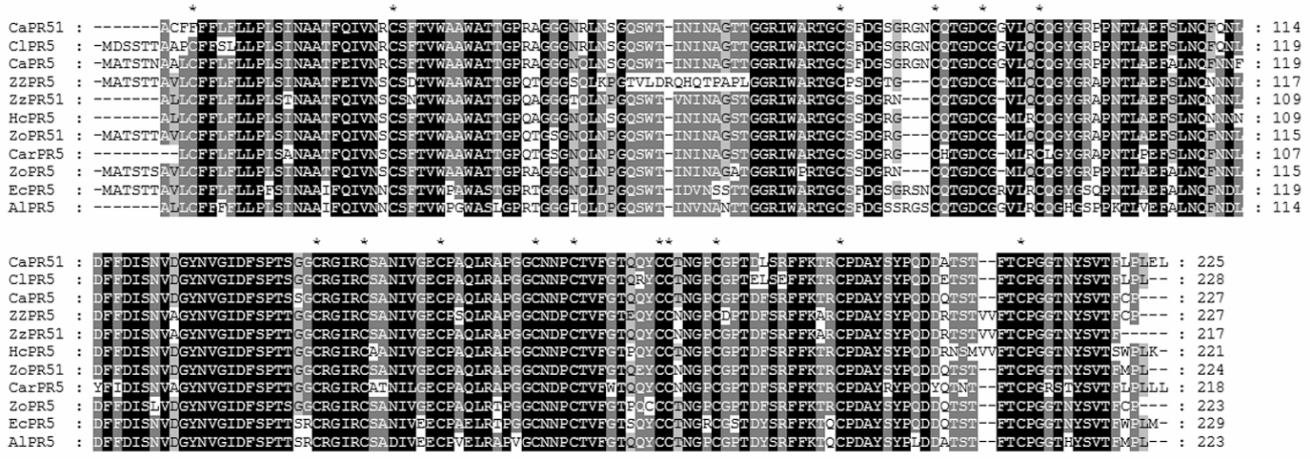

Fig. 1—Multiple amino acid sequence alignments of PR5s from species of *Zingiberaceae* using the ClustalW program. Conserved residues are shaded in black. Asterisks show the conserved cysteine residues.

Table 1—Amino acid sequence comparison between the predicted full length PR5 cDNAs

PR5 proteins	Amplicon size (no. of nucleotides)	Protein size (no. of amino acids)	Homology in amino acids (%)		
			*Ca*PR5 (AEH41422.1)	*Zo*PR5 (AEH41423.1)	*Zz*PR5 (ACL80664.1)
*Al*PR5 (AFV46361)	670	222	83.33	75.67	66.21
*Ca*PR5-1 (AFV46355)	676	224	92.85	85.20	72.32
*Cl*PR5 (AFV46358)	685	228	88.15	82.06	68.28
*Car*PR5(AFV46359)	655	219	78.99	82.64	75.79
*Ec*PR5 (AFV46360)	687	227	85.02	81.61	70.48
*Hc*PR5(AFV46362)	665	223	82.51	85.65	80.26
*Zo*PR5-1 (AFV46356)	673	224	87.50	91.92	84.37
*Zz*PR5-1(AFV46357)	652	217	83.41	86.63	85.71

H. coronarium and *Z. zerumbet*, had 222, 219, 228, 227, 223 and 217 amino acids, respectively.

To understand better the evolution of TLP subfamily of PR5s, we conducted a comprehensive search of available genome sequences. Alignment analysis was done using 30 known TLPs sequences selected from other plant species. The phylogenetic tree was constructed using MEGA 4[11], in which the evolutionary distances were computed using the Poisson correction method. The topological pattern of the resulting tree indicates that the PR5 is conserved among different plant species, with the possibility of 3 sub groups (I-III), each containing proteins with relatively high homology. Group I contained representative sequences from *Solanum*, *Arabidopsis, Cucumis, Helianthus*, *Cocoa* and *Piper* and found far away from other two groups. The PR5 sequences from *Zingiberaceae* formed a distinct sub cluster in group I (Fig. 2). This phylogeny showed a paraphyletic grouping of *Zingiberaceae* PR5s with at least 3 distinct clades. The *Ca*PR5 and *Cl*PR5 were placed in close proximity to *Al*PR5 and *Ec*PR5. In this comparison, the *Car*PR5, *Hc*PR5 and *Zz*PR5 grouped into a single clade. The Phylogenetic analysis of PR5s from *Zingiberaceae* showed that all of them share significant homology. Group III only consisted of monocots, *viz.*, *Zea*, *Oryza* and *Hordeum*, while few other PR5s were localized in another clade of group II. The close evolutionary relationship between PR5s was also observed previously[19,20].

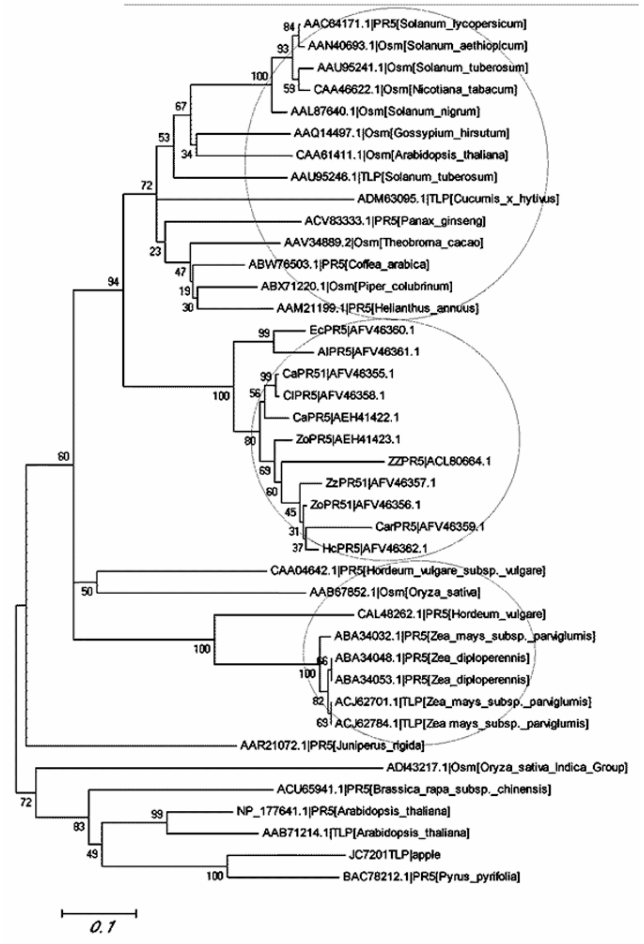

Fig. 2—Phylogenetic relationships between PR5s from species of *Zingiberaceae* with closely related other PR5s based on amino acid sequences.

Protein Parameters

Among the different PR5s of *Zingiberaceae*, *Ca*PR5 showed maximum homology of 92.85% with *Ca*PR5-1, followed by with *Cl*PR5 (88.15%) and *Zo*PR5 (87.50%); while least similarity was recorded with *Car*PR5 (78.99%) (Table 1). In case of *Zo*PR5, the homology was in the range 75.67 (*Al*PR5) to 91.92% (*Zo*PR5-1). However, the maximum homology for *Zz*PR5 (85.71%) was observed with *Zz*PR5-1 and the minimum with *Al*PR5 (66.21%).

The different physical and chemical protein parameters for PR5 proteins were analysed using the Expasy Protparam tool[13] and the results are presented in Table 2. The mol wt of PR5s varied only slightly and the lowest value (22988.4 Da) was observed for *Z. zerumbet* (ZzPR5-1), while the highest (24417.9 Da) value was noticed for *E. cardamomum* (*Ec*PR5). However, the isoelectric point of PR5 proteins considerably varied in the range of 4.56 (*Z. officinale*) to 8.14 (*C. aromatic*). The instability index of all the proteins were found to be well within the range of stability with all values being less than 40. Further, all the PR5 proteins had GRAVY value less than 0, ranging from -0.146 to -0.286; all negative values indicate their hydrophilic nature. The aliphatic index of a globular protein is related to its thermostability and is determined by the volume occupied by the aliphatic amino acids, such as, leucine, isoleucine, valine and alanine. The aliphatic index values of the given proteins were found to be in the range of 50-60 (Table 2). The instability index and aliphatic index that determine the stability of the proteins were similar for all the eight proteins, confirming that all the proteins were stable.

The disulphide bonds and their positions in the protein were detected using Rasmol software[21]. Most of the PR5s possess 16 conserved cysteine residues and form 8 disulfide bonds[20]. In the present study, the eight PR5s also had 16 cysteines and the presence of

Table 2—Protein properties of different PR5s (*Zingiberaceae*)

Protein	No. of amino acids	Mol wt (Da)	IP	II	AI	GRAVY	NR	PR
*Al*PR5	222	23968.8	5.01	27.7	61.04	-0.009	17	13
*Ca*PR5	228	24233.1	5.26	23.94	50.13	-0.179	13	12
*Ca*PR5-1	224	23839.5	7.69	26.69	56.65	-0.176	12	13
*Car*PR5	219	23825.1	8.14	28.89	53.52	-0.170	10	13
*Cl*PR5	228	24357.2	6.10	28.33	53.07	-0.286	14	13
*Ec*PR5	227	24417.9	4.68	26.7	50.70	-0.272	17	13
*Hc*PR5	223	23872.8	7.48	23.33	51.66	-0.212	12	13
*Zo*PR5	223	23646.4	5.18	26.14	53.86	-0.115	12	11
*Zo*PR5-1	224	23847.5	4.56	25.09	53.62	-0.146	14	10
*Zz*PR5	227	24077.8	4.68	28.19	57.18	-0.150	17	12
*Zz*PR5-1	217	22988.4	5.19	23.68	52.63	-0.192	12	11

IP, Isoelectric point; II, Instability index; AI, Aliphatic index; NR, Negative residues; PR, Positive residues

8 disulphide bonds were detected with CYS 179-184 and CYS 151-161. The formation of 8 disulfide bonds between 16 cysteines represents a very thermostable and pH-stable compound[22]. The disulfide bridges formed by these conserved cysteines help stabilize the molecule and allow for correct folding and high stability under extreme thermal and pH conditions[23] as well as for resistance to protease degradation[24].

Molecular Modelling

Three dimensional (3-D) model of the PR5 protein sequences were created using Modeller 9v8 software. Banana (*Musa acuminata*) thaumatin-like protein 1Z3Q was taken as a template. It has 63.3 (*Ec*PR5) to 70.9% (*Ca*PR5) similarity with all the eight PR5s. The crystal models obtained for all the 8 species have the characteristic thaumatin-like fold. Analysis of the secondary structure among PR5s revealed the presence of 1-2 α-helices and 14-17 β-strands (Table 3). Since these are the most variable regions among comparisons of homologous proteins, such differences are often found in acceptable structural models[25]. Molecular modeling revealed that PR5 proteins of *Zingeberaceae* species possessed the same 3-D folds and core structures that are conserved in other PR5 proteins. Three distinct domains are evident in the 3-D model of the studied eight PR5s (Fig. 3). The present results are found consistent with the previously characterized PR5 proteins[4]. Domain I form the central core of the molecule, which corresponds to the N terminus of the protein. It is made up of two bundles of antiparallel β-sheets connected by loops to form a flattened β-sandwich. Domain II comprises a main α-helix associated with shorter helical segments, and domain III consists of β-strands linked by a loop. A prominent deep cleft that

Table 3—Secondary structure analysis of different PR5s

Protein	No. of α helices	No. of ß sheets
*Al*PR5	2	16
*Ca*PR5	2	15
*Ca*PR5-1	1	17
*Car*PR5	-	16
*Cl*PR5	1	15
*Ec*PR5	1	14
*Hc*PR5	1	17
*Zo*PR5	2	16
*Zo*PR5-1	2	14
*Zz*PR5	2	15
*Zz*PR5-1	1	17

transverses the protein surface is found at the interface of domains I and II. The presence of a long and deep cleft that traverses the surface of the proteins is an important feature of these proteins[26]. In all PR5 proteins with known antifungal activity, this cleft has been found to be acidic because of five highly conserved amino acids (arginine, glutamic acid & 3 aspartic acid residues). This acidic cleft is assumed to be relevant to their specific receptor binding for an antifungal activity[27-29].

Further, the modeled proteins were used in docking studies using (1,4)-β-D-glucan as the ligand. (1,4)-β-D-glucans are important constituents of fungal and bacterial cell wall. Suggestions that the PR5 proteins might exert their antifungal activity at the fungal cell wall level have been supported by observations that barley thaumatin-like proteins bind to (1,3)-β-D-glucans[30], which is commonly found in fungal walls. The level of affinity calculated *in silico* between the cleft of a PR5 protein and a (1,4)-β-D-glucan molecule is a good predictor of the proteins' (1,3)-β-D-glucanase activity and antifungal efficacy *in vivo*[26,31]. The docking studies between the PR5

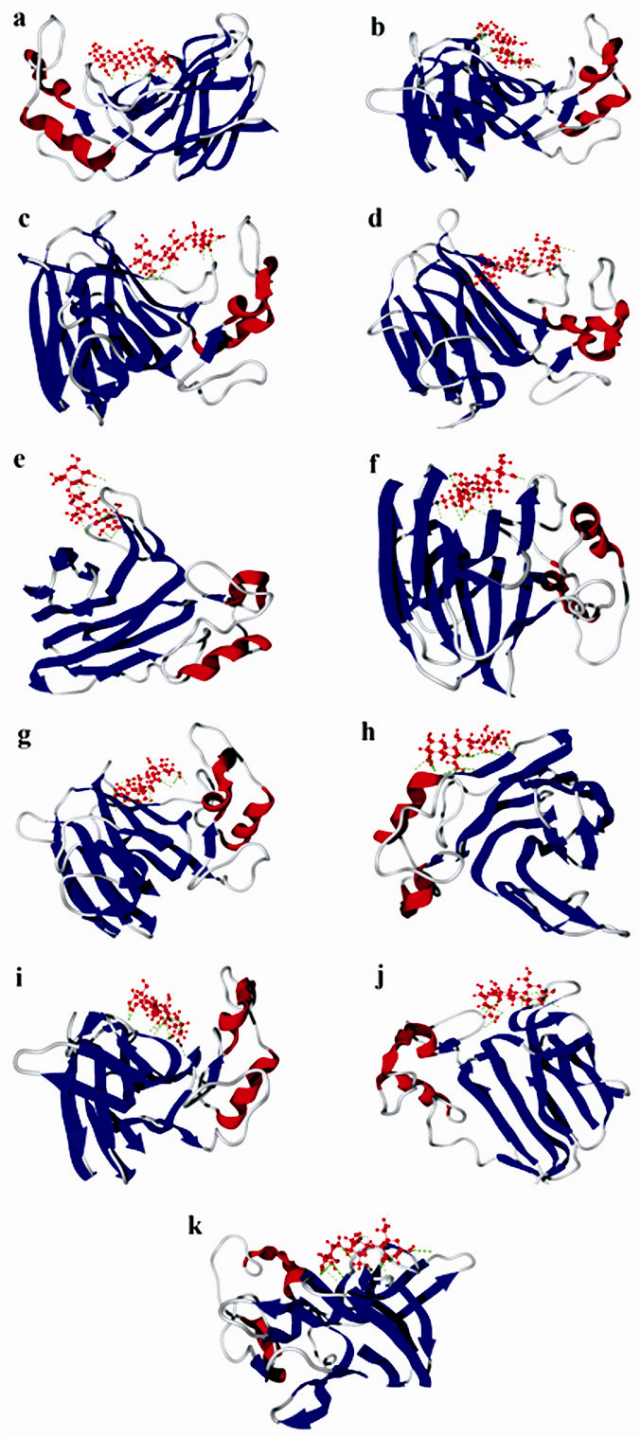

Fig. 3 (a-k)—A ribbon representation of PR5s, showing the three domains and hydrogen bond interactions between the different PR5 proteins and the ligand (1,4)-β-D-glucan: a. *Ca*PR5, AEH41422.1; b. *Ca*PR5-1; c. *Zo*PR5, AEH41423.1; d. *Zo*PR5-1; e. *Zz*PR5, ACL80664.1; f. *Zz*PR5-1; g. *Car*PR51; h. *Cl*PR51; i. *Ec*PR51; j. *Hc*PR51; & k. *Al*PR51. The ligand molecule and interacting residues of PR5 are shown as stick models.

proteins and the ligand (1,4)-β-D-glucan helps in determination of the efficient interactions. The MolDock scores in all the PR5 protein-ligand interactions ranged from -53.3755 (*Zz*PR5) to -94.5192 (*Al*PR5), providing the evidence for efficient interactions (Table 4). PR5 proteins are believed to disrupt proper assembly of the fungal cell wall during hyphal extension by binding to nascent (1,4)-β-D-glucan molecules[26]. The interactions are mainly by means of hydrogen bond. The hydrogen bond interactions between the protein and the ligand in the present study varied from 12 to 20 and were found to be strong enough for a stable interaction.

Antimicrobial Peptide Prediction

The MotifMaker online tool was used to find the conserved motifs with putative antimicrobial activity in the eight PR5 sequences resulting in three such motifs. Of these, two motifs exhibited putative antimicrobial activity and the remaining one did not show any such activity. The two antimicrobial motifs detected were, Motif 1: FFLFLLPLSINAATFQIVNSCSFTVWAAWATTGP RAGGGNQLNPGQSWTI, and Motif 2: QLRAP GGCNNPCTVFGTQQYCCTNGPCGPTDFSRFFK TRCPDAYSYPQDD (Fig 4a). The antimicrobial motifs were then subjected to ClustalW program for multiple sequence alignment, which led to the interpretation that these motifs are conserved throughout, in spite of changes in other residues (Fig 4b). Thus we can presume their possible antimicrobial role.

The present study has led us to a better understanding of PR5 proteins in *Zingiberaceae*. The isolated PR5 genes from eight species of *Zingiberaceae* have shown high homology with their homologous genes in other species. Present analyses based on the docking studies have shown that the biological functions of PR5 proteins are relative to disease resistance. It has led to the identification of antimicrobial peptides, which remain conserved among all the cloned PR5 genes. Further studies characterizing these motifs as well as the promoter sequences of these eight PR5 genes would pinpoint their exact role in disease resistance. Furthermore, the characterization of transgenic plants overexpressing these PR5s would help clarify the relationships between their structures and functional roles in environmental stress tolerance.

Table 4—Results for docking analysis with RMSD and number of hydrogen bonds

Protein	MolDock score	Rerank score	RMSD	Interaction	H bond	No. of H bond
*Al*PR5	-94.5192	-42.5316	19.7446	-141.063	-18.0315	19
*Ca*PR5	-69.1080	-78.0976	34.1077	-110.610	-16.4175	15
*Ca*PR5-1	-71.6656	-75.0238	27.8765	-109.005	-15.0244	16
*Car*PR5	-95.4948	-96.8880	28.9540	-135.321	-16.9651	21
*Cl*PR5	-90.2616	-86.5170	30.7104	-136.441	-13.1127	16
*Ec*PR5	-73.7165	-80.7036	32.5848	-111.315	-19.4750	18
*Hc*PR5	-79.1163	-88.2443	27.3523	-136. 219	-20.5190	17
*Zo*PR5	-76.4397	-85.4078	39.6182	-118.684	-10.8213	16
*Zo*PR5-1	-73.7774	-77.0580	32.9796	-115.684	-11.6028	19
*Zz*PR5	-53.3735	21.8662	12.0225	-97.9361	-8.09007	17
*Zz*PR5-1	-74.2175	-78.191	37.2837	-116.526	-19.5051	20

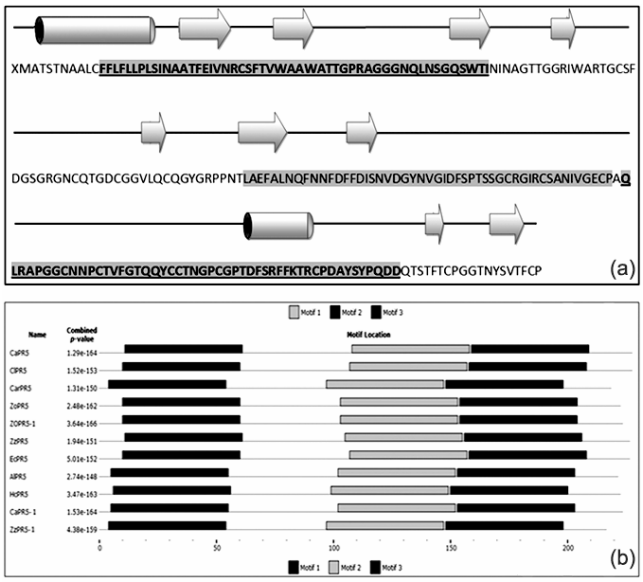

Fig. 4 (a & b)—Comparison of the antimicrobial motif in PR5: a. Secondary structure depicting the three conserved motifs in *C. amada* [Bold highlighted motifs indicate the antimicrobial peptides, while the underlined ones are found with no such activity]; b. Combined block diagram of different PR5s for conserved motifs.

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