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IN-SILICO ANALYSIS OF PATHOGENESIS-RELATED PROTEINS (PR-1) IN BARLEY (*HORDEUM VULGARE* L.)

Krishna Kumar Yadav[1], Pradeep Kumar1,[2*], Alamgir[1, 2], Ajay Kumar Singh[1], A. Radhakrishna[1, 3], Praveen Kumar Yadav[1], Ayush Yadav[1], and Krishna Kant Kushwaha[1]

[1]ICAR- Indian Grassland and Fodder Research Institute Jhansi U.P. 284003, India
2 S V P University of Agriculture and Technology, Meerut -250110, U.P. India
3 ICAR- National Institute of Abiotic Stress Management, Baramati, Pune, Maharashtra, India
*Corresponding Author E-mail: pradeepkumarbadal@gmail.com
https://orcid.org/0000-0002-3150-1321
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ABSTRACT

A voide range of proteins is induced in the plant, once the defensed is alarmed including pathogenesis related proteins (PRs). The aim of my research was to study understand the role and possible function of PRs in plant defense. The studies are mainly focused on PR-1 CAP domain. The CAP domain was confirmed as being highly conserved in all these PR-1s. These PRs accumulated in both leaves and roots of barley seedlings as a response to infection of the respective tissues. Phylogeny-based inference exposed that PR-1 proteins clustered into five major groups, with the majority of PR-1s distributed in Group A (1 out 15), Group B and C (2 PR-1 each out of 15), Group D (4 out 15) and the Group E (6 out 15) respectively. PRs defense system of plants may respond and could be categorized into various families according to similarities in their sequences, enzyme-related properties and structural aspects. PRs appear to be part of a preformed defense since PR-1 was constitutively present in both xylem and phloem tissues and the root epidermis. These results suggest that different induction strategies may be active in Hordeum vulgare seedlings depending on the primary site of infection by *B. sorokiniana*.
Keywords **:** Pathogenesis-related proteins, PR-1, plant defense, barley, *Hordeum vulgare*, microscopy.

Introduction

Barley (*Hordeum vulgare* L.) is the fourth largest cereal & fodder dual purpose crops worldwide in case of planting area with a share approximately 7.0 % of the global cereal production (Pal *et al*., 2012). The greatest share of the world's Barley grain is used for animal feed, followed by malting, seed production and human food. Cultivated Barley is self-pollinating, diploid (2n=2x=14) species of the tribe Triticeae, family poaceae.

Barley is predominantly grown under rainfed conditions in the northern hill with very low inputs used by farmers (Verma *et al*., 2005). Green forage yield at the proper vegetative stage and grain yield after vegetative growth prepares the plant to form the developing head and yield components. Grain yield is largely determined by grain size & number, which is set by reproductive tiller and fertile spikelet number and survival. Grain-filling starts about five to ten days after pollination and continues until the grain is ripe. In India, Barley is mainly used as cattle and poultry feed after that its utilization for malting and beverages. 5 % of total production consumed by human as energy drinks like Bournvita, Horlicks, and biscuits prepared from malt extract. Though, India barley production fluctuated substantially in recent years, it tended to decrease through 1970-2018 period ending at 1.74 million tons in 2017-2018. In 2018, 1.78 million tons of barley was produced in India from 0.74 million ha land area with productivity of 2679 kg/ha. In Uttar Pradesh, barley covered an area of about 1.70 lakh ha with a production of 2.87 lakh tons and productivity of 1690 kg/ha. It is also known as poor man's crop

because of its low input requirement and better adaptability to drought, salinity, alkalinity and marginal lands (Gyawali *et al*., 2018; Kumar *et al.,* 2023).

A number of biotic and abiotic stresses pose a challenge to increase the production of barley. Barley leaf spot blotch is caused by *Bipolaris sorokiniana* (Telomrph = *Cochliobolus sativus*) and powdery mildew is caused by *Blumeria graminis* f. sp *hordei*. These are serious fungal diseases with wide geographical distribution. The defense strategy of plants against stress factors involves a multitude of tools, including various types of stress proteins with putative protective functions. A group of plant-coded proteins induced by different stress stimuli, named "pathogenesis related proteins" (PRs) were assigned an important role in plant defense against pathogenic constraints and in general adaptation to stressful environment. A large body of experimental data has been accumulated and changing views and concepts on this hot topic have been evolved. Plants have no immune system, though; coevolution of plants and pathogens has created a multifaceted relationship, resulting from the exchange of molecular information between the species (Benhamou, 1996). Based on this, plants have developed a complex defense mechanism. Pathogens, on the other hand, possess strategies to overcome the defense system and colonize plants. The difference between success and failure of plant defense is most likely dependent on the time it takes for the plant to recognize a potential pathogen and subsequently activate the defense system (Garcia-Brugger *et al*., 2006). Once the defense is alarmed a wide range of proteins is induced in the plant; among these the pathogenesis-related proteins (PRs) (van Loon *et al*., 2006). The specific function PRs proteins strong antimicrobial activity; PR1 proteins in vitro against different phytopathogens including oomycetes fungi and bacteria invasion. PR proteins comprise of seventeen groups, which are mostly induced by pathogen infection and elicitor treatment; and they contribute to disease resistance in many plant species. Several PR proteins belonging to the group 5 (PR5) exhibit amino acid and structural similarity to an intensely sweet tasting protein, thaumatin, from the fruits of the West African shrub, *Thaumato coccusdaniellii*, and hence are called thaumatin-like proteins (TLPs). PR5 family includes basic and acidic members according to their isoelectric points, although they show similar activity. Some of the recombinant PR5 proteins that have been purified are antifungal. PR5 proteins generally exert their antifungal activity through a very fast and dramatic increase in the permeability of the pathogen's plasma membrane, by disrupting the lipid bi-layer and creating trans-membrane pores. Hence, some of them are also called permatin.

Over-expression of PR5 proteins in transgenic plants have generally resulted in enhancing disease resistance in some plant species. For example, potato osmotin enhances resistance to potato late blight pathogen *Phytophtohra infestans*; the rice *TLP-D34* enhances resistance to the sheath blight pathogen, Rhizoctonia *solani*; rice *TLP* enhanced resistance to the head blight pathogen *Fusarium graminearum*. Constitutive expression of *Vitis vinifera* TLP, VVTL-1 plays an important role in grape resistance to anthracnose and transgenic grapes expressing VVTL-1 exhibit sustained resistance to several fungal pathogens such as *Uncinulanecator* and *Botrytis cinerea*. Upon pathogen infection, plants initiate a large spectrum of defense responses involving several pathways including the deployment of PR proteins. Further, plants also synthesize small secondary metabolites such as phytoalexins that are fungi toxic and are associated with disease resistance. The present study was aimed to understand the pathogenesis related proteins in Barley (*Hordeum vulgare* L.).

Materials and Methods

The study was carried out at the ICAR-Indian Grassland and Fodder Research Institute (IGFRI), Jhansi, Uttar Pradesh during 2021-2022. The present study was aimed to understand the pathogenesis related proteins in Barley (*Hordeum vulgare* L.) and i*n-silico* characterization of Genome-wide identification of pathogenesis-related protein-1 (PR-1) genes in Barley (*Hordeum vulgare* L.)

Identification of Pathogenesis-Related Proteins (PR-1)

The PR-1 nucleotide sequence of maize and wheat was retrieved from NCBI database. For the identification of pathogenesis-related protein (PR-1) group in Barley (*Hordeum vulgare* L.) CAP domain of maize and wheat was used as a query sequence to search against local Hordeum vulgare available on BOGE protein database with an E value cutoff of 0.001. Preliminarily identified PR-1 protein sequences were also checked for the presence of CAP domain using NCBI Conserved Domains Databases (CDD) batch search. Sequences were lacking the whole or incomplete domain were discarded from our analysis. The domain was visualized using Tbtool. The molecular weight (MW), isoelectric point (pl) and protein length etc were determined using an online tool available at protparam (https://web.expasy. org/ protparam/).

Multiple Sequence Alignment

To understand the conservedness of the PR-1 proteins, the CAP-derived peptide (CAPE) of PR-1 proteins was used for multiple sequence alignment in *Hordeum vulgare* L. using Clustal W programme in MEGA (Molecular Evolutionary Genetic Analysis) version X software.

Phylogenetic Analysis

The unrooted phylogenetic tree of identification of PR-1 proteins in *Hordeum vulgare* L. was constructed by using MEGA (Molecular Evolutionary Genetic Analysis) version X. The reliability of the obtained phylogenetic tree was tested using a bootstrap value of 1000 iterations.

Structure of PR-proteins

The 2° and 3° structure was prediction using Phyre 2 (https://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) with default parameters.

Characterization of Motif Analysis in PR-1 Proteins

Organization of conserved motifs of PR-1 protein was identified by MEME (Multiple Expectation Maximization for Motif Elicitation) software. (http:meme-suite.org/intex.html) MEME is a searching tool for discovering motif in a group of related DNA or protein sequences. The default parameters were used in MEME and maximum ten motif. The motif was visualized using Tbtool.

Results and Discussion

Pathogenesis related proteins (PRs) are a group of plant-coded proteins induced by different stress stimuli and play an important role in plant defense against pathogenic constraints and in general adaptation to stressful environment. PR proteins comprise of seventeen groups, which are mostly induced by pathogen infection and elicitor treatment; and they contribute to disease resistance in many plant species. *PR-1* genes also play important roles in response to abiotic stresses. In rice, stress associated proteins 1 (OsSAP1) induce the endogenous stress-related genes such as aminotransferase (OsAMTR1), SCP/TAPS or pathogenesis-related-1 class of protein (OsSCP). Liu *et al* (2013) showed that a transcription regulator, *Di19* (Drought-induced) gene induced up-regulation of pathogenesis-related *PR-1*, *PR-2* and *PR-5* genes expressions in *Arabidopsis*. Seo *et al* (2010) reported that plasma membrane-tethered NAC (NAM/ATAF1/2/CUC2) transcription factor NTL6 induced some *PR* genes by directly binding to promoter sequences of cold-responsive *PR* genes, *PR-1*, *PR-2* and *PR-5*.

Identification of Pathogenesis-Related Proteins (PR-1)

In order to identify the putative PR-1 genes in Barley (*Hordeum vulgare* L.), previously identified CAP protein sequences of maize and wheat were used as query sequences to BLAST search *Hordeum vulgare* genome available at CoGe (https://genomevolution.org). The positions of the genomic sequences and coding sequences were retrieved in FASTA format. Also, search was carried out using HMM search, using the CAP super family seed sequence (PF00188) from Pfam database against the *Hordeum vulgare* genome database. The putative sequences were further BLAST searched for CAP domain using NCBI Conserved Domain Database (CDD) tool. The 15 sequences obtained from the *Hordeum vulgare* genome were found to have the CAP domain (Figure 1). Among the crop species (Table 1) garlic showed the lowest PR-1 genes (5) while wheat genome was found to have the highest PR-1 genes (54). A total of 15 *PR-1* genes/proteins, details of which were presented in Table 2 were identified in the genome of barley. It was determined that all 15 Barley PR-1 proteins contained a CAP superfamily (cysteine-rich pathogenesis-related 1 proteins (CAP) domain structure (PF00188) based on the Pfam database and NCBI conserved domains database suggesting well conserved domain structures in the PR-1 proteins. Molecular weights of the PR-1 proteins were found between 14.38 and 23.01 kDa. Akbudak *et al*. (2020) reported that PR proteins were generally low-molecular weight proteins of about 13.48 and 22.83 kDa in tomato, while Loon *et al* (1994) reported about 6–43 kDa proteins, which is in agreement with our findings.

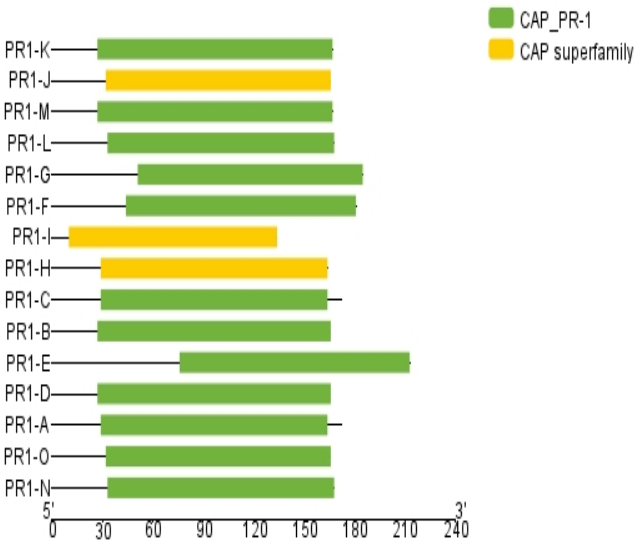


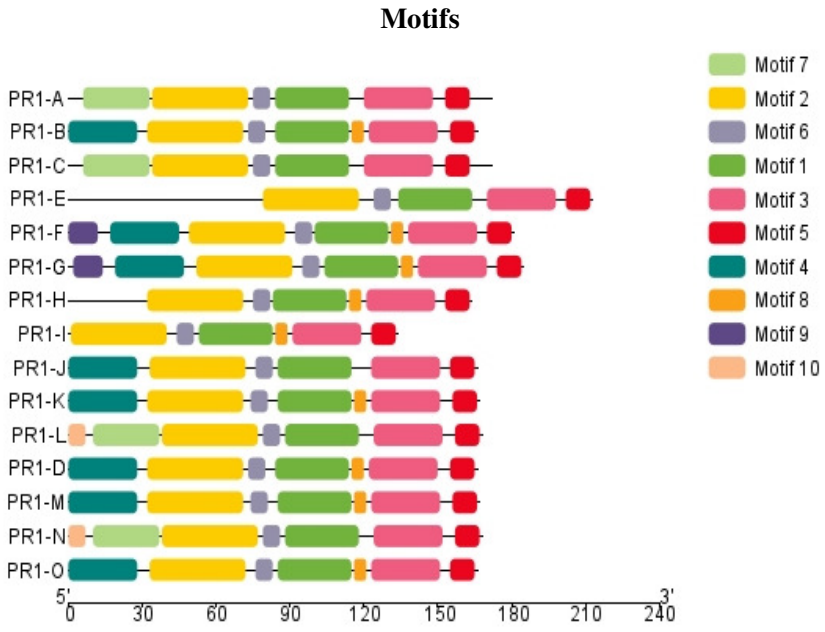
Fig. 1 : The schematic representation of CAP PR-1 subfamily protein structure. The schematic representation focuses on the structure composition of conserved CAPs sequences.

Analysis and prediction were done on the PR-1s properties, such as protein size, MW, pI, & GRAV. The protein lengths ranged from 134 to 213 amino acid residues. In view of the *pI* data, it is understood that four of the 11 PR-1 proteins are acidic while 4 out of 15 have a basic character. These variations in the *pI* data may be related with functional diversities of the PR-1 proteins in barley. The size and molecular weight of PR1-I (134 amino acids and 14.38 kDa) were much lower than those of other PR1s proteins, suggesting that they might have antimicrobial functions and undergone different evolutionary processes.

Table 1 : This table representation of PR-1s superfamily with Gene of PR-1-A to PR-1-O, Transcript ID, localized with different chromosomes, with protein length, PI, GRAVY, Aliphatic index and Instability index.

S. No.	Gene	Transcript ID	Chr. No	Mol. Weight (kDa)	Theoretical pI	Protein length (aa)	GRAVY	Aliphatic index	Instability Index
1	PR1-A	HORVU7Hr1G040730.1	7	18.67	9.32	172	-0.347	61.92	32.11
2	PR1-B	HORVU5Hr1G106010.1	5	17.70	4.56	166	-0.263	70.00	44.18
3	PR1-C	MLOC_63125.1	7	18.67	9.32	172	-0.347	61.92	32.11
4	PR1-D	MLOC_72965.1	5	17.70	4.56	166	-0.263	70.00	44.18
5	PR1-E	HORVU6Hr1G083390.1	6	23.01	6.64	213	-0.066	72.63	50.52
6	PR1-F	HORVU3Hr1G114820.1	3	19.76	5.45	181	0.029	80.39	37.62
7	PR1-G	HORVU0Hr1G007430.1	Un	19.84	5.04	185	0.046	79.19	33.07
8	PR1-H	HORVU3Hr1G114790.1	3	18.06	5.02	164	-0.142	60.06	29.37
9	PR1-I	HORVU0Hr1G026520.1	Un	14.38	4.94	134	-0.117	66.27	43.2
10	PR1-J	HORVU0Hr1G026530.1	Un	17.83	4.71	166	-0.023	76.51	33.24
11	PR1-K	HORVU5Hr1G106020.1	5	17.87	5.71	167	-0.137	71.92	39.76
12	PR1-L	HORVU7Hr1G022230.1	7	17.96	8.15	168	-0.074	64.58	30.09
13	PR1-M	MLOC_43156.1	5	17.87	5.71	167	-0.137	71.92	39.76
14	PR1-N	MLOC_14514.1	7	17.96	8.15	168	-0.074	64.58	30.09
15	PR1-O	HORVU0Hr1G026540.1	Un	17.92	4.52	166	-0.056	75.84	40.77

Most of the PR-1 proteins possessed four different chromosomes and 4 show unknown chromosome (Table-1). Multiple sequence alignment of the PR-1 protein sequences showed conserved CAP-derived peptide (CAPE) and caveolin-binding motif (CBM) in all proteins (Figure 2). Cysteine-rich secretory protein, antigen 5 (Nath *et al.,* 2015) and pathogenesis-related (CAP) domain structures were found to be well-conserved in all the barley PR-1 proteins.



Location	Gene
111160316-111160834	PR1-A
623107592-623108092	PR1-B
121706443-121706961	PR1-C
518543389-518543889	PR1-D
551156226-551156867	PR1-E
691823794-691824339	PR1-F
42224607-42225164	PR1-G
691668843-691669337	PR1-H
139996952-139997356	PR1-I
140005804-140006304	PR1-J
623130527-623131030	PR1-K
32314888-32315394	PR1-L
518872422-518872925	PR1-M
34403828-34404334	PR1-N
140015659-140016159	PR1-O

Fig. 2 : Conserved motifs identified in barley PR-1 proteins. (A) Distribution of conserved motifs in barley PR-1 proteins and location of gene in genome of that particular motifs identified in PR-1 proteins (supplementary file 1).

To provide insight into the evolution of PR-1 proteins phylogenetic analysis was carried out. In this study, the neighbor-joining method with 100 boot-strap using the protein sequences of 15 PR-1 genes was performed to construct the phylogenetic tree. All the 15 PR-1 genes were classified into five groups based on the predicted CAP domain among which, groups E had the largest branch with 6 PR-1 while group D have 4 PR proteins but group B and C had 2 PR-1 proteins members each & group A had only one PR-1 proteins members respectively (Figure 3).

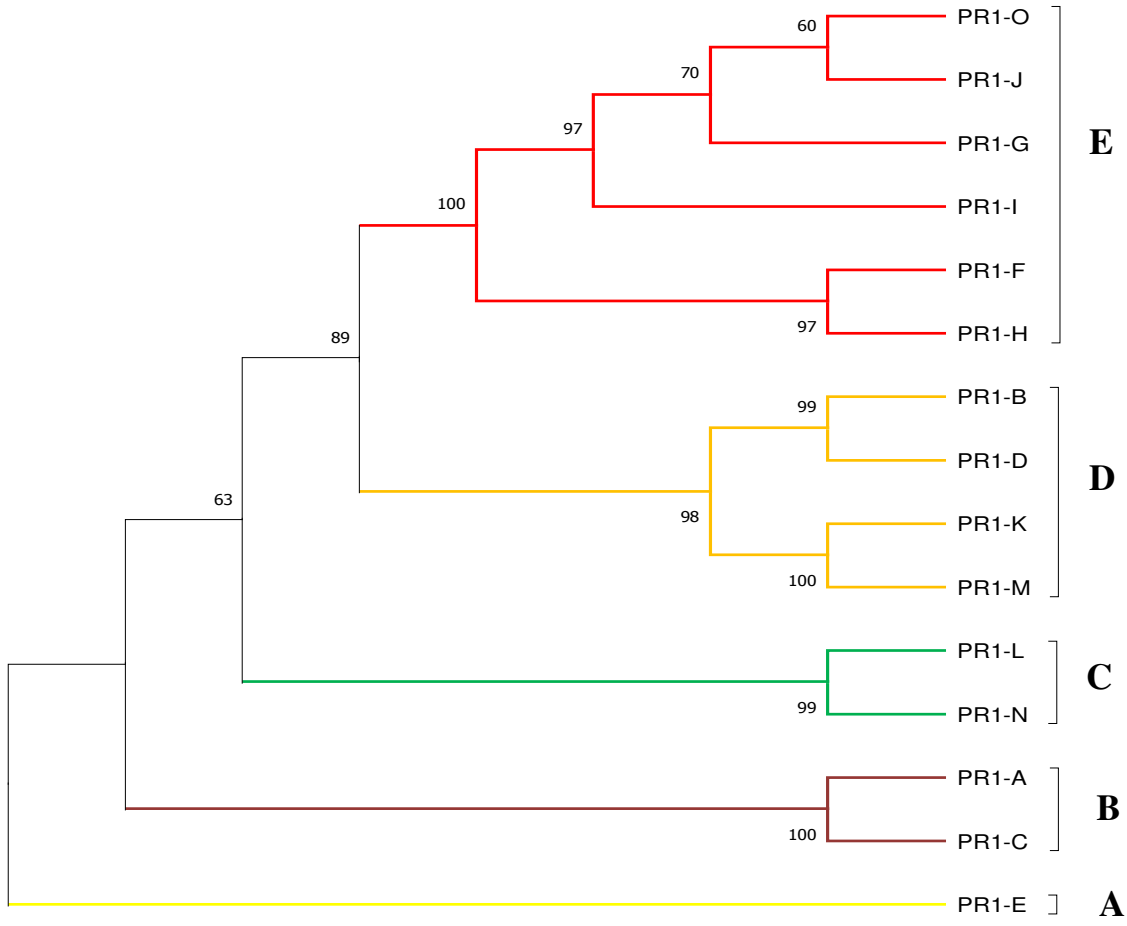


Fig. 3 : Phylogenetic relationship of PR-1 proteins from barley, maize and *wheat.* The phylogeny was constructed by MEGA 11, using the neighbor-joining (NJ) method.

To obtain insights into the diversity of PR-1 genes in barley, the MEME (Multiple Expectation maximizations for Motif Elicitations) programme was used to assess the motif compositions in 15 PR-1 proteins. These motifs are represented in their relative location within the protein. Motif 1 was uniformly observed in all PR-1 proteins and was confirmed to be the conserved CAP domain. Moreover, the barley PR-1 proteins in each group had several special motifs at C-terminal regions, suggesting that they had a similar function. The motifs identified by MEME software are represented by colored boxes and their consensus sequences are shown in Table 3

According to the homologous modeling results, most *H. vulgae* PR-1s were distinguished by a secondary structure similar to d1cfea model (PR1 I, PR1 J, & PR1 K) & c1cfeA model based on template (Figure 4). Similar to the template, α-helixes and β-sheets with highest percentage is 37%, seen in PR1-K, PR1-M, and PR1-O, while the lowest percentage is 20%, and highest percentage β-sheets 23%, found in PR1-I, and the lowest is 15%, seen in PR1-A, PR1-C, and PR1-E (Table 2). But, variation in the protein sequence generated compositional change to their secondary structure.

The analysis highlighted that PR-1 E show the greatest level of disorder at 28% implying a higher degree of flexible regions within the proteins. Whereas the lowest percentage of disordered indicated as more ordered structure. The highest transmembrane content was found in PR1-K, PR1-L, and PR1-M at 10 %, signifies the presence of membrane-spanning protein domain consist of one or several alpha-helices or a transmembrane beta barrel. Almost all proteins indicate perfect confidence level range 99.80% to 100.00% i.e. ensuring good reliability in predicted PR-1s proteins. Protein coverage demonstrate comprehensive alignment with reference sequence, and lowest coverage show the more gaps or mismatches in its sequence alignment. Whereas PR-1H indicate higher (95%) coverage and PR-1E show lowest (63%) coverage of PR-1s proteins.

Table 2 : Overview of the secondary structure of PR-1 proteins and their variations

S.N.	PR name	Alpha Helix	Beta sheet	Disorder	TM	Confidence	Coverage
1	PR1-A	36%	15%	5%	9%	99.90%	78%
2	PR1-B	36%	19%	4%	-	99.80%	81%
3	PR1-C	36%	15%	5%	9%	99.90%	78%
4	PR1-D	36%	19%	4%	-	99.80%	81%
5	PR1-E	30%	15%	28%	-	99.90%	63%
6	PR1-F	30%	17%	8%	9%	99.90%	74%
7	PR1-G	31%	17%	15%	9%	99.80%	72%
8	PR1-H	27%	22%	8%	-	99.90%	95%
9	PR1-I	20%	23%	6%	-	100.00%	90%
10	PR1-J	36%	19%	5%	-	100.00%	79%
11	PR1-K	37%	16%	4%	10%	100.00%	81%
12	PR1-L	34%	17%	11%	10%	99.90%	80%
13	PR1-M	37%	16%	4%	10%	99.80%	81%
14	PR1-N	34%	17%	11%	10%	99.90%	80%
15	PR1-O	37%	16%	5%	-	99.80%	81%

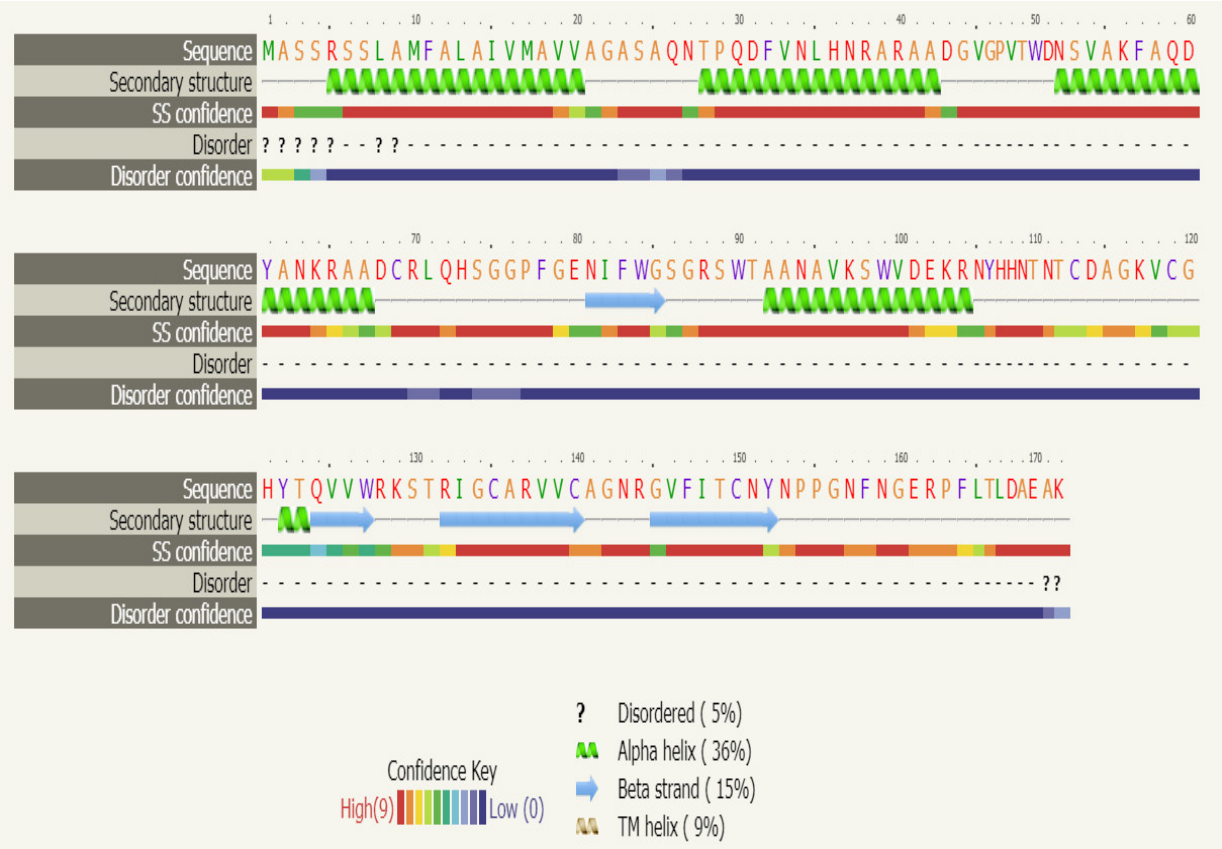


Fig. 4 : Figure representation of local spatial conformation of the polypeptide backbone and TM helix, confidence key, disordered and disordered confidence. (supplementary file 2)

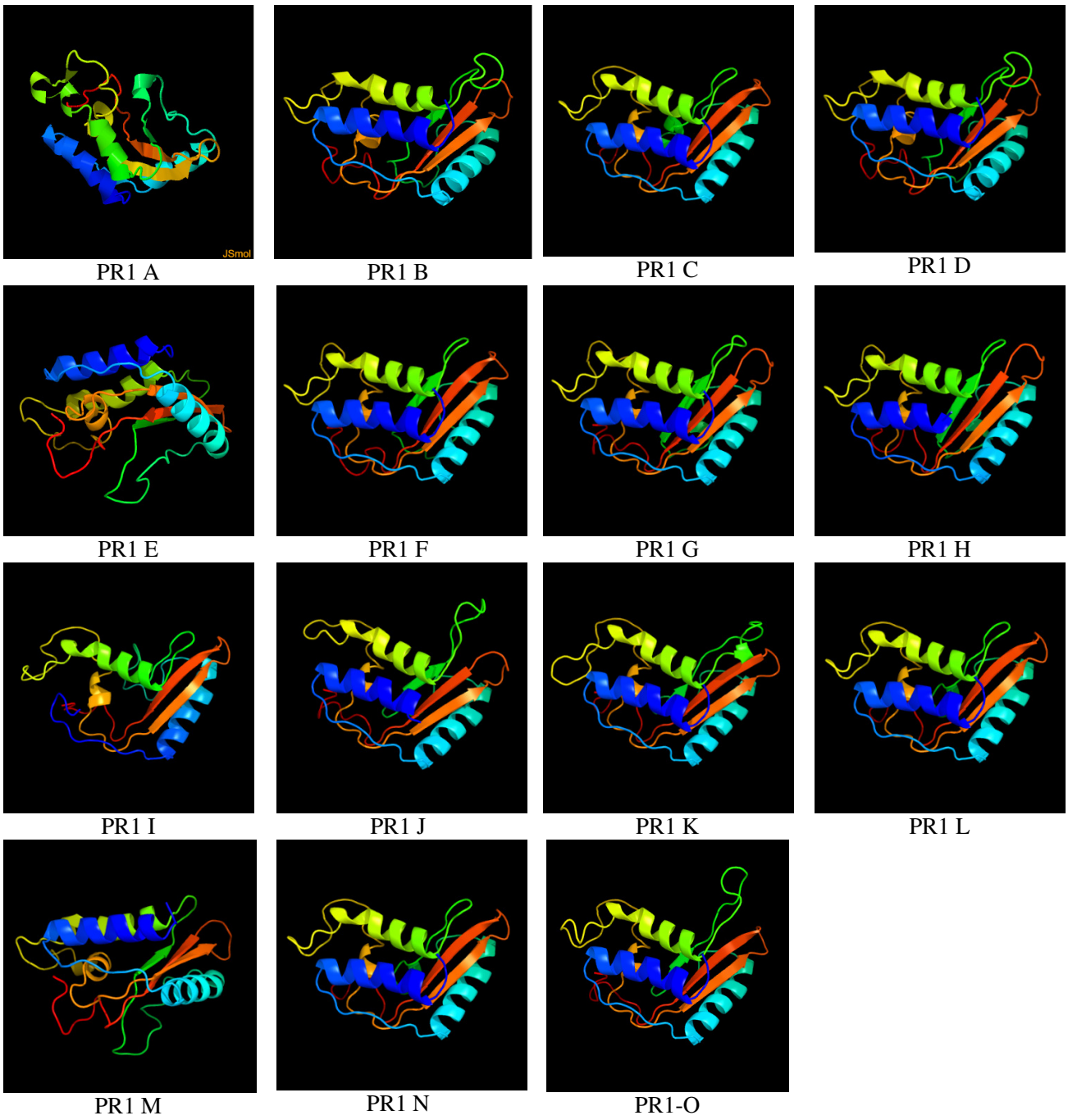


Fig. 5 : Three-dimensional structure determination by phyre2 tools of barley PR-1s. Structure prediction was performed by homologous modeling, using the Phyre 2 server, for which the structure of the maize and wheat pathogenesis-related protein with d1cfea (PR1 I, PR1 J & PR1 K) & c1cfeA model based on template coloured by rainbow N → C terminus.

Conclusions

The defense strategy of plant against stress factors involves a multitude of tools, including various type of stress protein with putative protective functions. A groups of plants coded proteins induced by different stress stimuli, named pathogenesis related proteins (PRs) where assigned an important role in plants defense against pathogenic constraints and in general adaptation to stressful environments. A number of biotic and abiotic stresses pose a challenge to increase the production of Barley. Upon pathogen infection, plants initiate a large spectrum of defense responses involving several pathways including the deployment of a class of proteins known as pathogenesis-related (PR) proteins. The pathogenesis-related protein 1 (PR-1) gene family plays important roles in the plant

metabolism in response to biotic and abiotic stresses. The present study aimed genome-wide identification and *in-silico* characterization of *PR-1* genes in Barley (*Hordeum vulgare* L.). The analyses resulted in the identification of 15 novel *PR-1* genes, each of which produces a protein belonging to the CAP superfamily. Molecular weights of the PR-1 proteins were found between 14.38 and 23.01 kDa. The protein lengths ranged from 134 to 213 amino acid residues with four of the 11 PR-1 proteins are acidic while four out of 15 have a basic character. Aliphatic index of Pathogenesis related proteins -1 were found between 61.92 to 80.39 and instability index verge 29.37 to 50.52, whereas GRAVY is negative of PR1 variants. All the 15 PR-1 genes were classified into large and small five groups based on the predicted CAP domain among which, groups E had the largest branch with five PR-1 members, while smallest group A had one member, but group B and C had two members each, while group D had four members respectively. The secondary structure of PR-1 proteins was analyzed and the functional proteins have antimicrobial function in nature. This is the first study to report the PR-1 genes in Barley at genome-wide scale and the present study offers novel insights into the evolution of PR-1 genes and provides a better understanding of their function.

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Conflicted of interests

No Conflicted of interests of authors.

Reference

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