

Genome-Wide Analysis of *PR-1* Genes From *Piper Nigrum* Reveals Critical Role in Defense Response During *Phytophthora Capsici* Infection

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Research Article

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Abstract

Background: Black pepper is a prominent spice which is an indispensable ingredient in culinary and traditional medicine. *Phytophthora capsici*, the causative agent of foot rot disease causes drastic constraint in black pepper cultivation and productivity. To counterattack various biotic and abiotic stresses plants employ a broad array of mechanisms one such includes the accumulation of pathogenesis-related (*PR*) proteins. Several studies have reported the role of *PR-1* proteins in triggering the plant defenses during plant-oomycete interaction.

Results: Through the genome-wide survey, eleven *PR-1* genes that belongs to a CAP superfamily protein with Caveolin-Binding Motif (CBM) and CAP-derived peptide (CAPE) were identified from *P. nigrum*. Despite the critical functional domains, *PnPR1* homologs differ in their signal peptide motifs, and core amino acid sequence composition in the functional protein domains. The GO, biological function analysis reveals their role in defense responses and response to biotic stimulus whereas the KEGG functional annotation predicted their function in the plant-pathogen interactions. Furthermore, transcriptome-assisted FPKM analysis revealed *PnPR-1* genes mapped to *P. nigrum* - *P. capsici* interaction pathway. The differentially expressed pathogen-responsive *PR-1* gene was validated through qRT-PCR. Subsequent analysis revealed the structural details, phylogenetic relationships, conserved sequence motifs and critical cis-regulatory elements of *PnPR-1* genes.

Conclusion: This is the first genome-wide study that identified the role of *PR-1* genes during *P. nigrum* - *P. capsici* interactions. The detailed *in silico* experimental analysis revealed the vital role of *PnPR-1* genes in regulating the first layer of defense towards *P. capsici* infection in Panniyur-1 plants.

Introduction

Plant immunity involves multiple layers of defense responses. The first layer of defense is triggered by the detection of microbe-associated molecular patterns (MAMPs) through plant-, pathogen- or pattern-recognition receptors (PRRs), which activates PAMP / pathogen / pattern - triggered immunity (PTI) [1]. Plants defense machinery was forced to evolve continuously to combat a wide range of challenges project through harmful pathogens and pests. These challenges activate an array of induced mechanisms such as hypersensitive response (HR) which involves a series of events including the production of reactive oxygen species (ROS), synthesis of antimicrobial molecules and pathogenesis related (PR) proteins. PR proteins induces programmed cell death which further inhibits the spread of infection contribute to systemic acquired resistance (SAR) [2–5]. In the second layer of defense, pathogens suppresses the PTI by secreting the effector proteins which are later recognized by plant resistance (R) proteins leading to effector-triggered immunity [1].

Salicylic acid and the subsequent activation of PR genes are necessary for the establishment of SAR in the distant regions of infections [6]. Arabidopsis mutants deficient in Nonexpressor of Pathogenesis Related 1 (*NPR1*) protein, a key SAR regulator showed less PR gene expression which in turn increased the susceptibility to pathogens [7]. Various plant species overexpressing Arabidopsis *NPR1* in other plant species displayed enhanced disease resistance to a variety of pathogens [8–10]. Till date 17 families of PR proteins have been classified and characterized [11]. PR proteins are generally functionally diverse proteins that are inducible during pathogen attack, and are regulated by the signalling compounds such as abscisic acid (ABA), ethylene (ET), jasmonic acid (JA) and salicylic acid (SA) [12, 13].

PR-1a, the first member of *PR-1* family was identified in *Nicotiana tabacum* plants infected with Tobacco Mosaic Virus. *PR-1* proteins belong to the group of most abundantly produced proteins during plant defense responses, and they are ubiquitous across plant species [14]. *PR-1* proteins are involved in the cell wall thickening and thereby prevents the spread of the pathogens in the apoplast (Wang et al., 2013). The induction of certain members of *PR-1* proteins upon pathogen infection have been studied in many plants [15]. Apart from the biotic stresses, the role of *PR-1* in abiotic stresses [16–18] were also reported. The upregulation, abundance and the localization in the apoplast make them a potential candidate for broad antimicrobial activity [19]. An enhanced tolerance to fungi [20], oomycetes [21] and bacterial infections [22] were demonstrated with overexpression of *PR-1* in the transgenic plants. Apart from its role in various biotic and abiotic stresses *PR-1* and *PR-1 like* proteins have reported to be involved in plant flowering [23] and seed development [24] respectively.

PR-1 proteins are widely reported across the plant kingdom. These are the members of cysteine-rich secretory protein, antigen 5, and pathogenesis-related-1 (CAP) protein superfamily. Stress signalling peptide such as CAPE1 (Cap-derived peptide 1) are embedded within *PR-1* proteins. CAPE-1 peptide that comprises of last 11 amino acids from the C-terminus of the *PR-1* protein was reported from tomato plants subjected to wounding and methyl jasmonate treatment [25]. A caveolin-binding motif (CBM) in the CAP region which is involved in sterol binding is responsible for antimicrobial activity against oomycetes, including *Phytophthora* and *Pythium* species which require exogenous sterols for basic metabolism. Plants with enhanced *PR-1* expression are particularly well protected against oomycete pathogens [26]. The in-depth structural and biochemical analysis *PR-1* proteins can provide more insights into their function during defense signalling in crop plants.

In the world of spices, black pepper (*Piper nigrum* L., family Piperaceae) is considered as 'The King of spices' due to its pungent constituent. It is a major additive in many ayurvedic medicinal preparations. The major hindrance in the black pepper production is the destructive foot rot or quick wilt disease caused by an oomycete *Phytophthora capsici* [27, 28]. In this work, to extend our knowledge on the defense mechanisms underlying PR function in *P. nigrum*, we carried out a comprehensive genome-wide analysis and validation of *PR-1* genes from *P. nigrum*. Transcriptome assisted analysis and expression profiling revealed the differential expression of *PR-1* genes during *P. capsici* infection in *P. nigrum* variety Panniyur-1.

Results

Genome wide identification and analysis of *P. nigrum* PR-1 genes

Eleven potential *PnPR-1* gene candidates were obtained from the genome-wide analysis in comparison to the *A. thaliana* PR genes. The number of exons harboring in each *PnPR-1* gene is one, except in Pn2.357 which has two exons. The length of *PnPR-1* proteins ranged from 127 to 357 amino acid residues with molecular weight ranging from 14.38 to 38.49 kDa. The theoretical isoelectric point (pI) data categorized *PnPR-1* proteins into acidic (Pn21.1032 and Pn36.35) and others as basic in nature (Table 1). Furthermore, the signal peptide regions and cleavage sites were also identified in all the *PnPR-1* protein sequences except Pn11_1637 and Pn31.171 (Table 2).

Table 1
Sequence characteristics and physio-chemical properties of the *PR-1* proteins in *Piper nigrum*.

Genome CDS Id	Genome Scaffold No.	Exon No.	Start	Stop	Strand	Protein length (AA)	Molecular weight (kDa)	Theoretical pI	
Pn2.460	Pn23	1	1315655	1316146	-	155	17.09488	4.82	Acidic
Pn2.459	Pn23	1	1320928	1321419	-	192	21.91361	5.8	
Pn2.433	Pn23	1	1493563	1494054	-	168	18.35074	7.58	Basic
Pn2.357	Pn23	2	2226595	2229193	-	127	14.37881	7.62	
Pn2.340	Pn23	1	2402850	2403341	-	185	19.80756	9.1	
Pn21.1032	Pn3	1	3202437	3202904	+	163	17.85002	9.15	
Pn31.171	Pn15	1	26854949	26855332	-	163	17.87807	9.3	
Pn36.35	Pn25	1	5754975	5755553	-	163	17.88005	9.3	
Pn11.1637	Pn4	1	33322945	33323502	-	176	19.63337	9.37	
Pn8.549	Pn8	1	25779763	25780269	-	163	17.96421	9.44	
Pn14.1312	Pn14	1	5932334	5932864	+	357	38.49596	11.3	

Table 2
Signal peptide region detected from the *PnPR-1* proteins

Genome Id	Cleavage site position	Sequence position	Probability	Protein type	Signal Peptide (Sec/SPI)	Other
Pn21.1032	16 and 17	CNA-QN	0.9541	Likelihood	0.9981	0.0019
Pn2.340	24 and 25	AQA-QN	0.8849	Likelihood	0.9984	0.0016
Pn2.357	24 and 25	AQA-QN	0.8898	Likelihood	0.9986	0.0014
Pn2.433	24 and 25	AQA-QN	0.8892	Likelihood	0.9987	0.0013
Pn2.459	24 and 25	AQA-QN	0.8898	Likelihood	0.9986	0.0014
Pn2.460	24 and 25	AQA-QN	0.8892	Likelihood	0.9987	0.0013
Pn36.35	29 and 30	ASS-SP	0.6292	Likelihood	0.9813	0.0187
Pn14.1312	31 and 32	TNA-AL	0.4399	Likelihood	0.7324	0.2676
Pn8.549	30 and 31	TLA-QN	0.3454	Likelihood	0.5106	0.4894
Pn31.171	-	-	-	Likelihood	0.0029	0.9971
Pn11.1637	-	-	-	Likelihood	0.2691	0.7309

All the eleven *PnPR-1* protein sequences were identified as potential members of CAP superfamily with CAP domain structure indicated in green line, along with caveolin-binding motif (CBM; marked in yellow) and CAP-derived peptide (CAPE; marked in blue) motif (Fig. 1). Comparison of *PnPR-1* sequences with various other monocot and dicot plants showed distinct CAP domain with CBM and CAPE (Fig. S1). The signal peptide regions

(Pink colored boxes) of *PnPR-1*, were also represented in Fig. 1 and the cleavage site predicted was indicated by the arrowhead.

Sequence conservation of PnPR-1 genes

The nucleotide and protein sequence conservation of all the *PnPR-1* candidates were checked using Mega 7. The subsequent phylogenetic analysis of all eleven *PR-1* nucleotide and protein sequences through the maximum-likelihood method with 1000 bootstraps (Fig. S2A & S2B) revealed two main clusters leaving one outnumbered group (Pn21.1032). A total of ten conserved motifs were identified using MEME server. Motif 1 and 2 were conserved in all the deduced *PR-1* proteins whereas motif 3 and motif 6 are conserved in all *PnPR-1* proteins except in Pn31.171 and Pn2.357 respectively. Motif 8, motif 9 and motif 10 were preserved in Pn36.35 along with Pn14.1312, Pn31.171, Pn1.1637 respectively (Fig. 2).

GO and KEGG pathway analysis of PnPR-1 genes

Gene ontology (GO) analysis carried out using PANNZER2 online server yielded five biological processes, four molecular functions and four cellular components. Based on the GO enrichment analysis, predicted PR proteins have their roles in all the three classes. Most of the *PR-1* genes have their role in defense responses and response to biotic stimulus in biological function. In terms of molecular function, it has protein kinase activity, adenyly nucleotide and purine ribonucleoside binding activities. Cellular component showed its role in extracellular region (Table 3). Kegg pathways analysis categorized its role in Environmental Information Processing- Signal transduction pathways like MAPK signaling pathway - plant (04016), Plant hormone signal transduction (04075) and Plant-pathogen interaction (04626).

Table 3
The gene ontology (GO) term distribution of *PnPR-1* proteins

GO Id	GO Domain	Function Description
GO:0006952	Biological process	Defense response
GO:0009607	Biological process	Response to biotic stimulus
GO:0048544	Biological process	Recognition of pollen
GO:0006468	Biological process	Protein phosphorylation
GO:0010274	Biological process	Hydrotropism
GO:0004672	Molecular function	Protein kinase activity
GO:0030554	Molecular function	Adenyly nucleotide binding
GO:0035639	Molecular function	Purine ribonucleoside triphosphate binding
GO:0032555	Molecular function	Purine ribonucleotide binding
GO:0005576	Cellular component	Extracellular region
GO:0016020	Cellular component	Membrane
GO:0031224	Cellular component	Intrinsic component of membrane

Secondary and 3D structure of PnPR-1 protein

A varied percentage of α -helices (15.6–36.88%), extended strand (10.64–23.78%), beta turn (3.65–5.88%) and random coil (37.42–67.79%) were found in the *Pn-PR1* proteins (Fig. S3). 3D-structures of the *Pn-PR1* genes were generated using Phyre2online server and pocket structures were predicted (Fig. 3). The distinct structural variations were detected among the *PnPR-1* proteins. At the same time, proportion of disordered regions of *PR-1* proteins ranged from 4.5–28.1%.

Cis-regulatory elements of PnPR-1 genes

The cis-elements were found to be distributed over the 2.0 kb upstream promoter region of *PnPR-1* genes (Fig. 4). The length of these cis-elements varied from 9 to 42 bp in Softberry database whereas 4 to 24 bp in NewPlace database. Among the ten *PR-1* gene loci, the typical TGA binding site LS7, WBSI, G-box and C- motif were found in the promoter regions of Pn36.35. Meanwhile, GT motif and Zc2A/T-2 were found in the Pn2.340, Pn2.433 and Pn2.460. The hormone signalling elements such as ABI4 and GCC-box were present in Pn2.459 and Pn2.340, Pn2.433 respectively. The stress responsive MYB was also detected in the promoter regions of *PnPR1* genes such as Pn21.1032, Pn31.171 and Pn36.35. Among 154 cis-elements detected from New Place database, CAAT box (CAAT), E-box (CANNTG), DOFCOREZM (AAAG) regions were found to be widely distributed across the *PnPR-1* promoter regions (Fig. S4).

Expression of PR-1 genes during *P. capsici* infection in *P. nigrum*

The assembled transcriptome of RNA-seq data from control and *P. capsici* infected *P. nigrum* plants revealed 60437 transcripts (Communicated). From the assembled data seven transcripts of *PnPR-1* genes were mapped to the *P. capsici* - *P. nigrum* interaction pathway. The transcripts length ranges from 391-1015bps. Differential expression of these transcripts was assessed from their corresponding FPKM values among control leaf (CL), infected leaf (IL) and infected root (IR). CL10113.C1/2 and Unigene17664 (Fig. 5A-C) that respectively mapped to the Pn23 and Pn8 scaffolds were highly expressed (4.57 to 5.7-fold) in the infected leaf during pathogen infection. Meanwhile Unigene11116, Unigene15555, Unigene26912 and Unigene693 were moderately expressed in IL (Fig. 5D-G). All the transcripts, except Unigene693 were downregulated drastically (2 to 7-fold) in IR compared to IL (Fig. 5 main figure).

RT-qPCR validation of PnPR-1 genes

The expression of *PnPR-1* gene was further validated using RT-qPCR from *P. capsici* infected *P. nigrum* plants. In agreement with the transcriptome results, a high upregulation of *PnPR-1* gene CL10113.C2 was detected from the infected leaf. Furthermore, the temporal expression at 6 hrs, 12 hrs and 24hrs of *P. capsici* infection revealed sequential increase in expression of *PnPR-1* genes from 6 to 12 hours of infection (Fig. 6A). A drastic upregulation of *PnPR-1* expression was observed in the leaves at 24hours after infection compared to the mock control plant (ML). The trypan blue staining has enabled us to discrimination between intact-viable and damaged regions on leaves. Viable cells were colorless whereas infected tissue was stained in blue (Fig. 6B).

Discussion

PR proteins are defense-related signaling molecules induced by phytopathogens which play a vital role in resisting the entry of invading pathogen. PR proteins have been classified into many families based on their function, molecular weight, amino acid sequence and other properties [29]. In tobacco PR proteins were initially

classified as five major classes (PR1, PR2, PR3, PR4 and PR5) [30, 31]. However, later studies in tobacco and tomato have grouped them into 11 families [3]. The members belonging to PR family can be either acidic or basic in nature. Basic PR proteins are located intracellularly in the vacuole regions and are constitutively expressed to some extent and are also induced by stress signals, whereas acidic types were produced extracellularly and only triggered by specific stress signals [32]. In our current study, both acidic and basic *PR-1* proteins which have critical role during *P. nigrum*: *Phytophthora* interactions were identified.

KEGG orthology analysis revealed *P. nigrum PR-1* genes mapped to the plant-pathogen interaction (04626), MAPK signaling pathway (04016) and Plant hormone signal transduction (04075). *PR-1* family mainly possess antifungal and anti-oomycetes activities [29]. Overexpression of PR1 or like proteins in various plants leads to enhanced disease resistance to a wide variety of pathogens [15] especially the oomycetes [21, 33]. The anti-oomycete properties of PR-1 proteins such as P14c and PR-1 were demonstrated against the sterol auxotroph pathogen *Phytophthora brassicae* [26].

As previously reported [19, 26], *P. nigrum PR-1* proteins possess the established features such as the CAP tetrad, CBM involved in Sterol binding [34] and CAPE involved in plant immune signalling [25]. The property of *PR-1* family of proteins to bind sterols contributes towards their antimicrobial activity towards *Phytophthora* species, a major plant pathogen belonging to sterol auxotroph [26]. The CAPE 1 peptide has the consensus motif PxGNxxxxxPY, that is conserved between the monocots and dicots. Highly conserved and distinct similarity in domains of PR1 protein structure was observed in *PnPR-1* proteins which might account to a general strategy in responding to various biotic stresses as reported in other studies [35]. The role of CAPE-1 peptide in the defense signalling was demonstrated from the previous study on *Pseudomonas syringae* pv tomato (Pst) strain DC3000 interaction in tomato. A diverse set of defense-related genes were induced in the tomato plants pretreated with CAPE-1 peptide. Furthermore, a non-canonical pathway other than PAMP triggered immunity (PTI) signalling was suggested for the CAPE-1 mediated defense responses, as CAPE-1 did not induce the WRKY TRANSCRIPTION FACTOR53 (WRKY53) [25]. The pathogen effectors ToxA and Tox3 proteins from *Parastagonospora nodorum* were found to interact with wheat *PR-1* proteins [36, 37].

Promoters are the regulators of gene at the transcriptional level [38]. Various computational methods are thoroughly being used for the identification of different cis-elements in the promoter region that are responsible for the regulation of the genes [39]. A range of cis-elements were predicted from *P. nigrum PR-1* genes which were likely related to the regulation of plant growth, development and response to various stresses. A high frequency of CAAT box (CAAT), E-box (CANNTG), DOFCOREZM (AAAG) regions were found in the *PnPR1* promoter regions. A high occurrence of CAAT box was previously reported in the *A. thaliana* PR proteins [40]. As previously reported, hormone regulating sequence motifs [40] such as LS7, GCC-box, ABA responsive elements (ABREs; also termed as G-box) [41] and ABI4 were detected from the upstream of certain *PnPR1* genes. LS7 which contains a TGA binding site is reported as the key activator of *PR-1* expression, NPR1 [42]. Ethylene-responsive factor binds to GCC-box (ethylene-responsive element) and responses to various biotic and abiotic factors in *Arabidopsis* [43]. ABA-INSENSITIVE-4 (ABI4) transcription factor was reported to be involved in ascorbate-dependent plant growth [44]. AC elements in the promoter region of Leucoanthocyanidin reductase gene in Proanthocyanidins pathway promoter harbors binding site for MYB2 Myb-like transcription factors [45]. Subsequently WER-binding site (WBSI) and NonaLS are also reported in *PnPR1* promoter region. WBSI was detected in the *CAPRICE* (*CPC*) promoter of *Arabidopsis*, WEREWOLF (WER) is a MYB protein and gene transcription activator during the specification of epidermal cell fates [46]. NonaLS (Nona like sequence - GATCGGACG) is the positive cis-acting element of histone

wheat, tomato, and Arabidopsis [47]. Induction of both biotic and abiotic responsive Cis-acting regulatory elements in *PnPR-1* indicates that these reported *PR-1* genes from *P. nigrum* may play a key role in regulating resistance against *P. capsici* and other abiotic stresses.

Even though *PR-1* proteins belong to the group of abundant proteins expressed in plant pathogen interaction, all the members of the family were not uniformly upregulated [19]. Likewise, transcriptome assisted analysis revealed high upregulation of two basic *PR-1* genes ($p > 7.35$) such as CL10113.C1/2 and Unigene17664 in *P. nigrum* upon *P. capsici* infection. As *PR-1* genes belong to the group of multigene families, they differ widely in their properties.

In addition to the host defense response during pathogen infection, *PR-1* proteins were also reported to have role in abiotic stress stimuli [17, 48–50]. Studies show the rise in *PR-1* expression levels both transcriptionally and translationally upon pathogen infections [51, 52]. Same pattern of expression was observed in our study where the increased transcript levels were observed in CL10113.Contig1_All, CL10113.Contig2_All, Unigene17664_All. The qRT-PCR expression studies of CL10113.Contig2_All also proved the same. The trypan blue staining clearly shows the necrotic region as the defense responses of the host plant to the pathogen and its further effect to inhibit the growth of pathogen to the surrounding regions. Till date only few studies were carried out on role of *PR* proteins in *P. nigrum* or related Piperaceae species. The activity of PR protein chitinase, β -1,3-glucanase and their related enzymes in *P. capsici* infected *P. nigrum* were reported by [53–55]. The present study contributes a significant advancement in the understanding of the molecular function of *PR-1* proteins in black pepper. *PnPR-1* genes are found to have key role in the early defense such as PTI towards *P. capsici* infection in Panniyur-1 plants. It may be possible that the key genes in the subsequent effector triggered immunity act as critical players of defense response in Panniyur-1 plants. Therefore, future studies on identification of the potential *P. capsici* effectors coupled with *PR-1* functional studies will ascertain the in-depth mechanisms of defense signal amplification and anti-oomycete properties of this enigmatic proteins in black pepper.

Conclusion

The genome-wide survey identified eleven *P. nigrum PR-1* gene homologs mapped to seven distinct genome scaffolds. A subsequent transcriptome analysis of *P. capsici* infected black pepper plants showed the expression of *PR-1* genes from all the mapped loci. Meanwhile a significantly different expression was detected for CL10113.C2 mapped to the *P. nigrum* scaffold 23. Detailed *in silico* analysis revealed cis-regulatory elements such as phytohormone responsive, transcription activators etc. in the promoter regions. The structural analysis revealed similar binding pockets in the predicted 3D structures of all *PnPR1* proteins, except Pn31.171. The differential expression of certain *PnPR-1* homologs revealed their crucial role during early defense response in *P. capsici* - *P. nigrum* interaction. Further in-depth functional studies on *PnPR-1* genes, promoter cis-regulatory elements and the pathogen-specific effectors can provide us the exact molecular mechanism of the susceptibility /tolerance of black pepper cultivars to Phytophthora infection, which in turn contribute towards the novel disease protection strategies in *P. nigrum* plants.

Methods

Identification and analysis of *PR-1* genes from *P. nigrum* genome

PR-1 genes of *Arabidopsis thaliana* were downloaded from TAIR (The Arabidopsis Information Resource) database (<https://www.arabidopsis.org/index.jsp>) and did tblastn search against the *P. nigrum* genome assemblies [56]. The coding sequences were translated and aligned by multiple sequence alignment using BioEdit Sequence Alignment Editor [57]. The domain structure prediction was carried out using NCBI- Conserved Domain Database (CDD) [58].

The molecular weight and pI of *PnPR-1* proteins were estimated by ExPASy ProtParam tool (<https://web.expasy.org/protparam/>). The potential signal peptide regions and the cleavage sites were also predicted using Signal-P 5.0 server (<http://www.cbs.dtu.dk/cgi-bin>). Subsequently the conserved motifs of *PnPR-1* proteins were predicted using MEME (Multiple Em for Motif Elicitation) (<http://meme-suite.org/tools/meme>) [59].

GO & KEGG analysis

Gene ontology (GO) was classified into biological processes, cellular components, and molecular function. *PnPR-1* genes were analyzed for their role in GO using PANNZER2 web server (<http://ekhidna2.biocenter.helsinki.fi/sanspanz/>) [60]. KEGG (Kyoto Encyclopedia of Genes and Genomes) tool, BlastKOALA (KEGG Orthology and Links Annotation) server (<https://www.kegg.jp/blastkoala/>) [61] was used for the individual characterization of gene functions.

Secondary and tertiary structure prediction

Secondary structure prediction of *PnPR-1* proteins was predicted using Self-Optimized Prediction Method with Alignment (SOPMA) server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) [62]. Predicted 3D structures were build using Protein Homology/analogY Recognition Engine v2 (Phyre2) server (<http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index>) [63]. The CASTp (Computed Atlas of Surface Topography of proteins) tool (<http://sts.bioe.uic.edu/castp/calculation.html>) was used to predict the active site pockets and topology of *PnPR-1* proteins structures [64].

Role of *PnPR-1* in *P. capsici* infected *P. nigrum*

Control *P. nigrum* (uninfected) leaf transcriptome data (SRA050094), *P. capsici* infected *P. nigrum* leaf (SRX853366) and root transcriptome data (SRX856639) were reanalyzed and the final assembled data was used for the expression studies. *PnPR-1* sequences curated from the *P. nigrum* genome [56] were mapped to the transcriptome assembly files. The obtained transcripts differential regulation checked using FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values.

Plant-pathogen infections, staining and RT-qPCR

Virulent, pure cultures of *P. capsici* and root cuttings of *P. nigrum* cultivar panniyur1 were procured from College of Agriculture, Vellayani. 24hr old *P. capsici* culture was infected to the fully expanded second leaf of *P. nigrum* plant. The treatment of mock infection was done with plain PDA discs. To check the temporal expression of *PnPR-1*, the infected leaf samples were collected at 6hr, 12hr and 24hr after infection. Mock infected plats were used as a control. To detect and visualize the tissue damage in the leaf region after pathogen infection, trypan blue staining was performed [65]. Three biological replicates were used for all the studies.

RNA was isolated from the collected leaf samples using mirVana miRNA isolation kit (Invitrogen, Cat No: AM1560) according to the manufacturer's instructions. The quality and the quantity of RNA samples were checked by Colibri Microvolume Spectrometer. RNA was reverse transcribed into cDNA using High-Capacity cDNA synthesis Kit (Applied Biosystems, Cat No: 4374966). RT-qPCR was carried out using Applied Biosystems 7900 HT sequence detection system (ABI) using SYBR Green qPCR Master Mix (ABI). Each qPCR reaction was conducted in 10 μ L volume containing 1 μ L of diluted cDNA (10ng/ μ L), 5 μ L of SYBR green and 5 pmol of forward (5'CCCACAAGACTTCCTCAAC3') and reverse (5'CGTAATTCGTGCGTAGG3') primers with the following conditions: 40 cycles of 95 °C for 15 s DNA denaturation, annealing at 60 °C for 15 s and elongation at 72 °C for 30 s. 5.8S RNA was used as an endogenous control [28]. The relative quantification was analyzed by comparative CT method using the formula $2^{-\Delta\Delta CT}$ and standard deviation was represented as the error bar [66].

Prediction of *cis*-acting regulatory elements in Promoter regions

The genomic sequences of 2.0 kbp upstream of the CoDing Sequence (CDS) region of each *PnPR-1* gene were extracted from *P. nigrum* genome assembly. Promoter regions of each *PR1* genes were scanned for the presence of functional motifs using Softberry (http://www.softberry.com/berry.phtml?topic=case_study_plants&no_menu=on) and New Place (<https://www.dna.affrc.go.jp/PLACE/?action=newplace>) [67].

Declarations

Ethics approval and consent to participate

Not Applicable

Consent for publication

Not Applicable

Availability of data and materials

Control *P. nigrum* (uninfected) leaf transcriptome data, *P. capsici* infected *P. nigrum* leaf and root transcriptome data can be accessed from NCBI SRA accession numbers SRA050094, SRX853366 & SRX856639 respectively.

Competing interests

The authors declare no competing interests.

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Author contributions

K.D, A.S. and E.V.S conceived the research plans and designed the experiments. D.K. and A.S. performed the experiments, analyzed the data and wrote the article, E.V.S. made critical revisions. All the authors read and approved the manuscript.

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Figures

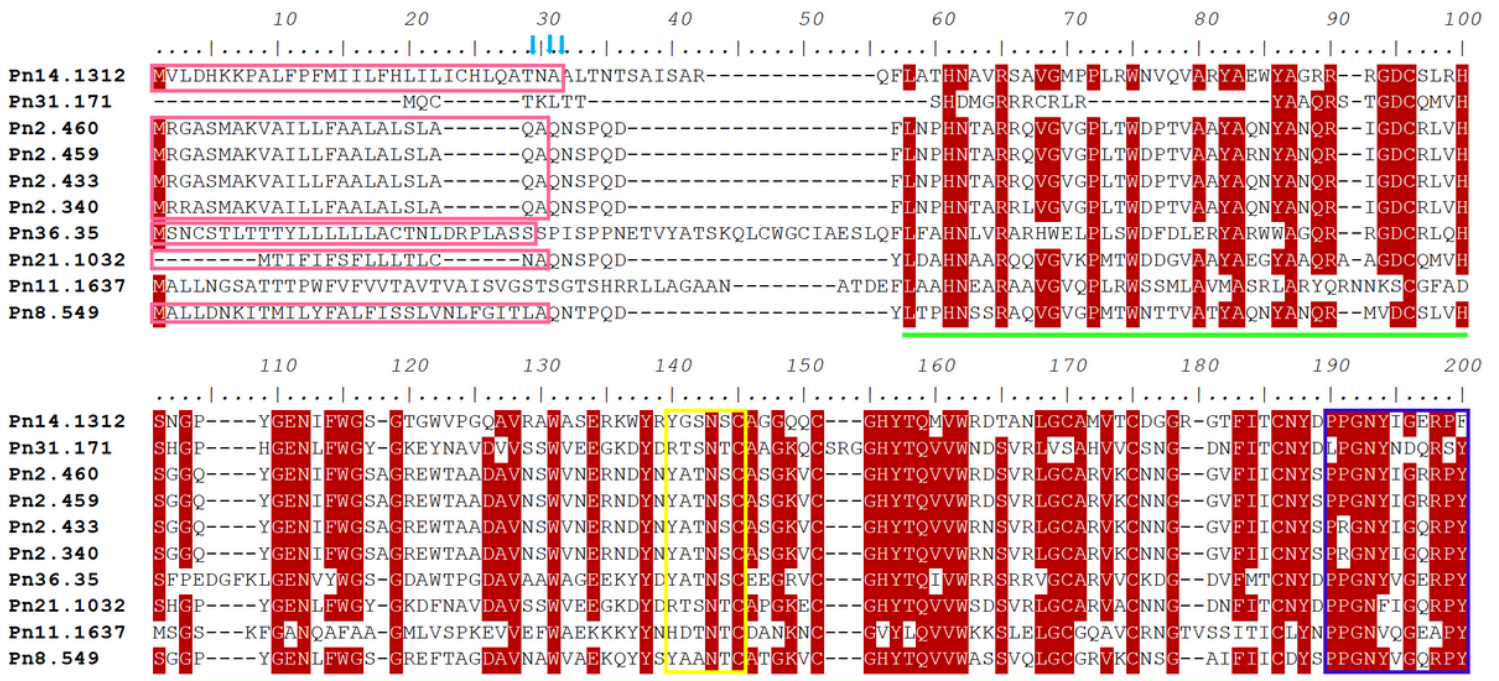
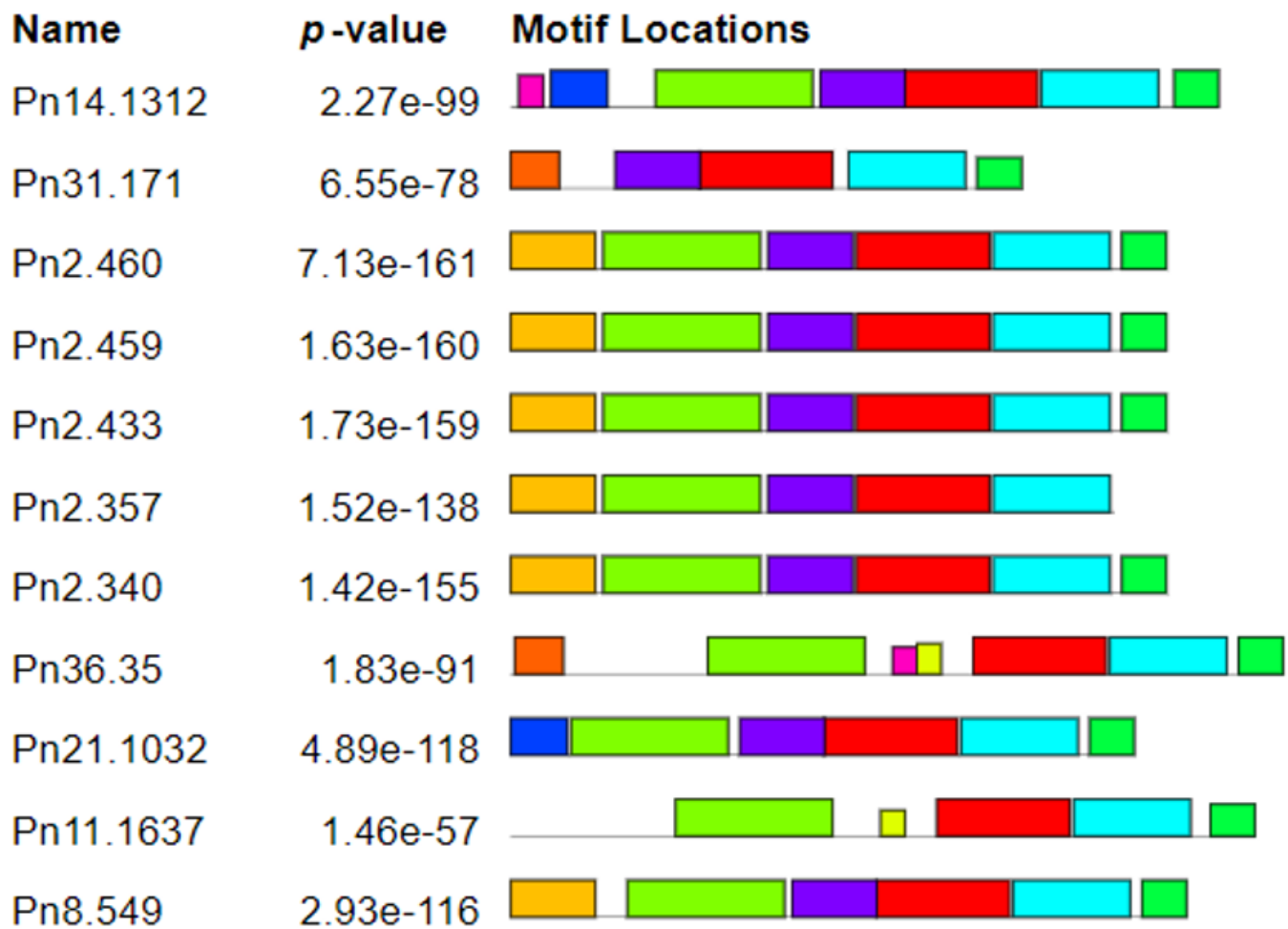


Figure 1

Multiple sequence alignments of the PnPR-1 protein sequences. Pink colored rectangle boxes indicate the signal peptide regions, cleavage site predicted were indicated in blue arrowheads. The green line indicates CAP domain structure of ~150bp. Yellow and blue color rectangles show the caveolin-binding motif (CBM) and CAP-derived peptide (CAPE) respectively.













Motif	Symbol	Motif Consensus
1.		GREWTAADAVNSWVNERKDYN YATNSCASGKVC
2.		HYTQVVWRDSVRLG CARVKCNNGGVFIIC
3.		AQNSPQDFLNPHTARRQVGVGPLTWDPTVAAYAQN YAN
4.		IGDCRLVHSGGQYGENI FWGS
5.		MRGASMAKVAILLFAALALS L
6.		PPGNYIGZRPY
7.		F P F F I F L F H L J L J C
8.		LDHKFP
9.		MNCSKLTTS HDL
10.		EDGPKF

Figure 2

Conserved motifs identified from Piper nigrum PR-1 protein homologs. A total of 10 conserved motifs were identified. Each color represents different motifs with consensus sequences.

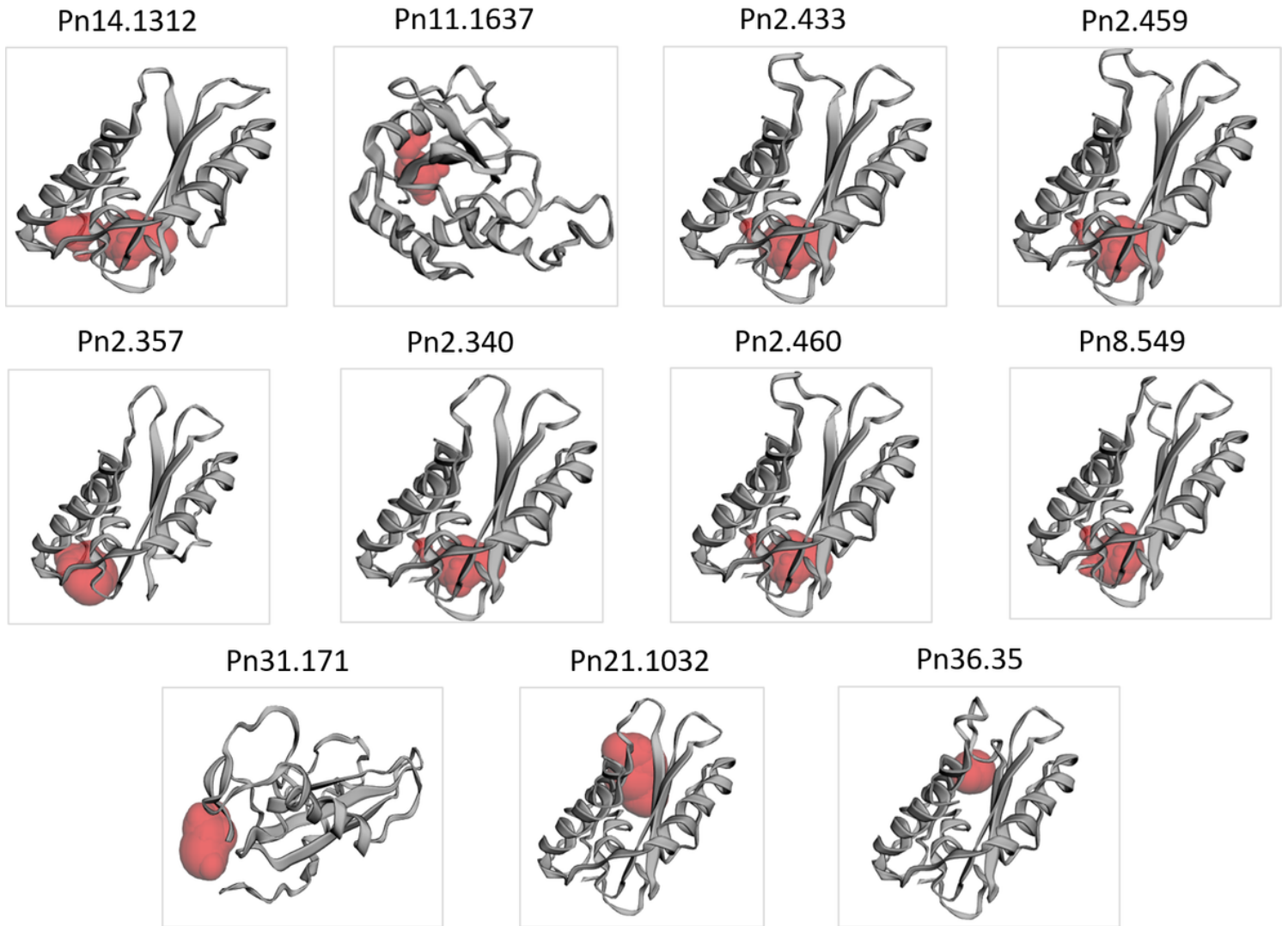


Figure 3

The predicted 3D structures of PnPR-1 proteins generated using the Phyre2 server and binding pockets identified by the CASTp 3.0 server (red color).

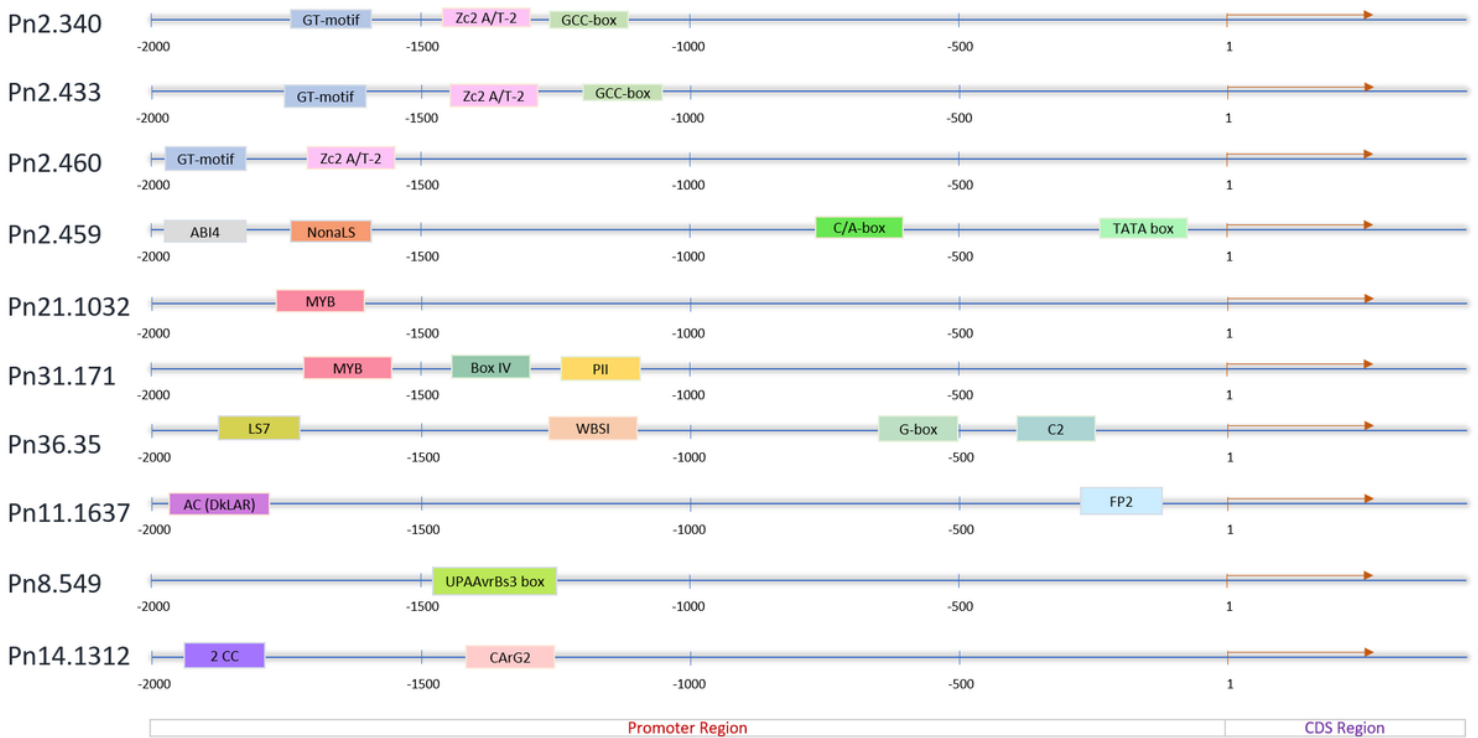


Figure 4

Analysis of cis-acting elements of the PnRR-1 promoter regions (2kb upstream from the CDS region) using the Softberry Software. Each element was represented with different colors.

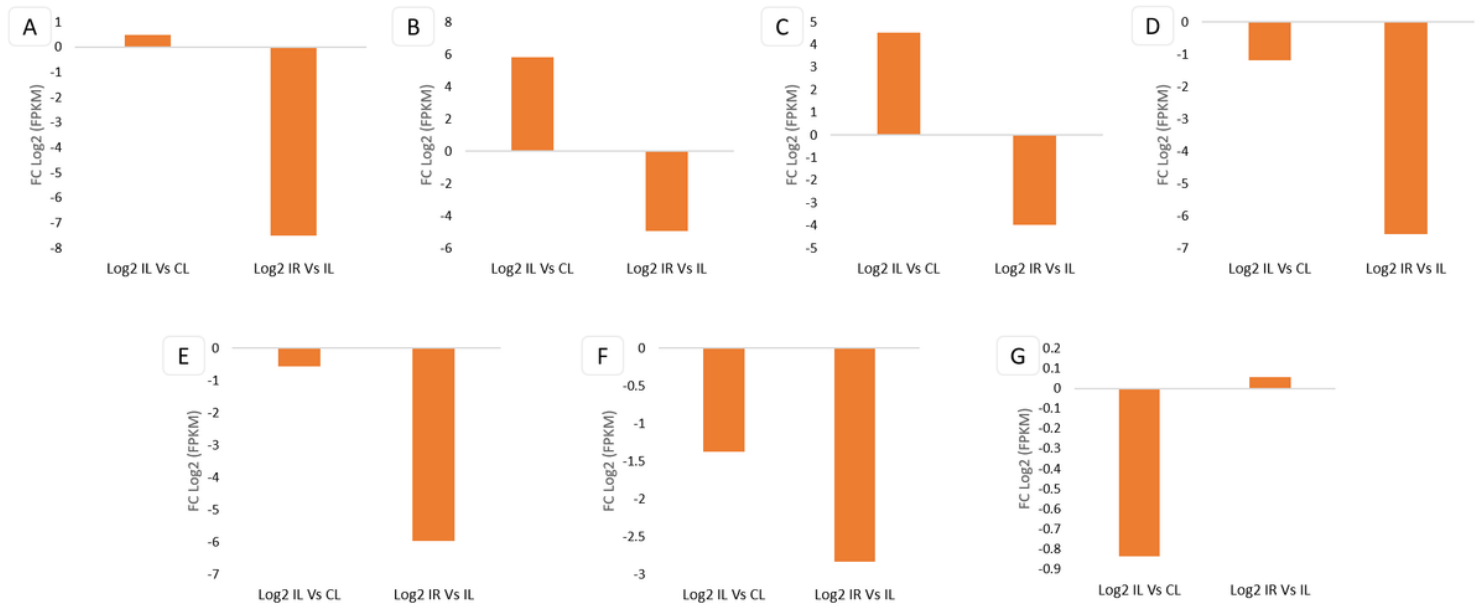


Figure 5

Expression analysis of the PnPR-1 transcripts in *Phytophthora capsici* infected *P. nigrum* using FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values. (A) CL10113.C1 (B) CL10113.C2 (C) Unigene17664 (D) Unigene11116 (E) Unigene15555 (F) Unigene26912 (G) Unigene693. Positive and negative numbers on the X axis indicate fold changes Log2 gene expression levels.

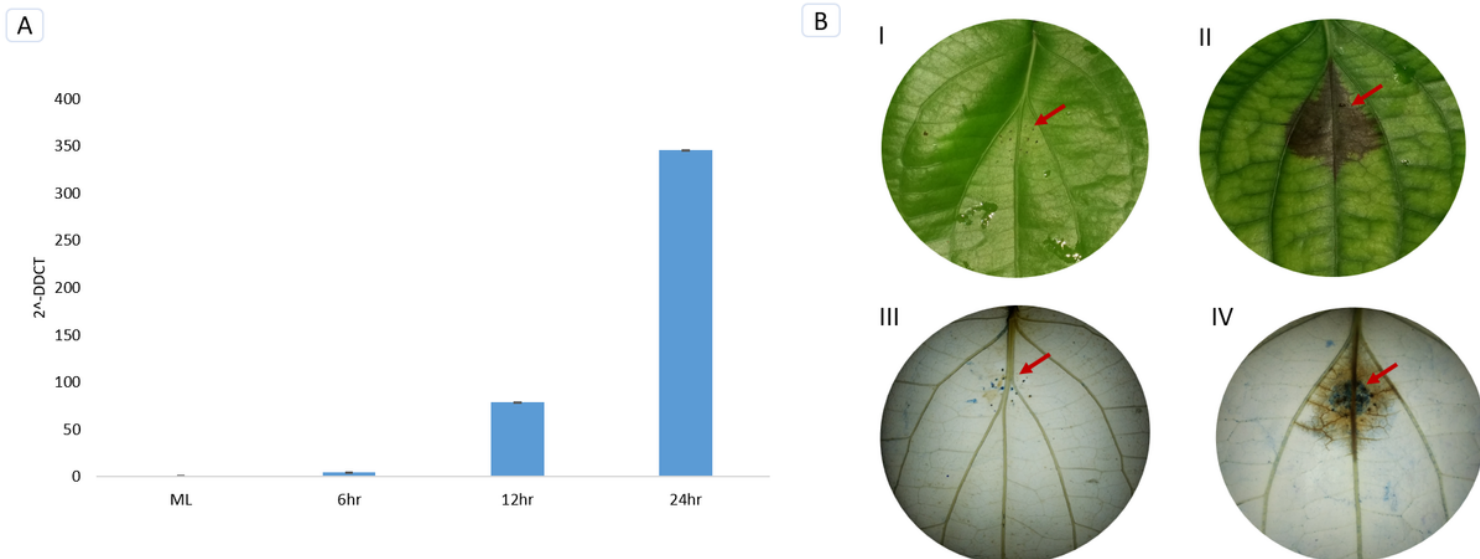


Figure 6

(A) Temporal expression validation of the PnPR-1 gene in *Phytophthora capsici* infected *Piper nigrum* plants by RT-qPCR. (B) Mock and *Phytophthora capsici* infected *Piper nigrum* leaves after 24 hrs of infection. (I) Mock infected Leaf, (II) *Phytophthora capsici* infected *Piper nigrum* leaf, (III) Trypan blue-stained mock infected leaf & (IV) Trypan blue-stained infected leaf. Red colored arrows indicate the infected regions.

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