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Resistance Genes in *Piper colubrinum*: In Silico Survey From Leaf Transcriptome and Expression Studies Upon Challenge Inoculation with *Phytophthora capsici*

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Abstract The oomycetes, *Phytophthora capsici*, cause foot rot disease in black pepper. *Piper colubrinum* Link, a distant relative of cultivated black pepper, is highly resistant to this destructive pathogen. Identification of resistance (R) genes in *P. colubrinum* and the study of its expression profile during interaction with the pathogen can help in understanding the resistance mechanism involved. In the present study, 1289 R gene-related transcripts were mined from *P. colubrinum* transcriptome, clustered, and classified according to the conserved motifs and domains. Transcripts belonging to four major R gene classes were identified in *P. colubrinum*, but TIR-NBS-LRR-type R genes were absent. The relative expression of 12 selected R genes was studied using two virulent isolates of *P. capsici*, and these were found to be upregulated in the initial hours of plant pathogen interaction. The R genes studied were expressed even in aseptically maintained tissue-cultured plants and uninoculated greenhouse-grown plants at basal level suggesting that the plants are geared up with the R gene all the time and are under continuous surveillance for the pathogen and basal level of R gene expression do not require a pathogen trigger. *ACT*, *ATUB*, and *EIF3E* were identified as the most stable reference genes that can be used for real-time PCR study. The present study identified promising R genes in *P. colubrinum* which can be used in developing *Phytophthora*-resistant black pepper.

Keywords *Piper colubrinum* · R gene · *Phytophthora capsici* · Gene expression · Real-time PCR · Reference genes · Resistance

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Introduction

Black pepper (*Piper nigrum* L.), also referred to as the king of spices, is one of the major economic spices across the world. However, its yield is affected by many diseases, of which the *Phytophthora* foot rot or quick wilt caused by the oomycetes *Phytophthora capsici* is the most devastating. *Phytophthora* infection affects almost all parts of the plant, namely, the leaves, berries, spikes, stem, and root, eventually leading to the death of the vine, and the symptoms can vary depending on the site and the severity of infection [1]. *P. capsici* causes a compatible host-pathogen interaction in *P. nigrum* leading to the death of the plant, whereas it results in an incompatible (host-resistant) interaction in its wild relative, *Piper colubrinum*, which is completely resistant to *P. capsici* infection [2, 3]. Black pepper varieties with high level of *Phytophthora* resistance are yet to be developed; hence, *P. colubrinum* is the only potent plant that can serve as donor of resistance property to cultivated black pepper.

Disease outbreak in plants is due to the evasion of host defense mechanisms by plant pathogens [4]. Plants have two lines of defense to overcome pathogenic invasion: basal-mediated and resistance (R) gene-mediated defense. Basal defense is elicited via conserved microbe-specific molecules, referred to as microbe- or pathogen-associated molecular patterns, MAMPs or PAMPs, respectively [5, 6]. This is a non-specific defense mechanism and acts in the plant system as a response to either pathogens or non-pathogens. Resistance gene-mediated or effector-triggered defense is a highly specific mechanism brought about by R genes that recognize the pathogen effector (Avr) protein [6]. The R protein-mediated resistance is often associated with a hypersensitive (HR) reaction which results in the death of the pathogen and the plant tissue surrounding the infection sites [7].

The plant R genes belong to a multigene family which recognizes the pathogen effector protein in a highly specific fashion [8]. The R genes are typically classified into five major classes based on their predicted protein structure [9, 10] and recently divided into eight major classes based on their amino acid motif organization and membrane-spanning domain [11]. The first class of the R gene is the CC-NBS-LRR-type gene which has a coiled-coil (CC) domain at the N terminal and leucine-rich repeat (LRR) at the C terminal along with the nucleotide-binding site (NBS). The second class of R gene, TIR-NBS-LRR, has NBS and LRR domains plus a TIR domain sharing similarity to human Toll interleukin-1 receptor (TIR). The third major class of the gene encodes a transmembrane domain which is connected to an extracytoplasmic leucine-rich repeat (eLRR). The fourth class has an extracellular LRR domain and an intracellular kinase (KIN) domain attached by a transmembrane domain. The fifth class of gene encodes a putative LRR domain, a short protein motif (ECS) together with a motif for protein degradation PEST (Pro-Glu-Ser-Thr). A transmembrane domain fused to the CC domain makes up the sixth class, and the seventh class is the R gene TIR, NBS, and LRR domains fused to NLS (nuclear localization signal) and WRKY domains. The last class of R gene is the enzymatic R gene which lacks either NBS or LRR domain [11]. The V, VI, and VII classes of R genes are not very widespread in plants, and only a few genes belonging to these classes have been characterized and studied.

The presence of a specific R gene in plants that recognizes the corresponding pathogen Avr gene results in plant resistance. The absence of either the Avr gene or the R gene against it triggers the onset of the disease [12–14]. Biotic resistance to pathogenic infection is usually observed in wild relatives of the cultivated plants, in which sophisticated mechanisms against pathogenic invasion are well evolved. *P. colubrinum*, the wild relative of black pepper, is highly resistant to a number of diseases including foot rot caused by *P. capsici* [2, 3]. Hence,

the identification of the R gene involved in plant immune response against *P. capsici* is important in providing insight into the molecular mechanism of resistance and in developing resistant cultivar through transgenic approaches.

The present work identified the abundance and diversity of R genes in *P. colubrinum* transcriptome. The pathogen-activated relative gene expression of selected R genes in *P. colubrinum* with two virulent isolates of *Phytophthora*, 05-06 and 98-93, was evaluated to identify the promising R genes in *P. colubrinum.* The work also examined the pathogen-independent expression of the R genes in the plants maintained under tissue culture and greenhouse condition. The stable reference genes for the real time study in *P. colubrinum*-*P. capsici* interaction were also determined.

Materials and Methods

Plant Material and *P. capsici* Inoculation

Three-month-old *P. colubrinum* cutting maintained at the greenhouse of ICAR-Indian Institute of Spices Research (IISR), Kozhikode, was used for the study. Two distinct and virulent *P. capsici* isolates were used for the challenge inoculation: 05-06 isolate and 98-93 isolate maintained at the National Repository of *Phytophthora*, ICAR-IISR.

For inoculation studies, the isolates were cultured on carrot agar medium at 28 °C for 72 h, and four mycelial plugs of 0.8 cm diameter taken from the periphery of growth was inoculated on the abaxial side of *P. colubrinum* leaf along with moist cotton. Pathogen-free carrot agar disc and moist cotton was used for mock inoculation of control. Inoculations and mock inoculation were made on the third to fifth fully opened leaves from the apex, and the leaves were harvested at 0.5, 1, 2, 4, 8, 16, and 24 hours post inoculation (hpi), immediately frozen in liquid nitrogen, and stored at − 80 °C. To identify the basal R gene expression in *P. colubrinum* without pathogen trigger, uninoculated/unchallenged leaves of aseptically maintained tissue culture plants and uninoculated/unchallenged leaves of greenhouse-grown plants were analyzed. The expression of selected R genes in these uninoculated leaves was also investigated by real-time quantitative PCR. For each condition, three leaves from three different plants were pooled and used as a biological replicate, and two such pooled biological replicates were used for each sample (uninoculated, inoculated, and mock inoculated).

In Silico Identification of R Gene Transcripts from *P. colubrinum*

P. colubrinum leaves were challenge inoculated with 72 h old *P. capsici* isolate 05-06 for different time points, and the RNA extracted from pooled leaf samples was taken for transcriptome sequencing using Illumina paired-end sequencing technology at the GA II platform. The raw reads obtained were processed and subjected to de novo assembly using Velvet software and Oases program followed by NCBI Blast search and annotation. The sequence data from *P. colubrinum* transcriptome have been deposited in NCBI Sequence Read Archive database (SRA ID: SRS1405512) [15].

P. colubrinum transcriptome was used as the local nucleotide database for the in silico identification of R genes using BioEdit software [16]. R gene homologs from *P. colubrinum* were mined from a transcriptome sequence as described by Barbosa-da-Silva et al. [17]. Amino acid sequences of 42 known R genes belonging to the major R gene classes

(Table 1) were used as query for tblastn alignment against the transcriptome with a low-stringency E-value cutoff e^{-5} on the BLOSUM62 scoring matrix in BioEdit. The same query sequence was also used for tblastn alignment against the transcriptome with an E-value cutoff e^{-10} to analyze the effect of different E-values in mining R transcripts. An additional blastx analysis of the entire transcriptome was also carried out to identify transcripts with similarity to the R gene in the NCBI nr sequence database. The matching transcripts to the query sequence and the transcripts identified by blastx were selected, and the redundant sequences were further removed manually to make the sequences unique.

Functional Annotation of the Transcripts

Blast2GO PRO [18] with integrated InterProScan [19] was used for the functional annotation of the selected sequences. The expected E-value from the best blast hit was set to e^{-5}, and the minimum alignment size (HSP length) was set to 33 to carry out the blastx search. This was followed by the InterProScan to identify the conserved domains and important sites in query against the signatures in the InterPro consortium; the sequences were then mapped and annotated.

The coding region within the R gene transcripts were identified using TransDecoder (https://transdecoder.github.io/) which also predicts the amino acid sequence in the FASTA format. Only sequences above the 100-aa length were taken for further analysis. Hierarchical homology clustering of the translated R gene sequences was carried out using h-cd-hit on CD-HIT Suite [20] using multiple CD-HIT runs with sequence identity cutoffs at 0.9, 0.6, and 0.3, respectively. The homology clusters were then manually categorized to different R gene classes based on the Blast2GO annotation and the InterPro IDs of the gene in it. Signatures from the databases PROSITE, PRINTS, PFAM, GENE3D, SMART, PIR SuperFamily, COILS, and PANTHER were considered for classification.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from the leaves of *P. colubrinum* using TRIzol (Invitrogen) reagent according to the manufacturer's instructions, and residual DNA was removed using DNA-free kit (Ambion). RNA concentration and purity were measured using BioPhotometer Plus (Eppendorf) at 260/280 and the RNA integrity checked using Agilent 2100 Bioanalyzer.

In the present study, only RNA samples with absorbance ratio between 1.8 and 2.07 and the RIN (RNA integrity number) value above 7 indicating the good quality and integrity of the RNA were used for complementary DNA (cDNA) synthesis (Supplementary Fig. 1).

Five hundred nanograms of DNA-free RNA was used for cDNA synthesis using Super-Script III Reverse Transcriptase (Invitrogen) anchored by oligo (dT_{18}) in a total volume of 20 μl according to the manufacturer's protocol; no reverse transcriptase control (NRT) was also prepared for the sample.

Candidate Gene Selection and Primer Design

R genes with high transcript abundance indicated by Fragments Per Kilobase of transcript per Million mapped reads (FPKM) in pathogen-challenged *P. colubrinum* transcriptome [15] were chosen for the expression study. Eight commonly used candidate reference genes, namely, β-actin (*ACT*), alpha tubulin (*ATUB*), eukaryotic initiation factor 3E (*EIF3E*), elongation factor 1

Table 1 Details of 42 R genes sequences (amino acids) used as query for mining R genes from the *P. colubrinum* transcriptome database by tblastn approach

Class of R gene	Accession number	Source species	Sequence size (aa)
I: TIR-NBS-LRR	AEE83824.1	*Arabidopsis thaliana*	154
	AEE86624.1	*Arabidopsis thaliana*	1607
	CDN40096.1	*Prunus persica*	219
	AEE34163.1	*Arabidopsis thaliana*	1017
	P93244	*Linum usitatissimum*	1305
	AAG09951.1	*Glycine max*	863
	AAM18462.1	*Arabidopsis thaliana*	1135
	AAF08790.1	*Arabidopsis thaliana*	1361
	ABF81411.1	*Populus trichocarpa*	333
II: CC-NBS-LRR	XP_009387615.1	*Musa acuminata* subsp. *malaccensis*	1062
	NP_176135.1	*Arabidopsis thaliana*	907
	NP_001067534.1	*Oryza sativa Japonica*	1021
	AGC12590.1	*Arabidopsis thaliana*	926
III: LRR-TrD	AAK70805.1	*Gossypium hirsutum*	328
	O50024	*Solanum habrochaites*	855
	CAA05274.1	*Solanum pimpinellifolium*	863
	CAB78434.1	*Arabidopsis thaliana*	869
	AAC78591.1	*Solanum lycopersicum* var. *cerasiforme*	968
	AFQ23178.1	*Nicotiana attenuata*	478
IV: LRR-TrD-KIN	XP_007047661.1	*Theobroma cacao*	1018
	NP_197965.1	*Arabidopsis thaliana*	1005
	XP_007155783.1	*Phaseolus vulgaris*	1018
	AAO22763.1	*Arabidopsis thaliana*	933
	AAF91322.1	*Glycine max*	1008
	AAO22764.1	*Arabidopsis thaliana*	882
V: TrD-CC	NP_566794.1	*Arabidopsis thaliana*	221
	XP_003611878.1	*Medicago truncatula*	615
	XP_003611817.1	*Medicago truncatula*	434
VI: TIR-NBS-LRR-NLS-WRKY	C4B7M5.1	*Arabidopsis thaliana*	1373
VII: VLRR-TrD-PEST-ECS	NP_001234474.2	*Solanum lycopersicum*	1053
	AAQ82053.1	*Solanum torvum*	1138
VIII: Enzymatic R gene	AAM81978.1	*Hordeum vulgare* subsp. *vulgare*	802
	AJ606013.1	*Triticum aestivum*	677
	ABK51312.1	*Hordeum vulgare*	808
	CDN24609.1	*Oryza sativa*	317
	AFC37605.1	*Piper colubrinum*	547
	AAZ15370.1	*Solanum peruvianum*	305
	AAB47421.1	*Solanum lycopersicum*	311
	AAZ15350.1	*Solanum chilense*	303
	AAD49772.2	*Lycopersicon esculentum*	364
	NP_192110.2	*Arabidopsis thaliana*	725
	XP_009416258.1	*Musa acuminata*	832

alpha (*EF1A*), fasciclin-like arabinogalactan protein 1 (*FLAP*), glyceraldehyde 3-phosphate dehydrogensase (*GAPD*), RNA-binding protein (*RNABP*), and ASK-interacting protein (*SKP1*), were selected based on previous studies in other plants to identify the most stable reference gene in the current experimental condition [21–23]. *P. colubrinum*-specific primers for candidate reference genes and R genes were designed using PrimerQuest tool (http://eu.idtdna.com/PrimerQuest/Home/Index) with the following parameters: amplicon length 80–

250 bp, melting temperature 58–62 °C, and GC content 40–60%. For each primer pair studied, the reaction efficiency was estimated from the standard curve generated by six-point serial dilution of pooled cDNA. The PCR efficiency (E), slope (M), R value, and R^2 value were calculated using the Rotor-Gene Q software.

Quantitative Real-Time PCR

Quantitative RT PCR (qPCR) was carried out in the 72-welled rotor in the Rotor-Gene Q system (Qiagen, Germany) using FastStart Essential DNA Green Master (Roche, Germany) in a three-step reaction. The sample maximization method was selected for the real-time experiment, where all the samples for a single gene were analyzed in the same run to minimize inter-run or technical variation between samples [24]. Each set of biological replicate was analyzed in different runs. Each 20-μl reaction comprised 10 μl 2X SYBR Green Master Mix, 7.5 ng of RNA equivalent cDNA and a final concentration of 0.5 μM of forward and reverse primers. The PCR parameters are as follows: 95 °C for 10 min, and 35 cycles of 10 s at 95 °C, 10 s at optimal annealing temperature, and 20 s at 72 °C. The melt curve analysis was done following the run to confirm the single product, by increasing the temperature stepwise by 1 °C in the range of 65–95 °C. Each PCR reaction was carried out in triplicate, and no template control (NTC) and NRT was analyzed for every primer pair. The products were run on 1.5% agarose gel to confirm specificity and the PCR products sequenced.

Determination of Reference Gene Stability and Optimal Number of Reference Genes

To investigate the candidate reference gene stability during *P. colubrinum-P. capsici* interaction, expression profiles of eight candidate reference genes were comprehensively evaluated in all the samples (uninoculated, mock inoculated, and challenge inoculated) taken for expression study using four different algorithms, geNorm [25], NormFinder [26], BestKeeper [27], and comparative ΔCt [28] tool, incorporated in the web-based analysis tool RefFinder. The GeNorm tool identifies the pairwise variation (V) of a reference gene relative to the other reference genes studied and also computes the gene expression stability measure (M) based on the average pairwise expression ratio. NormFinder is based on the variation of each gene in the expression level and calculates the stability value (SV). BestKeeper identifies Pearson's correlation coefficient (r) and the standard deviation to rank the genes. RefFinder analyzes the individual ranking from each algorithm and assigns a weight to these genes to calculate the geometric mean for the overall weight ranking [29]. Each candidate reference gene was ranked from most stable to least stable based on the different computational algorithms.

The V_n/V_{n+1} value was used to select the minimal number of genes for the correct analysis of the real-time data, with the recommended V value cutoff of 0.15. The pairwise variation (V_n/V_{n+1}) between the genes was calculated as the standard deviation of the log-transformed expression of the V_n gene with respect to V_{n+1} genes [25].

Data Analysis and Normalization of the R Gene Expression

The differential expressions of the R gene in *P. colubrinum* on challenge inoculation at different time intervals were calculated against their corresponding mock-inoculated control. The basal level R gene expressions without pathogen trigger in uninoculated tissue culture

plants were calculated against the uninoculated greenhouse samples. For expression analysis, the quantification cycle (Cq) values were converted to the relative quantity by the ΔCq method using the Cq value of the control as calibrator incorporating the amplification efficiency of the primer. The Cq values of the best-ranked reference genes were used for calculating the normalization factor, which is the geometric mean of the relative expression of the selected reference genes [25]. Fold change was calculated as the ratio between the relative quantities of the gene of interest to the normalizing factor [24]. The expressions of the R genes at each hpi were expressed as $\log_2$ values of the fold change. The genes were considered differentially expressed if the $\log_2$ relative expression varied by at least 2.0.

Results

Transcriptome Profiling of *P. colubrinum* Challenged with *P. capsici*

The transcriptome analysis of *P. colubrinum* leaf was carried out with the aim of understanding the genes involved in *Phytophthora* resistance. Illumina sequencing generated a total of 22,708,868 raw reads (approximately 72 bp) accounting for a total of 1,635,038,496 bp. The de novo assembly produced 62,619 contigs (> 100 bp) with an average contig length of 643.076 bp and a maximum length of 7555 bp. The maximum number of annotated contigs from *P. colubrinum* aligned with sequences from *Vitis vinifera* (12,895 contigs) followed by *Populus trichocarpa* (5709 contigs).

In Silico Identification of R Genes and Their Classification

Using the 42 known R genes from the NCBI database as seed sequence for tblastn (e^{-5}) against the transcriptome together with blastx analysis against the NCBI database, we could identify 1289 *P. colubrinum* transcripts showing similarity to resistance genes, whereas, using e^{-10}, we could mine only 1142 putative R gene transcripts. The length of these putative R gene transcripts ranged from 122 to 4126 nucleotides of which the majority had a GC content of 40–45%. The Blast2GO search and InterProScan revealed the presence of either complete or partial conserved domains or motifs in these mined sequences. The Blast2GO PRO identified the blast identity, first hit species, first hit gene, the InterPro ID, and the GO terms of the R gene transcripts. The maximum number of R genes in *P. colubrinum* showed similarity and aligned to the R genes and R gene analogues (RGA) in *Nelumbo nucifera* followed by *V. vinifera.* InterProScan revealed the presence of conserved sites and domains in the R gene transcripts; LRR (IPR032675 and IPR001611) was identified to be most abundant in the *P. colubrinum* R gene transcripts followed by the protein kinase domain (IPR000719 and IPR011009) (Supplementary Fig. 2).

Out of the 1289 putative R gene transcripts, 997 sequences had an amino acid ORF sequence length of at least 100, and these were further clustered by hierarchical clustering to form 91 homology clusters and 64 singletons. The hierarchical clustering of the sequences by CD-HIT helped to group the R gene based on their sequence similarity. Even though most clusters had transcripts representing single class, 10 clusters had representation from more than one class of the R gene. The R gene homology clusters identified by CD-HIT were manually classified based on conserved domains and sequences (Supplementary Table 1): NBS-LRR was the most abundant cluster (26) followed by 22 enzymatic R genes, 16 LRR-TrD, and 15

LRR-TrD-KIN clusters. Three CC NBS LRR clusters were also present among the 26 NBS LRR clusters. The clusters having representation from more than one R gene class were classified under MIX. In addition to the four major clusters of R gene identified, two RPW8 R genes (class V) and six *Verticillium* (Ve) resistance genes (class VI) were also mined and annotated from the *P. colubrinum* dataset. The RPW8 genes remained as singleton, and Ve genes clustered together with the LRR TrD class on homology clustering.

Twelve R gene (Table 2) candidates were selected from the 1289 transcripts for the expression study based on their transcript abundance in the *P. colubrinum* transcriptome [15] indicated by high Fragments Per Kilobase of transcript per Million mapped reads (FPKM) value (data not given). One R gene transcript representing the CC-NBS-LRR class (*R1-1644*) and sharing similarity to already reported *Phytophthora* resistance gene in other plants, nine representing the NBS-LRR class (*R3-2277*, *R4-2905*, *R5-413*, *R7-1670*, *R8-33941*, *R9-4669*, *R10-36038*, *R11-7014*, and *R12-4018*), and one transcript each from LRR-TrD-KIN (*R6-3203*) and LRR-TrD (*Hspro*-type gene, *R2-1990*) were chosen for the expression study.

Amplification Efficiency and Specificity of the Candidate Genes

Primers for the eight candidate reference genes and the 12 candidate R genes studied were highly specific and yielded distinct peaks on the real-time dissociation curve. They also gave single amplification products of expected size on agarose gel. The amplicons were sequenced by Sanger sequencing which confirmed 100% identity to the corresponding transcript. A standard curve of six-point serial dilutions using pooled cDNA was generated for each primer pair to calculate the slope (M), correlation coefficient (R^2), and PCR efficiency (E) (Supplementary Table 2). The serial decrease in the Cq value at each dilution point and the superior M value confirmed the absence of PCR inhibitors in the cDNA. The presence of genomic DNA contamination was ruled out by using no reverse transcriptase (NRT) control for each candidate gene amplified. And non-specific amplification was ruled out using no template control (NTC) of the genes studied.

Transcript Profiling of *P. colubrinum* Reference Gene Stability

The expression stability of eight candidate reference genes in *P. colubrinum* on *P. capsici* biotic stress was evaluated by four different statistical approaches: geNorm, NomFinder, BestKeeper, and the ΔcT method. The web-based analysis tool, RefFinder finally ranked the candidate reference genes based on the entire stability ranking. The Cq value of the reference genes studied ranged between 15 and 25 in the samples analyzed, and most Cq were distributed between 17 and 21 and *ACT* was the most highly expressed gene of the set with a mean Cq of 17.4 (Supplementary Fig. 3).

GeNorm software ranked *ATUB* and *ACT* (M = 0.512) as the most stable reference gene followed by *EIF3E* and *SKP1* across all the samples. The M value of all the candidate reference genes, calculated by stepwise exclusion of least stable gene, were well below the GeNorm threshold of 1.5 indicating higher gene expression stability under the given stress condition (Supplementary Fig. 4a). NormFinder identified *ATUB* (SV = 0.404) followed by *ACT*, *RNABP*, and *EIF3E* as the most stable reference gene. *ACT* (SD = 0.45, r = 0.804) was selected as the most stable reference gene by the BestKeeper software followed by *EFI3E*, *ATUB*, and *SKP1*. The gene ranking with the ΔcT method was identical to the NormFinder

Table 2 *P. colubrinum* R gene transcripts selected for real-time expression analysis showing the Blast hit, GO term, InterPro ID, and classification as identified by Blast2GO

Sequence Name	Transcript length	Sequence description	Top-hit species	Blast hit GOs	InterPro IDs	Classification based on Blast2GO and clustering
R1-1644	3008	Disease resistance RPM1-like	*Phoenix dactylifera*	GO:0043531 (ADP binding)	PR00364 (PRINTS) IPR032675 (G3DSA:3.80.10.GENE3D) IPR002182 (PFAM) IPR027417 (G3DSA:3.40.50.GENE3D) PTHR23155:SF592 (PANTHER) PTHR23155 (PANTHER) PTHR23155 (PANTHER) IPR032675 (SUPERFAMILY) IPR027417 (SUPERFAMILY)	CC-NBS-LRR
R2-1990	1048	Nematode resistance-like HSPRO2	*Nelumbo nucifera*	GO:0006952 (defense response)	IPR009869 (PFAM) IPR009743 (PFAM) PTHR34795 (PANTHER) SSF140959 (SUPERFAMILY)	LRR-TrD (Hspro)
R3–2277	1067	Disease resistance RGA3	*Citrus sinensis*	GO:0043531 (ADP binding)	PR00364 (PRINTS) IPR002182 (PFAM) IPR027417 (G3DSA:3.40.50.GENE3D) PTHR23155 (PANTHER) PTHR23155:SF414 (PANTHER) IPR027417 (SUPERFAMILY)	NBS-LRR
R4-2905	631	Disease resistance RGA2-like isoform ×1	*Nelumbo nucifera*	No GO term	IPR032675 (G3DSA:3.80.10.GENE3D) SSF52047 (SUPERFAMILY)	NBS-LRR
R5-4130	724	Disease resistance RGA3	*Populus trichocarpa*	No GO term	IPR032675 (G3DSA:3.80.10.GENE3D) SSF52047 (SUPERFAMILY)	NBS-LRR
R6-3203	572	LRR receptor-like serine	threonine-kinase FLS2 Kinase fls2	*Nelumbo nucifera*	GO:0016020 GO:0043226 GO:0044464 GO:0004672 GO:0006952 GO:0009617 GO:0016310 GO:0044763 GO:0050794	

Table 2 (continued)

Sequence Name	Transcript length	Sequence description	Top-hit species	Blast hit GOs	InterPro IDs	Classification based on Blast2GO and clustering
						GO:0051179 GO:0051716 F:ATP binding P:protein phosphorylation F:protein kinase activity
IPR0007-19 (PFAM) G3DSA:1.10.-510.10		(GENE3D) PTHR27000 (PANTHER) PTHR27000:-SF16 (PANTHER) IPR011009 (SUPER-FAMILY)	LRR--TrD--KIN			
R7-1670	678	Disease resistance RGA3	*Populus trichoc-arpa*	GO:0043531	IPR032675 (G3DSA:3.80.10.GENE3D) PTHR23155:SF563 (PANTHER) PTHR23155 (PANTHER) IPR032675 (SUPERFAMILY)	NBS-LRR
R8-33941	358	Disease resistance protein rga2-like isoform ×1	*Citrus sinensis*	GO:0043531	PTHR23155:SF563 (PANTHER), PTHR23155 (PANTHER)	NBS-LRR
R9-46693	326	Disease resistance RGA3	*Sesamum indicum*	GO:0043531	PTHR23155 (PANTHER), SIGNAL_PEPTIDE (PHOBIUS), SIGNAL_PEPTIDE_N_REGION (PHOBIUS), SIGNAL_PEPTIDE_C_REGION (PHOBIUS), SIGNAL_PEPTIDE_H_REGION	NBS-LRR

Table 2 (continued)

Sequence Name	Transcript length	Sequence description	Top-hit species	Blast hit GOs	InterPro IDs	Classification based on Blast2GO and clustering
					(PHOBIUS), NON_CYTOPLASMIC_DOMAIN (PHOBIUS), TMhelix (TMHMM)	
R10-36038	309	Disease Disease resistance RGA3	*Citrus Nelumbo nucifera*	No GO term	PTHR23155:SF563 (PANTHER) PTHR23155:SF505 (PANTHER) PTHR23155 (PANTHER)	NBS-LRR
R11-7014	656	Disease resistance RGA3 isoform X1	*Beta vulgaris* subsp. *vulgaris*	GO:0043531	PR00364 (PRINTS) IPR027417 (G3DSA:3.40.50.GENE3D) IPR002182 (PFAM) PTHR23155 (PANTHER) PTHR23155:SF494 (PANTHER) IPR027417 (SUPERFAMILY)	NBS-LRR
R12-4018	1128	Disease resistance RGA4	*Prunus persica*	No GO term	IPR032675 (G3DSA:3.80.10.GENE3D) PTHR23155:SF554 (PANTHER) PTHR23155 (PANTHER) SSF52047 (SUPERFAMILY)	NBS-LRR

ranking with *ATUB* ranked as the most stable reference gene. *FLAP* and *GAPD* were identified to be the least stable reference genes by all computational algorithms (Supplementary Table 3). The comprehensive ranking according to RefFinder from the most stable to the least stable reference gene was in the order *ATUB*, *ACT*, *EIF3E*, *SKP1*, *EF1A*, *FLAP*, and *GAPD* (Supplementary Fig. 4b).

The pairwise variation of the reference genes was determined for the dataset, and a threshold value of 0.15 was adopted to identify the minimum number of genes to be used for accurate normalization of real-time data [25]. The $V_{3/4}$ ratio calculated for the reference genes in *P. colubrinum* was 0.15, whereas the $V_{2/3}$ ratio was above the recommended threshold of 0.15 (Supplementary Fig. 5). Hence, the optimal minimal number of the reference gene to be used in *P. colubrinum* under biotic stress to accomplish accurate qPCR data normalization was 3, and the combination of reference genes recommended in the current experimental condition is *ATUB*, *ACT*, and *EIF3E*.

Relative Expression Study of Twelve Selected R Genes

The relative expressions of 12 different R genes in *P. colubrinum* were calculated using the selected reference genes *ACT*, *ATUB*, and *EIF3E*. The expressions of R genes in uninoculated samples of *P. colubrinum* leaves were studied in tissue culture-maintained plantlets against greenhouse-grown plants to identify the R gene expression without pathogen trigger. The study identified that the selected R genes were expressed in both uninoculated tissue culture leaves and the greenhouse-grown plants. The relative expression levels of the selected R gene transcripts in tissue culture plants unexposed to pathogen trigger were lower or at par with the greenhouse-maintained *P. colubrinum* sample. Six of the 12 selected R genes were at least 1 fold ($\log_2$) downregulated in tissue culture plantlets in comparison with greenhouse-maintained plants, and the remaining were at par with the greenhouse plants (Fig. 1).

The challenge inoculation of *P. colubrinum* leaves with two virulent isolates of *P. capsici* resulted in the change in relative expression of R gene transcript levels in the plant. Most of the selected genes studied were induced in the initial hpi (0.5 and 2 h) invariable of the *P. capsici* isolate used for infection. The *R12-4018* (> 6-fold) gene showed the maximum relative

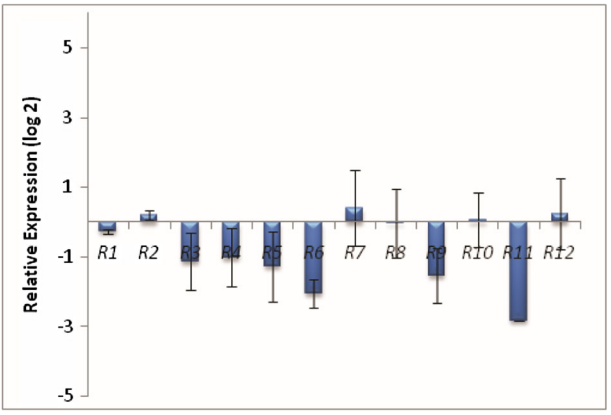


Fig. 1 Relative expression of 12 selected R genes in uninoculated *P. colubrinum* tissue culture-derived leaves in comparison with the uninoculated greenhouse-grown plants showing relatively lower basal R gene transcript accumulation in tissue culture plants

expression on interaction with either of the isolate, followed by *R2-1990* (> 5-fold). The gene *R3*-2277 was overexpressed (> 2-fold) at 0.5 and 2hpi when challenge-inoculated with the isolate 98-93 but not with *05-06*. The expression of all the selected R genes was downregulated at 1 and 16 hpi irrespective of the pathogen isolate used for study. Figure 2 shows the relative expression of the *P. colubrinum* R genes at different hpi on challenge inoculation with *P. capsici*.

Discussions

Numerous R genes have been identified and cloned in plants and classified based on their conserved domains. Even though many classifications of R genes were reported, the classification criteria are based on the presence or absence of conserved domains. In the present study on *P. colubrinum* transcriptome, we could obtain various transcripts belonging to different R gene classes which were expressed under the influence of *P. capsici.* The reverse alignment (tblastn) with known R genes together with blastx analysis facilitated the identification of 1289 R gene transcripts expressed in *P. colubrinum*. The blastx analysis of the transcriptome helped us to mine 221 exclusive non-redundant R gene transcripts which were not obtained by reverse alignment using seed sequences. Hence, an additional blastx analysis was found to be useful in mining additional R gene transcripts from a dataset. The use of more number of representative genes per class helped us to identify numerous transcripts sharing similarity to R genes than by using a smaller dataset (737 transcripts with 30 query sequences). Use of a large number of query sequences for mining R gene transcripts was also reported by Wanderley-Nogueira et al. [30, 31]. The use of lower e^{-5} helped to mine 147 extra R genes from *P. colubrinum* than e^{-10}; hence, lower stringency with e^{-5} was selected for reverse alignment to avoid the loss of important R gene transcripts from the dataset. It has been reported that the use of higher stringency e^{-50} in the blast analysis has resulted in the loss of R genes in sugarcane and the lowering of the E-value from e^{-20} to e^{-10} facilitated in the identification of extra R gene clusters [30]. But in the present study, though many R genes could be mined by the lowering of the E-value, it has also resulted in a few non-R gene transcripts aligning to the seed sequences which had to be manually removed after Blast2GO search. On reverse alignment mining, numerous *P. colubrinum* transcripts have aligned to more than one class of query sequence due to the sharing of domains by a different class. The alignment of a transcript to more than one class of seed sequence was also observed by previous researchers [32].

The *P. colubrinum* R gene transcripts were grouped into 91 homology clusters and 64 singletons on hierarchical clustering of amino acid sequence by the CD-HIT tool. Blast2GO and InterProScan revealed the presence of CC, NBS, LRR, KIN, and TrD domains in the transcripts, and the clusters were classified based on these. Maximum numbers of clusters were found to be in the class NBS-LRR followed by enzymatic R genes. Two clusters were identified, which did not have any conserved motifs and hence could not be classified. Six *P. colubrinum* NBS-LRR transcripts were found to have an atypical transmembrane motif, similar to the finding in eucalyptus TIR-NBS-LRR-related sequences [17]. LRR was the most abundant in *P. colubrinum* R genes; the abundance may be attributed to the presence of the LRR domain in more than one class of R gene.

R gene sequences from *P. colubrinum* were clustered into classes II, III, IV, and VIII. Two transcripts having a RPW8 domain (class V) and six *Verticillium* wilt genes (class VII) were also mined. One RPW8 R gene remained as singleton on clustering analysis, while the other

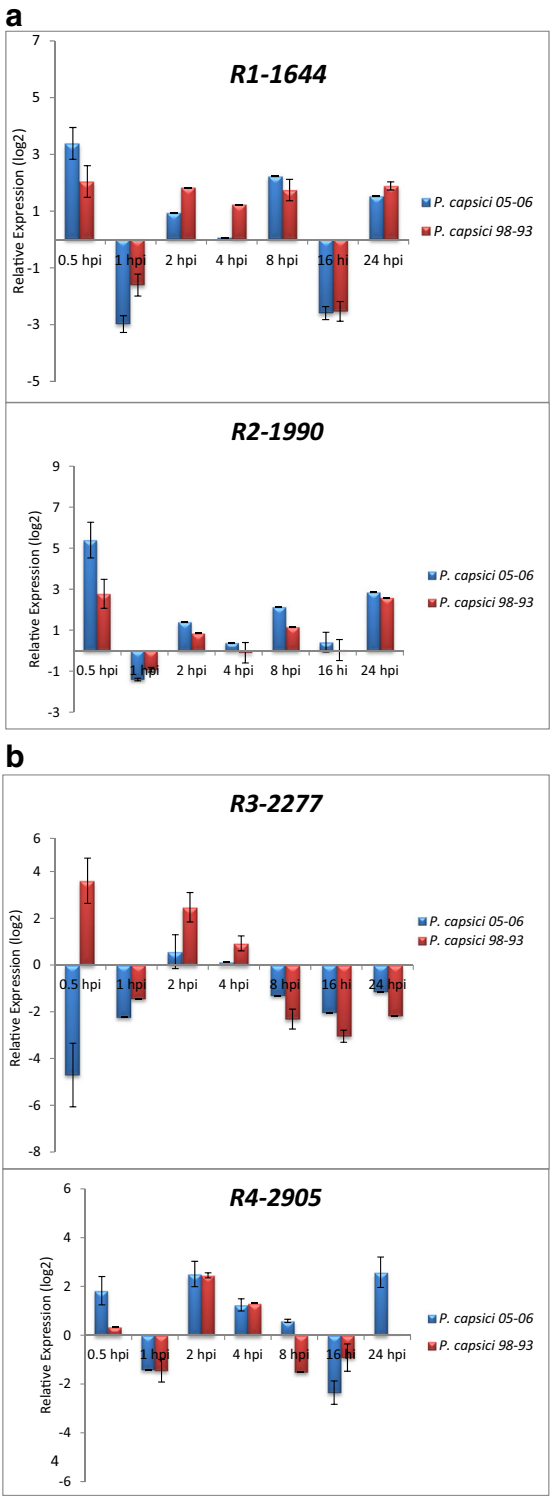

a
R1-1644
Relative Expression (log2)
7
5
3
1
-1
-3
-5
0.5 hpi
1 hpi
2 hpi
4 hpi
8 hpi
16 hi
24 hpi
P. capsici 05-06
P. capsici 98-93
R2-1990
Relative Expression (log2)
9
7
5
3
1
-1
-3
0.5 hpi
1 hpi
2 hpi
4 hpi
8 hpi
16 hi
24 hpi
P. capsici 05-06
P. capsici 98-93
b
R3-2277
Relative Expression (log2)
6
4
2
0
-2
-4
-6
-8
0.5 hpi
1 hpi
2 hpi
4 hpi
8 hpi
16 hi
24 hpi
P. capsici 05-06
P. capsici 98-93
R4-2905
Relative Expression (log2)
6
4
2
0
-2
-4
4
-6
0.5 hpi
1 hpi
2 hpi
4 hpi
8 hpi
16 hi
24 hpi
P. capsici 05-06
P. capsici 98-93

Fig. 2 Expression of 12 selected R genes transcripts in *P. colubrinum* at different hpi on challenge inoculation with two isolates of *P. capsici* (98-93 and 05-06) showing the relative accumulation of R gene transcripts in the initial hours of challenge inoculation and downregulation of the genes towards the later hours

could not be taken for clustering due to its limited size (< 100 aa), and this gene has an LRR repeat, which is uncommon in this class. The *Verticillium* wilt-resistant (Ve) type clustered together with the class III transcript owing to the presence of the LRR region, and hence was classified under MIX, and this Ve type was considered separate class by Gururani et al. TIR-NBS-LRR (class I) is reported to be present mostly in dicots and gymnosperms and absent in monocots and magnoliids [33]. No TIR-NB-LRR genes were identified in *P. colubrinum* which belongs to the magnoliids clade, confirming the previous report.

Real-time quantitative PCR (qPCR) is a very sensitive and reliable method for the identification of gene expression in the biological system [34]. Although relative gene expression studies using qPCR are gaining importance in plant biological research, the identification of stable internal reference genes is essential for valid qPCR analysis. Even though equalization of the initial RNA quantity and use of equal size of sample for isolation are the basic steps of RNA normalization, variation in amplification efficiency, disparity in the cDNA sample loading, and reverse transcriptase (RT) efficiency can have an effect on the expression analysis [35]. To overcome this, the real-time data should be normalized to a stable reference gene under any given set of condition. As many of the commonly used reference genes show differential expressions under different biotic and abiotic stress conditions, the identification of a stable reference gene during any biological study is considered inevitable. *ACT*, *ATUB*, and *EIF3E* were found to be the most stable reference genes in *P. colubrinum* under *P. capsici* biotic stress.

Twelve highly expressing R genes based on the FPKM value from pathogen-challenged *P. colubrinum* transcriptome were chosen for expression profiling. As majority of oomycetes resistance genes reported belong to the NBS-LRR class [11], we have chosen 10 transcripts representing NBS-LRR type for expression study. *Hspro* genes are reported to impart resistance against a number of pathogens including aphids [36], nematodes [37], and *Pseudomonas syringae* [38]. An *Hspro* type R gene, *R2-1990*, was also for taken expression analysis.

Uninoculated samples from greenhouse- and tissue culture-maintained plants were taken to evaluate the expression of R genes without pathogen trigger. Though greenhouse-grown uninfected plants alone may serve as a medium to identify basal expression of the R gene, the constant interaction of the plant with the soil and other microorganisms might influence the gene expression. Hence, tissue culture plants with minimal/zero contact with microorganisms or pathogens were used in our study for verification of R gene expression without any pathogen trigger. R gene transcripts studied were found to be expressed in both the plants indicating constitutive expression of R genes to a certain level which might help in triggering fast hypersensitive (HR) reaction upon pathogen entry. The lower expression of six R gene transcripts in uninoculated tissue-cultured plants than greenhouse plants suggests that the constant interaction of greenhouse plants with the environmental microflora might elevate the R gene expression in them. Basal-level transcript expression of R genes was also reported in uninoculated leaves of *Solanum* [39], *Arabidopsis* [40], and *Curcuma* [41], though the expressions of the R genes in tissue culture plants were not analyzed.

Eleven R genes studied were invariably upregulated in *P. colubrinum* during challenge inoculation irrespective of the isolate used for challenge inoculation, but *R3-2277* (NBS-LRR

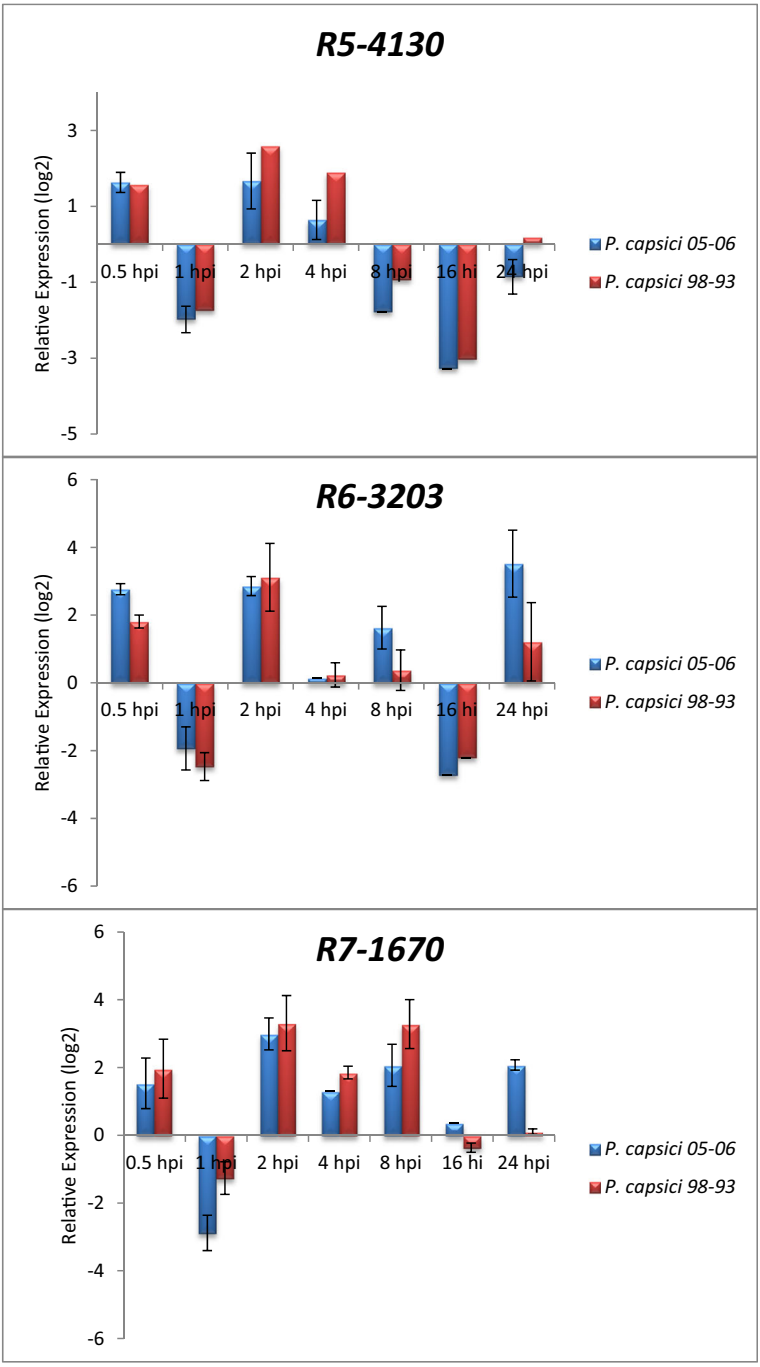
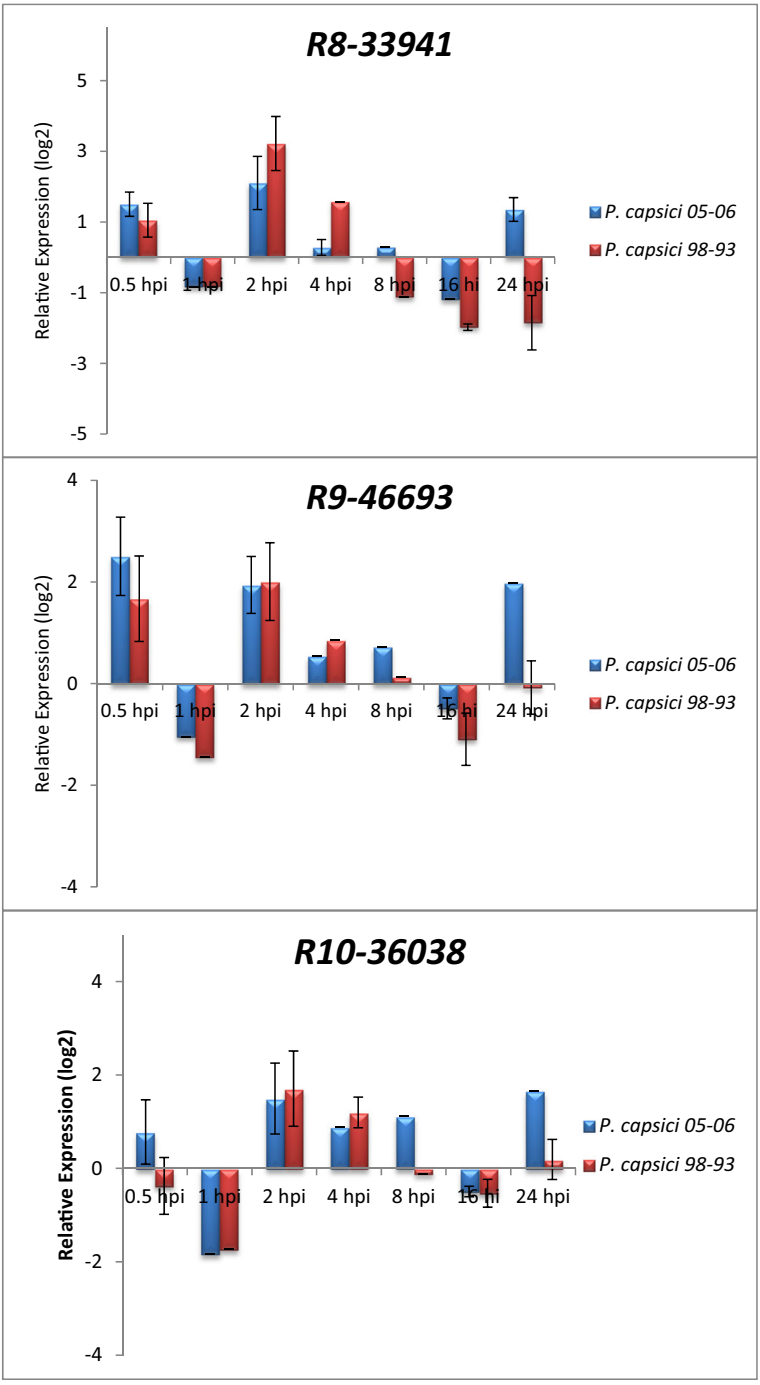


Fig. 2 (continued)



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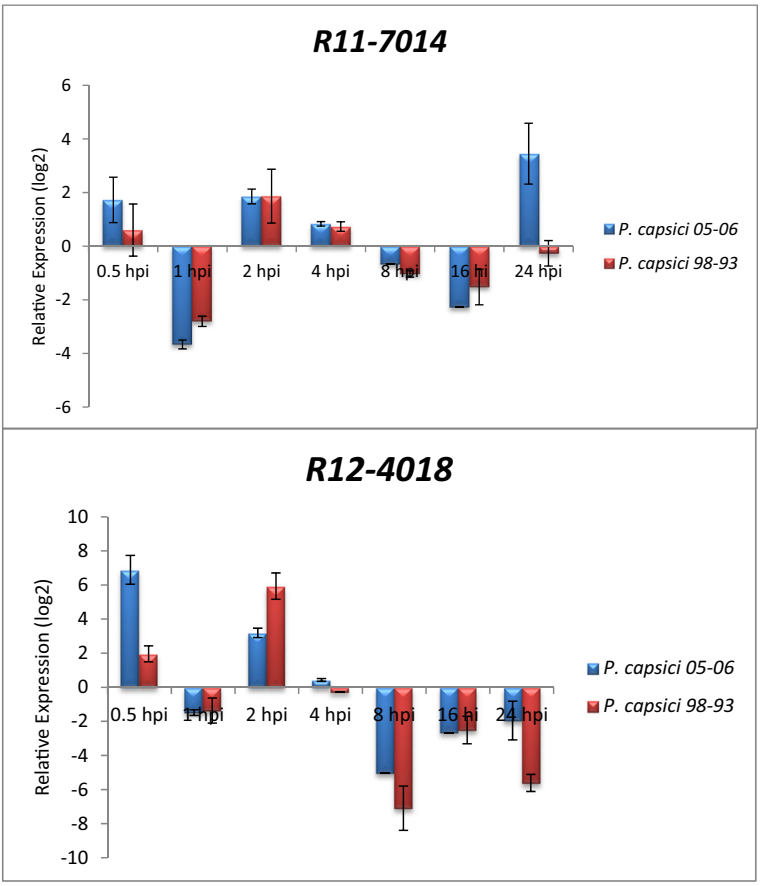

Fig. 2 (continued)

type) was upregulated only on interacting with the 98-93 isolate, indicating that this gene may be specifically triggered by this isolate. Although each R gene followed specific expression patterns when compared to the other, they exhibited a similar relative expression profile on using either of the isolates for challenge inoculation.

Eleven R genes studied were relatively upregulated in the initial hours of interaction (at 0.5 and/or 2hpi) with both the virulent *Phytophthora* isolates. The ability of resistance genes to identify more than one strain of virulent pathogen was also observed by a number of researches [39, 41, 42]. Early upregulation of the R genes could help the plant to initiate a faster HR reaction which may account for its increased resistance towards this pathogen. Kar et al. [41] also observed the upregulation of the R gene, *CzR1* in 3hpi, when *Curcuma zedoaria* was infected with four virulent strain of the *Pythium*. The increased relative expression of the *Hspro*-type gene, *R2-1990*, during inoculation study suggests that this *Hspro*-like R gene may also have a role to play in *Phytophthora* resistance in *P. colubrinum*. The transcript accumulation of more than one R gene in *P. colubrinum* in response to pathogen inoculation suggests the involvement of more than one R gene in pathogen perception and elimination.

In the present study, it was observed that the expressions of all the R genes were downregulated at 1 and 16 hpi when *P. colubrinum* was inoculated with both the isolates of *P. capsici*. It may be postulated that *Phytophthora* miRNA may be involved in the suppression of the defense mechanism at this time interval. miRNAs are involved in control of expression of many plant R genes [43, 44]; gra-miR482 family in cotton [45] and miR482/2118 from Solanaceae [46] are reported to target multiple NBS LRR genes. Plant and pathogens, though unrelated, can have conserved miRNA. Transgenic plants overexpressing miRNA from *Phytophthora infestans*, pi-miR1918, which was conserved across its host, tomato**,** suppress target gene**s** involved in defense mechanism in tomato [47]. Similar mechanisms of pathogen-induced miRNA-mediated suppression of host defense may be operating in this case, which has to be further investigated. Though the R genes analyzed in the present study belong to three different classes, the presence of the LRR domain in them may be a possible target of the small RNA leading to the miRNA-mediated downregulation of these R genes. The hemi-biotrophic life cycle of *P. capsici* is associated with the reprogramming transcription of immune-responsive genes in the host system in the early and transitional phases of the pathogen's life cycle [48]. The regulation of the *P. colubrinum* R gene by the *P. capsici* miRNA can contribute towards the downregulation of the genes at 1 hpi (initial infection phase) and 16 hpi (start of the transition phase).

To conclude, the abundance, diversity, and relative expression of R genes in the multi-disease-resistant *P. colubrinum* were identified by us in the study. We have mined and clustered more than a thousand R gene transcripts from *P. colubrinum* transcriptome expressed under the influence of *P. capsici* and classified them into four major R gene classes. For relative expression study, *ACT*, *ATUB*, and *EIF3A* were identified as the most stable references in *P. colubrinum*; these genes may be positively used for any qPCR data normalization in *P. colubrinum-Phytophthora* interaction studies in the future. The relative expression profiling of 12 selected R genes revealed the R gene transcript expression in uninfected tissue-cultured and greenhouse-grown plants, suggesting that the expressions of R genes may not require a pathogen trigger and are constitutively expressed and were found to be upregulated on challenge inoculation with two different virulent isolates of *P. capsici*. The upregulation of R genes in the initial hours during pathogen inoculation study implies that resistance of *P. colubrinum* against the pathogen can be due to the faster onset of R gene-mediated immune response in the plant.

This is the first attempt to study R genes in the promising disease-resistant plant *P. colubrinum* and can serve as an insight into the defense response triggered in *P. colubrinum* in response to *P. capsici*. The full-length characterization and the functional validation of these promising R genes, namely, *R1-1644*, *R12-4018*, and *R2-1990*, will be the next line of our study which can help in confirming their role in *Phytophthora* resistance.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that that they have no conflict of interest.

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