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Molecular characterization and functional analysis of *CzR1*, a coiled-coil-nucleotide-binding-site-leucine-rich repeat *R*-gene from *Curcuma zedoaria* Loeb. that confers resistance to *Pythium aphanidermatum*

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ABSTRACT

Rhizome rot disease caused by necrotrophic oomycete *Pythium aphanidermatum* is responsible for upto 60% of yield losses in turmeric (*Curcuma longa* L). However, *Curcuma zedoaria* L, a wild relative of turmeric, is resistant to *P. aphanidermatum* and has been proposed as a potential donor for rot resistance to *C. longa*. We used a previously isolated resistance gene candidate *Czp11* from *C. zedoaria* as a template to characterize a major resistance gene *CzR1* through candidate gene approach in combination with RACE-PCR strategy. *CzR1* encodes a 906 amino acid predicted protein with a calculated pI of 8.55. Structural and phylogenetic analyses grouped *CzR1* within the non-TIR (homology to Toll/interleukin-1 receptors) subclass of NBS-LRR *R*-genes. Reverse transcription PCR revealed specific transcript expression of *CzR1* only in *P. aphanidermatum* resistant lines of *C. zedoaria* and *Zingiber zerumbet*, another resistant wild species of the family *Zingiberaceae*. Semi quantitative RT-PCR analysis showed constitutive expression of *CzR1* which gets significantly upregulated in response to infection by different strains of *P. aphanidermatum*. Although, the expression of *CzR1* was reported in the root, leaf and rhizome tissues of *C. zedoaria*, the relative transcript expression was highest in the rhizomes. Elucidation of these molecular characteristics of *CzR1* will pave way towards a broad spectrum rhizome rot resistance development in the cultivated turmeric.

1. Introduction

Turmeric (*Curcuma longa* Loeb; *Zingiberaceae*) is one of the most important herb in the tropical and sub-tropical countries. Its rhizome is used as a spice, food preservative, coloring agent, and in the traditional systems of medicine [1]. Recent utility of turmeric by the pharmaceutical industries as a source of antioxidant, hepatoprotectant, anti-inflammatory in addition to its use in cardiovascular and gastrointestinal disorders has categorized it as a major industrially important crop of high demand [1]. The International Trade Centre, Geneva, has estimated an annual growth rate of 10% in world demand for turmeric [1]. However, crop losses upto 60% has been realized in the recent times mainly due to the infection by a necrotrophic oomycetic fungus *Pythium aphanidermatum* causing the rhizome rot disease in turmeric [2]. Utilization of chemical pesticide for the control of rhizome rot is highly unsatisfactory and growing cultivars with inherent resistance to *P. aphanidermatum* can be the most cost-effective and environment friendly method of protecting turmeric plants. However, the obligatory asexual nature and high stigmatic incompatibility of the extant turmeric lines prevents the establishment of a conventional breeding approach. A genetic transformation approach using foreign genes could be the only solution towards development of rhizome rot resistance in turmeric. Although the transformation technology in turmeric is ready [3], no resistance genes have been cloned and transferred to susceptible turmeric cultivars against the most destructive turmeric diseases.

Utilization of the plant disease resistance genes (*R*-genes) has been the major means towards detection of the pathogen effectors and activation of the plant defense response. *R* gene-mediated recognition of specific pathogen virulence factors as invasion signals results in the activation of a series of rapid cellular defense signaling often leading to swift local cell death at the infection site through hypersensitive response (HR) [4]. Around 70 different plant *R* genes grouped into five major classes have been isolated

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and characterized from different plant species during the last 15 years for resistance to a wide spectrum of pathogens, including bacteria, viruses and fungi [5]. Among them, the largest class of *R*-gene encodes proteins that have a putative amino-terminal signaling domain, a nucleotide binding site (NBS) and a series of carboxy-terminal leucine rich repeats (LRRs) [6]. These genes have been classified into the TIR subclass and the nonTIR subclass on the basis of the presence/absence of an N-terminal Toll/interleukin receptor (TIR) domain [7]. Genes in the TIR group are known among both monocotyledonous and dicotyledonous species while the non-TIR group typically includes a coiled-coil (CC) sequence or putative leucine zipper (LZ) at the N terminus among the monocots [8]. The LZ domain is believed to facilitate the formation of CC structure to promote oligodimerization with a wide variety of proteins although its actual task in *R*-gene function is still unknown [9]. The NBS region is thought to regulate signal transduction through nucleotide triphosphate (NTP) hydrolysis and conformational changes [10,11]. The LRRs are the major sites for protein–protein interaction and determines the specificity for the pathogen avirulence factor(s) [12].

Our laboratory has been engaged in characterizing resistance related sequences in turmeric against *P. aphanidermatum* through candidate gene approach. The genetic variation for disease resistance is poorly developed in the cultivated turmeric [13]. Our earlier attempt to clone resistance related sequences from wild turmeric genotypes resulted in the isolation of expressive resistance gene candidates (RGCs) from *Curcuma aromatica*, *Curcuma angustifolia* and *Curcuma zedoaria* [14]. Interestingly, the expression of *Czp11* RGC from *C. zedoaria* was uniquely found associated only with *P. aphanidermatum* resistant lines. This is in accordance to the earlier report that *C. zedoaria* L, a wild relative of turmeric show exclusive resistant against *P. aphanidermatum* [15]. In the present report, we have cloned and characterized a *P. aphanidermatum* responsive NBS-LRR *R*-gene *CzR1* in *C. zedoaria* using *Czp11* RGC as the reference, analyzed its phylogeny, expression pattern and discussed the possible function of the *R*-gene encoded protein in regulating defense mechanism in *C. zedoaria.*

2. Materials and methods

2.1. Plant material and pathogen inoculation

A *C. zedoaria* accession (Accn. No. Cze512-11), resistant to *P. aphanidermatum* was used for isolation of the *R*-gene. In addition, four resistant *C. zedoaria* accessions (Cze516-04; Cze522-01; Cze527-13; Cze533-06), five susceptible *C. zedoaria* accessions (Cze102-03; Cze107-01; Cze112-09; Cze121-07; Cze123-02), a wild accession of *Zingiber zerumbet* resistant to *P. aphanidermatum* and a susceptible cultivated turmeric line *C. longa* cv. Surama were used for functional analysis. Four virulent strains of *P. aphanidermatum* (CBT-201 collected from Indian Institute of Spices Research, Kerela; CBT-27 collected from Pottangi, Orissa; CBT-113 collected from Gangtok, Sikkim and CBT-153 collected from Guwahati, Assam) were used for plant inoculation. Rhizomes harvested from mature plants were sprouted in earthen pots and the pseudostems were allowed to grow for 3 months. Inoculation was done using a field isolate of *P. aphanidermatum* (CBT-201) obtained from Indian Institute of Spices Research (IISR), Calicut, Kerala, India. The pseudostems were pin-pricked and inoculated by pouring 500 μl of zoospore suspension upon the poked region according to Kavita and Thomas 2008 [16].

2.2. DNA and RNA isolation and RT-PCR

Rhizome from inoculated plants was frozen in liquid nitrogen and grounded into fine powder. Total Genomic DNA was then extracted by using the protocol described by Doyle and Doyle (1990) [17] with required modifications. RNA was isolated from the rhizome using TRI reagent (Sigma–Aldrich, USA) following the manufacturer's instructions. It was treated with RNAse free DNAse I (Promega) to remove genomic DNA contaminants. The quality and concentration of DNA and RNA samples were examined by ethidium bromide-stained agarose gel electrophoresis and spectrophotometric analysis.

cDNA was synthesized from 1ug of total RNA using a GoScript reverse transcription system (Promega). A RGC specific primer pair designed previously from the conserved region of *Czp11* RGC of *C. zedoaria* was used for reverse transcription polymerase chain reaction (RT-PCR). PCR amplification was performed in a final volume of 25 μl reaction mixture containing 2 μl of 25 mM $MgCl_2$, 0.2 μl of 10 mM dNTP mix, 5 μl of 5× reaction buffer, 1 μl of synthesized cDNA, 1U of *Taq* DNA polymerase and 1 μM of each of the RGC specific primers (forward, 5′ CCCTGGCGAAAATTGTGTAT 3′; reverse, 5′ GGCAGGCCCAGCACAATAAT 3′). The reaction conditions were 5 min at 94 °C, followed by 35 cycles of denaturing at 94 °C for 1 min, annealing at 57 °C for 30 s, and elongating at 72 °C for 2 min followed by a final extension at 72 °C for 7 min. The RT-PCR reaction included a positive control for turmeric *Actin 1* gene and a negative control with RNA instead of cDNA as template. Amplicons were separated on a 1.2% agarose gel. The amplified products were electrophoresed, gel eluted (SV gel purification kit, Promega), cloned into pTZ57R/T vector (Insta clone T/A cloning kit, Fermentas, Germany) and sequenced.

2.3. Isolation of full length cDNA

To obtain the full length sequence of the cDNA, 5′ and 3′ rapid amplification of cDNA ends (RACE) reactions were performed using 5′/3′ RACE amplification kit (Invitrogen, USA) following the manufacturer's instructions with minor modifications. Based on the available partial cDNA sequence of Czp11, primers were designed for 5′ and 3′ RACE reactions. Gene specific primers Cz5PR1 and Cz5PR2 were utilized for 5′ RACE. Cz3PR1 and Cz3PR2 were used for 3′ RACE PCR (Table 1). The first round of PCR was followed by a nested round of PCR. PCR amplification was programmed on a Veriti Thermal Cycler (Applied Biosystems) with 35 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 2 min with a final extension of 10 min at 72 °C. The RACE products were fractionated on agarose gels, eluted (SV gel purification kit, Promega), cloned using pTZ57R/T vector (Insta clone T/A cloning kit, Fermentas, Germany) and

Table 1
Primers used in the study.

Primer	Description	Sequence (5′-3′)
P1F	Degenerate primer forward	GGIGGIRTIGGIAARACIAC
P2F	Degenerate primer reverse	WTIARIGYIARIGGIARICC
Czp11F	RGC specific primer forward	CCCTGGCGAAAATTGTGTAT
Czp11R	RGC specific primer reverse	GGCAGGCCCAGCACAATAAT
3PR1	3′ RACE primer, Forward outer	CCAGGATAGCAGCCTGAACACCGAA
3PR2	3′ RACE primer, Forward nested	GAACAGCTGCTGAACATGATGGAT
3 PR-AP	3′ RACE Adaptor primer	GGCCACGCGTCGACTAGTACT$(T)_{16}$
3 PR-AUAP	3′ RACE Abridged universal amplification primer	GGCCACGCGTCGACTAGTAC
5PR1	5′ RACE primer, reverse outer	GCAGCGCAATGTTCAGGCTTTCCCAC
5PR2	5′ RACE primer, reverse nested	CACGCGCCGGTGCGCCACACATCAT
5PR-AUAP	5′ RACE Abridged Universal Amplification Primer	GGCCACGCGTCGACTAGTAC
CzR1F	Gene specific primer forward	CTAGTCTGCACTCGGCGTTACGCA
CzR1R	Gene specific primer reverse	CACCGGTCCTTGCAAGTGCAGACA
CzRTF	Gene specific primer forward	GCAAAATTGAAAGCCTGAGC
CzRTR	Gene specific primer reverse	AATCTGCACCAGCTGGCTAT
CzR2F	Gene specific primer forward	CGATACCTGGGCCTACCCATTTGT
CzR2R	Gene specific primer reverse	TAGGCCCAGTTATCTGAGGCCCT

sequenced using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer) on an ABI Prism 310 genetic analyzer (Applied Biosystems). The full length cDNA of the *R*-gene was deduced by aligning 5′ and 3′ RACE product sequences with the partial cDNA fragment cloned earlier.

Gene specific primers CzR1F and CzR1R were designed based on sequence information of inserts from multiple clones derived from 5′ and 3′ RACE products and was used to amplify the full-length cDNA. PCR was carried out using the XT-5 PCR system (Merck Biosciences) in a total volume of 20 μl containing 2.0 mM $MgCl_2$, 200 μM dNTP mix (Fermentas), 10× PCR buffer, 10pM each of forward and reverse primers and 1.5 units of XT-5 Taq DNA polymerase using 2 μl of cDNA as template. The cycling conditions were 94 °C for 2 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 2 min. The full-length amplicon was eluted, cloned, and sequenced as described above. We designated the cDNA of the *R*-gene cloned from *C. zedoaria* as *CzR1*. Sequence data from this article have been deposited at the National Center for Biotechnology Information (NCBI) under accession number JX912230. Two gene-specific primers, CzR2F and CzR2R primer designed based on the cDNA sequence was used to PCR amplify the genomic clone of the *R*-gene using *C. zedoaria* genomic DNA as template. PCR was carried out using 50 ng of genomic DNA in a 25 μl reaction volume containing 2.0 mM $MgCl_2$, 200 μM dNTP mix (Fermentas), 10× PCR buffer, and 1.5 units of Taq DNA polymerase (Merck Biosciences). The PCR conditions were 94 °C for 2 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 2 min and a final extension of 15 min at 72 °C.

2.4. Genomic southern blot analysis

10 μg of *C. zedoaria* genomic DNA was digested with restriction endonucleases: *EcoRV, XbaI, BglII* and *SacI* (Fermentas, Germany) respectively. The digests were electrophoretically separated on 1% agarose gel, capillary-blotted onto a nylon membrane filter (Hybond-N+, Amersham Pharmacia Biotech) with 0.5 N NaOH transfer buffer and baked for 2 h at 80 °C. Digoxigenin labeled probes were prepared from purified DNA of *CzR1* gene using a digoxigenin DNA labeling and detection kit (Roche Diagnostics, Basel, Switzerland). A 297 bp fragment at the 3′ end (83 bp of the 3′UTR and 214 bp of the coding region) of the *CzR1* gene was PCR amplified as DIG-labeled probe using a mixture of DIG-labeled and standard dNTPs in 1:3 ratio. The membrane was blocked for 1 h at 62 °C with DIG Easy Hyb (Roche) before hybridization. It was then hybridized with DIG-labeled probes at 65 °C for 15 h in a hybridization chamber followed by two washes with 0.1× SSC (15 mM NaCl, 1.5 mM Na-citrate, pH 7.0) containing 0.1% sodium dodecyl sulfate at 65 °C for 20 min. Detection of the hybridized probe was done according to the manufacturer's instructions.

2.5. Sequence analysis

Homology sequence search of the *CzR1* gene sequence was performed with BLASTn and BLASTp with non-redundant GenBank database (http://www.ncbi.nlm.nih.gov). Protein translations of the gene sequence was performed using (DNA/RNA to protein) Translate tool at ExPASy Server (http://www.expasy.ch). The protein comprises 906 amino acids. A multiple sequence alignment of the following *CzR1* protein with that of other previously reported *R* proteins such as *Mla1* from *Hordeum vulgare* (AAG37356), *Pi36* from rice (ABI64281), *Lr10* from wheat (AAQ01784) and *RPM1* from *Arabidopsis thaliana* (A57072) was performed using CLUSTALX multiple sequence alignment tool at European Bioinformatics Institute (http://www.ebi.ac.uk), whereas the phylogenetic analysis was performed based on neighbor-joining (NJ) method using Molecular Evolutionary Genetics Analysis (MEGA) package version 5 [18]. The degree of support for particular grouping patterns in the tree was checked by 1000 bootstrapping replicates. Conserved motif structures of *CzR1* resistance protein was analyzed using the Multiple Expectation Maximization for Motif Elicitation (MEME) [19].

2.6. Expresion analysis and relative quantification by qRT-PCR

Total RNA was isolated from 45 days old rhizome tissue of five resistant *C. zedoaria* accessions, five susceptible *C. zedoaria* accessions, a wild accession of *Z. zerumbet* resistant to *P. aphanidermatum* and a susceptible cultivated turmeric line *C longa* cv. Surama using the SV total RNA isolation system (Promega). cDNA synthesis was carried out using GoScript reverse transcription system (Promega) according to manufacturer's instructions. PCR amplification was performed using a 25 μl reaction mixture containing 2 μl of 25 mM MgCl2, 0.2 μl of 10 mM dNTP mix, 1 μM of each forward and reverse primer, 5 μl of 5× reaction buffer, 1 μl of synthesized cDNA and 1U of *Taq* DNA polymerase. A set of gene specific primers CzRT-F (5′GCAAAATTGAAAGCCTGAGC3′) and CzRT-R (5′AATCTGCACCAGCTGGCTAT3′) were designed to amplify a putative 722 bp fragment of CzR1 gene. The RT-PCR reaction included a positive control for turmeric *Actin 1* gene and a negative control with RNA instead of cDNA as a template. The reaction conditions were 5 min at 94 °C, followed by 35 cycles of denaturing at 94 °C for 1 min, annealing at 55–57 °C for 30 s, and elongating at 72 °C for 2 min followed by a final extension at 72 °C for 7 min. Amplicons were separated on a 1.2% agarose gel.

Mature plants of *C. zedoaria* accession Cze512-11 were inoculated with four virulent strains of *P. aphanidermatum*. Total RNA was also extracted from leaf, root and rhizome tissue using TRI reagent (Sigma–Aldrich, USA) at 3, 6, 12, 24, 48 and 96 h post inoculation (hpi) and just before inoculation (0 hpi) and was treated with RNase-free DNase (Promega) as per the manufacturer's protocol. cDNA synthesis was performed as discussed earlier. Gene specific primer CzR2F and CzR2R capable of amplifying the entire coding region (2.8 kb) were used for quantitative real-time polymerase chain reaction (qRT-PCR). qRT-PCR was set in a total volume of 25 μl containing 12 μL SYBR Green PCR Reagent (Applied Biosystems), 2 μL diluted cDNA, and 200 nM each of the gene specific primers in a StepOne real time PCR system (Applied Biosystems). The PCR conditions were as follows: The initial incubation at 95 °C was carried out for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 40 s. For quantification of the PCR products, the house keeping gene *Actin 1* amplified by using primers CAct1-F and CAct1-R was used as an endogenous control. Five technical replicates were taken for each biological sample. The relative gene expression was calculated using the threshold cycle ($2^{-\Delta\Delta C_T}$) method [20]. The ratio of gene-specific expression to house keeping gene signals was defined as the relative expression.

3. Results

3.1. Isolation of full length cDNA of CzR1

In our attempt to clone a *P. aphanidermatum* responsive resistance gene from *C. zedoaria*, a pair of gene specific primer Czp11F and Czp11R were designed to amplify the pathogen responsive resistance gene candidate Czp11. Using RT-PCR, a single cDNA fragment of 538 bp was obtained. A forward gene specific primer 3PR1 designed according to the sequence information of the partial cDNA fragment and a 3′ RACE Adaptor primer was used for the amplification of 3′-end cDNA of *CzR1*, resulting in a single and specific fragment of about 2107 bp (Fig. 1). The RACE product was further confirmed by amplification with the nested primer 3PR2 and 3′ RACE Abridged universal amplification primer (Table 1).

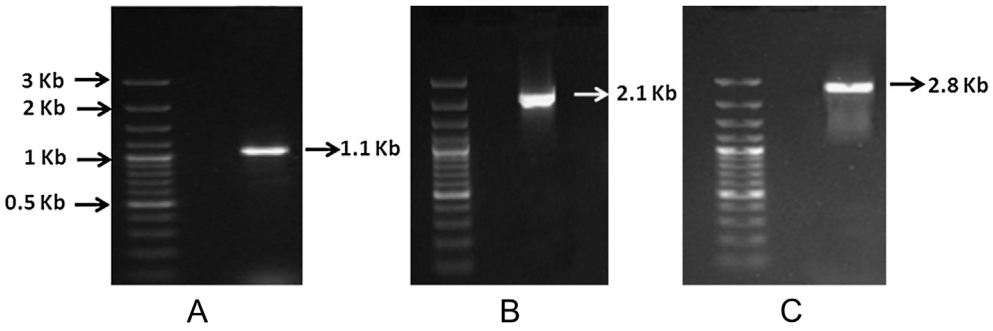

Fig. 1. Electrophoresis gels of the fragments of the middle region. A) Amplicon of 1.1 kb obtained from 5′ RACE-PCR. B) Amplicon of 2.1 kb obtained from 3′ RACE-PCR. C) Full length amplification of *CzR1* gene.

Likewise a fragment of about 1160 bp was obtained with 5′ RACE by a reverse gene specific primers 5PR1 and 5′ RACE Abridged Anchor Primer which was further confirmed by amplification with the nested primer 5PR2 and 5′ RACE Abridged Anchor Primer. The two types of sequences were aligned to obtain the overlapping regions and the full length cDNA of *CzR1* was deduced. Based on the sequences of RACE products, two gene specific primers CzR1F and CzR1R were designed and the full length cDNA was amplified and sequenced, which was identical to the deduced cDNA (Fig. 1). *CzR1* cDNA was submitted to NCBI Genbank database under the accession number JX912230.

3.2. Sequence analysis of CzR1

The full-length cDNA of *CzR1* is 2985 bp long including the open reading frame (ORF), 5′ and 3′ untranslated (UTR) regions (Supplementary Fig. S1). The cDNA contained a 2721 bp open reading frame (ORF) encoding a protein of 906 amino acids. The reading frame of the cDNA had the translational initiation codon (ATG) at nucleotide 171 and translational stop codon (TAG) at nucleotide 2888. The 5′ untranslated region constitutes a 170 bp sequence upstream from the start codon and 3′ untranslated region constitutes a 93 bp downstream of the stop codon thereby flanking the ORF. Both the 5′ UTR and 3′UTR region possessed moderate G + C content (48.23% and 52.12% respectively), and no polyadenylation signal sequence (AATAAA) was found within 3′UTR region. The conceptual protein has a predicted molecular weight of 102473.57 Da and a calculated isoelectric point (pI) of 8.55 (ExPaSy pI/Mw tool).

A GenBank BLASTp search revealed the conceptual gene product to have homology with the reported NBS-LRR type resistance proteins of *Hordeum vulgare, Oryza sativa, Triticum aestivum, Aegilops tauchii* and *Dasypyrum breviaristatum* besides several reported eukaryotic and prokaryotic proteins possessing leucine rich repeats. The *CzR1* encoded gene product exhibited the highest similarity of 62% to *Mla1* resistance protein from *Hordeum vulgare* (AAG37354) followed by 51% with CC-NBS-LRR *Pi36 R* protein from *Oryza sativa* (ABI64281) (Table 2).

3.3. Multiple alignment, phylogeny and functional motif analysis

Sequence alignment of the predicted amino acid residues of *CzR1* with known resistance gene products using ClustalX indicated that it contains all the conserved domains (N-terminus, NBS and LRR region) of known NBS-LRR resistance proteins described previously (Supplementary Fig. S2). Phylogenetic tree developed based on the neighbor joining method between *CzR1* and known resistance protein using MEGA 5 software, showed clustering of the predicted sequence with CC-NBS-LRR type *R*-proteins (Fig. 2). *CzR1* shared 62% similarity with *H. vulgare Mla1* (AAG37354) protein encoding powdery mildew resistance. While, it had 51%, 44% and 41% similarity with *Oryza sativa Pi36* (ABI64281), *Pi-ta* (AAK00132) and *Pib* (BAA76281) proteins, respectively, *CzR1* protein showed 41% and 38% resemblance with the wheat *Lr10* (AAQ01784) and *Arabidopsis RPM1* (Q39214). Likewise, the tomato *I2* (AF118127) protein shared 26% percent identity, while the common rust resistance protein *Lr21* (ACO53397) from wheat and *Rp1-D* (AAD47197) from maize exhibited 21% similarity each. On the other hand, *Arabidopsis* TIR-NBS-LRR protein *RPP5* (AAF08790) had 12% identity while both tobacco mosaic virus resistance protein *N* (AAA50763) and flax rust resistance protein *L6* (U27081) showed 10% similarity.

Conserved motif analysis of *CzR1* using the Multiple Expectation Maximization for Motif Elicitation (MEME) revealed several regions of similarity to the polypeptide encoded by the known resistance genes (Fig.3). The N-terminal region of ≈200 amino acids has a coiled-coil domain (leucine zipper) to facilitate protein–protein interaction. A 25 amino acid pre P-loop sequence, similar to *Arabidopsis RPM1* (Q39214) was identified in *CzR1* protein with the consensus ANNLVGID (158–165). Within the NBS domain, kinase-1a (P-loop) motif involved in interaction with phosphates and Mg^{2+} ions, was identified with conserved sequences of VVGGVGKTT (187–195), RNBS-A motif (257–279) and consensus sequence KRYLLVFDDVW (280–290) corresponding to kinase-2a motif, known to function in phosphotransfer reaction, were also identified (Fig. 3). Kinase 2a motif contained four consecutive

Table 2
Sequences showing significant homology to *CzR1* protein of *Curcuma zedoaria* in BLASTp search.

Sl no.	Accn no. & size	Blast sequence	% homology	E-value
1	AAG37354	*MLA1* resistance protein *(Hordeum vulgare subsp. vulgare)*	62%	0.0
2	ABI64281	CC-NBS-LRR *Pi36* resistance protein (*Oryza sativa* Indica group)	51%	1e-161
3	ACZ65507	NB-ARC domain containing resistance protein *MLA1 (Hordeum chilense)*	45%	1e-152
4	ADX06722	*Mla1* like CC-NBS-LRR resistance protein (*Triticum monococcum)*	44%	2e-119
5	ACC64518	*Y10* like resistance protein (*Dasypyrum breviaristatum)*	38%	1e-111
6	AAM69841	LZ-NBS-LRR class *R* protein (*Aegilops tauchii*)	34%	5e-116
7	ADY39265	Stripe rust resistance protein YR10 (*Triticum aestivum*)	34%	5e-103

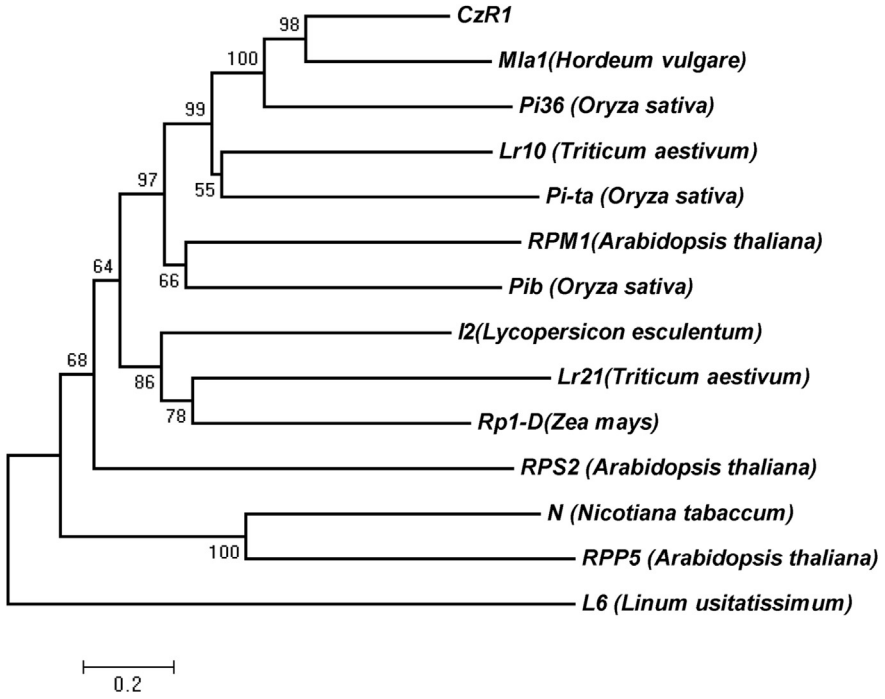

Fig. 2. Neighbor-joining phylogenetic tree of *CzR1* and NBS-LRR class *R*-genes from other plant species. The protein sequences of the known *R*-genes used for construction of the tree are listed in the GenBank database under the following accession number: *Mla1* (AAG37356), *Pi36* (ABI64281), *Lr10* (AAQ01784), *Pi-ta* (AAK00132), *RPM1* (Q39214), *Pib* (BAA76281), *I2* (AF118127), *Lr21* (ACO53397), *Rp1-D* (AAD47197), *RPS2* (Q42484), *N* (AAA50763), *RPP5* (AAF08790), *L6* (U27081). Numbers on the branches indicate the percentage of bootstrap replications.

hydrophobic amino acids (LLVL) followed by a conserved aspartate (D) and tryptophan (W) at the end site. RNBS-B motif with consensus SRVIVTTRI (309–317) and RNBS-C motif involved in purine or ribose binding was found with an argenine (R) residue (337–351). Consensus GLPLA (amino acid 374–378), corresponding to Domain2 (hydrophobic domain) and RNBS-D domain, CLLYLSLFP (432–440) were also identified. The eighth major conserved motif called MHDV, which is highly conserved in most CC-NBS-LRR genes, with minor modification in TIR-NBS-LRR proteins, was identified with the consensus CRMHDVLVDL at 501-510 positions. Eleven imperfect leucine rich repeats (LRR) regions with an average size of 24 amino acids were identified in the C-terminal region (583–897). The conserved aliphatic residue of the β-turn- β-sheet domain (xxLxLxx) was located within the imperfect repeat.

3.4. Genomic structure analysis of CzR1

Southern blotting was performed to obtain a rough estimation of copy number of *CzR1* in *C. zedoaria* genome. *C. zedoaria* genomic DNA was digested with *EcoRV, XbaI, BglII* and *SacI* and subjected to hybridization using 3′-end partial coding sequence (83 bp 3′UTR and 214 bp of initial coding region carrying the pre P-loop) of *CzR1* as a probe. The partial coding sequence did not harbor restriction sites of any of the above enzymes used. *CzR1* showed the hybridization pattern representing four copies in the genome of *C. zedoaria* (Fig. 4). This can be explained by the fact that *C. zedoaria* is pentadecaploid (15×) having 105 chromosomes and have most likely originated through allolopolyploidic interaction of lower polyploids of *Zingiberaceae*. Thus, one band corresponds to the *CzR1* gene form one genome while the other three bands in southern presumably belong to its ortholog from other other genome. However, it is still unclear which are the two lower polyploids of the family *Zingiberaceae* that has resulted in the development of *C. zedoaria*.

A genomic clone of *CzR1* was amplified using a pair of gene specific primers, CzR2-F and CzR2-R designed based on the sequence of the 5′ and 3′-UTR of the full length *CzR1* cDNA using *C. zedoaria* genomic DNA as template. A 3902 bp fragment was obtained encompassing 2806 bp cDNA sequence and interrupted by 1095 bp introns. The genomic structure of the *CzR1* gene was established by the alignment with the corresponding transcript, which revealed that coding region of *CzR1* contained three exons and two introns (976 bp and 119 bp each) (Table S1). 5′ and 3′ splice junctions of both the introns showed canonical consensus dinucleotide sequence GT–AG which is a typical structural characteristic of plant introns. Further, A high AT content was found in both the introns (69% for intron 1, 65% for intron 2).

3.5. Expression analysis of CzR1

To determine the transcript expression profile of *CzR1*, a set of five resistant and five susceptible *C. zedoaria* accessions along with a resistant wild ginger and a susceptible cultivated turmeric line were subjected to reverse transcription-PCR analysis. The mRNA transcript encoding *CzR1* was expressed in five *C. zedoaria* accessions (Cze512-11; Cze516-04; Cze522-01; Cze527-13; Cze533-06) resistant to *P. aphanidermatum* but not in plants susceptible (Cze102-03; Cze107-01; Cze112-09; Cze121-07; Cze123-02) to the fungal pathogen (Fig. 5). Similarly, *Z. zerumbet*, a well known wild ginger resistant to *P. aphanidermatum* also revealed a positive transcript profile while the common cultivated turmeric *C longa cv Surama* resulted in no amplification. This suggest that the expression of *CzR1* is largely limited to genotypes resistant to *P. aphanidermatum*

To determine the expression pattern of *CzR1* in response to *P. aphanidermatum* infection, a quantitative RT-PCR was carried out using RNA samples harvested at various time intervals from rhizome tissues of resistant *C. zedoaria* accession-Cze512-11

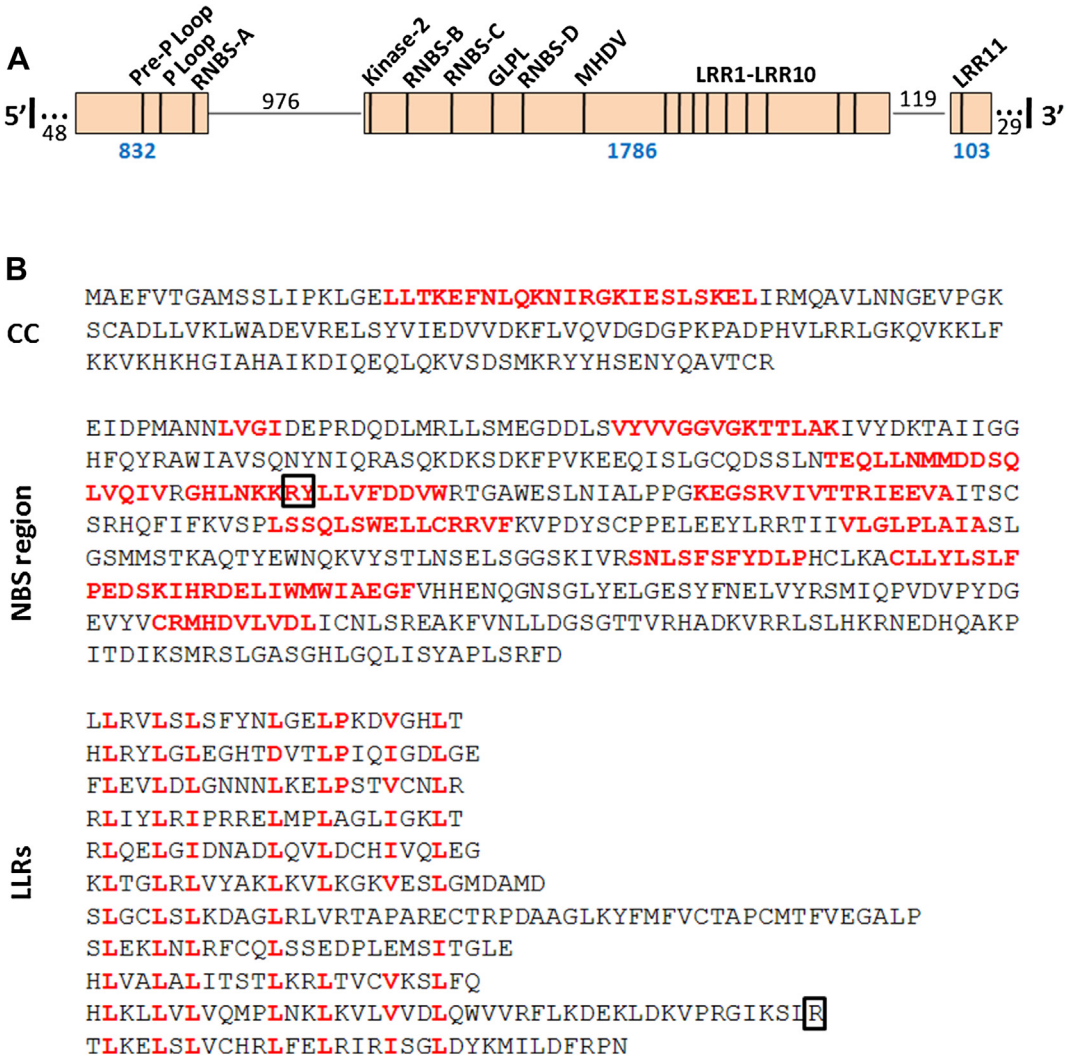

Fig. 3. A) Graphical representation of *CzR1* gene structure consisting of three exons and two introns. Exons are represented by closed boxes and the introns by dark lines, the dotted lines represented the 5′ and 3′UTRs respectively. The individual exons, introns and UTRs length are given in base pairs. B) Amino acid sequence of the *CzR1* gene. The CC, NBS and LRR domains are indicated. The NBS domain has sequences in the following order marked in red: Pre P-loop, P-loop, RNBS-A, kinase2, RNBS-B, RNBS-C, GLPL, RNBS-D, and MHDV. The aliphatic (A) residues in the consensus (xxAxAxx) region of the LRR domain are marked in red. The boxed amino acids (RY in the NBS domain and R in the LRR region) represents the position of the two introns. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

after inoculation with four distinct virulent strains of *P. aphanidermatum*. The gene expression was found to be constitutive and uniformly similar in the resistant *C. zedoaria* under inoculation with all the four pathogenic strains (Fig. 6A). *CzR1* expression was upregulated within 3hr post inoculation (PI) and the mRNA level was significantly elevated (greater than two fold the basal level) at 12hr after treatment. The qRT-PCR analysis recorded maximum expression of *CzR1* at 48hr PI with all the four strains before declining at 96hr. Among the four pathogenic strains, CBT-113 strain of *P. aphanidermatum* from Gangtock, Sikkim showed the highest level of expression for *CzR1*. Although there was significant variation in the expression pattern of *CzR1*, no remarkable difference in morphology could be seen between the inoculated and uninoculated resistance plants during the course of the experiment.

To determine whether the expression profile of *CzR1* was influenced by tissue type, total RNA was extracted from different tissues (roots, stems and leaves) of resistant *C. zedoaria* at 48hr PI with four virulent strains of the pathogen and just before inoculation (0 hpi) and subjected to qRT-PCR. As expected, *CzR1* showed marginal expression in all the tested tissues of *C. zedoaria* in absence of pathogen attack. However, the expression of *CzR1* was notably upregulated in the root, leaf and stem tissues of *C. zedoaria* after pathogen attack albeit in a variable pattern. The mRNA level was significantly higher (fourfold higher than the basal level in root) in the rhizome while the least expression of *CzR1* was realized in the *C. zedoaria* roots (Fig. 6B).

4. Discussion

Rhizome rot, caused by *P. aphanidermatum* is the most devastating disease accounting for upto 60% of losses in turmeric productivity [2]. Control of rhizome rot in India and other countries relies extensively on fungicide infection. The obligatory asexual nature and availability of poor genetic information prevent the traditional crop breeding approaches for host resistance development in turmeric. *C. zedoaria*, a wild relative of turmeric has been lately identified as a source of resistance against the oomycete infection. In the present study, we used a candidate gene approach to isolate, clone and characterize a full-length resistance gene *CzR1* from *C. zedoaria* that exhibit significant expression in response to *P. aphanidermatum*.

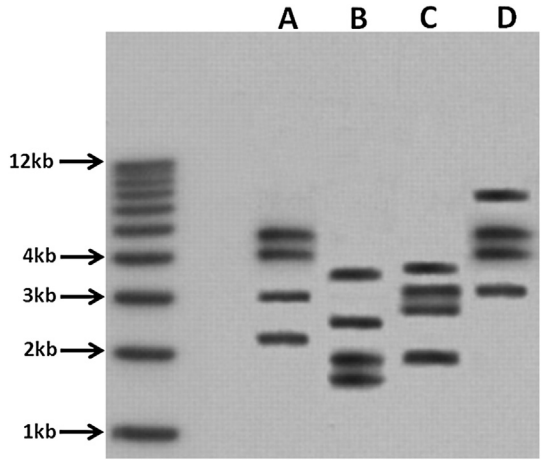

Fig. 4. Southern blot analysis of *CzR1* in *Curcuma zedoaria*. The restriction enzymes used are (A) *EcoRV*, (B) *XbaI*, (C) *BglII* and (D) *SacI*. *Curcuma zedoaria* genomic DNA was digested with the indicated restriction enzymes, fractionated in 1% agarose gel, blotted on to a nylon membrane and hybridized with *CzR1* 3′-UTR fragment as a probe.

A previously isolated NBS-LRR type resistance gene analogs *Czp11* from *C. zedoaria* was used as a template for *CzR1* characterization. Linkage and mapping studies have shown that resistance gene analogs are linked with the known *R*-genes, and at least 21 resistance loci were linked with RGAs in *Arabidopsis thaliana* [21]. *R*-gene *Lr10* was also isolated from wheat by RGA screening [22]. A full-length cDNA of 2985 bp long was isolated for *CzR1* includng the open reading frame (ORF), 5′ and 3′ untranslated (UTR) regions. In most cases the coding sequence of characterized *R*-genes is about 3 kb [23–25]. Blast analysis predicted an amplicon of about 2 kb from 3′ RACE and 1 kb amplicon from 5′ RACE-PCR. As desired, 3′ and 5′ RACE-PCR yielded amplicons corresponding to 2.1 kb and 1.16 kb, respectively without any unspecific banding suggesting the accuracy of the primer in isolating the *R*-gene. Many *R*-genes are generally organized as genetically defined clusters in most of the plant species which may be simple consisting of homologous *R*-gene sequences from a single gene family or complex one consisting of *R*-gene sequences derived from different unrelated families [25–27]. Similarly, southern blotting revealed four copies of *CzR1* in *C. zedoaria* genome suggesting that it might be a member of a multicopy *R*-gene family. Analyzing the genomic sequence of *CzR1* showed that it contains two exons and an intron within the NBS region. Introns interrupting the NBS domain of *R* genes are quite common in monocotyledonous species especially cereals. Six putative *R*-genes in rice including the rice blast resistance CNLs (*Pi-ta* and *Pi36*) carry introns in their NBS domain [27]. Wheat rust resistance gene *Lr10* also has one intron of 1171 bp located in the NBS domain at the N-terminus of the kinase-2 motif [28].

CzR1 encodes a polypeptide of 906 amino acids that exhibit a tripartite arrangement of modules comprising a predicted N-terminal coiled-coil structure, a central ATP/GTP binding NBS domain [10], and 11 imperfect C-terminal LRRs. CC domain is believed to facilitate the formation of coiled-coil structure to promote either dimerization or specific interaction with other proteins. NBS domain is responsible for the binding and the hydrolysis of ATP and GTP [29] while LRRs are typically involved in protein–protein interactions, and various studies indicate that the LRR motif is at least partly responsible for recognition specificity [30]. The 11 LRRs lies in the C-terminal region of the *CzR1* but the repeats are mostly imperfect with only few having any consensus sequence. Similar results has been found with other known CNL type *R*-genes such as *Pi36, Pi9, Pib* [31] from rice and *RB* from *Solanum* [32] and *Lr10* from wheat [28]. It is still unclear, whether the non-distinguishable repetitive structures of imperfect LRRs have any part in determining the specificity for particular pathogen types.

Phylogenetic analysis revealed that *CzR1* is more closely related to CNL class *MlaI* protein of *Hordium vulgare* (41% amino acid identity, 62% similarity over 906 aa) than it is to any other known *R* protein [33]. *CzR1* also exhibited optimum similarity with three CNL *R*-genes from rice (*Pi36, Pi-ta* and *Pib*) that show resistance to rice blast disease although it has a limited similarity with the protein of *Lr21* (19% identity, 37% similarity over 1080aa), a CNL *R*-gene derived from *T. aestivum* that confers hypersensitive resistance to wheat rust fungus [34]. Majority of NBS-LRR *R*-genes involved in the disease resistance response share a common structure and assume to have a common evolutionary origin. Recent studies on genome sequencing, diversity and population genetics in different plants have thrown new insights on the

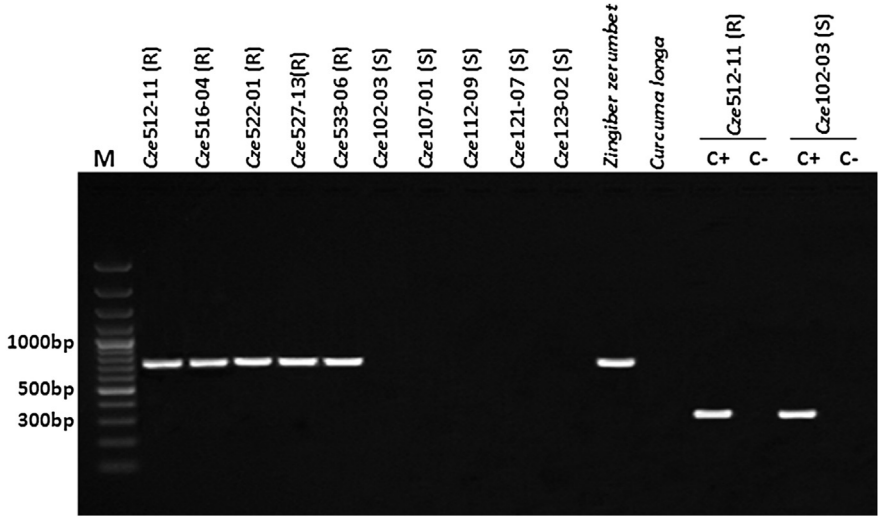

Fig. 5. Reverse transcriptase PCR analysis of CzR1 gene in rhizome tissues of different genotypes resistant or susceptible to *Pythium aphanidermatum*. *CzR1* resulted in a positive expression product in five resistant genotypes of *C. zedoaria* (Cze512-11(R); Cze516-04(R); Cze522-01(R); Cze527-13(R); Cze533-06(R)) and wild ginger (*Zingiber zerumbet*). The five susceptible genotypes of *C. zedoaria* (Cze102-03 (S); Cze107-01(S); Cze112-09(S); Cze121-07(S); Cze123-02(S)) and the cultivated turmeric genotype (*C. longa*) showed no expression. C+ denotes a positive control and show the expected ≈300 bp fragment amplified from turmeric *actin 1* cDNA. C− denotes the negative control which show no amplification of turmeric *Actin 1* amplification due to lack of reverse transcription. M denotes the molecular weight marker.

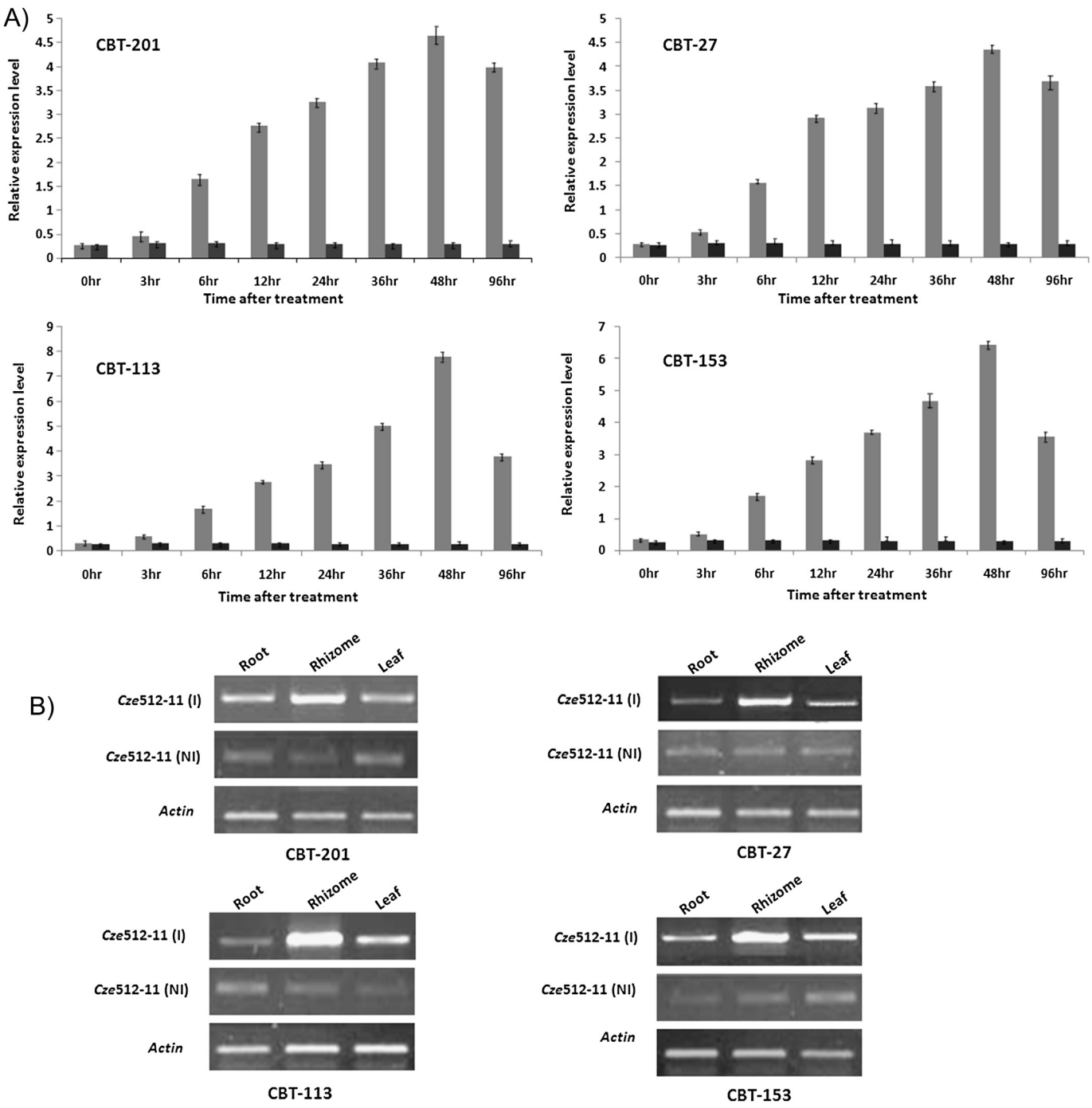

Fig. 6. A) Relative transcript level changes of *CzR1* gene obtained from quantitative real-time PCR in *Curcuma zedoaria* accession Cze512-11 with and without inoculation with four virulent strains (CBT-201; CBT-27; CBT-113; CBT-153) of *Pythium aphanidermatum*. Representative images are shown. The grey bar and the blue bar represents the transcript level of the inoculated and uninoculated tissue at different time intervals. Expression levels are shown as percentage of the level of control (0 h). Error bars show standard deviations for three independent experiments. B) Expression patterns obtained from semi-quantitative reverse transcriptase PCR in different tissues of *C. zedoaria* accession Cze512-11 with and without inoculation with four *Pythium aphanidermatum* strains. Turmeric *Actin* gene was used as a reference gene for the expression analysis of *CzR1*.

molecular evolution of *R*-genes [35]. Although many CNL type *R*-genes have been isolated from vegetatively propagated plants [36–39], *CzR1* from *C. zedoaria* isolated in this study is the first one that exhibit significant upregulation under pathogen challenge. Thus, it can be used as a model towards analysis of genomic diversification and molecular recombination towards R-gene evolution in these groups of plants. Multiple alignment of the amino acid sequences of *CzR1* with other *R*-genes has demonstrated that nonconservative residue substitution was less in the NBS domain and has high frequency in the LRR domain. This support the general opinion about LRRs as the domain involved in diversifying selection and responsible for functional specificity [40].

The functional analysis of *CzR1* through quantitative real time PCR revealed low level of expression of *CzR1* transcript even in the unchallenged plants. Similar pattern has been observed in majority of cloned *R*-genes [32,41,42]. This suggest that *CzR1* is expressive even

in the absence of corresponding Avr-expressing pathogen as like other *R*-genes involved in pathogen surveillance and exhibit a constitutive ability to recognize the pathogen infection and induce downstream defense responses [42,43]. *CzR1* expression was upregulated within 3hr post inoculation (PI) with *P. aphanidermatum* and the upregulation in its expression continued upto 48hr PI before declining at 96hr PI. This suggest that *CzR1* could be acting at an early step in defense signaling pathways towards oomycete resistance in *C. zedoaria*, either as primary recognition molecules or accessory proteins as true for other *R*-genes [44,45]. Furthermore, evidences also shows that the expression of several NBS-LRR-encoding and related genes gets altered during defense response to pathogen attack [42,46]. Thus, we can conclude that *CzR1* gene expression is regulated during plant defense response and causes the induction of enhanced levels of defense-related surveillance in response to *P. aphanidermatum* infection in *C. zedoaria*.

The present study also demonstrated that *CzR1* showed upregulated expression in presence of all four tested virulent strains of *P. aphanidermatum*. It has been already reported that a typical NBS-LRR *R*-protein may have the ability to mark the presence of more than one *Avr* proteins [43]. Grant et al., 1996 [47] has reported the dual specificity of *RPM1* gene for two non homologous *Avr* genes from *Pseudomonas syringae*. Xiao et al., 2001 [48] reported upregulated expression of RPW8, a coiled-coil domain carrying *R*-gene against four different powdery mildew pathogens in *Arabidopsis*. Further, Song et al., 2003 [32] have shown that *RB,* a CC-NBS-LRR *R*-gene from potato exhibit resistance to six different isolates of *Phytopthora infestens*. The upregulation of *CzR1* in response to multiple virulent strains suggest that it may have the ability to recognize conserved *Avr* molecules from different isolates of the pathogens. A variable pattern of *CzR1* expression was also realized in different tissues of *C. zedoaria* after pathogen attack. The rhizomes had a significantly higher level of mRNA level than the leaf and root tissues. Wan et al., 2012 [49] reported 13 resistance gene candidates from pepper having variable levels of expressions in different plants organs. This tissue specific differential expression may attribute to the functional role of *CzR1* with respect to pathogens that attacks particular parts of the plants. The oomycete *P. aphanidermatum* enters the plants through the rhizome tissues causing the rhizome rot and then spread into other parts of the plants. Tan et al., 2007 [42] also found that 14 known NBS-LRR-encoding *R*-genes exhibited tissue specific gene expression in *Arabidopsis*. Likewise, the tomato NBS-LRR-encoding *R*-gene, *I-2*, has the highest level of relative mRNA expression at the site of lateral root formation although it has marginal expression in other parts of the plants [41].

In conclusion, our study resulted in the identification of a potential CC-NBS-LRR class *R*-gene *CzR1* involved in the basal defense of *C. zedoaria* against *P. aphanidermatum*. Expression profiling revealed low level of *CzR1* transcript in unchallenged plants while it had an upregulated expression during the plant defense response to different strains of pathogen attack beside having a tissue specific transcriptional variability. Currently, none of the major cultivated turmeric varieties grown in India contain resistance to *P. aphanidermatum*. Revelation of the molecular characteristic of *CzR1 from C. zedoaria* together with its tissue specific expression abilities and recognition of multiple virulent strains may pave the way towards deployment of the cloned CzR1 gene, through genetic engineering, to provide broad spectrum rhizome rot resistance in the cultivated turmeric.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pmpp.2013.05.003.

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