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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