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Research note

Molecular cloning, characterization and transcriptional variability study of resistance gene candidates from wild *Curcuma spp.* for resistance against *Pythium aphanidermatum*

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Abstract

The genetic base of preferred turmeric (*Curcuma longa* L.) genotypes has eroded due to their continuous domestication through exclusive vegetative routes thus making them highly susceptible to various pests and pathogens. Molecular cloning of resistance related sequences from wild genotypes can result in efficient turmeric improvement by evolving more effective resistance specificities compared to cultigens. In this study, a PCR mediated approach has been made using degenerate primers designed on conserved region (P-loop and GLPL) of the NBS domain from *R*-genes that provides the source for cloning analogous sequences called resistance gene candidates (RGCs) from three wild turmeric- *Curcuma aromatica*, *Curcuma angustifolia* and *Curcuma zedoaria.* Twenty-one wild turmeric RGCs were isolated and grouped into four phenetic classes. A strong amino acid identity ranging from 41 to 53% together with presence of internal conserved motifs provided evidence that the isolated RGCs belong to the non-toll interleukin receptor (non-TIR) NBS-LRR *R*-gene sub-family. Southern hybridization showed a high copy representation of turmeric RGCs. Expression variability of wild turmeric RGCs was analyzed through reverse transcription PCR in root tissues of the three wild turmeric plants resistant or susceptible to *Pythium aphanidermatum. Cap12* and *Can12* showed a constitutive expression in both resistant and susceptible plants of *Curcuma aromatica* and *Curcuma angustifolia* respectively while *Czp11* expression was realized only in *Pythium aphanidermatum* resistant lines of *Curcuma zedoaria* as well as *Curcuma longa L.* This result can pave way towards the identification and characterization of a potential *Pythium aphanidermatum* resistance gene in turmeric.

Keywords: Multiple sequence alignment; NBS-LRR; Resistance gene candidates (RGCs); wild turmeric, Transcriptional variability analysis.

Abbreviations: RGCs-resistance gene candidates; RGAs-resistance gene analogues; *R*-genes-resistance genes; NBS-Nucleotide binding sites.

Introduction

About 75% of plant *R*-genes encode proteins with a nucleotide-binding site and leucine-rich repeat (NBS-LRR) domains that confer resistance to various pests and pathogens including bacteria, fungi, viruses, insects and nematodes (Dangl and Jones, 2001). The C-terminal LRR acts as a site for pathogen recognition only while the N-terminal NBS initiate signaling that activates signal transduction pathways leading to disease resistance (Belkhadir et al., 2004). The NBS-LRR *R*-genes are abundant in plant genomes with approximately 150 and 600 isolated from *Arabidopsis* and rice respectively (Meyers et al., 2003; Zhou et al., 2004). The NBS-LRR class *R*-genes are divided into two distinct subclasses- TIR NBS–LRR and non-TIR NBS–LRR based on the presence or absence of a TIR domain (*Drosophila* Toll and mammalian Interleukin-1 receptor homology region) at the N terminus of the protein. While the TIR subclass is restricted to the dicot species, the non-TIR subclasses comprises a coiled-coil structure and is widely distributed both among monocot and dicot species (Pan et al., 2000). The NBS and LRR domain being highly conserved, degenerate oligonucleotide primers designed from these domains can be used in a candidate gene approach to isolate NBS–LRR genes or resistance gene candidates (RGCs) from plant species. This strategy have been successfully employed in many plant species for the isolation of homologous resistance sequences by heterologous amplification (Leister et al., 1996; Kanazin et al., 1996; Mago et al., 1999; Xiao et al., 2006; Nair and Thomas, 2007). Genetic mapping and phylogenetic analysis have revealed that many of the RGA either co segregate with or are closely linked to known disease resistance loci. Thus, the RGCs can not only act as useful tools for the isolation of full-length resistance genes conferring both qualitative and quantitative resistance to different pathogens but also provide vital information about the organization, expression and evolution of *R*-genes (Pan et al., 2000; Bai et al., 2002; Meyers et al., 2003; Deng et al., 2003; He et al., 2004). In our earlier work, we had isolated five non-TIR NBS-LRR RGCs from *Curcuma longa* cultivar surama (Joshi et al., 2010). However, RT-PCR analysis showed all the five sequences to be non-expressive (data not shown). This suggests that the genetic variation for disease resistance is poor in the cultivated *Curcuma*. Utilization of wild turmeric genotypes for isolation and characterization of RGCs can be a possible alternative because they can evolve resistance specificities more efficiently than cultigen as seen in many other plants (Ebert and Hamilton, 1996). In the present study, a PCR based method using *R*-gene specific degenerate primers were adopted for cloning and sequence characterization of RGCs of the NBS type from three wild turmeric genotypes- *Curcuma aromatica*, *C. angustifolia* and *C. zedoaria*. Further, a functional characterization of selected wild

Curcuma RGCs was carried out to see their expression profiles.

Results

Amplification and cloning of RGCs of the NBS-type from wild Curcuma spp.

Using the three *R*-gene specific degenerate primers, a ≈530bp fragment was amplified between the P-loop and GLPLA motifs of the NBS region of the NBS-LRR class of *R*-genes from the three wild turmeric genotypes (see supplementary material). An additional band of 100/250/300bp was also observed in a few samples. Different PCR conditions, including the concentrations of template DNA, Mg^{2+} and *Taq* DNA polymerase, were tested to further confirm the PCR amplification. Although the 530-bp band could be reproduced under all conditions tested, the DNA bands of other sizes could not. Therefore, the 530-bp band was cloned and sequenced. Nine amplicons of 530-bp in size (Supplementary Fig. S1) were cloned and 38 clones were isolated that comprised of 10, 17 and 11 clones respectively from *Curcuma aromatica, Curcuma angustifolia* and *Curcuma zedoaria*. BLASTx search revealed 21 (nine, five and seven from *Curcuma aromatica, Curcuma angustifolia* and *Curcuma zedoaria* respectively) out of 38 clones having high homology to the NBS domain of known *R*-genes or RGCs from other plant species. These sequences have been submitted in the GenBank database (http://www.ncbi.nlm.nih.gov) with accession number from JN426969 to JN426989. The rest of the 17 clones showed homology to putative polyprotein and hypothetical leucine-rich repeats and were excluded from further analysis.

Sequence analysis of RGCs from wild Curcuma spp.

The 21 nucleotide sequences were translated using ExPaSy translate tool and the deduced amino acid sequences that showed a high level of sequence similarity with more than 100 amino acids to the NBS regions of disease resistance *R*-genes were published in the GenBank database (Table 1). The level of identity of these sequences to RGCs isolated from other plant species ranged from 51% between *Cap11* and NBS-LRR resistance like protein from *Coffea arabica* (e-value: 4e-35) to 63% between *Czp21* and NBS-containing disease resistance-like protein from *Musa acuminata subsp. malaccensis* (e-value: 1e-35). The level of sequence identity of isolated sequences with RGCs isolated from other zingiberaceous species ranged from 85% between *Czp21* and resistance like protein KRGC1 from *Kaempferia galanga* (e-value: 3e-103) to 99% between *Cap21* and *Kaempferia galanga* resistance like protein KRGC4 (e-value: 1e-04). BLASTP searches of deduced amino acid sequences of the 21 clones revealed the presence of NB-ARC (nucleotide binding and similarity to *Apaf-1*, R genes and *Ced-4*) domain and significant homology to well characterized RGCs and *R*-genes from angiosperms. Analysis of the sequences using the ORF finder at the NCBI server (http://www.ncbi.nlm.nih.gov/projects/gorf) revealed that all the 21 sequences could be translated into open reading frame (ORF) with more than 100 amino acids. Southern blot analysis of selected resistance gene candidates resulted in hybridization pattern consisting of multiple bands for each of the three RGC tested (Fig.1) suggesting that RGCs occurs as multiple copies in the turmeric genome. The RGCs *Cap12, Czp11* and *Can12* showed the hybridization pattern representing seven, six and five copies in the genome of *Curcuma aromatica, Curcuma zedoaria* and *Curcuma angustifolia* respectively. Such types of RGCs attribute to be a member of the multi resistance gene family. Analysis of the putative amino acid sequences of the wild *Curcuma* RGCs using CLUSTALX program revealed the presence of appropriately located consensus P-loop/Kinase-1a (GGVGKTT), kinase-2 (LLVFDDVW), RNBS-B (GSRVIVTTRI) and GLPL motifs characteristic of the NBS domain of *R*-genes (Fig.2). The non-TIR motif (WVxxIRELAYDIEDIVDxY) associated with non-TIR subclass of NBS sequences was also present in the N-terminal region of all the 21 wild C*urcuma* sequences. In addition, the analysis showed a tryptophan (W) residue at the end of kinase-2 motif, which is also a characteristic feature of non-TIR subclass of NBS-LRR *R*-genes. Sequence comparisons of the wild *Curcuma* RGCs (using the region spanning the P-loop and GLPLA motifs of the NBS domain) revealed a high amino acid identity among them. The amino acid identity ranged from 85% (between *Czp11 and Cap12, Cap21*) to 99% (between *Cap21 and Cap11*; between *Cap22* and *Cap23, Cap24, Cap31 and Cap32*; between *Can11 and Can12, Can21*; between *Can22* and *Can31*; between *Czp11 and Czp12, Czp21*) (Table 2). Similarly, the amino acid identity between wild *Curcuma* sequences and known *R*-genes varied from 41% between *Can11, Can12, Can21, Can31* and *Linium L6* (U27081) to 53% between *Cap23* and *Arabidopsis RPS2* (A54809). Likewise, the amino acid identity among *R*-genes utilized in the study ranged from 42% (between *RPS2, N* and *L6*) to 53% (between *L6* and *N*).

Phylogenetic analysis of wild turmeric RGCs

A phylogenetic tree was constructed using the Neighbor Joining algorithm implemented in the Molecular Evolutionary Genetics Analysis (MEGA) software package version 2.1 to examine the relationships of wild *Curcuma* RGCs among themselves and to the *R*-genes and RGCs from other plant species. The analysis was performed using only the NBS domain (P-loop to GLPLA) as it contains numerous conserved motifs that facilitate proper alignment. The resulting tree consisted of two major branches- TIR and non-TIR NBS-LRR disease resistance proteins (Fig. 3). The TIR branch consisted of seven known TIR-NBS-LRR genes namely *L* (AAD25976), *L6* (U27081), *M* (P93244), *P2* (AAK28806), *N* (U15605), *RPP4* (NM117790) and *RPP1* (NM114316). All the wild *Curcuma* RGCs clustered with the non-TIR subclass of NBS sequences, which supports our earlier results that the turmeric RGCs lacks a TIR domain at the N-terminal region. The wild *Curcuma* RGCs were distributed into four classes. 12 out of 21 RGCs were in class A and 3 RGCs each in class B, C and D. The distribution of wild *Curcuma* RGCs in different branches of the non-TIR-NBS cluster reflects a high level of sequence divergence for these sequences. However, in all the subtrees, the *Curcuma* RGCs were clustered with RGCs of known function from other members of the family Zingiberaceae such as *Zingiber officinale* and *Kaempferia galanga*.Thus, the 21 sequences identified from three taxa can be considered as resistance gene candidates based on their high level of sequence identity to R-genes/RGCs and presence of true open reading frames with desired conserved motifs.

Transcript expression analysis of the wild turmeric RGCs

Total RNA was isolated from the healthy plants of three wild turmeric genotypes either resistant or susceptible to *Pythium aphanidermatum* and subjected to RT-PCR to study the

Table 1. Results of the search for similarity between wild turmeric NBS-sequences and GenBank accessions carried out using the BLASTX algorithm.

Curcuma NBS clone	GenBank protein accession from Zingiberaceae showing the highest similarity	Amino acid identity (%)	E value	Other GenBank protein accession showing the highest similarity	Amino acid identity (%)	E value
Cap11	*Kaempferia galanga* NBS-LRR protein (ADZ73428)	98%	2e-123	Disease resistance like protein, *Coffea arabica* (CAC82610)	51%	4e-35
Cap12	*Kaempferia galanga* NBS-LRR protein (ADZ73428)	98%	5e-122	Disease resistance like protein, *Coffea arabica* (CAC82610)	53%	3e-36
Cap21	*Kaempferia galanga* NBS-LRR protein (ADZ73428)	99%	1e-04	Resistance like protein, *Vitis davidii* (ABS50350)	52%	2e-35
Cap22	*Kaempferia galanga* NBS-LRR protein (ADZ73431)	98%	5e-124	Resistance like protein, *Vitis davidii* (ABS50350)	52%	7e-39
Cap23	*Kaempferia galanga* NBS-LRR protein (ADZ73429)	98%	1e-126	NBS containg R-gene [*Musa acuminata* subsp. malaccensis] (ABY75804)	55%	1e-38
Cap24	*Kaempferia galanga* NBS-LRR protein (ADZ73430)	98%	3e-129	NBS containg R-gene [*Musa acuminata* subsp. malaccensis] (ABY75804)	60%	3e-37
Cap31	*Kaempferia galanga* NBS-LRR protein (ADZ73428)	96%	3e-120	NBS-LRR type resistance protein [Cynodon x magennisii] (ADC54190)	60%	1e-37
Cap32	*Kaempferia galanga* NBS-LRR protein (ADZ73430)	97%	1e-121	NBS-LRR type resistance protein [Cynodon x magennisii] (ADC54190)	59%	1e-36
Cap33	*Kaempferia galanga* NBS-LRR protein (ADZ73431)	89%	2e-126	NBS-LRR type resistance protein [Cynodon x magennisii] (ADC54190)	56%	2e-36
Can11	*Kaempferia galanga* NBS-LRR protein ADZ73429	89%	2e-112	NBS containg R-gene [*Musa acuminata* subsp. malaccensis] (ABY75804)	56%	4e-35
Can12	*Kaempferia galanga* NBS-LRR protein (ADZ73428)	87%	5e-107	NBS containg R-gene [*Musa acuminata* subsp. malaccensis] (ABY75804)	56%	5e-32
Can21	*Kaempferia galanga* NBS-LRR protein (ADZ73428)	86%	2e-104	NBS containg R-gene [*Musa acuminata* subsp. malaccensis] (ABY75804)	55%	8e-34
Can22	*Kaempferia galanga* NBS-LRR protein (ADZ73429)	88%	1e-108	NBS-LRR type resistance protein [Cynodon x *magennisii*] (ADC54190)	56%	2e-32
Can31	*Kaempferia galanga* NBS-LRR protein (ADZ73429)	88%	2e-107	NBS-LRR type resistance protein [Cynodon x *magennisii*] (ADC54190)	54%	3e-30
Czp11	*Kaempferia galanga* NBS-LRR protein (ADZ73429)	87%	4e-109	NBS containg R-gene [*Musa acuminata* subsp. malaccensis] (ABY75804)	57%	3e-36
Czp12	*Kaempferia galanga* NBS-LRR protein (ADZ73431)	86%	9e-106	NBS containg R-gene [*Musa acuminata* subsp. malaccensis] (ABY75804)	57%	5e-36
Czp21	*Kaempferia galanga* NBS-LRR protein (ADZ73428)	85%	3e-103	NBS containg R-gene [*Musa acuminata* subsp. malaccensis] (ABY75804)	63%	1e-35
Czp22	*Kaempferia galanga* NBS-LRR protein (ADZ73429)	93%	2e-123	NBS containg R-gene [*Musa acuminata* subsp. malaccensis] (ABY75804)	58%	2e-39
Czp23	*Kaempferia galanga* NBS-LRR protein ADZ73429	89%	2e-112	NBS containg R-gene [*Musa acuminata* subsp. malaccensis] (ABY75804)	59%	5e-39
Czp31	*Kaempferia galanga* NBS-LRR protein (ADZ73430)	87%	3e-109	NBS containg R-gene [*Musa acuminata* subsp. malaccensis] (ABY75804)	59%	1e-38
Czp32	*Kaempferia galanga* NBS-LRR protein (ADZ73428)	87%	3e-104	NBS containg R-gene [*Musa acuminata* subsp. malaccensis] (ABY75804)	59%	3e-36

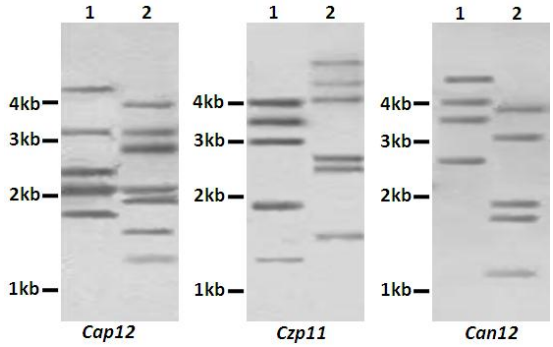

Fig 1. Southern hybridization of three selected resistance gene candidates Cap12, Czp11 and Can12 of *Curcuma aromatica*, *Curcuma angustifolia* and *Curcuma zedoaria* plants respectively. The restriction enzymes used are (1) *EcoRI* and (2) *HindIII*. Molecular weight marker values are indicated in the left side of each blot. Hybridization pattern consisting of multiple bands was realized for each of the three RGC tested with both *EcoRI* and *HindIII* suggesting that the RGCs belongs to a multi-copy gene family in the turmeric genome.

Table 2. Percentage amino acid identity derived from pairwise comparison between wild turmeric resistance gene candidates and the NBS motif of NBS-LRR *R*-genes.

	Cap 11	Cap12	Cap21	Cap22	Cap23	Cap24	Cap31	Cap32	Cap33	Can11	Can12	Can21	Can22	Can31	Czp11	Czp12
Cap 11		99%(1e-102)	99%(9e-104)	97%(3e-100)	97%(9e-100)	97%(2e-1000	97%(1e-99)	97%(5e-98)	97%(4e-99)	87%(3e-90)	87%(3e-90)	87%(2e-88)	88%(6e-88)	87%(4e-88)	86%(1e-87)	86%(2e-87)
Cap12			99%(5e-104)	97%(4e-100)	96%(3e-100)	96%(2e-99)	96%(1e-99)	96%(5e-98)	97%(4e-99)	87%(4e-89)	87%(4e-89)	86%(6e-89)	87%(7e-88)	86%(6e-87)	85%(1e-87)	85%(2e-87)
Cap21				97%(4e-100)	96%(1e-99)	96%(1e-100)	96%(1e-99)	96%(5e-98)	97%(4e-99)	87%(1e-90)	87%(9e-91)	86%(2e-88)	87%(7e-88)	86%(5e-88)	85%(1e-87)	85%(2e-87)
Cap22					99%(4e-106)	98%(1e-103)	98%(8e-104)	99%(5e-105)	99%(4e-104)	90%(2e-95)	90%(2e-95)	89%(1e-94)	89%(3e-92)	89%(2e-91)	88%(7e-93)	88%(9e-93)
Cap23						98%(4e-103)	98%(2e-103)	98%(2e-102)	99%(1e-103)	89%(5e-95)	89%(4e-95)	89%(8e-95)	89%(1e-91)	89%(5e-91)	87%(2e-92)	87%(3e-92)
Cap24							99%(5e-107)	98%(3e-102)	99%(9e-104)	88%(1e-93)	88%(3e-94)	88%(7e-92)	88%(2e-89)	87%(9e-90)	86%(2e-90)	86%(6e-90)
Cap31								98%(2e-102)	99%(5e-104)	88%(8e-93)	88%(2e-93)	88%(4e-92)	88%(1e-89)	87%(8e-89)	86%(1e-90)	86%(4e-90)
Cap32									99%(6e-105)	89%(8e-92)	89%(6e-92)	88%(5e-91)	88%(9e-89)	88%(7e-88)	87%(2e-89)	87%(3e-89)
Cap33										89%(6e-93)	89%(2e-93)	89%(3e-92)	89%(7e-90)	88%(6e-89)	87%(6e-91)	87%(2e-98)
Can11											99%(6e-114)	99%(1e-111)	88%(1e-89)	88%(9e-90)	87%(5e-90)	87%(4e-90)
Can12												99%(1e-111)	88%(1e-89)	88%(9e-90)	87%(2e-90)	87%(5e-90)
Can21													89%(1e-90)	88%(1e-89)	86%(3e-89)	86%(3e-89)
Can22														99%(1e-105)	88%(2e-89)	88%(3e-89)
Can31															87%(1e-88)	87%(2e-88)
Czp11																99%(4e-113)
Czp12																
Czp21																
Czp22																
Czp23																
Czp31																
Czp32																
Pi-ta																
RPS2																
N																
L6																

Czp21	Czp22	Czp23	Czp31	Czp32	Pi-ta	RPS2	N	L6
85%(2e-87)	91%(9e-98)	88%(4e-90)	88%(4e-90)	87%(5e-88)	52%(1e-15)	51%(4e-10)	44%(6e-05)	45%(6e-07)
85%(3e-86)	91%(1e-96)	87%(3e-90)	87%(3e-90)	88%(4e-89)	48%(5e-16)	52%(2e-10)	43%(9e-05)	45%(6e-07)
85%(2e-87)	91%(9e-98)	88%(4e-90)	87%(4e-90)	88%(4e-89)	48%(5e-16)	52%(2e-10)	43%(9e-05)	45%(6e-07)
87%(2e-91)	94%(7e-103)	90%(2e-95)	90%(3e-95)	89%(2e-93)	54%(8e-19)	51%(1e-11)	43%(2e-05)	44%(2e-07)
87%(5e-91)	92%(2e-102)	89%(7e-95)	89%(9e-95)	89%(7e-93)	53%(2e-18)	53%(3e-11)	43%(3e-05)	44%(5e-07)
86%(8e-90)	93%(3e-101)	89%(1e-92)	88%(6e-93)	88%(2e-90)	54%(1e-18)	51%(8e-12)	43%(4e-05)	44%(7e-07)
86%(6e-89)	93%(3e-100)	89%(1e-92)	88%(5e-93)	88%(1e-90)	54%(9e-19)	51%(8e-12)	43%(5e-05)	44%(1e-06)
86%(4e-88)	93%(3e-99)	89%(7e-92)	89%(8e-92)	88%(1e-89)	50%(3e-17)	51%(4e-11)	42%(8e-04)	45%(2e-07)
87%(4e-89)	94%(2e-100)	90%(6e-93)	89%(2e-93)	89%(9e-91)	50%(4e-18)	51%(4e-11)	43%(2e-04)	45%(4e-07)
86%(1e-89)	93%(6e-101)	89%(7e-93)	89%(1e-92)	88%(1e-90)	48%(4e-17)	47%(5e-10)	42%(8e-04)	41%(3e-06)
86%(1e-89)	93%(5e-101)	89%(7e-93)	89%(3e-93)	88%(1e-90)	48%(1e-17)	47%(5e-10)	42%(9e-05)	41%(4e-06)
86%(1e-87)	94%(6e-101)	88%(4e-92)	88%(6e-92)	89%(1e-91)	48%(3e-17)	45%(2e-10)	42%(8e-05)	41%(2e-06)
87%(3e-88)	95%(2e-101)	90%(1e-91)	90%(3e-92)	90%(2e-91)	48%(2e-13)	44%(2e-08)	44%(0.001)	42%(7e-06)
87%(2e-88)	95%(1e-101)	90%(6e-91)	90%(1e-91)	90%(1e-90)	48%(9e-13)	43%(7e-08)	42%(6e-04)	41%(3e-06)
99%(7e-114)	93%(4e-110)	93%(3e-99)	93%(2e-99)	92%(3e-97)	48%(2e-16)	45%(1e-11)	44%(4e-06)	42%(2e-07)
99%(8e-114)	93%(5e-100)	93%(4e-99)	93%(7e-99)	92%(4e-97)	48%(7e-16)	45%(2e-11)	44%(4e-06)	42%(1e-07)
	92%(7e-100)	93%(5e-98)	92%(5e-98)	91%(6e-96)	48%(4e-16)	44%(1e-11)	44%(2e-06)	42%(8e-08)
		95%(1e-102)	95%(1e-102)	95%(2e-102)	47%(4e-17)	47%(1e-10)	44%(2e-05)	42%(2e-07)
			99%(3e-111)	99%(1e-108)	51%(3e-16)	47%(1e-09)	44%(2e-05)	42%(6e-06)
				98%(2e-108)	50%(2e-16)	47%(1e-09)	44%(1e-05)	42%(6e-06)
					49%(7e-15)	44%(1e-08)	44%(1e-04)	43%(3e-05)
						44%(1e-10)	49%(1e-09)	42%(7e-07)
							42%(2e-14)	45%(3e-16)
								53%(1e-76)

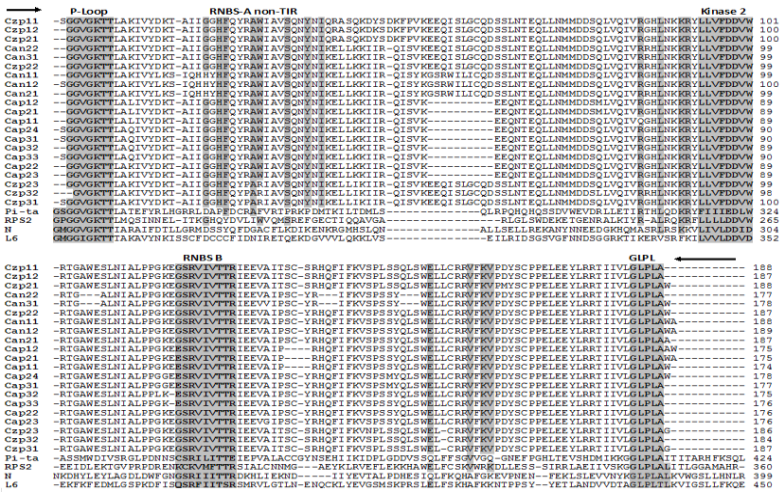

Fig 2. CLUSTAL_X multiple alignment of deduced amino acid sequences of the NBS region of wild Curcuma sequences and analogous domains in known *R*-genes. *RPS2* (A54809) confers resistance to *Pseudomonas syringae* in *Arabidopsis thaliana*, *N* (U15605) confers resistance to Tobacco mosaic virus in *Nicotiana tabaccum*, *L6* (U27081) confers resistance to *Melampsora lini* in *Linum usitatissimum* and *Pi-ta* (AAK00132) confers resistance to *Magnaporthe grisea* in rice. Conserved motifs are shaded and indicated above the alignment. The arrows indicate the position and orientation of degenerate primers.

transcript expression profiles. *Cap12* and *Can12* resulted in constitutive amplification of PCR product in both resistant and susceptible accession of *Curcuma aromatica* and *Curcuma angustifolia*. However, in case of *Curcuma zedoaria*, the mRNA encoding *Czp11* expressed in the plant resistant to *Pythium aphanidermatum* but not in plants susceptible to the fungal pathogen (Fig. 4). Similar results were also obtained with cultivated turmeric (*Curcuma longa* L.); *Czp11* was detected in as many as seven resistant accessions (CBT no. C106, C109, C110, C122, C123, C134, C137) but not in susceptible lines (data not shown). This suggests a transcriptional variance of *Czp11* might account for resistance against *Pythium aphanidermatum*. Control reactions were set up to ensure that the PCR products were not amplified from genomic DNA. PCR was done carrying a positive control with actin-specific primers and negative control with actin primers but with RNA instead of cDNA as the template. Amplification of the expected cDNA fragment (≈300bp) in the positive control for *Actin 1* gene and no amplification in the negative control confirmed that the mRNA samples were free of genomic DNA contamination.

Discussion

There has been a rapid rise in the global demand and production of turmeric in recent times, owing to its huge commercial and medicinal importance (Selvan et al., 2002). The rapid cultivation and domestication has eroded the genetic base of most of the turmeric cultivars resulting in heavy pathogen infestation thus incurring huge crop losses. High genetic sterility and stigmatic incompatibility impede the traditional crop breeding approaches for host resistance development in turmeric cultivars (Damayanti et al., 2003). Genetic transformation offers the most likely opportunity for improvement of turmeric cultivars by transferring specific resistance traits without compromising on other important traits of interest. Although natural sources of resistance against these diseases have been reported in wild turmeric (Rao et al., 1992) the non-availability of a true turmeric resistance gene has been an impediment in the genetic transformation of turmeric for resistance development. The obligatory asexual nature added to poor genetic information makes it impossible to develop a segregating population or to construct a genetic map for isolation of *R*-genes. In these circumstances, candidate gene approach seemed a suitable strategy by which a full-length resistance gene can be isolated, cloned and characterized in turmeric. With this in mind, RGCs were isolated from three wild turmeric genotypes.The co-amplification of non-specific DNA fragments from the degenerate primers is a common feature and has been absorbed in various other species (Di Gaspero and Cipriani, 2002; Lopez et al., 2003). Thus, it is necessary to sequence many clones to ascertain the full range of RGAs that could be amplified in any crop by a given pair of specific primers designed to target the RGAs. 38 clones were generated from the three turmeric taxa using the three selected degenerative RGC primer pairs. The cloned sequences are considered as RGCs if they exhibit high sequence identities to known *R*-genes/other RGCs, show the presence of conserved NBS-LRR domain found in *R*-genes and are represented by open reading frame (ORF) of more than 100 amino acids uninterrupted with stop codons (Noir et al., 2001). Based on the above criteria, 21 out of 38 (55%) sequences were characterized as wild turmeric RGCs. Similar studies in other plants such as citrus (Deng et al., 2000) ginger (Nair and Thomas, 2007) and alfalfa (Cordero and Skinner, 2002) showed that 75%, 49%, and 55% of sequence characterized were RGCs respectively. This concludes that the number of RGCs isolated varies from one plant to another. The percentage identity and e-value of turmeric RGCs to RGCs from other plant species ranged from 51% to 63% and 4e-35 to 1e-35, respectively. Likewise, the identity with RGCs from other zingiberaceous species ranged from 85% to 99% (3e-103 to 1e-04). A possible explanation for very high level of percentage identity in many of the clones obtained could be due to sequencing error. Often, the cloning of the same length fragment using the same set of primers results in the amplification of repeat sequences. Cross sequencing of the cloned fragments can overcome such drawback. In our case, even cross sequencing resulted in high sequence identity making us believe that, the amplified clones with high homology belong to a particular sub-group. In previous studies, NBS sequences identified in other plant species also showed a comparable range of identities of *R*-genes/RGCs such as 30-45% in *Phaseolus vulgaris* (Rivkin et al., 1999), 31-51% in grape (Di Gaspero and Cipriani, 2002), 27-50% ginger (Nair and Thomas, 2007) and 38 to 82% in *Kaempferia galanga* (Joshi et al., 2012). Significant sequence identity was also detected with dicot and eudicot plants such as *Coffea arabica and Vitis davidii*. Joshi et al (2012) also found significant sequence identity of 11 *Kaempferia* RGCs with dicot sequences. This further strengthens the belief that, Zingiberaceous species are positioned at the transition point between dicots and monocots so far as resistance genes are concerned. However, a valid conclusion about the evolutionary origin of Zingiberaceous plants largely will depend on further characterization of NBS-LRR class *R*-genes between different members of this family. Amino acid alignment showed that the wild turmeric RGCs share significant homology with NBS regions of well-characterized *R*-genes from other plants. Several features of the wild turmeric RGCs isolated in this study suggest they are non-TIR-NBS-LRR class resistance gene sequences. Moreover, the lengths of the isolated RGCs are also similar to the lengths of the non-TIR domain that range from 200-220 amino acids from the coding region until the beginning of the NBS domain (P-loop) (Bai et al., 2002; Zhou et al., 2004). No TIR-RGCs were obtained in wild *Curcuma* genotypes in the present study, which is in accordance with the earlier reports on the absence of such groups among the *R*-genes/*R*GCs of Zingiberaceae (Nair and Thomas, 2007; Joshi et al., 2010; Joshi et al., 2012). The phenetic analysis showed a high correspondence between the RGC sequence and the primer used as all the RGCs were grouped together or separately irrespective of the primer from which they are amplified. However, the groups mostly included the RGCs from the same turmeric genotypes. This suggests that the RGCs isolated from each species exhibit high degree of conservation within themselves. Many numbers of RGC classes have also been identified in other plant species such as six classes in apricot (Soriano et al., 2005), five classes in ginger (Nair and Thomas, 2007) and four classes in *Kaempferia galanga* (Joshi et al., 2012). The cluster analysis supported the classification of the turmeric RGCs into the non-TIR subclass. The TIR domain has also not been found in the NBS-LLR *R*-genes of other important monocots such as rice (Monosi et al., 2004; Zhou et al., 2004), wheat (Dilbirligi and Gill, 2003) and maize (Xiao et al., 2006). Pan et al. 2000 has earlier hypothesized that the loss of the TIR domain from the NBS-LRR genes in monocots may have occurred during the divergence of the monocots and dicots. The functional analysis of the wild turmeric RGCs suggests that the CC-NBS-LRR RGCs isolated in the present study are not inducible but expressed in a constitutive manner. Northern analysis of various known NBS-LRR *R*-genes also

Table 3. Resistance related degenerative primers used for the PCR amplification of *Curcuma* resistance gene candidates (RGCs) and number of clones derived from the three wild *Curcuma* taxa.

Primers			
Primer type	P1	P2	P3
Sequence (5'-3')*F	GGIGGIRTIGGIAARACIAC	GGTGGGGTTGGGAAGACAACG	CCGGGITCAGGIAARACWAC
R	WTIARIGYIARIGGIARICC	CCACGCTAGTGGCAATCC	CCCGAAGGAAACCRISRACWARA
Targeted domain	NBS-LRR (P-loop/HD)	NBS-LRR (P-loop/HD)	NBS-LRR (P-loop/HD)
Amplified products (Kb)	0.5	0.5	0.5
Reference	Rivkin et al 1999	Kanazin et al 1996	Joshi et al 2011
Wild *Curcuma* species	**Number of clones isolated**		
Curcuma aromatica	2	4	3
Curcuma angustifolia	2	2	1
Curcuma zedoaria	2	3	2

*Primers are based on *N, L6, RPM1* and *RPS2* resistance genes; P1: Primer set 1, P2: Primer set 2, P3: Primer set 3.

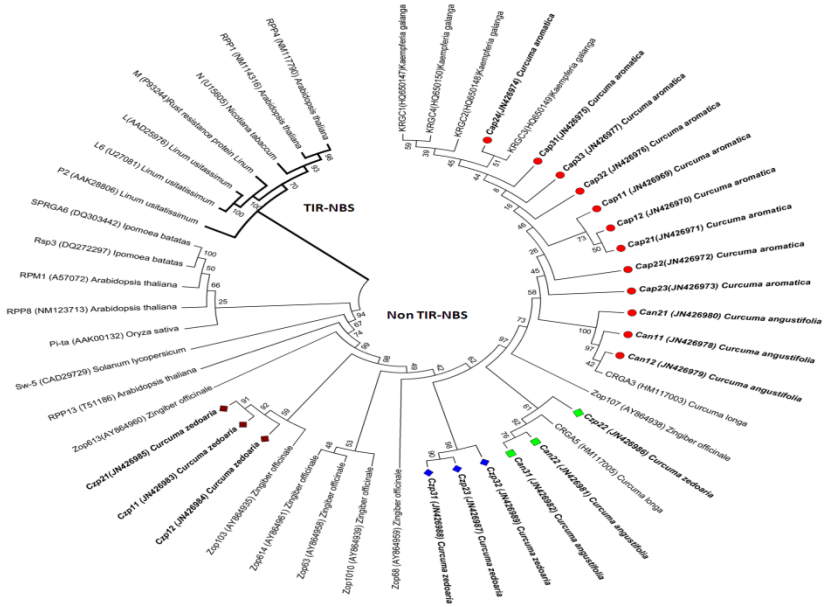

Fig 3. Neighbor-joining tree based on amino acid sequence alignment of resistance gene candidates isolated from wild *Curcuma spp*., other genotypes of Zingiberaceae and NBS sequences of *R*-genes from other plant species. The wild turmeric RGCs are represented with bold letters and classified into four phenetic classes with colour marks- class 1 (red); class 2 (green); class 3 (blue) and class 4 (brown). Numbers on the branches indicate the percentage of 1000 bootstrap replications supporting the particular nodes.The bold branches indicate the TIR-NBS cluster.

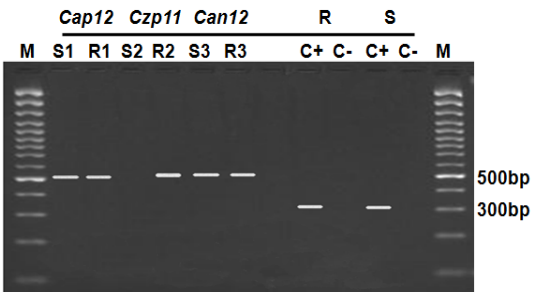

Fig 4. RT-PCR analysis of three selected resistance gene candidates *Cap12, Czp11* and *Can12* in root tissue of *Curcuma aromatica, Curcuma zedoaria* and *Curcuma angustifolia* plants resistant (R1, R2, R3) or susceptible (S1, S2, S3) respectively to *Pythium aphanidermatum*. *Cap12* and *Can12* showed a constitutive expression in both resistant (R1, R3) and susceptible (S1, S3) plants of *Curcuma aromatica* and *Curcuma angustifolia* while *Czp11* expression was realized only in resistant line of *Curcuma zedoaria (R2)* but not in susceptible line (S2). C+ denotes a positive control and show the expected ≈300bp 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 1kb ladder plus molecular weight marker.

exhibit similar results (Parker et al., 1997; Milligan et al., 1998; Mes et al., 2000). Thus, the assumption that NBS-LRR proteins acts as preformed receptors that recognize a pathogen in a gene-for-gene correlation for disease resistance in plants is practically true (Van der Biezen and Jones, 1998). Constitutive expression of the *R* protein might be an adaptation to protect every cell of the plants from pathogen attack in the absence of a true circulatory system. The polymorphic expression of *Czp11* can be an interesting finding for resistance development against *Pythium aphanidermatum.* The expression of *Czp11* in seven resistant turmeric accession and non-expression in the susceptible lines further confirm their role in resistance development against the fungal pathogen. In conclusion, the present study resulted in the isolation of 21 resistance gene candidates (RGCs) from three wild turmeric genotypes- *Curcuma aromatica, Curcuma angustifolia* and *Curcuma zedoaria* through a degenerative PCR approach. The isolated RGCs showed the characteristic features of non-toll interleukin receptor (non-TIR) NBS-LRR *R*-gene subfamily. *Czp11,* an RGC from *Curcuma zedoaria* specifically expressed in response to *Pythium aphanidermatum* infection. The full-length cDNA sequencing of the expressive *Czp11* is in progress, which will subsequently be used for transforming susceptible turmeric plants to test its ability in conferring *Pythium aphanidermatum* disease resistance. The potential role of the isolated wild turmeric RGCs in disease resistance could further be tested using other advanced methods such as RNAi and BIBAC (Binary bacterial artificial chromosome) technology to reveal new informations on the organization, function and evolution of the NBS-LRR-encoding resistance genes in asexually reproducing plants.

Materials and methods

Plant material and DNA isolation

Accessions of three wild species of the genus *Curcuma*- *Curcuma aromatica* (CBT no. Car211-3), *Curcuma angustifolia* (CBT no. Can732-5) and *Curcuma zedoaria* (CBT no. Cze512-11) were used in this study. Samples were collected from their natural habitat after through taxonomic verification of the rhizome size, leaf structure and flowering patterns. Rhizome from 46-day-old plants were frozen in liquid nitrogen and stored at -80^{0}C until use. DNA was isolated by grinding with a mortar, pestle in extraction buffer (0.2 M Tris–HCl pH 7.5, 0.25 M NaCl, 25 mM EDTA pH 8.0, 0.5% SDS), and incubated at 65^{0}C for 10 min. It was thrice treated with phenol: chloroform: isoamyl alcohol (25:24:1) for removal of non-nucleic acid compounds. DNA was precipitated using isopropanol and resuspended in 100 µl of 10 mM Tris, pH 8.0 with 10 ug RNaseA. The quality and quantity of the DNA were determined with a Thermo Scientific UV-Vis spectrophotometer.

PCR and gene sequence cloning

Three pairs of NBS-LRR encoding R-gene specific degenerate primers (Table 3) were used to amplify NBS-LRR gene sequences from wild turmeric. The design of the degenerate primers was based on the NBS and membrane spanning motif sequences of two cloned *R* genes, *N* and *L6*, from the TIR NBS–LRR class and two cloned *R* genes, *RPS2* and *RPM1* from the non-TIR NBS–LRR class. PCR was carried out using 50ng of genomic DNA in a 50ul reaction volume containing a 200uM dNTP mix (Fermentas), 1X PCR reaction buffer (16 mM $(NH)_2SO_4$, 67 mM Tris–HCl) with 15 mM $MgCl_2$, 20 pM of each primer (forward and reverse) and 1 unit of *Taq* DNA polymerase (Bangalore Genie, INDIA). Amplification was programmed on a Veriti Thermal Cycler (Applied Biosystems) for an initial denaturation at 94^{0}C for 5 min, followed by 40 cycles of denaturing at 94^{0}C for 1 min, annealing at 50/60^{0}C for 1 min, and elongating at 72^{0}C for 2 min. Differential PCR conditions were also maintained through variable concentrations of template DNA (15/25/35/45 ng), Mg^{2+} ion (10/15/20/25 mM) and *Taq* DNA polymerase (0.5/1/1.5 unit) to confirm the amplification of desired PCR product. The PCR products were separated by electrophoresis on a 1.2% (wt/vol) agarose gel. Desired amplified bands were gel-eluted and purified, using the Wizard SV gel and PCR cleanup system (Promega, USA) and cloned with pGEM-T easy vector system (Promega, Madison, USA) as per manufacturer's instructions. Recombinant DNA was transferred into competent *Escherichia coli JM109* strain cells and was plated on the Luria Broth (LB) agar blue and white selective medium. The clones were named with the first letter of the genus and the first letter of the species followed by primer code and the clone number. For *Curcuma angustifolia*, the name consists of the first letter of the genus and first two letters of the species followed by the clone number.

Southern blot analysis

10ug of genomic DNA isolated from young rhizome of three wild turmeric genotypes was digested separately with *EcoRI* and *HindIII.* The digests were electrophoretically separated on 1.2% 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^{0}C. Digoxigenin labeled probes were prepared from purified DNA of the identified RGCs (*Cap12, Czp11 and Can12*) using a digoxigenin DNA labeling and detection kit (Roche Diagnostics, Basel, Switzerland). The hybridization membrane was blocked for 1 h at 62^{0}C with DIG Easy Hyb (Roche) before hybridization. Hybridization was carried out

with DIG-labelled probes at 65^0C for 15 h in a hybridization chamber followed by two washes with 0.1 X SSC (15 mM NaCl, 1.5 mM Na-citrate, pH 7.0) containing 0.1% sodium dodecyl sulfate at 65^0C for 20 min. Detection of the hybridized probe was done according to the manufacturer's instructions.

Plasmid purification, DNA sequencing and phylogenetic analysis

Clones were grown overnight in LB medium containing 100ug/ml ampicillin. Plasmid DNA was purified using the Wizardplus miniprep DNA purification system (Promega, USA). Briefly, a single bacterial colony harboring the desired plasmid was inoculated in 2 ml Luria broth (LB) liquid medium (containing suitable antibiotics) and was incubated overnight at 37 °C under continuous shaking at 200 rpm. The overnight grown culture was transferred to a cold centrifuge tube and centrifuged at 5000 x g for 8min to spin down the bacteria. The bacterial pellet was resuspended in 400 µl of cell resuspension solution (Tris-HCl, EDTA and RNAse A). The sample was then transferred to a 1.5 ml vial, incubated on ice for 5 min. 400 µl of the freshly prepared lysis solution (1% SDS, 0.2 M NaOH) was mixed with the sample and placed on ice for 5 min. 400 µl of ice-cold 1.3 M potassium acetate (pH 4.8) was mixed gently to the sample and incubated further for 5 min on ice. This preparation was then transferred into a minicolumn and centrifuged at 10,000 rpm for 10 min at 4 °C to separate the supernatant from the the cell debris and chromosomal DNA. The supernatant was then transferred to a fresh vial and 0.6 volumes of Iso-propanol was added to it. The mixture was incubated at room temperature (25 °C) for 10 min. The plasmid DNA was precipitated by centrifugation at 15000 rpm for 15 min at 4 °C. The plasmid pellet was finally dissolved with 50 µl of sterile TE buffer. The plasmid DNA was sequenced using the BigDye Terminator Cycle Sequencing kit (Perkin Elmer) with SP6 and T7 forward and reverse primer. Sequences of the PCR product were determined on the ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequences were edited using GeneDoc software, to remove the primer and vector sequences. Database searches were performed using the National Center for Biotechnology Information Center's BlastX to search the similarity of the RGA to the NBS-LRR-encoding *R* genes and RGA cloned in plants. Amino acid sequences of resistance genes from other plant species were added to the set of NBS sequences, and cluster analysis was carried out using the CLUSTALX program version 1.81 (Thomson et al., 1997) with the default settings. The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) using the NJ algorithm implemented in the Molecular Evolutionary Genetics Analysis (MEGA) software package version 2.1 with the Poisson correction (Kumar et al. 2001). NBS sequences of the following *R*-genes were included in the phylogenetic analysis: *N* (U15605) from *Nicotiana tabaccum*, *L* (AAD25976), *L6* (U27081) and *P2* (AAK28806) from *Linum usitatissimum*, *RPP1* (NM114316), *RPP4* (NM117790), *RPP8* (NM123713), *RPS2* (U12860), RPS5 (NM101094), *RPM1* (AAD41050), *RPP13* (T51186), *RPP8* (NM123713) from *Arabidopsis thaliana*, *Sw-5* (CAD29729) from *Solanum lycopersicon*, *Pi-ta* (AAK00132) from *Oryza sativa* in addition to RGCs from *Zingiber officinale* (AY864935, AY864938, AY864939, AY864958-AY864961), *Ipomea batatas* (DQ272297, DQ303442), *Curcuma longa* (HM117003, HM117005) *and Kaempferia galanga* (HQ650147- HQ650150). Searches for ORF were done using ORF finder at the NCBI server (http://www.ncbi.nlm.nih.gov/projects/gorf). The RGA sequences that did not give ORF were defined as "pseudogene," according to Deloukas and associates (2001).

RT-PCR analysis

Total RNA was isolated from 45 days old rhizome tissue of all the three wild turmeric genotypes by 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 25ul reaction mixture containing 2ul of 25mM $MgCl_2$, 0.2ul of 10mM dNTP mix, 1uM of each forward and reverse primer, 5ul of 5X reaction buffer, 1ul of synthesized cDNA and 1U of *Taq* DNA polymerase. Three sets of gene specific primers were designed one each for the amplification of a specific RGC from the three wild turmeric genotypes: For *Cap12*, F5'-TTTGAGATGGCGAGTAGAAC-3' and R5'-ATGAGGG-AAGAGAGGAGAAG-3'; for *Can12*, F5'-ACACAACA-TTCAGTTTAGCAC-3' and R5'-TCCCTATTCTTTCCTC-TCG-3'; for *Czp11*, F5'-TATCCTCCCTGGTCGTTT-3' and R5'-GATTCCCTTTCCTTTCTTTTG-3'. 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 94^0C, followed by 35 cycles of denaturing at 94^0C for 1 min, annealing at 55-57^0C for 30 sec, and elongating at 72^0C for 2 min followed by a final extension at 72^0C for 7 min. Amplicons were separated on a 1.2% agarose gel. The amplified products were gel purified, cloned into pGEMT easy vector and sequenced.

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