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ORIGINAL PAPER

Isolation, characterization and expression studies of resistance gene candidates (RGCs) from *Zingiber* spp.

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Abstract Ginger (*Zingiber officinale* Rosc.) production is seriously affected by many fungal and bacterial diseases to which no resistant source is available in the cultivated germplasm. Degenerate primers based on conserved motifs of plant resistance (R) genes were used to isolate analogous sequences called resistance gene candidates (RGCs) from cultivated and wild *Zingiber* species. Cloning and sequence characterization identified 42 *Zingiber* RGCs, which could be classified into five classes following phenetic analysis. Deduced amino acid sequences of *Zingiber* RGCs showed strong identity, ranging from 16 to 43%, to non-toll interleukin receptor (non-TIR) R-gene subfamily. Non-synonymous to synonymous nucleotide substitution (dN/dS) ratio for the NBS domains of *Zingiber* RGC classes showed evidence of purifying selection. RT-PCR analysis with 15 *Zingiber* RGC-specific primers demonstrated 8 of the 15 *Zingiber* RGCs to be expressed. The present study reports for the first time the isolation and characterization of RGCs from ginger and its wild relatives, which will serve as a potential resource for future improvement of this important vegetatively propagated spice crop.

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Introduction

Ginger (*Zingiber officinale* Rosc.), a herbaceous perennial of the family *Zingiberaceae* is valued the world over as culinary herb, spice, home remedy and medicinal crop (Afzal et al. 2001). India is the largest world producer and exporter of the finest quality ginger and accounts for approximately 24% of world production (FAOSTAT 2005). Continuous domestication of preferred genotypes coupled with their exclusive vegetative propagation seems to have eroded the genetic base of this crop and as a result all the cultivars available today are equally susceptible to all major diseases such as soft rot caused by *Pythium aphanidermatum* (Edson) Fitz. and bacterial wilt caused by *Pseudomonas solanacearum* (Smith) Smith, incurring heavy crop loss (Dake 1995; Anandaraj et al. 2001). Ginger is completely sterile and is propagated exclusively by vegetative means using rhizome. Other species in the genus *Zingiber* are wild, and contrary to ginger, sexual reproduction also occurs in them contemporaneous with asexual reproduction. Wild relatives of many other plants have been used as an important source of genetic variation for disease resistance (Xiao et al. 1998; Zamir 2001) since they can evolve resistance specificities more efficiently than cultigen (Clay and Kover 1996; Ebert and Hamilton 1996). In this context molecular characterization of resistance-related sequences from ginger and its wild relatives may provide a lead towards retrieving resistance specificities suitable for the improvement of ginger.

Plant resistance genes (R-genes) are an important component of the genetic resistance mechanism in plants (Flor 1971; Dangl and Jones 2001). Disease resistance (R) genes have been cloned from wide range of plant species either by map-based cloning (Johal and Briggs 1992; Grant et al. 1995; Dixon et al. 1998) or transposon tagging (Whitham

et al. 1994; Anderson et al. 1997) from model systems or species with a long history of genetic research. These approaches are not feasible for the isolation of R-genes in a species with poor genetic information, particularly in obligatory asexuals where the development of segregating populations and construction of genetic maps are not possible. Comparative analysis of the cloned R-genes from different plants and providing resistance to different pathogens have revealed certain conserved amino acid domains that include nucleotide-binding sites (NBS), leucine-rich repeat (LRR), transmembrane domains (TM) and serine/threonine protein kinases (PK's) arranged in different combinations (Hammond-Kosack and Jones 1997; Dangl and Jones 2001). Based on the arrangement of these domains, five R-gene classes have been identified: NBS–LRR, LRR–TM, LRR–TM–PK, PK and those devoid of these signature domains. Degenerate PCR primers designed to these conserved domains have been successfully employed for the isolation of homologous sequences called resistance gene candidates (RGCs) from many plant species by heterologous amplification (Leister et al. 1996; Kanazin et al. 1996; Aarts et al. 1998; Song et al. 1997; Shen et al. 1998; Rivkin et al. 1999; Liu and Ekramoddoullah 2003). RGC sequences amplified following candidate gene approach have yielded vital information about the organization, distribution and evolution of R-genes/RGCs (Kanazin et al. 1996; Pan et al. 2000; Bai et al. 2002; Meyers et al. 2003; Irigoyen et al. 2004). More importantly, RGCs have served as promising tools for the isolation of full-length resistance genes from crop plants such as wheat (Feuillet et al. 1997), common bean (Ferrier-Cana et al. 2003), soybean (He et al. 2003) and citrus (Deng and Gmitter 2003).

In the present study we followed this PCR-based strategy using R-gene specific degenerate oligonucleotides to (1) isolate and sequence characterize RGCs from ginger and its wild relatives and that included four *Zingiber* taxa: a wild accession of *Z. officinale*, cultivar kuruppampady and one accession each of the wild species; *Z. zerumbet* and *Z. cernuum* and (2) functional characterization of selected *Zingiber* RGCs to see their expression profile.

Materials and methods

Plant materials

Accessions of the wild species of genus *Zingiber*, *Z. zerumbet* (RGCB collection no. 2010–1) and *Z. cernuum* (RGCB collection no. 2011–5) collected from their natural habitat and three accessions of *Z. officinale*: cv. kuruppampady, cv.Rio-de-Janeiro and a wild accession (obtained from National Bureau of Plant Genetic Resources NBPGR, Trichur, India; Number:IC324523) were used in this study. Genomic DNA was isolated using GenElute Plant Genomic DNA Purification kit (Sigma–Aldrich, USA) following manufacturer's instructions.

Primers and PCR conditions

Three R-gene specific degenerate primers (Table 1), successfully amplifying RGCs in other crops, were obtained from published literature. PCR reaction was carried out in a volume of 20 µl containing 1 unit Taq DNA polymerase (NEB, USA), 1× PCR buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 µM of each primer and 20 ng template DNA. Amplification was carried out in iCycler (BioRad, California) programmed for an initial denaturation at 94°C for 5 min, followed by 35 amplification cycles (94°C for 1 min, 60°C for 1 min and 72°C for 1 min) and a final extension step at 72°C for 5 min.

Cloning and sequencing of PCR products

The amplicons were gel-purified using GFX^{TM} Gel Band Purification kit (Amersham Biosciences, USA), cloned using pGEM-T vector system II (Promega, Madison, USA) as per manufacturer's instructions and transformed into competent *Escherichia coli* JM109 cells. Plasmid DNA was purified using Wizard Plus Plasmid Minipreparation Kit (Promega) according to the instructions of the manufacturer. The clones were grouped according to their restriction profiles obtained following digestion with 4- or 5-bp cutting enzymes; *Rsa*I, *Taq*I (NEB, USA) and *Hinf*I (Genei, Bangalore, India). A minimum of one clone from each restriction group was sequenced using BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA) cycle sequencing kit in an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The clones were named with the first letters of the taxa name followed by primer code and the clone number.

Sequence analysis

The sequence data were subjected to GenBank searches with BLAST (Altschul et al. 1990) and BLASTX algorithm (Gish and States 1993). Multiple alignment of amino acid sequences were performed using CLUSTALX program of BioEdit software (Hall 1999). Phylogenetic analyses (Saitou and Nei 1987) were performed using the NJPLOT module of CLUSTALX software (Thompson et al. 1997). Robustness of clustering was checked by bootstrapping 1,000 replicates. NBS sequences of the following R-genes were included in the phylogenetic analysis: *N* (U15605), *L6* (U27081), *RPS2* (U12860), *RPS5* (AF074916), *I2* (AAU90295), *RPP13* (AAF42831), *RPM1* (AAD41050) and *Pt20* (AAN08174). Searches for ORF were done using

Table 1 Resistance-gene specific oligonucleotide primers used for the PCR amplification of *Zingiber* resistance gene candidates (RGCs) and number of clones derived from the amplicons from each of the four *Zingiber* taxa

Primers			
Primer details	P2	P6	P10
Sequence ($5' \rightarrow 3'$)[a]	GGIGGIGTIGGIAAIACIAC ARIGCTARIGGIARICC	GGACCTGGTGGGGTTGGGAAGACAAC CAACGCTAGTGGCAATCC	GGTGGGGTTGGGAAGACAACG CCACGCTAGTGGCAATCC
Domain/Motif targeted[b]	NBS-LRR/P-loop; NBS-LRR/HD	NBS-LRR/P-loop; NBS-LRR/HD	NBS-LRR/P-loop; NBS-LRR/HD
Reference	Kanazin et al. (1996)	Ohmori et al. (1998)	Mago et al. (1999)
Amplicon size (kb)	0.5	0.6	0.6
Zingiber species and their origin	Number of clones isolated		
Z. officinale cv. kuruppampady; KAU Vellayani	6	32	40
Z. officinale (wild); NBPGR, IC324523	0	41	39
Z. zerumbet (Wild); Kallikad, Kerala, India, 2010–1	30	40	37
Z. cernuum (Wild); Ponmudi, Kerala, India, 2011–5	31	41	39

[a] Degenerate IUB code: *I* inosine; *R* A or G; *W* A or T; *Y* C or T; *X* G or C; *Z* T or G; *D* A, G, T; *N* A, C, G or T

[b] Primer pair P10 and reverse primer of P2 is based on *N* and *RPS2* genes; forward primers of P2 based on *N*, *RPS2* and *L6* gene; P6 based on *RPS2*

ORF Finder at the NCBI server (http://www.ncbi.nlm.nih.gov/projects/gorf/). Rates of non-synonymous and synonymous substitution (dN/dS) values were determined among RGCs of each of the phylogenetic classes using codeml (Yang 1997) of PAML 3.15 software package under the F3 × 4 model of codon substitution (Goldman and Yang 1994) and also using Nei and Gojobori's method (Nei and Gojobori 1986) with Jukes and Cantor correction as implemented in MEGA 3.1 software (Kumar et al. 2004).

Zingiber RGC-specific primers

Primer pairs were deduced from the RGCs isolated from *Z. officinale* cv.kuruppampady, *Z. zerumbet*, *Z. cernuum* and *Z. officinale* (wild) using the software Primer 3.0. Altogether 15 RGC-specific primers were designed following separate multiple alignment of RGCs from each of the five phylogenetic class obtained. Using these primers, conditions for PCR amplification were standardized with genomic DNA from the respective taxon and annealing temperatures were identified.

RT-PCR analysis

Total RNA was isolated from the leaves of *Z. zerumbet* and ginger cultivar, Rio-de-Janeiro by the method of Salzman et al. (1999). Total RNA was treated with DNase I (Promega) to remove any traces of genomic DNA. For cDNA synthesis, 2 µg of DNA free RNA was primed with 15-mer oligo dT (Promega) and the first strand cDNA synthesized using 200 units of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega). The cDNA from *Z. zerumbet* and Rio-de-Janeiro were amplified using 15 *Zingiber* RGC specific primers. Control reactions included a positive RT-PCR with actin-specific primers besides a negative control with actin primers but with RNA instead of cDNA as template to test for genomic DNA contamination. Amplicons were electrophoresed on 1.2% agarose gel. The bands after elution using GFX^{TM} Gel Band Purification System (Amersham Biosciences) were cloned into pGEM-T vector system II (Promega) and sequenced.

GenBank submissions

The sequences derived from the four *Zingiber* taxa using the three R-gene specific degenerate primers have been deposited in the GenBank database under the accession numbers: AY864934-AY865018. The expressed *Zingiber* RGCs identified using 15 RGC-specific primers have been submitted to the GenBank EST database with the following accession numbers: EL645415-EL645434.

Results

Amplification and cloning of RGCs from *Zingiber* spp.

Using the three R-gene specific degenerate primers, RGCs were isolated from two accessions of *Z. officinale*: cv.kuruppampady and a wild accession (IC324523) and one accession each of two wild species of the genus *Zingiber viz*., *Z. zerumbet* (2010–1) and *Z. cernuum* (2011–5). PCR amplification with genomic DNA resulted in the production of amplicons of the predicted sizes based on previously published RGC sequences (Fig. 1; Table 1). The amplicons were cloned and a total of 376 clones were isolated that comprised of 78, 80, 107 and 111 clones respectively from *Z. officinale* cv.kuruppampady, *Z. officinale* (wild), *Z. zerumbet* and *Z. cernuum*.

Following restriction analysis with four base-cutter enzymes (*Rsa*I and *Taq*I) or five base-cutter enzymes (*Hinf*I), the 376 clones isolated were separated into 51 restriction groups. A minimum of one clone from each of the restriction groups identified, amounting to a total of 85 were sequenced. Following homology searches with BLASTX algorithm, 69 out of the 85 clones analyzed showed homology to NBS domain of known R-genes or RGCs cloned from other plant species. The 69 clones comprised 32, 11, 16 and 10 from kuruppampady, wild ginger, *Z. zerumbet* and *Z. cernuum*, respectively. The remaining 16 clones showed homology to putative pentacotripeptide (PPR) repeat containing protein, phosphoprotein-related protein, putative polyprotein, putative map-kinases (MAPK's) or hypothetical leucine-rich repeat protein.

Sequence analysis of *Zingiber* RGCs

The 69 selected sequences showed a high level of sequence identity to comparable regions of disease resistance genes published in GenBank, supported by low e-values (supplementary material A). The level of identity of these sequences to RGCs isolated from other plant species ranged from 27% between ZzP616 and NBS–LRR like protein from *Hordeum vulgare* (e-value: 9e-14) to 50% between ZwP1028 and disease resistance-protein from *Oryza sativa* (e-value: 8e-04). The level of sequence identity between *Zingiber* sequences and known R-genes varied from 35 to 45% between ZwP1012 and ZzP633 respectively to *Arabidopsis RPP*13 (accession number: AAS93952).

BLASTP searches of deduced amino acid sequences of the 69 selected sequences 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 R genes from angiosperms. Domain searches at NCBI server (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) confirmed the presence of NB–ARC domain. Further analysis of the sequences using ORF Finder at the NCBI server (http://www.ncbi.nlm.nih.gov/projects/gorf/) revealed that 42 out of the 69 could be translated into a single open reading frame (ORF) of considerable length of more than or equal to 100 amino acids. Further analysis of these 42 RGCs revealed the presence of stop codons or frameshift mutations in 18 out of 42 *Zingiber* RGCs.

Multiple alignment of the deduced amino acid sequences of the selected 42 sequences and the NBS domains of R-genes using CLUSTALX revealed the presence of the conserved resistance nucleotide binding site (RNBS-A) non-TIR, kinase-2, RNBS-B and RNBS-C motifs internal to P-loop and GLPL that were used to design the primers (Fig. 2). Among these conserved motifs, the P-loop, RNBS-B and GLPL motifs are conserved in both TIR and non-TIR NBS–LRR proteins, while RNBS-A non-TIR has been found only in the non-TIR NBS–LRR proteins (Meyers et al. 1999). 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. Pairwise comparisons of *Zingiber* RGCs and NBS

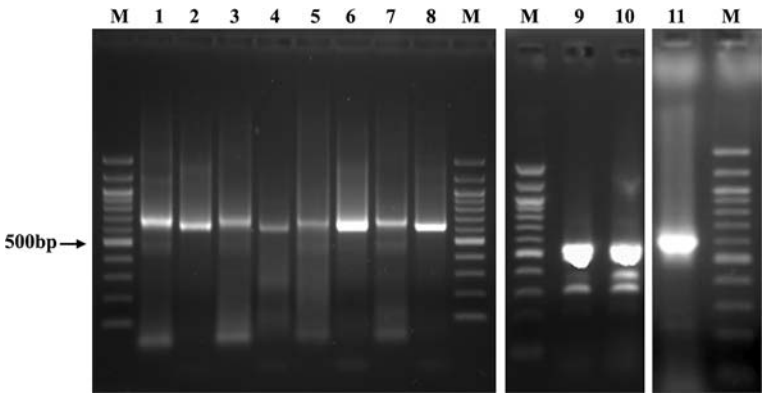

Fig. 1 PCR amplification products generated by the three selected R-gene specific primer pairs in *Zingiber* species. *Lanes 1*, *2* and *11* amplicons generated by primers P6, P10 and P2 respectively in *Z. officinale* cv.kuruppampady; *Lanes 3*, *4* and *10*- P6, P10 and P2 in *Z. cernuum*; *Lanes 5*, *6* and *9*- P6, P10 and P2 in *Z. zerumbet*; *Lanes 7* and *8*- P6 and P10 in *Z. officinale*, wild. *M* 100 base pair DNA ladder (NEB)

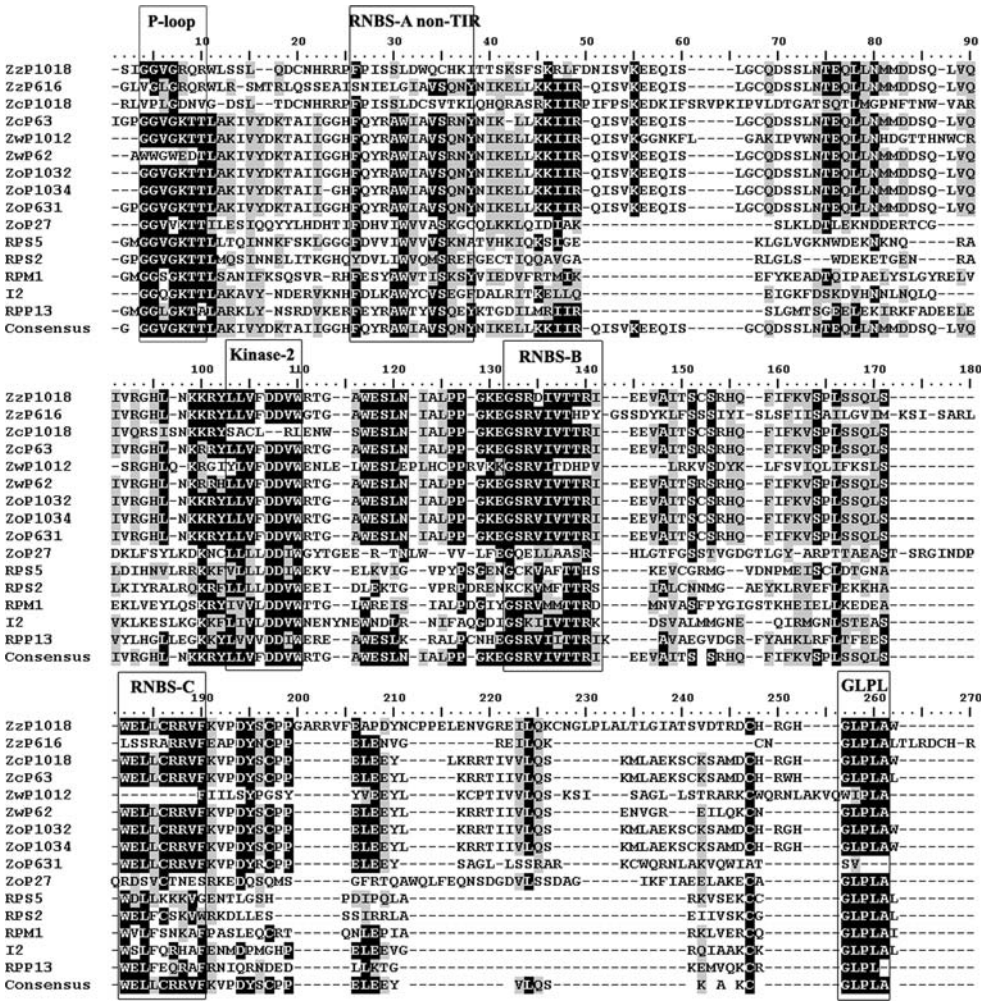

Fig. 2 Amino acid sequence alignment of representative *Zingiber* RGCs with NBS domains of R-genes: *RPS*5 (Accession number: AF074916), *RPS*2 (Accession number: U12860), *RPM*1 (Accession number: AAD41050), *I*2 (Accession number: AAU90295) and *RPP*13 (Accession number: AAF42831) R-genes. R gene specific resistance nucleotide binding site (RNBS) and kinase-2 motifs are highlighted. P-loop and GLPL are the priming sites

domain of R-genes revealed amino acid identities ranging from 12 to 99% among *Zingiber* RGCs and 16–43% between the R-genes and *Zingiber* RGCs. Percentage identities between the R-genes examined ranged from 25 to 45%. Phylogenetic analysis was carried out to examine the relationships of *Zingiber* RGCs among themselves and to R-genes from other plant species. The resulting tree consisted of two major branches: one consisting of TIR NBS-LRR and the other consisting of non-TIR NBS–LRR disease resistance proteins (Fig. 3). All *Zingiber* RGCs were in the non-TIR (CC-NBS–LRR) branch, which was supported by a very high bootstrap value. In the non-TIR branch, the *Zingiber* RGCs were distributed into five classes. A majority of the tree nodes were supported with >80% of the 1,000 replicates in bootstrap analysis; 28 out of the 42 RGCs were in class D, 8 were in class C, 4 in class E and one each in class A and B (Fig. 3). Thus the 42 sequences identified from the four taxa were treated as resistance gene candidates (RGCs) based on their high level of sequence identity to R-genes/RGCs, considerably long open reading frame and the presence of conserved motifs.

Variation and evolutionary analysis of *Zingiber* RGCs

Rates of non-synonymous (dN) to synonymous (dS) nucleotide substitution were calculated among RGCs for each of the three classes and the overall mean values were computed using both MEGA and codeml of PAML package. These two methods produced essentially the same results (Table 2); dN/dS ratio is an indicator of evolutionary pressures on a class of genes (Michelmore and Meyers 1998). A dN/dS ratio greater than 1 reflects diversifying selection and a value less than 1 suggests purifying selection (Parniske et al. 1997). The dN/dS ratio for all the three RGC classes analyzed was less than 1, indicating purifying selection (Michelmore and Meyers 1998).

Expression analysis

Following alignment of the deduced amino acid sequence of RGCs from each class, 15 RGC-specific primer pairs (Table 3) were designed to *Zingiber* RGCs. All the 15 primers (Table 3) were then used to amplify both genomic

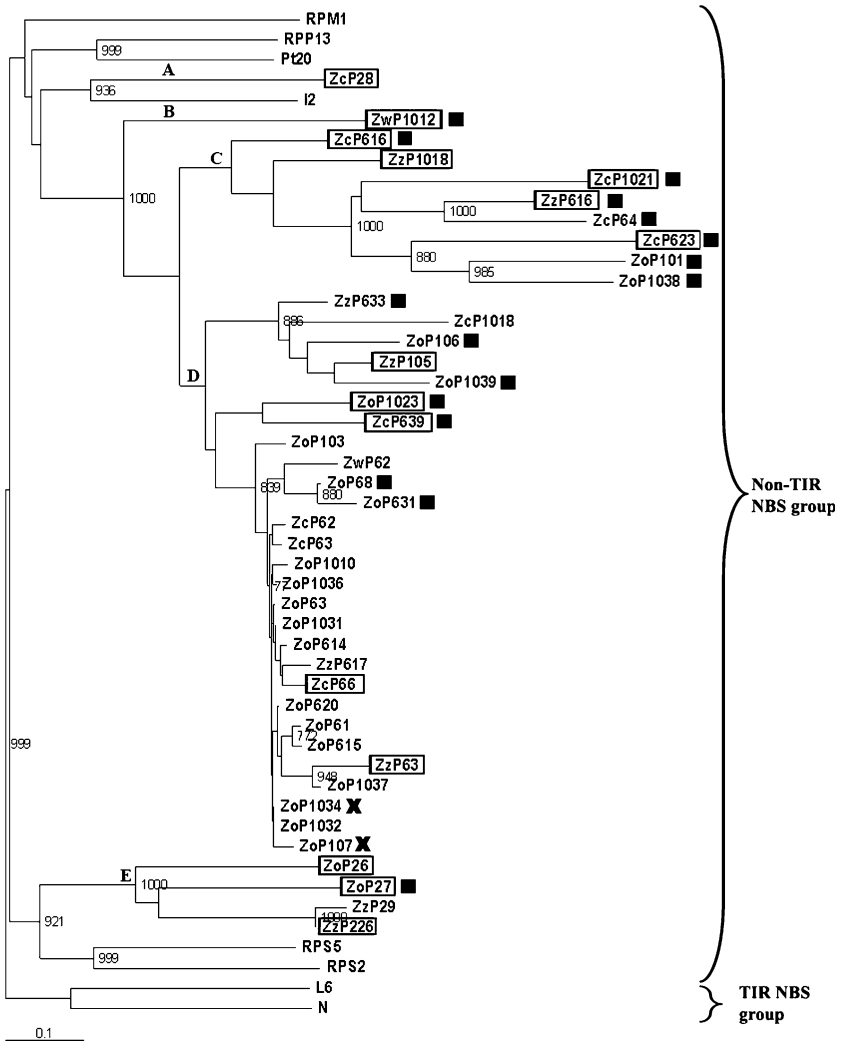

Fig. 3 Neighbor joining tree based on amino acid sequence CLUSTALX alignment of *Zingiber* RGCs and NBS sequences of R-genes from other plant species. Bootstrap values greater than 700/1000 and the scale of genetic distance as computed from the pairwise distance in CLUSTALX are indicated. Major phylogenetic groups identified are designated A–E and presence of stop codons (*multi sign*) or frameshift mutations (*filled square*) are also indicated. Sequences in *box* indicate those used for primer designing

Table 2 Rates of non-synonymous (dN) and synonymous (dS) nucleotide substitutions among *Zingiber* RGCs

	Class C	Class D	Class E
dN/dS[a]	0.758	0.387	0.266
dN/dS[b]	0.794	0.279	0.272

[a] dN/dS calculated using Nei and Gojobori method implemented in MEGA software

[b] dN/dS calculated using codeml of PAML software package

DNA and cDNA from two taxa *viz*., *Z. officinale* cv.Rio-de-Janeiro and *Z. zerumbet*. . The 15 primers were found to amplify fragments of predicted size from the genomic DNA of the respective source species. However, when used in RT-PCR experiments, out of the 15 primers only 10 yielded a product in both Rio-de-Janeiro and *Z. zerumbet*. The expression profiles obtained are shown in Fig. 4a, b. The primers, ZP1, ZP13, ZP14 and ZP15 failed to yield a product in both the species. The primer ZP6 that yielded a product in *Z. zerumbet* did not amplify a product in cultivar whereas the primer ZP11 amplified only in cultivar. All amplification products were almost identical in size to the products generated following the amplification of the genomic DNA of the respective taxa using the same primers. Negative controls confirmed that the mRNA samples were free of genomic DNA contamination. The amplicons were gel purified, cloned, sequenced and the sequence data were used for homology searches. Of the ten primers that amplified a product, eight yielded products homologous to disease resistance protein in both Rio-de-Janeiro and *Z. zerumbet* (supplementary material B). The product amplified by ZP5 was homologous to resistance-related protein in *Z. zerumbet*, while in cultivar it was homologous to a hypothetical protein. The ZP12 derived amplicon showed homology to tyrosine phosphatase protein in *Z. zerumbet* while in cultivar it was showing homology to *Zingiber* RGC clone, ZoP26. ZP11 yielded a product with homology to S-adenosyl methionine synthetase in Rio-de-Janeiro and ZP6 amplified a product homologous to kinase domain containing protein in *Z. zerumbet*. BLASTN algorithm was

Table 3 List of *Zingiber* RGC specific primers used for expression studies with expected amplicon sizes and the annealing temperature

Clone name	Accession number	Primer code	Sequence forward (5′→3′)	Sequence reverse (5′→3′)	Amplicon size (bp)	Temp (°C)
ZoP1023	AY864944	ZP1	caacgttggctaagatagtctatg	gtcgggcactttaaatactcttc	476	55
ZzP226	AY864989	ZP2	ggggaagaccaccgtattgaagagc	agacgacgactttacgctgctgctg	326	60
ZcP28	AY865006	ZP3	gacgactctggctcagcttgtttac	gctaggggggagcccgtcaacttcg	501	60
ZzP616	AY864980	ZP4	gggaagacaacgttggctaagg	gaccctacttccttctttacctgg	352	60
ZoP26	AY864966	ZP5	ggacaagaattgctttgctgc	gggctaggggggaggcctgcacattc	321	55
ZoP27	AY864965	ZP6	cgatgacatttggggatacactgg	gggggaggcctgcacattctttagc	392	55
ZwP1012	AY865010	ZP7	gggaagacaacgttggctaag	tctggaggacaactatagtcgggc	525	56
ZcP616	AY865001	ZP8	cgagcttggattgcagtgtcacg	gtggatccattgccttttgcaag	536	53
ZcP623	AY865003	ZP9	gaggccatttccaatatcgagc	ggcaatccattgcacttttgc	556	57
ZcP66	AY864999	ZP10	tggattgcagtgtcacga	gctctggaggacaactatagttggg	429	56
ZzP63	AY864977	ZP11	ggccatttccaatatccgagcttgg	ctgggtggcaatgcaatgttcaag	476	59
ZzP1018	AY864971	ZP12	ccaccccaaccctctgttgcaaccg	ccgttaggtaacgtgaaaacgttct	600	56
ZcP1021	AY864993	ZP13	caagactgcaatcatcggaggcc	gggggcaatgccaatgtttcaagc	303	58
ZcP639	AY865004	ZP14	ctgcaatcatcggaggcc	cccctacttcttctttacctg	312	59
ZzP105	AY864968	ZP15	tcatcccgaggccatttcc	agtccggcgcttcaaatactc	483	59

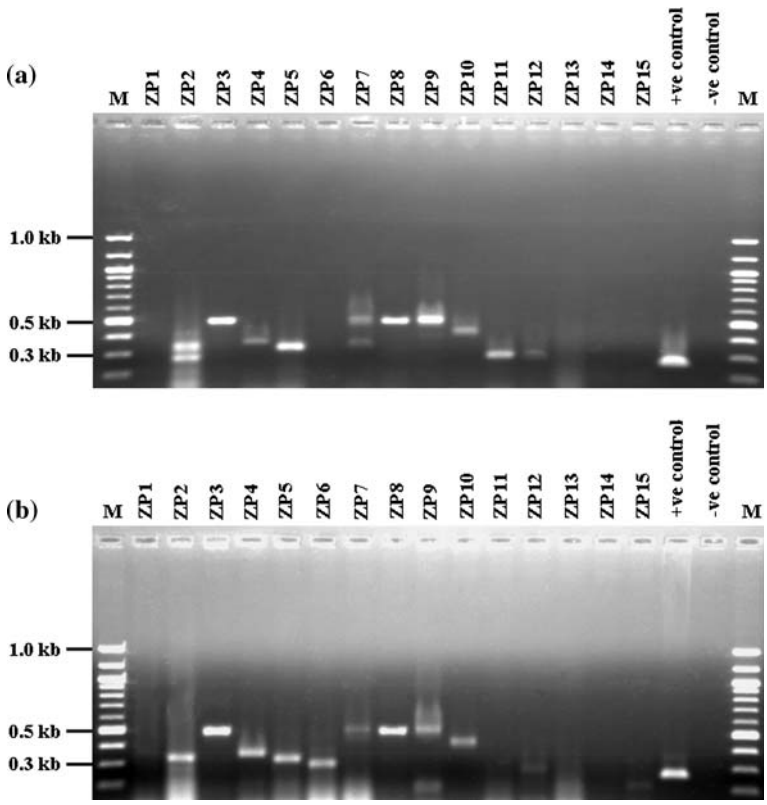

Fig. 4 RT-PCR analysis of **a** ginger cultivar, Rio-de-Janeiro and **b** *Z. zerumbet*, using *Zingiber* RGC specific primers. Primer code as given in Table 3. Actin was used as control

used to search for sequences with similarity to expressed genes from dbEST. Expressed *Zingiber* RGCs from both Rio-de-Janeiro and *Z. zerumbet* showed homology to ESTs from different plant sources elicited in response to biotic or abiotic stress stimuli (supplementary material B). The amino acid sequences of the amplicons homologous to resistance-related proteins were aligned with the corresponding *Zingiber* RGCs that served as the basis for primer designing to identify the conserved motifs and a phylogenetic tree was constructed (Fig. 5). In the case of three primers, ZP2, ZP3 and ZP9, the amplicons generated by each of them in both Rio-de-Janeiro and *Z. zerumbet* were

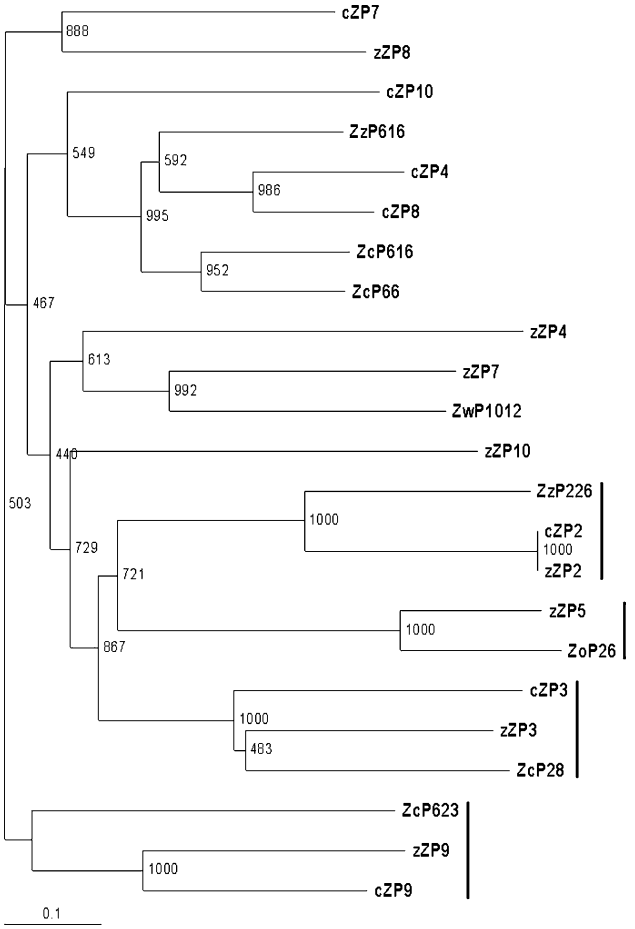

Fig. 5 Phylogenetic tree generated following alignment of amino acid sequences of RT-PCR products and the corresponding *Zingiber* RGCs. Bootstrap values are given at the nodes and the expressed products clustering with the corresponding RGCs are also indicated

clustered together with the RGCs that formed the basis for primer design. The amplicons generated by the primer pair ZP5, which yielded resistance related protein only in *Z. zerumbet* also clustered with the respective RGC. In the case of other primers, one of the amplicons, generated in either Rio-de-Janeiro or *Z. zerumbet*, was clustered with the respective RGCs, while the other amplicons clustered distantly.

Discussion

Ginger production is seriously affected by many diseases like soft rot, bacterial wilt and leaf spot for which no resistant source is available in the cultivated ginger germplasm. In these circumstances, utilizing the plant's own defense mechanism from the secondary gene pool is a good alternative as has been shown in many crop plants like potato, tomato, rice, etc., wherein alien introgression has yielded pathogen resistance. Conventional methods for breeding resistance in ginger is often encountered with such impediments as obligatory vegetative mode of propagation and its totally sterile nature. In ginger natural seed set is a rare phenomenon and it is attributed to its stylar and stigmatic incompatibility (Dhamayanthi et al. 2003). In the present scenario, adoption of transgenic technology is the only alternative and for this certain biotechnological tools have to be developed. Research on R-genes over the past few decades has revealed that the bottlenecks in conventional gene isolation methods can be transcended by exploiting the sequence similarities between different cloned R-genes with different pathogen specificities. In this context, the candidate gene approach constitutes an ideal strategy for developing molecular tools that will assist in future R-gene isolation programs in this scarcely studied spice crop. With this objective, RGCs were isolated from ginger and its wild relatives.

The heterogeneous nature of the DNA fragments within an amplicon generated by R-gene specific primer pairs is a common feature (Lopez et al. 2003) and has been observed previously in many other species (Kanazin et al. 1996; Leister et al. 1996; Mago et al. 1999). Thus, it is necessary to isolate many clones to ascertain the full range of RGCs that can be amplified by a given pair of primers. To this end a total of 376 clones generated from the four *Zingiber* taxa using the three selected primer combinations were taken for initial screening. However, sequencing of this large number of clones not being practical but at the same time to identify as many diverse clones as possible, they were subjected to a heterogeneity test using restriction enzymes with four or five base pair recognition sequences. Following restriction analysis a total of 51 restriction groups were identified among the clones derived from the three primers in the four *Zingiber* taxa examined. This is suggestive of the presence of multi-gene families with sequence similarities at selected structural domains but being different partly at other regions. Earlier workers have used cross-hybridization to classify RGC sequences. But for the present analysis, this approach was not followed since an extensive literature survey revealed that the results vary with the stringency of post-hybridization washes (Shen et al. 1998) and thus may not be successful in identifying minor sequence differences among the clones. The PCR derived sequences are identified as RGCs or fragments of R-genes based on following features: high sequence identities to known R genes/RGCs from other species; presence of conserved motifs characteristic of NBS-LRR R genes and uninterrupted ORFs of considerable length (Noir et al. 2001; Deng et al. 2000). Based on these criteria it was observed that 42 out of the 85 (about 49%) characterized sequences were RGCs. In similar studies in alfalfa (Cordero and Skinner 2002) and citrus (Deng et al. 2000), about 54 and 75%, respectively, of the sequences characterized were RGCs while in coffee only

15% corresponded to RGCs (Noir et al. 2001). Thus, conclusions can be made that the number of RGCs identified varies among plants. The amplification of sequences unrelated to resistance genes by the three selected primers may be the result of amplification on the basis of P-loop alone that are observed in the receiver domain of MAPK's and proteins of phosphotransfer pathways (Rigden et al. 2000). It could also be hypothesized that the RGCs may be interspersed with these defense-related sequences as has been observed in the *Pto* loci (Salmeron et al. 1996; Mysore et al. 2002).

The percentage identity and e (expected) value of *Zingiber* RGCs to RGCs from other plant species ranged from 27 to 50% and 9e-14 to 8e-04, respectively. NBS sequences identified in other plant species with a similar approach showed a comparable range of identities to R-genes/RGCs following BLASTX searches: 30–45% in *Phaseolus vulgaris* (Rivkin et al. 1999), 31–56% in *Brassica oleracea* (Vicente and King, 2001), 30–39% in soybean (Graham et al. 2000), 18–48% in citrus (Deng et al. 2000), 19–44% in lettuce (Shen et al. 1998) and 31–51% in grape (Di Gaspero and Cipriani, 2002).

The presence of NB-ARC domain in the sequences following BLASTP analysis in the GenBank database further shows them to be analogous to plant R-gene products and to the human *Apaf*-1 and *Caenorhabditis elegans Ced*-4 proteins, whose activation results in cell death (van der Biezen and Jones, 1998). Amino acid alignment showed that the *Zingiber* RGCs share homology with NBS regions of well-characterized R genes from other plants. The last residue of kinase-2 motif of identified RGCs can be used to predict with 95% accuracy whether they belong to the TIR or non-TIR subclass of NBS–LRR R-genes (Meyers et al. 1999). A tryptophan residue (W) is expected at the end of kinase-2 motif in non-TIR NBS-LRR sequences while an aspartic acid (D) or asparagine (N) residue is expected for TIR NBS–LRR sequences. Using this criterion, it was observed that all the *Zingiber* RGCs amplified by the three primers belonged to the non-TIR NBS–LRR subclass. Additionally, the RNBS-A and RNBS-B non-TIR specific motifs within the NBS that distinguishes the non-TIR group of NBS sequences (Pan et al. 2000; Meyers et al. 1999) were also found in *Zingiber* RGCs. It is noteworthy that no TIR-type RGC sequences were isolated from any of the three *Zingiber* species surveyed and this is very well in consistence with earlier reports on the absence of this subclass of R-genes in monocotyledons (Meyers et al. 1999; Pan et al. 2000; Cannon et al. 2002). Phylogenetic analysis also clearly separated all the *Zingiber* RGCs into non-TIR NBS–LRR subclass. Results therefore suggest that the nature of RGCs amplified by any given R-gene specific primer depends on the plant species since the selected primers were known to amplify both subclasses of NBS–LRR sequences. For instance, the primer P2 designed by Kanazin et al. (1996) had yielded TIR NBS–LRR sequences from soybean while in *Brassica oleracea* it yielded only non-TIR NBS–LRR sequences (Vicente and King 2001) and in citrus both TIR as well non-TIR subclasses were amplified (Deng et al. 2000).

In order to visualize the relatedness between *Zingiber* RGCs and R-genes/RGCs from other plant species, a phylogenetic tree was constructed based on amino acid sequence alignment. The tree distinguished the two subclasses of NBS–LRR sequences and separated *Zingiber* RGCs into five classes. Phenetic analysis revealed a loose correspondence between RGC sequence and the primer combination used, as the clones derived from P2 were forming unique classes. The number of RGC classes identified in *Zingiber* species (after analyzing 376 clones and 85 sequences) was comparable to the number of RGC classes identified in other plant species. For example, Kanazin et al. (1996) found nine classes in soybean (analyzing 450 clones and one to five sequences from each class), Cordero and Skinner (2002) found six in alfalfa (analyzing the sequences of 94 clones), Di Gaspero and Cipriani (2002) found three classes in grape (analyzing 71 clones and 29 sequences) and Soriano et al. (2005) could differentiate six classes in apricot (analyzing 200 clones and 86 sequences).

The presence of multiple stop codons in 39% (27 out of 69 sequences) of the amplified RGC sequences suggests these to correspond to non-functional genes/pseudogenes. Since we were interested in considering the maximal number of *Zingiber* RGCs, we included all the 42 RGCs isolated for further analysis despite the occurrence of stop codons or frameshift mutations in 18 of the 42 sequence translations. In a similar study on *Pinus monticola* (Liu and Ekramoddoullah 2003), almost half of the cloned RGCs were pseudogenes. Such non-coding RGCs have been reported in the paralogs of R genes *Xa*21, *Cf*9, *Pto* and *Dm*3 (Song et al. 1997; McDowell et al. 1998) and in PCR experiments (Kanazin et al. 1996; Leister et al. 1996; Aarts et al. 1998; Vicente and King 2001). Detection of pseudogenes in paralogs of plant R genes suggests their role in evolution of new specificities by recombination and gene conversion (Michelmore and Meyers 1998). It may be noted that pseudogenes are also called *poto*genes (Brosius and Gould 1992) as they have the potentiality for becoming new genes.

Ratio of non-synonymous (dN) to synonymous substitutions (dS) is an indication of the evolutionary pressures on a class of genes. A dN/dS ratio greater than 1 reflects diversifying selection, meaning that there is a selective advantage for amino acid diversity and a value lower than 1 suggests purifying selection (Parniske et al. 1997) meaning that there is not much selective pressure on the region. The estimated dN/dS ratio for NBS domain of three *Zingiber* RGC classes, ranging from 0.272 to 0.794, are similar to those

observed in NBS domains of known angiosperm R-genes, suggesting that these sequences are subject to purifying selection rather than diversifying selection (Michelmore and Meyers 1998). As suggested by Michelmore and Meyers (1998) and Stahl et al. (1999), R-genes might not be evolving rapidly in order to keep pace with changes in the pathogen, but are rather evolving fairly slowly to provide resistance against pathogen populations that are heterogeneous in space and time.

In all plants studied till now, the functional role of NBS–LRRs seems to be exclusively devoted to defense responses. However, the level of expression of RGCs or R-genes is an area that is still under discussion due to their low transcript levels (Graham et al. 2002) and only few reports can be seen in the literature on R-gene expression. The results on expression studies of many R-genes have demonstrated that they are expressed constitutively at a very low level, before pathogen challenge (Hammond-Kosack and Jones 1997; Hulbert et al. 2001) or induced following pathogen inoculation (Yoshimura et al. 1998; Wang et al. 1999). While studying RGC expression, 11 out of 29 RGCs in *Brassica* (Fourmann et al. 2001), 4 of 8 RGC classes in common bean (Rivkin et al. 1999), and 4 of 6 RGCs in *B. oleracea* (Vicente and King, 2001) were reported to be expressed. However, in a survey of *A. thaliana* databases, Meyers et al. (1999) found only rare EST sequences matching the NBS area of R-genes.

Elucidation of *Zingiber* RGC expression was carried out with primers specific to selected RGCs representing the five *Zingiber* RGC classes as well as originating from the four taxa examined viz., *Z. zerumbet*, *Z. cernuum*, *Z. officinale* cv.kuruppampady and *Z. officinale*, wild. While studying RGC expression in two *Zingiber* species, *Z. zerumbet* and cultivar Rio-de-Janeiro, a corresponding cDNA was identified for 10 out of 15 RGC sequences. The lack of a cDNA signal for five of the RGCs may be indicative that they correspond to pseudogenes. It could also indicate that probably these genes were not either expressed or sufficiently expressed. Sequence analysis of these products revealed that in both Rio-de-Janeiro and *Z. zerumbet*, 8 out of 10 were homologous to resistance-related proteins. BLASTN searches revealed that these sequences are homologous to ESTs originating from tissues harvested under different stress conditions or after pathogen inoculation in other plant species. The experiments therefore provided qualitative evidence of expression for 8 out of the 15 RGCs, suggesting these to be functionally significant. Phylogenetic analysis using these expressed products derived from cultivar, Rio-de-janeiro and wild *Z. zerumbet* accession revealed clustering of products generated by four primers namely, ZP2, ZP3, ZP5 and ZP9, with the RGCs that formed the basis for primer design. Thus the analysis reveals the potential of these RGCs as probes for future R-gene isolation programs. This is the first study aimed at isolating and characterizing RGCs from the genome of cultivated and wild *Zingiber* species. The most significant payoff from this endeavor will involve development of strategies for isolating resistance genes for ginger improvement and will thereby constitute an attractive model for vegetatively propagated plants.

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