Evaluation of resistance gene (R-gene) specific primer sets and characterization of resistance gene candidates in ginger (*Zingiber officinale* Rosc.)

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Ginger (*Zingiber officinale* Rosc.), an obligatory asexual spice crop, is extremely vulnerable to bacterial and Oomycete pathogens. Resistance gene candidates (RGCs) holds much promise to investigate features of resistance-related loci in ginger. Fourteen oligonucleotide primers, designed to the conserved regions of four classes of cloned resistance genes (R-genes) were evaluated to examine their efficiency to yield RGCs in ginger. Clones derived from altogether 17 amplicons, generated by 12 successful primers were sequence characterized. Clones derived from three primers showed strong homology to cloned R-genes or RGCs from other plants and conserved motifs characteristic of non-TIR sub-class of NBS–LRR R-gene superfamily. Phylogenetic analysis separated ginger RGCs into two distinct subclasses corresponding to clades 3 and 4 of non-TIR NBS sequences described in plants. This is the first report on the identification of primer sets to amplify RGCs in ginger. Our study provides a base for future RGC mining in ginger and valuable insights into the characteristics and phylogenetic affinities of non-TIR NBS–LRR R-gene subclass in ginger genome.

Keywords: Ginger, non-TIR NBS–LRR sequences, resistance gene candidates, *Zingiber officinale*.

GINGER is an important cash crop in tropical and subtropical countries. Ginger rhizome is valued world over both as a spice and as a medicine. India is the largest producer of ginger in the world, contributing to 33% of world production, followed by China, Nigeria, Indonesia, Bangladesh and Thailand[1]. It is not amenable to genetical studies and conventional crop improvement protocols due to two major reasons. Ginger is completely sterile and never set seeds[2]; it is propagated exclusively by vegetative means using rhizome. Secondly, all the ginger cultivars available today are equally susceptible to all major diseases such as soft rot caused by *Pythium* spp. and bacterial wilt caused by *Pseudomonas solaracearum*[3]. Crop improvement programmes of ginger are therefore confined only to clonal selections[2,3]. Theoretical considerations predict a total decay of genotypic diversity in exclusive asexuals over a long evolutionary period[4]. Empirical evidences have been provided for reduced genetic diversity in self-pollinating species[5] and in chromosomal segments with restricted recombination in outbreeders[6]. However, empirical data on the nucleotide diversity of asexual crops are extremely scanty in literature. The conserved motifs in proteins encoded by resistance genes (R-genes) cloned from different plant species have facilitated PCR amplification of analogous sequences, called resistance gene candidates (RGCs), from heterologous plant species using degenerate primers designed to these conserved motifs[7–16]. Investigations using RGCs have provided vital insight into the organization, distribution and evolution of R-genes in plants[17–19], and more importantly, the RGCs serve as vital tools for the isolation of full length R-genes in plants[20–22]. RGCs hold much promise in the genome analysis of genetically less amenable clonal crops like ginger. The results may help to depict the consequences of exclusive reliance on asexual propagation on the resistance loci in ginger and, perhaps, a clue to the reasons for the monotonous susceptibility of its cultivars to diseases. Such initiatives may ultimately help to design strategies for aligning the component of resistance traits in ginger through transgenic methods, as it is the only alternative for crop improvement in ginger. Ability of R-gene specific primers to yield RGCs varies from species to species[14,23] and thus becomes essential to evaluate several primer combinations to identify successful combinations in any given species. As part of a long-term programme to understand ginger genome, we assessed the ability of 14 R-gene specific primer pairs selected from published literature to yield RGCs in ginger. Based on the nature of their conserved domains, most of the cloned R-genes are grouped into four classes: nucleotide binding site (NBS)–leucine-rich repeat (LRR) class; LRR–transmembrane (TM) class; LRR–TM–kinase class, and receptor kinase class[17,24]. Primers designed to R-genes encompassing all the four classes were included in this study. Characteristics of RGCs yielded by successful primer pairs are discussed.

Fourteen R-gene specific oligonucleotide primers that had previously been used in other taxa were selected from published literature (Table 1). The genes and their conserved motifs, which formed the basis for primer design are given in Table 1. The selected set mostly comprised the primers reported during early years of RGC research (Table 1) and tested later in diverse plant taxa by several investigators[10,12,15,16,25,26]. Primer selection was performed primarily based on three criteria: (i) Primers based on R-genes with specificities against fungal, bacterial and viral pathogens; (ii) Primers based on R-genes belonging to different classes, and (iii) Primers based on different combinations of motifs conserved in an R-gene class. Class membership of the R-genes used for primer designing in the present study is as follows: *N*, *L6*, *RPS2* and *RPP5* of NBS–LRR class; *Cf*-9 and *Cf*-2 of LRR–TM class; *Xa21* of LRR–TM–kinase class, and *Pto* of kinase class. Primer pair P4 was from Feuillet *et al.*[27], designed to the domains conserved among serine/threonine kinase family of genes. Genomic DNA was isolated from the young

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Table 1. Degenerate R-gene specific primers used for screening of sequences analogous to resistance genes from *Zingiber officinale* cv. kuruppampady

Primer code	Sequence[a] (5′→3′)	Conserved motif/domain	R-genes[b]	Reference	Amplicon size (kb)	Annealing temperature (°C)
P1	GGIGGIRTIGGIAARACIAC	P-loop	*N*, *RPS*2, *L6*	33	0.5	50
	WTIARIGYIARIGGIARICC	GLPL	*N*, *RPS*2			
P2*	GGIGGIGTIGGIAAIACIAC	P-loop	*N*, *RPS*2, *L6*	34	0.35 and 0.5	50
	ARIGCTARIGGIARICC	GLPL	*N*, *RPS*2			
P3	GGIGGIGTIGGIAAIACIAC	P-loop	*N*, *RPS*2, *L6*	7	0.28, 0.4 and	50
	IARIGYIARIGGIARICC	GLPL	*N*, *RPS*2		0.8	
P4	GAYGTNAARCCIGARAA	Domain VI	Ser/thr kinase	27	0.5	55
	TCYGGYGCRATRTANCCNGGITGIC	Sub-domain VIII	Ser/thr kinase			
P5	CGCAACCACTAGAGTAAC	LRR	*RPS*2	35	0.9 and 1.2	45
	ACACTGGTCCATGAGGTT	LRR	*RPS*2			
P6*	GGACCTGGTGGGGTTGGGAAGACAAC	P-loop	*RPS*2	8	0.6	60
	CAACGCTAGTGGCAATCC	GLPL	*RPS*2			
P7	TGGGGTGGGAAGACAAC	P-loop	*RPS*2	36	0.35 and 0.7	55
	GTCAGTTTCGGGCATATGAG	LRR	*RPS*2			
P8	TTTTCGTGTTCAACGACG	LRR	*Cf*-9	34	0.9	45
	TAACGTCTATCGACTTCT	LRR	*Cf*-9			
P9	WXIAAYAARYTICAYGGICCIAT	LRR	*Cf*-2, *Cf*-9	8	0.35 and 0.95	45
	GCIARYTGTCZIGGIATYTCICC	LRR	*Cf*-2, *Cf*-9			
P10*	GGTGGGGTTGGGAAGACAACG	P-loop	*N*, *RPS*2, L6	9	0.6	60
	CCACGCTAGTGGCAATCC	GLPL	*N*, *RPS*2, L6			
P11	GGAATGGGNGGNGTNGGNAARAC	P-loop	*N*, *RPS*2	37	0.35 and 0.9	55
	YCTAGTTGTRAYDATDAYYYTRC	RNBS-B	*N*, *RPS*2			
P12	TAGGGCCTCTTGCATCGT	LRR	*N*	34	0.5	50
	TATAAAAAGTGCCGGACT	LRR	*N*			
P13	CCGTTGGACAGGAAGGAG	LRR	*Xa*21	34	NA[c]	NA[c]
	CCCATAGACCGGACTGTT	LRR	*Xa*21			
P14	GCATTGGAACAAGGTGAA	Kinase	*Pto*	34	NA	NA
	AGGGGGACCACCACGTAG	Kinase	*Pto*			

*Primers that amplified sequences with homology to R-genes or RGCs from other plants species.

[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]*N*, From tobacco against tobacco mosaic virus; *RPS*2 and *RPP*5, From *Arabidopsis* against *Pseudomonas syringae* pv. *phaseolica* and *P. parasitica* respectively; *L6*, From flax against *Melamspora lini*; *Cf*-9 and *Cf*-2, From tomato against *Cladosporium fulvum*; *Xa*21, From rice against *Xanthomonas oryzae* pv. *oryzae*; *Pto*, From tomato against *P. syringae* pv. *tomato*.

[c]Not amplified.

leaves using GenElute Plant Genomic DNA kit (Sigma), according to manufacturer's instructions. PCR conditions were initially standardized using the genomic DNA of a popular ginger cultivar 'kuruppampady' and the annealing temperatures were determined for each R-gene specific primer pair (Table 1). The standardized cycling conditions comprised an initial denaturation at 94°C for 5 min followed by 40 amplification cycles consisting of 94°C for 1 min, 45–60°C for 1 min (Table 1) and 72°C for 2 min and a final extension step at 72°C for 7 min. Amplification was carried out in an i-Cycler (Bio-rad) in a 50 µl reaction volume containing 1X buffer containing 1.5 mM $MgCl_2$, 200 µM dNTP, each primer at 1 µM, 2.5 U of *Taq* DNA polymerase (Bangalore Genie, India) and 50 ng of ginger genomic DNA. PCR products were separated by electrophoresis on a 1.2% agarose gel. Out of the 14 primer pairs tested, 12 pairs yielded reproducible amplification products (Table 1) while the remaining primers failed to give consistent results. Altogether 17 bands ranging from 0.28 to 1.2 kb were scored from successful primers.

The 17 amplicons yielded by 'kuruppampady' were gel purified using GFX Gel Band Purification kit (Amersham Biosciences), cloned using pGEM-T Easy Vector System I (Promega) and transformed in competent *Escherichia coli* JM109 cells. The clones so derived were named with the two letters, representing the first letter of the generic and specific name of the species studied followed by primer code as in Table 1 and a clone number at the end. A minimum of two clones derived from each amplicons were sequenced using BigDye terminator cycle sequencing kit (Perkin Elmer) on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Database searches were performed with the BLAST[28] algorithm in the non-redundant GenBank database and the results are given in Table 2. Clones derived from nine primers, viz. P1, P3, P4, P5, P7, P8, P9, P11 and P12 indicated no significant sequence identity with either RGCs or known R-genes from other plant species (Table 2). They were mostly homologous to genomic clones and retro-elements. Similar sequences are often encountered in RGC isolation initiatives in

Table 2. Results of BLAST searches of clones obtained following screening for RGCs from ginger cultivar kuruppampady using 12 R-gene specific degenerate primers. GenBank accession numbers obtained for the sequences isolated are given. Selected primers are in bold. The primer code is as in Table 1

Primer code/ approximate amplicon size (kb)	Clone code	Homology details of BLAST results	e-value	Clone length (bp)	GenBank accession numbers
P1/0.5	ZoP13	*Homo sapiens* genomic fragment	3.8	411	AY856482
	ZoP15	*Hepatitis* E virus genomic RNA	3.1	333	AY856483
P2/0.35	ZoP22	*Oryza sativa* (japonica cv-group) genomic DNA	9.00E–07	371	AY856484
	ZoP23	*O. sativa* (japonica cv-group) genomic DNA	6.00E–08	375	AY856485
	ZoP24	*Sulfolobus solfataricus* P2 section DNA	0.82	351	AY856486
	ZoP25	*H. sapiens* PAC clone	0.003	300	AY856487
P2/0.5	ZoP26	*Poncirus trifoliata* resistance protein (*RGA 24*)	0.88	373	AY864966
	ZoP27	*Saccharum officinarum* clone *RGA-Q3* resistance gene-like mRNA	2.2	636	AY864965
P3/0.4	ZoP33	*Thermoproteus tenax* GAPN gene	0.98	414	AY856488
P4/0.5	ZoP41	*Coenorhabditis elegans* guanine nucleotide exchange factor	4.4	473	AY856489
	ZoP43	Unknown sequence		432	AY856490
P5/0.9	ZoP51	*H. sapiens* BAC clone	0.41	670	AY856491
P5/1.2	ZoP52	*O. sativa* (japonica cultivar-group) BAC clone	0.066	419	AY856492
P6/0.6	ZoP61	*Solanum phureja* × *Solanum stenotomum* clone *SOL 01* resistance gene analog gene	0.14	615	AY864957
	ZoP63	*Arabidopsis thaliana RPS*2	0.004	618	AY864958
	ZoP68	*A. thaliana RPS*2	0.002	617	AY864959
	ZoP613	*S. pinnatisectum* gene encoding nucleotide binding site	0.034	621	AY864960
	ZoP614	*RPS*2 resistance protein (*A. thaliana*)	3.00E–04	618	AY864961
P7/0.35	ZoP72	*Citrus reticulata* microsatellite DNA	8.00E–04	338	AY856493
	ZoP73	*A. thaliana* unknown protein mRNA	4.00E–05	285	AY856494
	ZoP74	Human DNA sequence	0.2	336	AY856495
	ZoP76	*H. sapiens* BAC clone	0.23	393	AY856496
P7/0.7	ZoP78	*O. sativa* japonica cv-gp putative polyprotein	3.00E–04	412	AY856497
	ZoP79	*O. sativa* japonica cv-gp putative retroelement	8.00E–08	476	AY856498
P8/0.9	ZoP81	*Medicago truncatula* BAC clone	3.00E–04	495	AY856499
	ZoP82	*O. sativa* japonica cv-gp putative retroelement	4.00E–04	707	AY856500
P9/0.35	ZoP91	*Capsicum anuum* PR-protein-like gene, partial sequence	0.66	287	AY856501
0.95	ZoP92	*Mus musculus* BAC clone	2.5	273	AY856502
	ZoP93	*M. truncatula* BAC clone	7.00E–04	402	AY856503
	ZoP94	*C. elegans*, pseudogene	0.11	692	AY856504
P10/0.6	ZoP101	*Lycopersicon hirsutum* clone SA2_300 RGA marker sequence	0.001	699	AY864934
	ZoP103	*A. thaliana RPS*2	0.016	662	AY864935
	ZoP104	*L. hirsutum* clone SA2_300 RGA marker sequence	0.001	650	AY864936
	ZoP107	*L. hirsutum* clone SA2_300 RGA marker sequence	0.001	637	AY864938
	ZoP1010	*S. pinnatisectum* NBS sequence	0.004	633	AY864939
	ZoP1015	*A. thaliana* unknown protein	0.036	604	AY864940
P11/0.35	ZoP111	*H. sapiens* BAC clone	0.15	261	AY856505
	ZoP112	*O. sativa* T-DNA integration genomic sequence	9.00E–26	452	AY856506
P11/0.9	ZoP113	*C. elegans* cosmid sequence	1.6	654	AY856507
P12/0.5	ZoP122	*H. sapiens* BAC clone	3.6	386	AY856508
	ZoP124	Human DNA sequence from BAC clone	0.012	309	AY856509
	ZoP126	*A. thaliana* CC4-associated factor	0.021	538	AY856510

plants[11,15,25] and were not considered for further analysis. In contrast, of the 13 clones derived from an approximately 0.6 kb amplicons yielded by each of the other primers P2, P6 and P10; 12 showed significant similarity to RGCs or known R-genes from other species and one clone (ZoP1015) to an unrelated protein (Table 2). These clones were further analysed using ORF Finder at the NCBI server (www.ncbi.nlm.nih.gov/projects/gorf/). Nine clones had possible frames encoding polypeptides longer than 100 amino acids, uninterrupted by stop codons. The remaining three clones, viz. ZoP613, ZoP101 and ZoP104 contained multiple stop codons and are likely to be pseudogenes. Amino acid sequences deduced from ginger sequences were subsequently aligned with NBS domain encoded by cloned R-genes using CLUSTALW program of BioEdit software[29] in order to look for motifs characteristic of resistance proteins. In addition to P-loop and GLPL that were used to design primers, the alignment clearly revealed four more conserved motifs, RNBS-A (Resistance Nucleotide Binding Site) non-TIR (*Toll*/Interleukin Receptor homology), kinase-2, RNBS-B and RNBS-C (Figure 1), which are characteristics of the NBS domain encoded by NBS–LRR resistance gene family[18,19]. All the sequences examined invariably showed a tryptophan (W) residue at the end of kinase-2 motifs. *NBS–LRR* gene superfamily consists of two distinct sub-classes: one comprising of sequences encoding an amino terminal TIR domain, and the other either lacking this TIR domain or replaced with a coiled–coil (CC) domain[18,19]. Both TIR and non-TIR NBS–LRR sub-classes are present in dicots but in monocots only non-TIR sub-class is present and the other is completely absent[18,19]. The presence of RNBS-A non-TIR motifs and a tryptophan (W) residue at the end of kinase-2 motifs in ginger sequences isolated in this study (Figure 1) are in good agreement with the characteristics identified earlier for non-TIR sub-class[18,19], suggesting that these

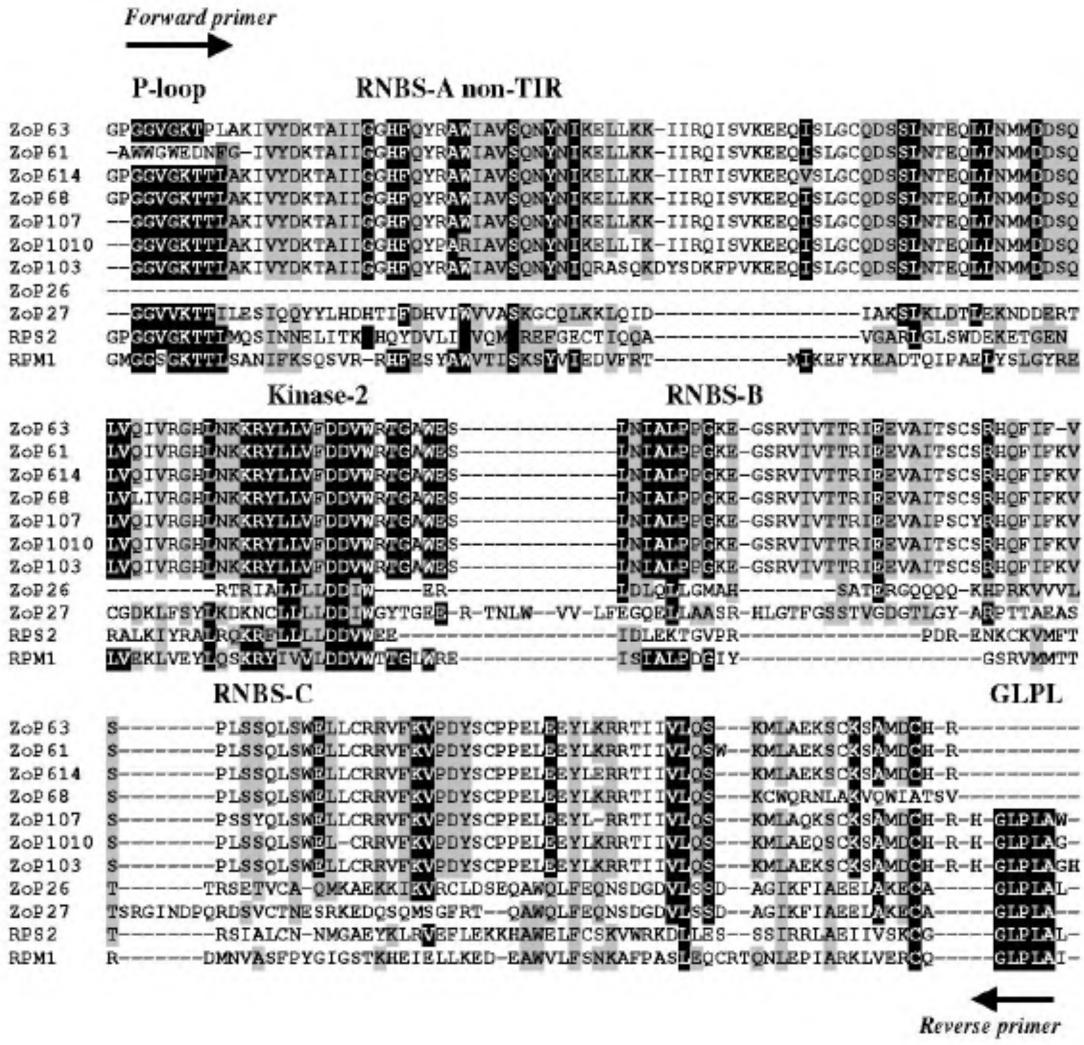

Figure 1. Alignment of deduced amino acid sequences of ginger RGCs with NBS domain of *Arabidopsis RPS2* (GenBank accession number: U12860) and *RPM1* (GenBank accession number: NM_111584) using CLUSTALW. Motifs identified according to Meyers *et al.*[18] are given at the top of the panel. Similar residues in the conserved motifs are highlighted. P-loop and GLPL are the priming sites.

sequences correspond to NBS region of non-TIR NBS–LRR sub-class of NBS–LRR super-family of genes, as expected for a monocot species.

In order to visualize the relative distance of ginger sequences to R-genes and RGCs from other species, we generated a neighbour-joining tree based on the multiple alignment of amino acid sequences. RGCs from other species included in the analyses comprised a minimum of two representatives from each of the four non-TIR clades identified earlier by Cannon *et al.*[19]. Phylogenetic tree demonstrated the existence of two distinct sub-classes in ginger RGCs: one comprising of P6 and P10 derived RGCs and pseudogenes, and the other comprising of P2 derived clones (Figure 2). The sub-class-1 was grouped with sequences belonging to non-TIR clade N4 identified by Cannon *et al.*[19], whereas the sub-class 2 was grouped with non-TIR clade 3. Based on an analysis of over 800 NBS–LRR RGCs from 10 monocots, 18 dicots and two gymnosperms, Cannon *et al.* observed a minimum of four deep splits within the non-TIR sub-family of *NBS–LRR* genes. Of the 148 non-TIR RGCs examined in this study from monocots, almost 95% were placed in phylogenetically ancient clades 1 or 2 and the remaining 5% in clade 3. We could not find a monocot non-TIR RGC from databases which belong to clade 4 in their study. The phylogenetic affinity of ginger RGCs to the phylogenetically recent and rarely seen non-TIR clades 3 and 4 holds great evolutionary significance and perhaps may have a bearing on the monotonous susceptibility of ginger cultivars. However, a large scale isolation and analysis of RGCs from ginger genome is essential to derive further conclusions. The tree also revealed the phylogenetically distinct positions for the P6 and P10 derived pseudogenes as compared to the RGCs derived by same primers. Subdivisions were also evident among pseudogenes: one group consisting of one pseudogene (ZoP101) and the other consisting of two (ZoP613 and ZoP104). Pseudogenes are commonly encountered during investigations of RGCs in plants[8,12,15] and sorted by different evolutionary forces[30]. Like in the case of gene super-families, different sub-families can be recognized among pseudogenes related to a multigene family[31]. Though it is difficult to predict functional relevance of sub-divisions of pseudogenes, it is generally interpreted as an indication of the conservation of stop codons, suggesting a function for stop codons[12]. Functional role of stop codons has been demonstrated at least in the case of resistance mediated by N-gene of tobacco through alternate splicing[32]. Results of pairwise comparisons of the translated products of ginger sequences supported the finding of phylogenetic analysis (data not shown). Three classes were discriminated at a 60% identity threshold value, corresponding to P6 and P10 derived clones, P2 derived clones and pseudogenes.

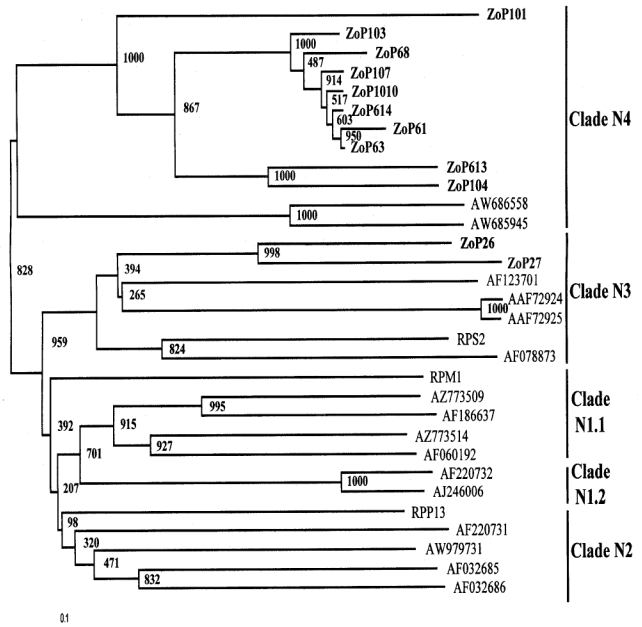

Figure 2. Phylogenetic tree based on alignment of deduced amino acid sequences of ginger sequences amplified by the primers, P2, P6 and P10 (indicated in bold) with NBS domain of R-genes, *Arabidopsis RPS2* (GenBank accession number: U12860), *RPM1* (NM_111584) and *RPP13* (AAF42831) and RGCs from *M. truncatula* (AW686558; AW685945; AZ773509; AZ773514), *M. sativa* (AAF72924; AAF72925), *Avena sativa* (AF078873), *Cajanus cajan* (AF186637), *Glycine max* (AF060192), *Oryza sativa* (AF220732; AF220731), *O. officinalis* (AJ246006), *Hordeum vulgare* (AF032685; AF032686), *Lycopersicon esculentum* (AW979731) and *Pisum sativum* (AF123701). The clades identified are as described by Cannon *et al.*[19]. Bootstrap values for 1000 replications are given at branch points.

This is the first study for the identification of primer sets to amplify RGCs in ginger. In the process, we identified three primer pairs designed to the conserved motifs of NBS domain of NBS–LRR R-gene class as most successful in isolating RGCs in ginger. Primers designed to NBS motifs are indeed most successful for isolation of RGCs in plant species. But, in order to maximize RGC mining from ginger genome, we tested primers based on other R-gene classes also in this study. However, such primers were found to be unsuccessful in ginger. Three primers identified in this study were highly efficient in amplifying RGCs in ginger, yielding nine RGCs out of 13 clones sequenced. Interestingly, the primers identified are selective in amplifying RGCs belonging to two distinct non-TIR NBS classes in ginger. The *NBS–LRR* gene superfamily is comprised of hundreds of paralogs in plant species[18,19]. Having identified the primer set, the task is to enrich the identified RGC pool and use them for genome analysis of ginger together with its wild relatives. Such an approach will not only help to understand the nature of genome evolution of obligatory asexual ginger, but also to target novel genomic resources in wild germplasm for the genetic improvement of ginger.

The sequences obtained from ginger have been deposited in the GenBank with the following accession numbers: AY856482–AY856510; AY864934–AY864936; AY864938–AY864940; AY864957–AY864961 and AY864965–AY864966.

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