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Functional genetic diversity at nucleotide binding site (NBS) loci: Comparisons among soft rot resistant and susceptible *Zingiber* taxa

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ABSTRACT

Fundamental problem confronting ginger production is uniform susceptibility of all cultivars to soft rot disease caused by *Pythium* spp. Resistance gene candidate sequences (RGCs) belonging to non-TIR (Toll-Interleukin receptor) NBS-LRR (Nucleotide Binding Site-Leucine Rich Repeat) class of resistance (R) genes was previously identified from cultivated and wild *Zingiber* taxa including *Zingiber zerumbet*, a putative resistance donor. Efficiency and sensitivity of SSCP (Single-strand conformation polymorphism) analysis over RFLP (Restriction fragment length polymorphism) analysis in discriminating *Pythium* susceptible and resistant *Zingiber* accessions is being described here. *Zingiber* RGCs detected 29.5% polymorphism in *Z. zerumbet*, 12.34% in *Zingiber cernuum* and no polymorphism in the analyzed ginger cultivars. Results are discussed in terms of their mode of propagation and R gene evolution in the wild and cultivated accessions. Four RGCs designated *Zz*P226, *Zc*P28, *Zo*P26 and *Zc*P623 that yielded *Z. zerumbet* specific bands constitute important genomic tools for isolation of full-length R-genes from resistant germplasm.

1. Introduction

Genus *Zingiber* (Family: Zingiberaceae) found throughout tropical and sub-tropical regions comprises of many economically important species including *Zingiber officinale*, a valuable spice and medicine (Khatun et al., 2003). India has a predominant position in ginger production and export with Indian ginger considered one of the best in world (Thanuja, 2006). A fundamental problem confronting ginger production is poor variability for resistance to bacterial and fungal diseases including soft rot caused by *Pythium* spp. and bacterial wilt by *Ralstonia solanacearum*, incurring heavy crop loss every year (Dake, 1995). Two reasons that could be attributed to this increased susceptibility include complete sterility (Valsala et al., 1996) and obligatory asexual propagation (Lawrence, 1984). South Asia being the center of origin of genus *Zingiber* (Selvan et al., 2002), greatest diversity resulting from various evolutionary processes is expected in the many eco-geographic niches. Thus programs were initiated to identify *Zingiber* germplasm resistant to *Pythium aphanidermatum* (Edson) Fitzp and subsequently *Zingiber zerumbet* was identified as a putative resistance donor to ginger (Kavitha and Thomas, 2007, 2008a, 2008b). Unlike ginger cultivars, mode of propagation of wild *Zingiber* species ranges from clonal to sexual (Kavitha and Thomas, 2008b). Sexual reproduction has a profound influence on maintaining evolutionary potential of the host plant (Clay and Kover, 1996). Thus elucidation of genetic diversity at functional loci in *Zingiber* spp. with varying mating patterns will provide insight into the adaptive variation of wild species especially during antagonistic host–pathogen interactions (Gibson et al., 2000; Strange and Scott, 2005).

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The extensively studied nucleotide binding site-leucine rich repeat (NBS-LRR) class of R-genes (Meyers et al., 1999, 2003; Fluhr, 2001) arranged in genome as large multi-gene clusters are attractive 'candidates' for studying genomic variation because pathogens are ubiquitous and exert high selective pressure on their hosts (Caicedo and Schaal, 2004; Ding et al., 2007). Within a gene cluster, NBS-LRR R-genes are structurally similar and probably result from duplications of a common ancestor (Richter and Ronald, 2000; Bergelson et al., 2001). Such duplication events followed by diversification allows the plant to broaden and adapt its defense response to new and evolving plant pathogens (Bergelson et al., 2001).

Differences in response to *P. aphanidermatum* infection by the cultivars and wild *Z. zerumbet* prompted us to investigate the relatively unexplored genetic variation in NBS regions using RFLP and SSCP that represent suitable tools for phylogenetic analysis targeting sequence variations in genomic loci (Orita et al., 1989). Extent of NBS diversity among cultivated and wild *Zingiber* spp. was analyzed using resistance gene candidate (RGC) probes targeting NBS loci and previously isolated and characterized from four *Zingiber* taxa: *Z. officinale* cv. kuruppampady, wild accession of *Z. officinale*, *Z. zerumbet* and *Z. cernuum* (Aswati and Thomas, 2007).

2. Materials and methods

2.1. Plant materials and DNA extraction

Plant materials used for the present study are shown in Table 1. Voucher specimens for representative samples of each of the wild *Zingiber* spp. *viz*., *Z. zerumbet* [2010-44 Wayanad (CALI)] and *Z. cernuum* [2011-12 Wayanad (CALI)] along with ginger cultivar kuruppampady (G. Thomas 95701 (CALI)) were deposited in the herbarium of University of Calicut, Kerala, India. The cultivar Maran is a variety released from Kerala Agriculture University (KAU), Kerala, India. Genomic DNA of *Zingiber* species for RFLP analysis were isolated and purified according to the method of Sharp et al. (1988). Genomic DNA for SSCP was isolated from 100 mg of tender leaf tissues using GenElute Plant Genomic DNA Purification Kit (Sigma, USA) following manufacturer's instructions.

2.2. Southern hybridization

Twenty μg of genomic DNA from cultivar, Rio-de-Janeiro, *Z. zerumbet* (2010-1) and *Z. cernuum* (2011-5) were digested with five restriction enzymes (*Eco*RI, *Eco*RV, *Hind*III, *Xba*I and *Taq*I) (Genie, Bangalore). Southern blots were hybridized with phylogenetically distinct radioactive *Zingiber* RGC and included ZwP1012 (AY865010), ZcP616 (AY865001), ZoP1031 (AY864947), ZoP614 (AY864961), ZcP28 (AY865006) and ZzP226 (AY864989). Random priming technique was used for labeling probes with α^{32}P dCTP using NEBlot kit (Amersham, UK), according to manufacturer's instructions. Hybridization was performed at 60 °C for 16 h in pre-hybridization buffer containing 500 mM Na_2HPO_4 (pH-7.2), 1% BSA, 7% SDS, 1 mM EDTA and 10 μg/mL denatured salmon sperm DNA. Membranes were washed twice with 2× SSC and 0.1% SDS and once with 0.5× SSC and 0.1% SDS at 60 °C and then exposed to phosphor screen in a phosphor cassette (BioRad) for a suitable period of exposure as judged by the intensity of radioactive emission.

2.3. Single strand conformation polymorphism (SSCP) analysis

A total of 15 oligonucleotide primers were designed (Table 2) to amplify RGC-sequence tagged sites (RGC-STS) considering the amino acid sequence variation of phylogenetically distinct RGCs from *Z. zerumbet*, *Z. cernuum*, *Z. officinale* cv. kuruppampady and cv. Maran. Each 20 μl PCR reaction contained 1× buffer with 1.5 mM $MgCl_2$, each dNTP at 200 μM, each primer at 0.2 μM, 1U Taq polymerase and 20 ng of template DNA. Thermal cycling was carried out with initial denaturation at 94 °C for 5 min followed by 25 cycles of 94 °C for 50 s, annealing at the appropriate temperature (Table 2) for 50 s and extension at 72 °C for 1 min. RGC-STS PCR products were cleaved using suitable restriction endonuclease (Table 3) identified

Table 1
Plant materials used.

Code	Species	Accession number	Source/Origin	Latitude	Longitude
1	*Z. zerumbet*	2010-4	Kollam	09° 0.5.549′N	077° 07.267′E
2		2010-13	Kallar	08° 44.089′N	077° 08.307E
3		2010-24	Kottayam	09° 36N	076° 34′E
4		2010-30	Thrissur	10° 32.592′N	076° 16.015′E
5		2010-44	Wayanad	11° 27′N	075° 47′E
6		2010-58	Vithura	08° 67.39′N	077° 07.279′E
7		2010-66	Konkalam	08° 52.24′N	077° 07.55′E
8	*Z. cernuum*	2011-2	Ponmudi	08° 67.39′N	077°7.279′E
9		2011-10	Ezhumalai	011° 58.112′N	075° 19.643′E
10		2011-12	Wayanad	11° 29′N	075° 48′E
11	*Z. officinale*	Maran	Kerala Agriculture University (KAU)	08° 44.28′N	077° 08.21′E
12		Kuruppampady	KAU (Vellayani)	08° 44.28′N	077° 08.21′E