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ORIGINAL ARTICLE

Phylogenetic analyses of peanut resistance gene candidates and screening of different genotypes for polymorphic markers

Osman E. Radwan [a,c,*], Talaat A. Ahmed [b], Steven J. Knapp [c]

[a] *National Soybean Research Center, 1101 W. Peabody drive, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA*
[b] *Department of Biological and Environmental Sciences, Qatar University, Doha 2713, Qatar*
[c] *Center for Applied Genetic Technologies, University of Georgia, Athens, Georgia 30602-6810, USA*

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Abstract The nucleotide-binding-site-leucine-rich-repeat (NBS–LRR)-encoding gene family has attracted much research interest because approximately 75% of the plant disease resistance genes that have been cloned to date are from this gene family. Here, we describe a collection of peanut NBS–LRR resistance gene candidates (RGCs) isolated from peanut (*Arachis*) species by mining Gene Bank data base. NBS–LRR sequences assembled into TIR-NBS-LRR (75.4%) and non-TIR-NBS-LRR (24.6%) subfamilies. Total of 20 distinct clades were identified and showed a high level of sequence divergence within TIR-NBS and non-TIR-NBS subfamilies. Thirty-four primer pairs were designed from these RGC sequences and used for screening different genotypes belonging to wild and cultivated peanuts. Therefore, peanut RGC identified in this study will provide useful tools for developing DNA markers and cloning the genes for resistance to different pathogens in peanut.

1. Introduction

The success of a pathogen in infecting a host plant depends on how rapidly the plant recognizes the pathogen and activates appropriate defence reactions. If the pathogen carries an *Avr* (avirulence) gene whose product is specifically recognized by product of the corresponding *R* (resistance) gene in the plant, resistance mechanisms are triggered rapidly, resulting in disease resistance. But, if either the *Avr* or the *R* gene is absent, the pathogen is not recognized rapidly, the defence responses are activated slowly, and disease ensues (Dangl and Jones, 2001, 2006). The largest class of these *R* genes code for proteins nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains (Hammond-Kosack and Jones, 1997; Hulbert et al., 2001). In the past several years, more than 40 genes

* Corresponding author. Address: National Soybean Research Center, 1101 W. Peabody drive, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA.
E-mail address: oradwan@illinois.edu (O.E. Radwan).

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Table 1 Genotype name, plant introduction number, scientific name and pathogen resistance of different peanut genotypes that were used as a DNA template for polymorphic marker screening.

Genotype	PI number	Scientific name	Resistant to
DUR-25	PI 475887	*Arachis duranensis*	Early leaf spot (*Cercospora arachidis*)
DUR35	PI 497483	*Arachis duranensis*	Rust (*Puccinia arachidis*)
BAT-6	PI 468324	*Arachis batizocoi*	Root-knot nematode (*Meloidogyne javanica*)
BAT-8	PI 468326	*Arachis batizocoi*	Tomato spotted wilt virus
AEQ-2	PI 497630	*A. hypogaea var. aequatoriana*	–
PRV-1	PI 502045	*A. hypogaea var. peruviana*	–
HIR-3	PI 576613	*A. hypogaea var. hirsuta*	–
FST-3	PI 497471	*A. hypogaea var. fastigiata*	–

conferring resistance to different pathogens, including bacteria, fungi, nematodes, and viruses, have been cloned in different plant species. Among the total number of cloned plant disease resistance genes, approximately 75% were from the NBS–LRR gene family (Hulbert et al., 2001). Thus, isolation and characterization of the NBS–LRR-encoding genes are extremely significant for understanding plant–pathogen interactions and development of novel approaches to effective control of plant pathogens in agriculture.

The NBS sequence of *R* genes are characterized by the presence of up to seven conserved domains including the P-loop, Kinase-2 and GLPL motifs (Meyers et al., 1999). The presence of these conserved domains has enabled rapid isolation of resistance gene candidate (RGC) from different plant species by using a polymerase chain reaction (PCR) with degenerate oligonucleotide primers designed from these domains. RGCs were isolated from several plant species, such as cotton (He et al., 2004), potato (Leister et al., 1996), soybean (Yu et al., 1996), lettuce (Shen et al., 1998), tomato (Pan et al., 2000), rice (Mago et al., 1999), barley (Leister et al., 1998), wheat (Seah et al., 2000), chickpea (Huettel et al., 2002), *Medicago truncatula* (Zhu et al., 2002) and Sunflower (Radwan et al., 2003, 2004, 2008).

Peanut or groundnut (*Arachis hypogaea* L.) is the fourth most important oil seed crop in the world, cultivated mainly in tropical, subtropical and warm temperate climates (FAO, 2004). It is an important crop for human food as well as animal feed. However, peanut yields are reduced around the world by fungal, bacterial, viral and root-knot nematode diseases. For example, root-knot nematode disease is causing losses of up to 12% in United States and India (Bailey, 2002). The first significant step for isolating NBS–LRR RGCs from the peanut genome was done by Yuksel et al. (2005) and Bertioli et al. (2003) who used the degenerate primers complementary to highly conserved sequences in the NBS domain.

In this current work, we describe a collection of peanut NBS–LRR resistance gene candidates (RGCs). Phylogenetic analyses were used to estimate the relationships within and among peanut RGCs as well as with cloned plant NBS–LRR-encoding *R* genes. Several genotypes belonging to wild and cultivated peanut were screened by using primers, which were designed from these RGC sequences.

2. Materials and methods

2.1. Plant materials and DNA extraction

Eight peanut genotypes resistant to different types of pathogens; two strains belonging to *Arachis duranesis*, two strains belonging to *A. batizocoi* and four varieties belonging to *A. hypogaea* (Table 1) were used for screening by different primers, which were designed from RGC sequences. Genomic DNAs were isolated from peanut genotype fresh leaf tissues using a modified CTAB (cetyltrimethylammonium bromide) method (Webb and Knapp, 1990).

2.2. Data base searching for peanut RGCs

The database of National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) was searched for the RGCs corresponding to NBS–LRR class of disease resistance genes. The keywords 'Peanut resistance gene candidates, Peanut NBS–LRR and Peanut NBS' were used for searching. We have chosen only the RGCs that have NBS domain, and cover the region between the P-loop and the GLPL motif.

2.3. Sequence analyses

The sequences were translated to suitable open reading frame using option 'DNA sequence translation in six-frames' available at http://cgpdb.ucdavis.edu/database/sms/translation.php. The translated sequences were compared with Gene Bank data base using Blast_N algorithms (Altschul et al., 1997). The nucleotide and amino acid sequences were aligned using Clustal_X (Thompson et al., 1997). The redundancy removal was carried out by 'Jalview' available at (http://www.ebi.ac.uk/~michele/jalview/download.html). The phylogenetic tree was constructed using the neighbor-joining method as implemented in Clustal_X with 1000 bootstrap sampling steps (Saitou and Nei, 1987).

2.4. PCR screening of different genotypes

Sequence specific primers (Table 2) were designed based on the sequences of peanut RGCs. The PCR (20 µl total) was carried out using 30 ng of DNA template, 0.65 U Taq polymerase (Qiagen, USA), 1 × PCR reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP and 0.16 µM of each primer. A 'touchdown' PCR protocol was used. The initial denaturation of 94 °C for 3 min was followed by 1 cycle of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 60 s. In each subsequent cycle, the annealing temperature was decreased by 1 °C till reached 58 °C. The amplification continued for 35 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 60 s. The final extension was carried out at 72 °C for 10 min. After verification of PCR product using 1.5% agarose gel, the single-strand conformational polymorphism (SSCP) was checked for PCR amplicons as described by Slabaugh et al. (1997).