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Mining of Resistance Gene Analogues for Anthracnose Disease in Greater Yam (*Dioscorea alata* L.)

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Abstract

Nucleotide-binding site- leucine-rich repeat (NBS- LRR) gene family holds the vast majority of resistance genes identified in plants. Primers based on the conserved motifs were used to isolate NBS type sequences in tolerant and susceptible greater yam (*Dioscorea alata*) cultivars. Cloning and sequencing of identified NBS-type sequences called resistance gene analogues (RGAs) depicted similarity to other cloned RGA sequences available in the public database. The deduced amino acid sequences of the RGAs showed the presence of conserved domains, viz. P-loop, RNBS-B, RNBS-C, Kinase-2 and GLPL, categorising them with the NBS–LRR class gene family. Amino acid sequence alignment of the *Dioscorea* RGAs with RGAs of other plant species grouped them with the non-toll interleukin receptor subclasses of the NBS sequences. The sequence comparison of RGAs in susceptible and tolerant variety showed very little difference between them. This study represents the first report of isolation and characterisation of RGAs from *Dioscorea alata* and will act as a stepping stone for future studies aiming at identifying candidate anthracnose resistance genes in *Dioscorea* spp.

Key words: Resistance gene, NBS-LRR, *Dioscorea*, *Colletotrichum*

Introduction

Anthracnose or dieback disease caused by *Colletotrichum gloeosporioides* is the most important foliar disease of *Dioscorea* spp (Orkwor, 1998). On susceptible yam varieties, symptoms develop as dark brown lesions on the surface of leaves and vine, resulting in tissue necrosis and vine die-back (Amusa, 1997). New shoots may arise from the planting material forming multiple stems in comparison to healthy plants bearing one or two. Once the infection is established, the consequent progress of disease depends on rainfall and host variety (Okigbo, 2005).

Plants defend themselves against its pathogens via the resistance (R) gene product recognising a pathogen avirulence (Avr) gene product resulting in defence activation (Flor, 1971). The vast majority of such plant R genes cloned to date contain nucleotide-binding sites (NBS) and a leucine-rich repeat (LRR). The sequence conservation of different NBS–LRR genes offers the opportunity for the use of PCR-based strategies to isolate and clone other R gene family members. This approach has been successfully used for cloning of putative NBS–LRR resistance gene analogues (RGAs) from many crops such as mango (Lei et al., 2014), taro (Nath et al., 2013), sugarcane (Que et al., 2009) etc. Identification of RGAs not only serve as useful markers for mapping disease resistance genes but also as reference materials for studying the evolution and organisation of the NBS–LRR gene family in different plant species (Joshi et al., 2012). Cloning and characterisation of *Dioscorea* RGAs may help in understanding the basic mechanism involved in anthracnose resistance which will aid in developing environment friendly management strategies to mitigate anthracnose disease. Therefore, the present research attempts to isolate the putative RGAs (NBS–LRR domains) from *Dioscorea* varieties Orissa Elite (susceptible)

and Sree Keerthi (tolerant). Cloning, sequence characterisation and the phylogenetic relation of *Dioscorea* RGAs to other reported RGAs are discussed in this paper.

Materials and Methods

Screening for disease susceptibility and tolerance

Greater yam cv. Sree Keerthi tolerant to anthracnose disease and Orissa Elite highly susceptible to anthracnose disease, maintained in the greenhouse of ICAR-Central Tuber Crops Research Institute, Thiruvananthapuram, India, was used in the present study. Young leaf samples from both the varieties were collected and washed under running tap water followed by sterile distilled water. In order to ensure the susceptible and tolerant properties of selected plant varieties, a detached leaf assay (Yang et al., 1991) was performed by artificially inoculating 25 μl of spore suspension containing approximately 2 x 10^6 spores/ml from a 7 days old culture of *Colletotrichum gloeosporioides* maintained in potato dextrose agar. Control leaves were also kept in the same assay plate by replacing spore suspension with sterile distilled water. On the third day of inoculation, leaflets were evaluated for symptoms of anthracnose.

DNA isolation

Total genomic DNA was extracted from the leaves of *Dioscorea alata* cultivars, Orissa elite (susceptible), and Sree Keerthi (tolerant). Young leaf samples were collected during early hours in the morning, washed in running tap water and immediately used for DNA isolation. A measure of 100 mg of fresh leaf tissue was ground in liquid nitrogen and DNA was extracted using the cetyl trimethyl ammonium bromide (CTAB) method with some modifications (Lodhi et al., 1994). The obtained DNA was dissolved in 100 μl of TE buffer (pH 8.0). DNA quality and quantity were assessed by means of 0.8% agarose gel electrophoresis followed by an ethidium bromide visualisation using 1 Kbp DNA ladder (Fermentas) as DNA size marker.

PCR amplification of RGAs

The RGAs in the selected greater yam varieties were amplified using a PCR based approach, using the genomic DNA as a template and a pair of reported degenerate primers based on the NBS–LRR region of other cloned R genes. The sequence of the primers were RGA 1F : 5' GGIGGIGTIGGIAAIACIAC- 3' and RGA 1R: 5'ARIGCTARIGGIARICC- 3' (Aswati and Thomas, 2007).

PCR was carried out in a 50 μl reaction mixture with 50 ng template DNA, 200nM of each forward and reverse primer, 200 ìM of Deoxyribonucleotide triphosphate mix, 10X PCR buffer (16mM $(NH)_2SO_4$, 67mM Tris-HCl, 15mM $MgCl_2$) and 1U Taq DNA polymerase (Merck Genie, India). PCR was performed in a Agilent Technologies Sure Cycler 8800 (Agilent Technologies, USA) using the following cycling conditions: initial denaturation at 94°C for 5 min followed by 35 cycles each consisting of DNA denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min and primer extension at 72°C for 1 min. The amplification was concluded with a final extension step at 72°C for 10 min. PCR products were subjected to electrophoresis on a 1.2% agarose gel containing 0.5 μg/ml ethidium bromide and visualised under UV light. Gel photographs were scanned through the Gel Doc System (Alpha Imager, Alpha Innotech, USA).

Cloning and sequencing

The purified PCR product was ligated to a cloning vector pTZ57R/T (Fermentas) and transformed into *E. coli* DH5α cells using a InsTAclone PCR cloning Kit (Fermentas). The transformed recombinant colonies were selected by blue-white screening and the presence of the insert was confirmed by colony PCR. The plasmid DNA was isolated from overnight culture using GeneJET™ Plasmid Miniprep Kit (Fermentas) and sequenced bidirectionally using M13 universal primers.

Sequence analysis

Sequences were processed to remove vector and poor-quality reads using the Geneious Pro software version 5.6. RGAs were identified on the basis of sequence similarity using the BLASTx algorithm against the GenBank non-redundant database (http://www.ncbi.nlm.nih.gov). Multiple alignments of nucleotide and corresponding deduced amino acid sequences was carried out by ClustalX module of BIOEDIT software (Hall, 1999) with default parameters.

Phylogenetic analysis

Phylogenetic analysis of putative yam RGA sequences with already well characterised R genes of different plant species was performed using a MEGA package v 6.0

(Tamura et al., 2013) based on the neighbour-joining method (Saitou and Nei, 1987). Bootstrap analysis was performed to determine confidence values on the clades within the phylogram.

Results and Discussion

On considering the economic and medicinal value of *Dioscorea*, it is high time that an effective anthracnose disease management strategy is to be developed. Exploiting the natural host resistance is regarded as the most promising and durable approach to mitigate anthracnose disease. To study the RGAs in greater yam, a susceptible and tolerant variety of yam was selected and the susceptibility and tolerance were confirmed using a detached leaf assay. Typical symptoms of anthracnose were developed on 3rd day post inoculation (Fig. 1) while no symptoms were observed on the control leaf samples. Dark brown spots and cankers were found in test leaves of both the susceptible and tolerant greater yam varieties, but the severity of disease was found to be less in tolerant variety compared to the susceptible variety.

The use of PCR approach with degenerate oligonucleotide primers designed from the NBS region of cloned disease resistance genes has led to the cloning and characterization of many resistance gene-like sequences in several plant species (Kanazin et al., 1996; Leister et al., 1996). This study is the first attempt for PCR-based isolation of R gene-like sequences or RGAs from *Dioscorea*. In the present study, a PCR-based approach using primer pairs designed from RGA conserved motifs of well-characterised plant species was used for amplifying greater yam RGAs. The primer pair produced a major band of the expected size (500bp) (Fig. 2). In addition to the major bands, other faint bands was also amplified with the primers. The faint bands were not considered for further studies and only the bands of expected size were cloned. The discovery of conserved motifs among the NBS-LRR class resistance genes opens the avenue for the use of PCR based strategy in isolating and cloning *R* gene analogues using degenerate or specific primers. This approach thus provides an alternative to the classical methods of transposon tagging and map-based cloning strategies used so far (Seah et al., 1998).

In order to confirm the presence of the cloned insert, random white colonies were picked and were analysed by colony PCR. The result showed the presence of insert sequence, suggesting that the cloning experiments were successful. The recombinant clones were sequenced using universal M13 primers. The representative sequences were deposited in the GenBank database (Accession no. KU749307 and KU749308). The deduced amino acid sequences of RGAs cloned in the present investigation were compared with other known *R* genes in the database using BLASTx and yam RGAs showed the highest similarity to *Elaeis guineensis* (Accession no: XP_010913302.1) for Orissa Elite and *Phoenix dactylifera* (Accession no: XP_008779586.1) for Sree Keerthi.

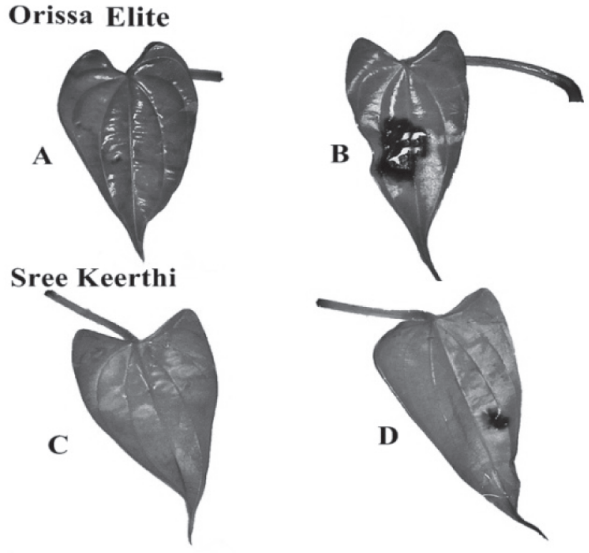

Fig. 1. Detached leaf assay (Observation on 3rd day).
(A) More damaged leaves of Orissa Elite and less damaged leaves of Sree Keerthi
(B) Control and inoculated leaf of Orissa Elite and Sree Keerthi.

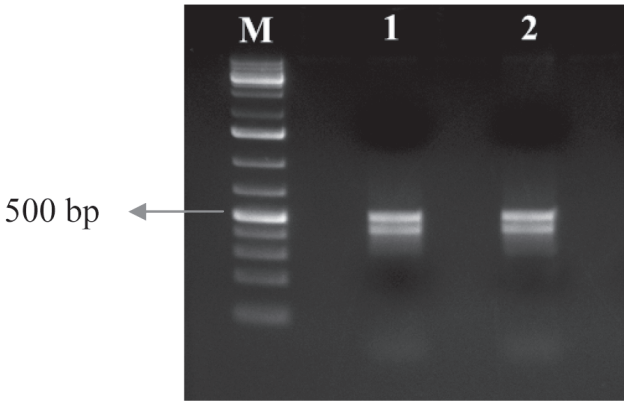

Fig. 2. PCR amplification of RGAs
Lane 1: Amplified RGA from Orissa Elite
Lane 2: Amplified RGA from Sree Keerthi
M: 1kb plus DNA marker.

A phylogenetic tree was constructed with similar sequences obtained through BLAST analysis. Phylogenetic analysis revealed that the amino acid sequences of Orissa Elite shows maximum similarity with the disease resistance protein of *Elaeis guineensis* and Sree Keerthi to disease resistance protein RGA3 of *Phoenix dactylifera* than with the other species used to construct the phylogenetic tree (Fig. 3).

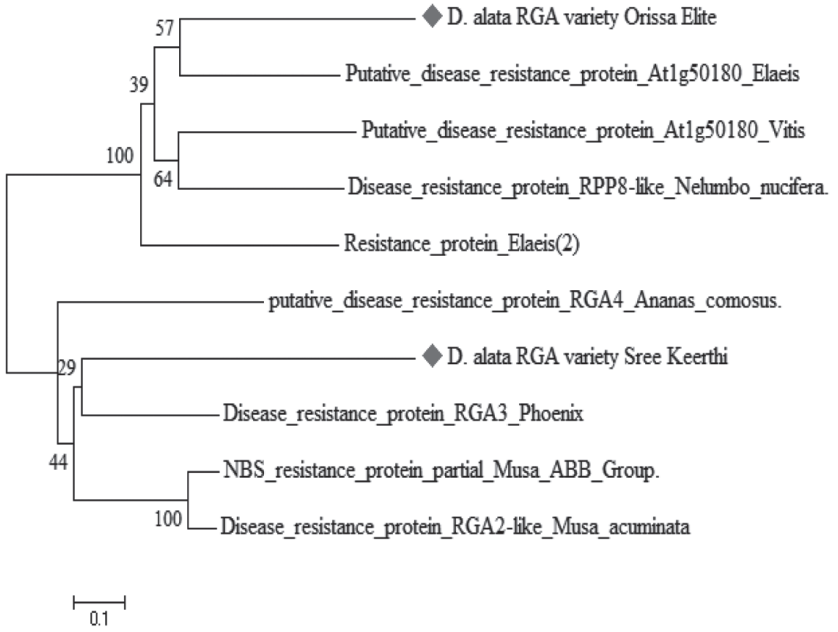

Fig 3. Phylogenetic tree based on the amino acid sequences of the RGAs of Orissa Elite and Sree Keerthi and other similar plant species available in the NCBI database constructed by Neighbor-Joining method. Numbers indicate the percentage support of branching based on bootstrap analysis (1000 replications). The scale bar indicates 0.1 substitutions/site.

Multiple alignments of amino acid sequences with already available database sequences were performed using the CLUSTALX program of BioEdit software. Multiple alignment results revealed the presence of conserved resistance nucleotide binding sites (RNBS-B and RNBS-C), kinase-2, P-loop and GLPL motifs (Fig. 4). In addition, the analysis also showed a tryptophan (W) residue at the end of kinase-2 motif, which is a characteristic feature of non-TIR subclass of NBS-LRR R-genes (Meyers et al., 1999).

The amino acid alignment showed that the *Dioscorea* RGAs share homology with NBS regions of well-characterized R genes from other plants. The last residue of kinase-2 motif of identified RGAs 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)

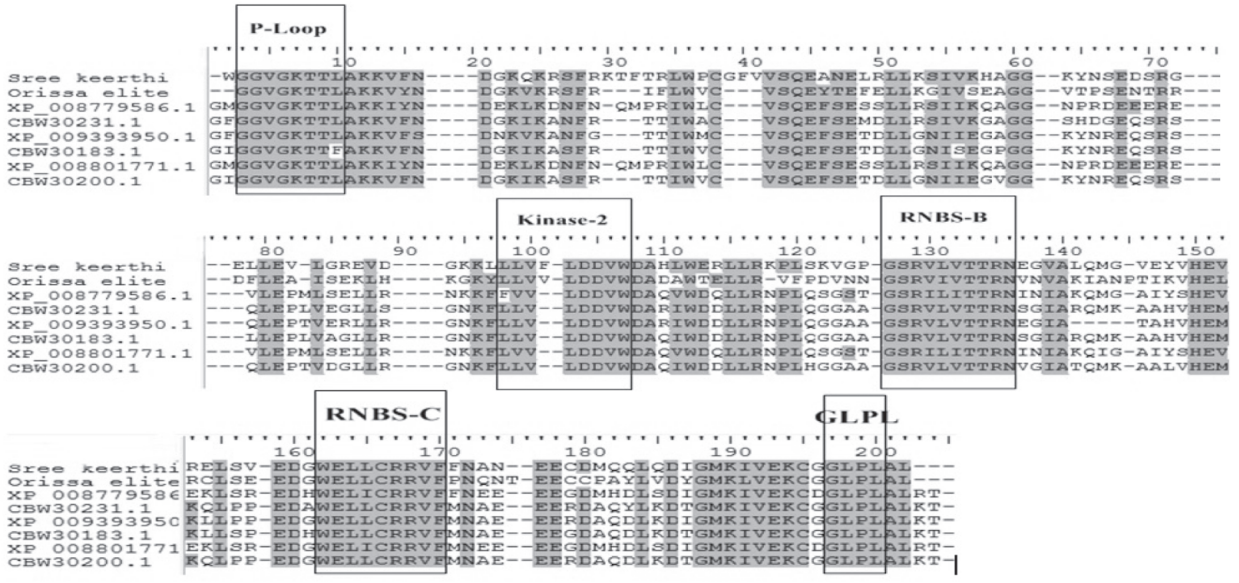

Fig 4. Alignment of amino acid consensus sequences of RGAs of Orissa Elite and Sree Keerthi with similar sequences available in the NCBI database. Conserved regions are highlighted in blue. Conserved domains of Kinase-2, R-gene specific nucleotide binding sites (RNBS-B and RNBS-C), P-Loop and GLPL are boxed. P-Loop and GLPL are the priming sites.

or asparagine (N) residue is expected for TIR NBS–LRR sequences. Using this criterion, it was observed that the *Dioscorea* RGAs amplified belonged to the non-TIR NBS–LRR subclass. Stop codons were identified in the RGA sequence of Sree Keerthi variety, suggests them to be non-functional genes or pseudogenes. In a related study on *Pinus monticola*, 50% of the RGAs cloned were identified to be pseudogenes (Liu and Ekramoddoullah, 2003). Discovery of pseudogenes in plant R genes suggests their involvement in the advancement of novel specificities by recombination and gene manipulations (Michelmore and Meyers, 1998).

In many plants studied till now, NBS-LRR genes seem to be exclusively devoted to defense responses. Development and use of resistant cultivars represent the most effective and economic approach to control plant pathogens. Therefore, identification of new sources of resistance has been the top priority in crop breeding programs for disease resistance. Structural similarity among different R genes conferring resistance to diverse pathogens indicates the highly conserved nature of the plant resistance mechanism (Dangl et al., 1996). RGAs have been utilized to develop molecular markers for tagging and mapping disease resistance traits in many plant species, for isolation of full-length R genes, and to analyze the evolutionary patterns of R-genes in different plant species. Utilizing plant R genes for development of disease resistant varieties is an ecofriendly alternative to the employment of chemical control measures for disease control (Gururani et al., 2012).The present study will serve as a stepping stone for future studies aiming at the cloning and characterization of Yam RGAs for exploring anthracnose resistance.

Conclusion

This study represents the isolation and characterisation of RGAs from *Dioscorea alata* and will act as a stepping stone for future studies aiming at identifying candidate anthracnose resistance genes in *Dioscorea* spp.

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