Identification of Putative Resistance Gene Analogues in *Coffea* and related *Psilanthus* taxa

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Abstract

Biotic stress related damage in plants could be managed by building disease resistance in cultivated genepool through breeding for plant resistance genes (R-genes). Recent studies show that R-genes conferring resistance to diverse pathogens in different plant species share a number of common functional motifs and are frequently clustered in the genome. This considerable conservation at the DNA sequence/protein level in R-genes has led to the development of novel PCR-based molecular approaches to isolate putative resistance gene analogues (RGAs) from new plant sources with considerable ease. We have successfully used one such approach to amplify putative RGAs from *Coffea* and four related endemic *Psilanthus* taxa with degenerate primer pairs designed from conserved P-LOOP and GLPL region of NBS-LRR type resistance gene.

To isolate RGAs, we tested both the total genomic DNA, as well as cDNA as templates in coffee and only genomic DNA for the four related taxa. The PCR-amplified products of expected size range of 500bp were cloned in to pMOS plasmid vector. The cloned fragments were amplified from the recombinant plasmid DNA using M13 universal primers and were sequenced for both strands on an automated DNA sequencer ABI3700 using fluorescence-dideoxy terminator chemistry. The sequences were partially characterized by BLASTn homology searches. Of the 434 sequences, thus analyzed, there were twenty-seven sequences that had all the characteristic features of the known NBS-LRR type of R-genes. Of these 27 putative RGAs, 24 had complete uninterrupted reading, while remaining 3 sequences showed one or more stop codons. The sequence comparisons with known reference RGAs of coffee and other plant species revealed considerable variability in the putative RGAs amplified in the study, especially for those from related, wild *Psilanthus* taxa. The data thus suggest the potential of the secondary genepool as donors of possible resistance genes to the present day cultivated species of coffee.

Introduction

The commercial cultivation of coffee (family: Rubiaceae) relies mainly upon two species, namely, *Coffea arabica* L (2n=4X=44) and *C. canephora* Pierre (2n=2X=22). *C. arabica* accounts for ~70% of world production of high quality coffee but is susceptible to several pests and disease causing pathogens. The latter remains one main concern in arabica

coffee cultivation that results in considerable losses to the global coffee industry. Breeding for disease resistance thus, has been one of the top priorities in coffee cultivar improvement programs worldwide. Conventional breeding efforts in this direction face limitations such as, lack of good resistance sources, poor understanding of the inheritance of resistance traits and need for long breeding cycles for transfer of genes to commercial cultivars. In this context, the new genetic tools in the form of molecular marker technologies provide promising alternatives to expedite the process of identifying, integrating and tracking of genes responsible for the biotic stress in crop plants.

Characterization of plant resistance genes is an important step in understanding plant defense mechanisms to combat biotic stress caused by a wide array of phytopathogens. Classical genetic and molecular data show that genes (R-genes) determining disease resistance in plants are frequently clustered in the genome. Data on such genes conferring resistance to diverse pathogens cloned from several species in recent years show that these encode proteins that share some common functional motifs. Most of these R-genes are found to encode a Leucine Rich Repeat (LRR) region comprising a highly conserved backbone of amino acid motifs. This considerable conservation at the DNA sequence/protein level in majority of disease resistance genes makes it possible to isolate resistance gene analogues (RGAs) from new plant sources via PCR approach with degenerate primers.Using similar PCR approach Noir et al. (2001) isolated 27 RGAs from cultivated coffee species. In the present study we have attempted to amplify and isolate RGAs from four wild related taxa of coffee belonging to the genus *Psilanthus* (endemic to India), in addition to the genomes of cultivated coffee and cDNA (originally isolated from moisture and high light-stressed leaves) of *C. arabica* var. Sln.12. The results on characterisation and diversity analysis of the putative NBS-LRR type RGAs and their relationship with few other known R-genes are reported and discussed.

Materials and Methods

Genomic DNA samples representing two cultivated varieties (*C. arabica var.*Sln12 and *C. canephora var.*BR9) and four endemic wild *Psilanthus* species viz., *P. bengalensis, P. khasiana, P. travencorensis, P. weightiana*, isolated from leaf materials originally obtained from Central Coffee Research Institute, Balehonnur, India, and cDNA obtained from light-drought stressed leaves of *C. arabica* var. Sln-12, formed the source material for this study.

The genomic DNA was isolated from fresh leaf samples according to Aggarwal *et al* (2002). Two sets of coffee specific non-degenerate primers (Noir et al., 2001) were used to amplify NBS containing RGA sequences from the source materials. PCR amplifications were performed in 20 µL reactions each comprising: 25 ng of genomic DNA/cDNA, 1 µM each primer, 150 µM dNTPs, 1 x PCR buffer II (Perkin Elmer), 2mM $MgCl_2$ and 1 unit of Ampli*Taq* DNA polymerase, on a PTC-200 thermolcycler (MJ Research). Amplification profile used was: initial denaturation step at 94^0 C for 10 min followed by 35 cycles of denaturation at 94^0 C for 45 sec, annealing at 50^0 C for 45 sec and elongation at 72^0 C for 1.5 min, and a final elongation step of 5 min at 72^0 C. PCR products were checked and sized on agarose gel before cloning in plasmid vectors pMOS (Amersham) and pCR2.1-TA (Invitrogen). Recombinant plasmid DNA was isolated manually by alkaline lysis method and cloned amplicons were amplified individually using universal M-13 primers for further sequencing. The amplified inserts falling in the size range of 450 – 600 bp were sequenced on the automated DNA sequencer ABI PRISM-3700 for both strands using the BigDye dideoxy terminator cycle sequencing chemistry (Applied Biosystems).

Results and Discussion

In total, 434 clones were sequenced and analyzed for their genetic content by sequence homology search. Majority of the amplified sequences were found to have no motifs characteristic of the target R-genes. However, BLASTn search revealed 27 sequences that showed high similarity to the NBS domains of R-genes. The 24 of these sequences could be translated to polypeptides without any stop codons, and they showed strong overall similarities to several plant R-gene sequences and many RGA sequences cloned from plant species using similar PCR-based approaches. The remaining 3 sequences showed similarity to cloned disease resistance genes at the DNA level, but contained one or more stop codons or frame shifts resulting in interrupted ORFs.

Comparative sequence analysis (performed using Clustal-X) of the newly amplified coffee RGAs with the reference R-gene nucleotide as well as peptide sequences retrieved from the NCBI database revealed that these shared high similarity with them. Of the 24 new translatable RGAs, 9 were found to be of considerable smaller size and thus not used in

further comparative analysis. Sequence analysis of the 15 newly amplified coffee RGAs with 12 already reported coffee RGAs and 5 RGAs from other plant taxa (from NCBI database) revealed high similarity especially, across the four motifs P-loop, kinase-2, kinase-3a and GLPL as also reported by Noir et al. (2001). These results suggest that newly isolated RGAs from *Coffea* and *Psilanthus* belong to the NBS-LRR resistance-gene super family. Further, these new RGAs of coffee and related *Psilanthus* species identified in this study showed considerable sequence variation within them. Despite the variation, all the RGAs were found closely related to one or several plant R-genes identified earlier in coffee and several other plant species.

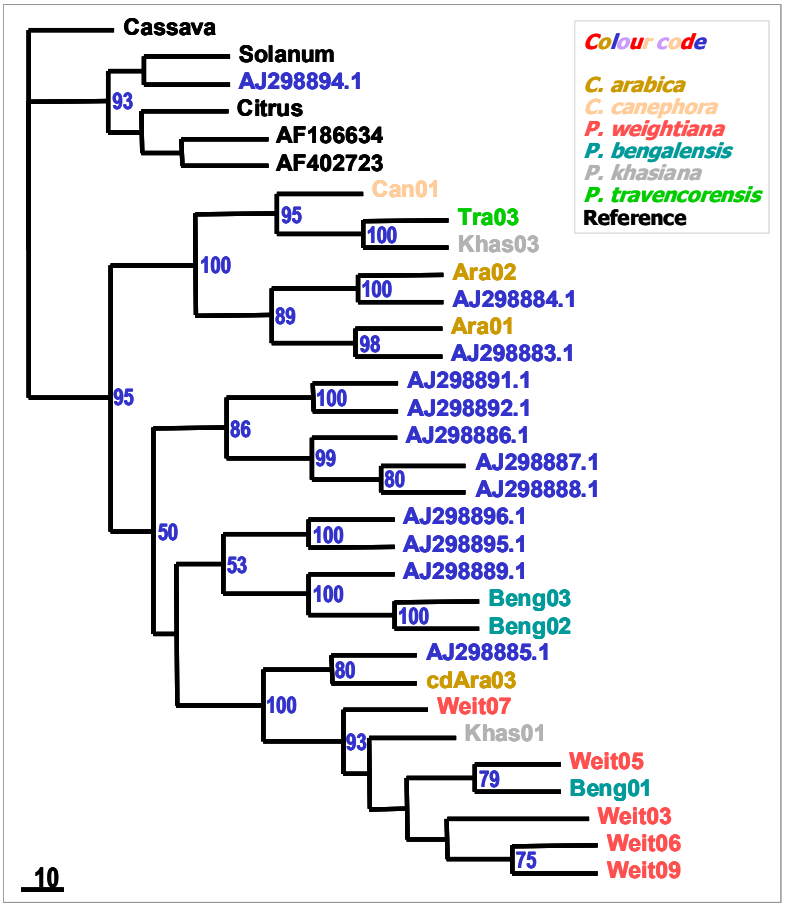

Figure: Neighour-Joining phenetic tree showing relationship of new RGA nucleotide sequences isolated from the cultivated coffee varieties (Sln12 and BR 9) and wild related Psilanthus spp., with the reference RGA sequences (identified by their GenBank accession numbers). The bootstrap values (only >50%) are shown at nodes of the clusters.

Phylogenetic analysis was performed to evaluate the relationship among the coffee RGAs and other plant R genes. The phylogenetic trees were constructed using both distance- and character-based algorithms for both nucleotide as well as putative peptide sequences using Phylip software package. The clustering outputs were also tested for reliability and confidence limits using bootstrapping. The analysis revealed: 1) new RGA to be most related to NBS-family reference RGA sequences described for coffee and other plant taxa; 2) considerable variation in the newly identified RGAs at the nucleotides level; and c) comparable similar relationships using peptide level variation that was generally lower than that seen in the nucleotide sequences. More significantly, the phenetic clustering revealed relatively many unique RGAs from the related the endemic *Psilanthus* spp species compared to that of cultivated varieties of *C. arabica* and *C. canephora* (Figure). The RGAs isolated from *P. weightiana* were found to be very distinct from others because of large deletions.

One RGA sequence could be isolated using cDNA source that was originally obtained from the abiotic stressed leaves of coffee. To our understanding, this observation is novel and may be suggestive of a role for RGA like gene(s) in abiotic stress tolerance, an interesting possibility that need to be explored further.

Many of the clones that didn't show homology to any known R-genes, showed homology to a number of important regulatory proteins like calcium binding proteins, GTP pyrophosphokinases, retro-transposons etc, which may be useful by-products of the RGA search by PCR approach.

Conclusion and Perspectives

The study demonstrates that new RGA can be successfully isolated by the indirect PCR-approach. More importantly, the results suggest that the related endemic taxa of coffee can serve as donor of disease resistance genes to present day cultivated varieties. Finally, identification of a transcribed RGAs from cDNA from abiotic stressed coffee leaves suggest newer interesting possibilities and need for exploring the role of R-genes abiotic stress tolerance in addition to their implied role in biotic resistance.

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References

Aggarwal RK, Shenoy VV, Ramadevi J, Rajkumar R, Singh L (2002) Molecular characterization of some Indian Basmati and other elite rice genotypes using fluorescence-AFLP. *Theoretical and Applied genetics*, 105: 680-690.

Noir, S, Combes, MC, Anthony F, Lashermes P (2001) Origin, diversity and evolution of NBS-type disease resistance gene homologues in coffee trees (*Coffea arabica* L.). *Molecular and General Genomics*, 265: 654-662.