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Biotechnology

Isolation of Putative Disease Resistance Gene Clones from Chickpea and Pigeonpea

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One of the major biotic constraints to crop yields is plant disease. It is essential to understand the development of disease and to implement control measures, irrespective of whether the causative agent is a fungus, bacterium or virus. Of the several disease-control measures available, making use of the genetic basis of disease resistance is the most important. Recent advances in molecular biology have paved the way for the genetic dissection of disease resistance.

The basis for the plant recognition system has been the classic genetic work of Flor (1971), according to which a single disease-resistance gene [R] in the plant recognizes the biotype of the pathogen with the corresponding complementary avirulence gene (Avr). Resistance genes are often present as gene clusters of different specificities in the plant genome. Recently several of these disease-resistance genes have been cloned. The availability of cloned disease-resistance genes permits studies of resistance-gene structure and function. The molecular cloning of disease-resistance genes will also have a major impact on agricultural practice. The two most common methods used for cloning have been chromosome walking (map-based cloning) and transposon tagging. Several recent reviews describe the developments in the study of disease-resistance genes (Baker et al. 1997; Hammond-Kosack and Jones 1997).

The majority of R genes cloned so far share DNA sequences encoding conserved amino acid motifs irrespective of whether they confer resistance to bacterial, fungal, viral, or nematode pathogens. In general, R genes can be grouped into five major classes based on their structural features (Baker et al. 1997) which are leucine-rich repeats (LRR), nucleotide binding site (NBS), and a serine/threonine protein kinase. These are considered to be

components of a signal transduction pathway. The first class encodes cytoplasmic receptor-like proteins that contain LRR domain and a nucleotide binding site (NBS), e.g., *RPS2* and *RPM1* from *Arabidopsis thaliana* conferring resistance to the bacterial pathogen *Pseudomonas syringae,* which has the Avr genes *AvrRps2* and *AvrRpm1*. In this class are included others like the *N* gene from tobacco, *L6* and *M* from flax whose amino-terminal domain shows homology to the *Drosophila* developmental gene Toll and the mammalian immune-response gene encoding the interleukin-1 receptor (IL-IR). The second class contains only a serine-threonine kinase, e.g., *Pto,* which confers resistance to the bacterial pathogen *P. syringae pv.* tomato containing *Avr Pto.* The third class encodes a putative transmembrane receptor with large extra cytoplasmic LRR domains, e.g., includes *Cf-2* and *Cf-9* from tomato which confer resistance to different races of *Cladosporium fulvum.* The fourth class encodes a putative transmembrane receptor with an extracellular LRR domain and an intracellular serine-threonine kinase domain, e.g., the rice *Xa 21* gene, which confers resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae.* The fifth class does not fit into any of the above classes and has an enzymatic function which is carried out without the involvement of the *Avr* component, e.g., the HM1, which confers resistance to the fungal pathogen *Cochliobolus carbonum* race 1. *HM1* encodes a reduced form of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reductase that inactivates toxin produced by *C. carbonum.*

The sequence similarity among the resistance genes from different plant species has made it possible to isolate such resistance-gene candidates (RGCs) from any plant species of interest using polymerase chain reaction (PCR) with oligonucleotide primers to the conserved domains of the resistance-gene classes mentioned above. This method has been successfully used to identify resistance-gene candidates in a variety of species including soybean, potato, and lettuce (Leister et al. 1996; Kanazin et al 1996; Shen et al. 1998).

We used the degenerate oligonucleotide primers designed to the conserved motifs in the NBS region to amplify DNA fragments from chickpea and pigeonpea. These fragments were cloned, sequenced, and screened for homology in the database. We report here the identification of several such disease-resistance gene candidates.

DNA was isolated from chickpea (cultivars - Annigeri, and JG 62) and pigeonpea (cultivars ICP 7119 and ICP 2376) by the standard protocol using alkyl trimethyl ammonium bromide (CTAB). The degenerate primers used were designed from the motifs within sequence encoding the NBS as described (Shen et al. 1998). PCR was performed with the different primer combinations using the cyclesμ described (Shen et al. 1998) in a total volume of 35μl. PCR products from different genotypes of a species were pooled and cloned into pGEM vector (Promega). About 12-16 clones were sequenced from each successful amplification consisting of about 80 clones using the Applied Biosystems model 377 PRISM automated sequencer. DNA homology searches were performed via the National Centre for Biotechnology Information web site (www.ncbi.nlm.nih.gov) using the BLAST algorithm.

Of the 13 combinations of pairs of primers tested on templates from pigeonpea and chickpea genomic DNA samples, only six yielded amplification products. The fragments obtained were approximately the expected size of 0.5kb with multiple templates and these products were cloned and sequenced. The BLAST search indicated that many of the isolated clones had DNA sequence similarity to the NBSs of known resistance genes. The maximum number of clones were obtained using the primers designed by Shen et al. (1998). The DNA sequence homology of the different families of RGC sequences was classified at 85% and 60% levels of DNA sequence similarity. Sequences with greater than 60% similarity were considered to belong to the same family. Based on similarity in nucleotide sequence these resistance-gene candidate clones could be put into 14 classes or families of RGCs, 8 in pigeonpea (RGCPP) and 6 in chickpea (RGCCP) (Table 1). Comparison of the deduced amino acid sequences of the RGC sequences to products of known resistance genes revealed that the RGC sequences are as similar to each other as they are to resistance

Table 1. Resistance-gene candidates (RGCs) isolated from chickpea and pigeonpea.

	Number of RGCs isolated[1]	
Primer combination	Chickpea	Pigeonpea
GLPL3-AA[2]	5/20	-
GLPL3-AG[2]	-	-
GLPL4-AG[2]	-	1/6
GLPL4-GA[2]	1/8	-
s1-as1[3]	-	4/5
s2-as3[3]	-	2/8
LM 637-LM 638[4]	-	1/1

1. No. of families based on less than 60% DNA sequence homology per total number of RGC clones isolated.
2. Shen et al. 1998.
3. Leister et al. 1996.
4. Kanazin et al. 1996.

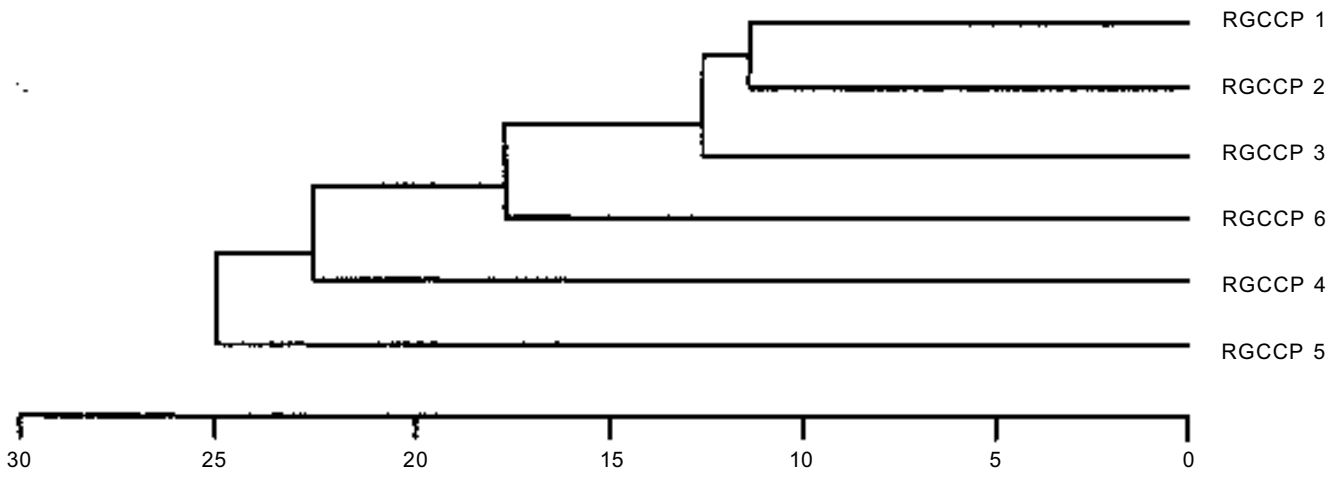

Figure 1. Phlyogenetic relationship of RGCCP genes based on the amino-acid sequence.

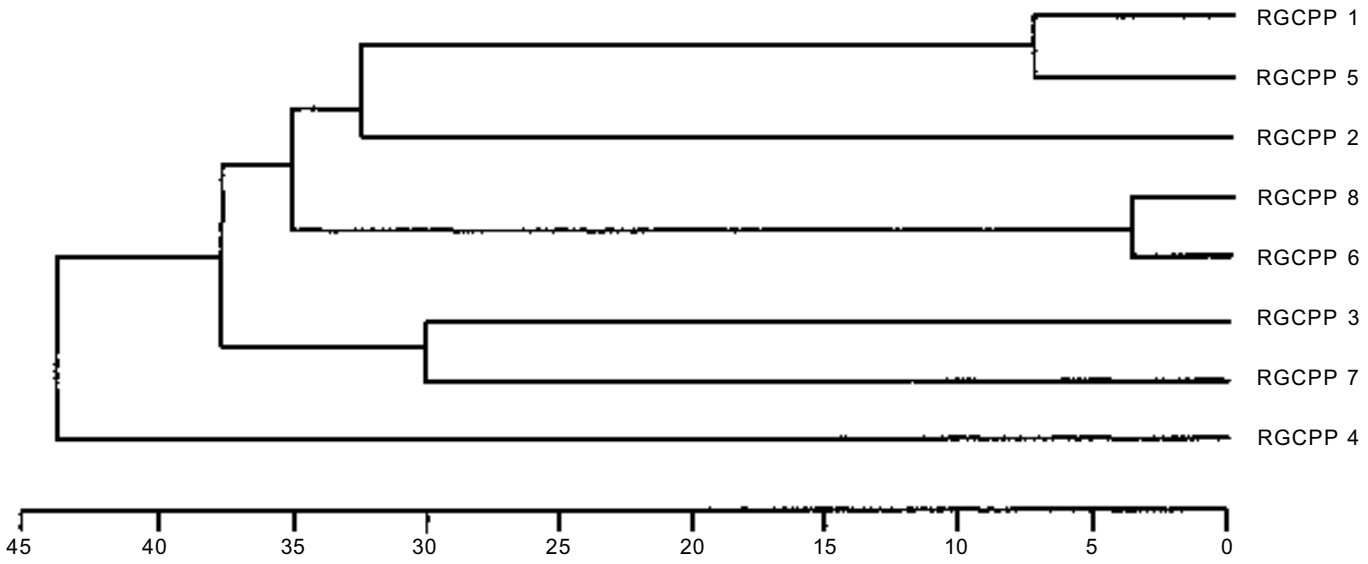

Figure 2. Phylogenetic relationship of RGCPP genes based on the amino-acid sequence.

genes from other species. A phylogenetic tree constructed based on the amino acid sequence revealed the homology among the different RGCs (Figs. 1 and 2). In both chickpea and pigeonpea the majority of the RGCs showed higher homology to *N* and *L6* resistance genes than *RPS2* and *RPM* (data not shown). In pigeonpea, one of the RGCs (*RGCPP5)* showed high homology to *Prf* from tomato. Two of the RGCs (*RGCPP4* and *RGCPP8)* showed both amino acid and nucleotide sequences quite different from the others. All the RGCs isolated from chickpea gave only monomorphic bands when hybridized with DNA from 10 of the elite cultivars on a Southern blot with *Eco*RI (data not shown). Definite proof that these sequences are resistance genes requires the isolation of full-length resistance gene clones and transgenic complementation. These also can be mapped to determine their relative position but at present no linkage map is available for these two legumes. With the availability of NILs or RILs differing for disease resistance the utility of these clones as markers for resistance will be revealed.

This PCR approach with degenerate oligonucleotide primers has great potential to amplify numerous resistance genes from diverse species. With the isolation of more

resistance genes, it is becoming possible to design primers that will be highly selective in amplifying resistance genes.

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Improved Efficiency in Chickpea Tissue Culture: Effects of Presoaking and Age of Explants on In Vitro Shoot Proliferation

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Genetic transformation and creation of elite germplasm require an efficient plant regeneration system. Chickpea (*Cicer arietinum* L.), a major grain legume, is susceptible to virulent strains of *Agrobacterium* (Mohapatra and Sharma 1991; Islam et ah 1994), but the lack of high-frequency regeneration has deterred the production of transgenic plants. In-vitro regeneration of plantlets from shoot meristems, immature cotyledons, and leaflet-derived callus has been reported in chickpea (Suhasini et al. 1994). We have found that benzyladenine (BA) with indoleacetic acid (IAA) or naphthaleneacetic acid (NAA) are effective growth regulators for shoot organogenesis from hypocotyl explants (Islam et al. 1994). However, only 6-8 shoots were obtained for each regenerating explant. This report describes the benefits of presoaking the hypocotyl explants in liquid B5 basal medium containing 5-15 µM BA for 24-60 h duration on their regenerative ability.

Table 1. Main effects of various treatments on percent cultures showing regeneration and average number of shoots per regenerating explant.[1]

Treatment	Cultures showing shoot formation (%)	Average number of shoot per explant
Effect of BA concentrations[2]		
BA 5 µM	60 a[5]	6.3 c
BA 10 µM	79 b	14.7 a
BA 15 µM	81 b	10.6 b
Effect of presoaking[3]		
Unsoaked	55 b	6.4 b
24 h soaking	60 b	6.8 b
36 h soaking	58 b	7.7 b
48 h soaking	85 a	12.8 a
60 h soaking	80 a	13.2
Effect of age of explants[4]		
7-day-old	85 a	22.0 a
14-day-old	75 b	6.8 b
21-day-old	60 c	2.8 c

1. The experiment was repeated twice and each treatment consisted of 12-15 explants.
2. Values for BA (benzyladenine) are the means of two sets of experiments, five presoaking periods and three ages of explants.
3. Values for presoaking are the means of two sets of experiments, three BA concentrations and three ages of explants.
4. Values for age of explants are the means of two sets of experiments, three BA concentrations and five presoaking periods.
5. Mean separation in groups within columns by Duncan's Multiple Range Test, 5% level.