LIST OF PUBLICATIONS


- Pranjan Barman, A.K. Handique, Rajiv Chandra Dev Goswami, Ellora Malakar, P.J. Handique and M.C. Kalita. Application of advanced instruments in Molecular Biology (June, 2011). *JASS* Vol. 52 No. 1,


- Pranjan Barman, A.K. Handique and Bhaben Tanti. Tagging SSR based Molecular Markers to Race-1 resistance to Fusarium Wilt (Caused by *Fusarium oxysporium* f. sp. *Ciceris*) in Chickpea (*Cicer arietinum* L.) (Communicated to Journal of General Plant Pathology-Springer)
Genomics approach towards cloning of R-gene for Fusarium wilt in chickpea (Cicer arrietinum L.)

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Domesticated chickpea (Cicer arrietinum L.) is the third most important grain legume (pulse) crop after bean and pea. It is produced worldwide in more than 50 countries and a good source of dietary protein and carbohydrates. Chickpea is also an important crop used for fixing atmospheric oxygen into soil and reducing pathogen inoculum for subsequent cereal crop cultivation.

Despite its agronomical importance and international efforts, productivity of the crop has not yet been significantly improved. The major difficulty in increasing yield is susceptibility to diseases viz. a foliar disease, ascochyta blight caused by Ascochyta rabiei and the vascular disease, Fusarium wilt, caused by Fusarium oxysporum f. sp. Ciceris. Yield losses due to Fusarium wilt is estimated to be 10-90% annually. Persistence of pathogen in soil and capacity to survive without even presence of host for years renders its control difficult. Soil applications of fungicides are costly and lead to indiscriminate killing of beneficial micro-flora. The disease in some extent can be managed by use of bio-control agents. However, the most efficient and eco-friendly means of controlling the disease is obtained by race-specific vertical resistance genes in the host. With the development of molecular marker system, genes involved are being tagged with closely linked markers. Markers assisted breeding and gene pyramiding leading to the development of multi-race resistant lines and cultivars. Establishment of lines with resistance to multiple strains of the pathogen as well as maintaining market qualities is of utmost importance for improving chickpea yield.

Disease resistance is currently a primary objective of most plant breeding programs. Until recently measures to control these diseases have relied on identification of resistant germplasm and development of resistant varieties through screening in controlled environment. New inventions in the molecular marker technology, emergence of genomics, proteomics and transcriptomics and the establishment of model legume crops such as Medicago truncatula, Lotus japonicus etc. have enhanced knowledge towards understanding molecular genetic basis of stress resistance and R-gene expression and manipulation.

Physical mapping is fundamental to any progress towards a more complete understanding of the structure, composition and function of genomes. Isolation of genes of agronomical importance (e.g., genes coding for receptor kinases, genes for signal transduction, transcription factors, regulatory elements, small regulatory RNAs or genes of defense pathways) inevitably necessitates a physical map. An elaborate genetic map is the first step in achieving this goal. In essence, the era of physical mapping in chickpea is beginning now. It will and has to be succeeded by an era of DNA sequence analysis. Never the less, the first few steps have already been made.

Here, we report tagging of race 1 resistance (rac 1) for chickpea in an intra-specific cross, WR315 (resistant cultivar) x C104 (late wilting cultivar) with SSR based STMS markers. A linkage map was generated using the scored STMS markers on 82 RILs of the cross along with the disease scores for reaction to the race 1 of Fusarium (for 1). The most closely linked marker, TA37 was identified at a distance of 0.2 cM from fin 1 in linkage group 2 (LG 2) which corresponds to LG 2 of Medicago truncatula.
This is to certify that Proj. Prasun Bose of Dept. of Biotechnology, Gauhati University participated and delivered invited talk / made oral / poster presentation in the National Symposium on "Biology of Infection, Immunity and Disease Control in Pathogen-Plant Interactions" in the 64th Annual Meeting of Indian Phytopathological Society organized during December 2-4, 2011 by the Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad - 500 046, India.

Prof. Appa Rao Podile
Organizing Secretary
In-silico determination of homolog of Melon R-gene R-FOM-2 in chickpea (Cicer arietinum L.) giving resistance against Fusarium wilt caused by the fungal pathogen Fusarium oxysporum f. sp. Ciceris.

ABSTRACT:

Fusarium wilt in chickpea and in Melon is caused by similar fungal pathogens. They are formae specialis of Fusarium oxysporum (f. sp. Ciceris in chickpea and f. sp. Melonis in melon). Resistance mechanisms in both the cases are thought to be similar as well as with the involvement of Avr (avirulence) and R-genes (resistance genes) interactions. R-genes for resistance against Fusarium wilt in melon has already been cloned and characterized to be a NBS-LRR kind of R gene. Most R-genes cloned are found to be of this class with a central Nucleotide Binding Site (NBS) domain, Leucine rich repeat (LRR) domain at the C-terminal end and a variable domain at the N-terminal end. Using R-FOM-2 as a reference sequence, databases were searched for putative chickpea sequence giving resistance to Fusarium wilt. Chickpea sequences were aligned with other NBS-LRR kind of genes using multiple sequence alignment tools and their domain structure was studied using domain search tools. Legume specific EST databases were searched to find out functional chickpea sequence against Fusarium wilt. A Phylogenetic tree was also constructed to see the relationship of chickpea sequences with other cloned NBS-LRR kind of genes.

Keywords: Chickpea, R-gene, NBS-LRR, R-FOM-2, BLAST, Multiple Sequence Alignment, ClustalW.
INTRODUCTION

Many commercial varieties of chickpea suffer from diseases such as Fusarium wilt, Ascochyta blight, Root rot, Botritis Grey Mould and soon resulting in yield losses (Kottapalli et al, 2009). Soil borne fungal pathogen Fusarium oxysporum cause wide spread vascular wilt in diverse species including legumes. It enters through the roots, colonizes the vascular tissue causing the blocking of water movement resulting in wilting of plants (Ratnaparkhe et al, 1998). Plants possess various mechanisms to counter pathogens growth and colonization. One of such mechanisms is achieved through R-genes in the hosts (Gachomo et al, 2003; Mishra et al, 2010). Increasing evidence suggest that plant defense mechanisms is as complex as the vertebrate immune system. But plants do not have a circulatory system and therefore cannot rely on specialized system. In plants each cell has to be equipped with the defense system. Plant R-genes are the appropriate answer to this. (Michelmore et al, 1998).

Phytopathogens enter into the plant parts and proliferate by assimilating nutrients from the host tissues. Specific defense mechanisms are employed by the plant parts to resist such invasion. The plant requires resistance genes (R-genes) which function in a cascade to prevent invasion and colonization. Various R-genes have been reported from different plant species for defense against invading plant pathogens (Hammond-Kosack et al, 2007; Michelmore et al, 1998). Upon infection, the pathogen produces some effector molecules (proteins) which are either directly recognized by host R-proteins or by other host protein guarded by R-proteins. Subsequently, various defense signaling networks are activated by R-protein phosphorylation, oligomerisation, and degradation or by conformational changes. The overall outcome of this process is the activation of a coordinated defense response against the invading plant pathogens (Hammond-Kosack et al, 2007).

Approximately, 40 R-genes have been cloned in different plant species (Hammond-Kosack et al, 2007), which confer resistance against bacterial and fungal pathogens. Out of 40 cloned R-genes, only 4 have been recessive in nature. The functioning of dominantly inherited R-genes immerging day by day but mechanism involved in recessive R-gene functioning is lacking (Hammond-Kosack et al, 2007). Most R-proteins contain a central Nuclotide Binding Site (NBS) domain, a Leucine Rich Repeat (LRR) domain and a variable domain at the amino terminal end.

Resistance genes are cloned in many other crops including model plant species such as Arabidopsis, Medicago, Tomato, Wheat, Flax (Gachomo et al, 2003, Peraza-Echeverria et al, 2009) and Melon (Joobeur et al, 2004). Plant resistance genes are known to be conserved throughout the evolution. Resistance genes cloned in other crops are now providing us with important information. These informations available in the public databases could be availed for finding homologues sequences in chickpea. From the conserved domains, Resistance gene Analogues (RGAs) based markers have been designed and mapped in genetic maps. RGAs have been reported from various crops such as maize, wheat, rice, potato, soybean, lettuce, apricot, cotton, oat and Arabidopsis (Kumar et al, 2007). Furthermore, from these homologous sequences R-genes from other crops could also be cloned. Here in this study, the information regarding resistance against Fusarium from other plants are being utilized to study chickpea sequences and predict a homolog of Fusarium wilt R-gene from melon (R-FOM-2) in chickpea. The predicted protein in chickpea is named as R-FOC-PB.

METHODS:

Sequence retrieval:

The complete nucleotide sequence for R-FOM-2 as well as the protein sequence was downloaded from Plant Resistance Gene database. Taking R-FOM-2 protein sequences as quarry, other NBS-LRR protein sequences were downloaded from Plant Resistance Gene database, NCBI, and many
other publicly available sequence databases by BLAST (Altschul et al., 1997). A list of sequence databases used for downloading the sequences is given in Table-1. Chickpea EST database at the Cool Season Legume Database was searched for putative chickpea sequences.

**Searching chickpea EST databases:**

Taking R-FOM-2 protein sequence as quarry, Cool Season Food Legume Genome database at http://www.gabcsfl.org/tools/blast was searched using BLASTn program for finding Chickpea EST sequences having homology with R-FOM-2. Best 10 chickpea hits are listed in Table-2. The selected EST sequences were checked manually from their GenBank entries. One single EST sequence was selected and was used for further studies. The EST sequence is mentioned in the results section of the paper.

**Translating EST sequence to Protein:**

The best EST sequence was then converted to a protein sequence using “Traslate” module at http://web.expasy.org/translate/. Default parameters were used for translating the EST sequence into 6 frame protein sequences. Each frame was checked for start and Stop codons and the protein sequence was selected from the best possible frame. The converted peptide (R-FOC-PB) sequence is mentioned in the results section as well.

**Pfam analysis:**

The predicted protein R-FOC-PB was subjected to protein motif/domain search at http://pfam.janelia.org/. SWISS-MODEL Workspace protein modeling software was used for predicting the structure of the peptide and obtaining other characteristics of the peptide. “ProtParam” module was used to get other parameters of the predicted peptide like the molecular weight, amino acid composition etc.

**Domain and Motif search:**

The converted protein sequence was searched for domains and motifs present in the sequence using pfam HMM module online at http://pfam.janelia.org/

**Multiple Sequence Alignment:**

A multiple sequence alignment of the NBS-LRR sequences (listed in the Table-1) along with the chickpea putative sequence (obtained from this study) was conducted using ClustalX version 2.1 software. The program was downloaded from the web and run locally using a LINUX pc. The output files were generated in three different forms viz. an Alignment file (.ANL file), a FASTA file and a PHYLIP (.PHY file) input file.

**Phylogenetic Analysis:**

A phylogenetic tree was generated using ClustalW program with default parameters. The downloaded NBS-LRR type of proteins from different plant species along with the predicted peptide from the present study was considered. The tree was viewed by TreeView X program.

**RESULTS**

From the chickpea EST database searches

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Name of the database</th>
<th>Website/ftp</th>
</tr>
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<td>1</td>
<td>Plant Resistance Gene Database</td>
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</tr>
<tr>
<td>3</td>
<td>Bioinformatics resources for legume researchers</td>
<td><a href="http://legumes.org/">http://legumes.org/</a></td>
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<tr>
<td>4</td>
<td>Medicago truncatula</td>
<td><a href="http://www.medicago.org/index.php">http://www.medicago.org/index.php</a></td>
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<tr>
<td>5</td>
<td>TIGR Plant Transcript Assemblies</td>
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</tr>
<tr>
<td>7</td>
<td>The Arabidopsis Information Resource (TAIR)</td>
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<tr>
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<td>Legume Information System</td>
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<tr>
<td>11</td>
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<tr>
<td>12</td>
<td>Arabidopsis Resistance Genes</td>
<td>http:// nbrs.ucdavis.edu/At_RGenes/</td>
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</tbody>
</table>

Table-1 List of databases searched for obtaining the NBS-LRR protein sequences. These databases were searched by taking R-FOM-2 protein sequence as quarry.
around 40 EST sequences were found to be homologous to the melon R-gene R-FOM-2. A single EST sequence with GenBank ID GR916471.1 was selected based on GenBank characteristics. The sequence had a homology with 37% identity to the melon protein R-FOM-2. The EST sequence is given below:

The translated product was found to be of 97 amino acids and is named as R-FOC-PB. The ProtParam output shows that the molecular weight of the predicted protein is 11100.8 and a theoretical pI at 10.15. The total number of negatively charged residues are found to be (Asp + Glu) 8 and total number of positively charged residues were found to be (Arg + Lys) 13. The predicted peptide sequence is given below:

> R-FOC-PB
MGRNPKIWNPTLFSPERFLGSEINFKGQNFQLTFPGESGRICPGMLAIRMLHTMGSLINSFDWKLESGDRDIDQLRAIPFRVVKYLYLDHAN

The 3D structure predicted by the SWISS-MODEL Workspace is shown in the Figure-1 and the other parameters of the predicted structure are shown in the Figure-2 and Figure-3. HMM pfam protein domain structure prediction shows a single conserved domain at the C-terminal end with similarity to p560 protein. The predicted domain structure is shown in the Figure-4. The multiple sequence alignment showed that the predicted peptide (R-FOC-PB) has some conserved region with various R-proteins of NBS-LRR kind. The clustalW output is shown in Figure-5.

The phylogenetic analysis of the predicted protein with other NBS-LRR proteins showed that the predicted protein from our study is associated with the disease responsive element RPM1 from Arabidopsis thaliana and disease resistance gene...
DISCUSSION

By evolution plants have acquired resistance genes (R-genes) that generally belong to multi-gene families. These genes respond to various elicitors in different mechanisms. By using conserved domains from cloned genes, several R-gene analogous in different plant species have been designed and mapped. Our effort in the present study was to use R-genes for *Fusarium oxysporum* infestation in other plant species to find putative homolog in chickpea. We could found out from chickpea ESTs a sequence homologous to R-FOM-2 from Melon in chickpea.

The predicted protein (R-FOC) shows a conserved domain of cytochrome p560 protein. At the same time the predicted protein has shown closeness to disease responsive element RPM1 of Arabidopsis and disease resistance gene homolog 1A from *Brassica napus* as evident from the phylogenetic tree (Figure-6). A similar study was conducted by Ashokan et al., 2011. From the results, it might be confirmed that domain p560 is also present in R-gene predicted in this study. The complete protein structure determination will provide us with more details.

![Figure-2 The SWISS-MODEL Workspace parameters of the predicted protein R-FOC-PB phylogenetic tree (Figure-6).](image)

![Figure-3 The SWISS-MODEL Workspace parameters of the predicted protein R-FOC-PB](image)

![Figure-4 The domain structure of the predicted protein as is obtained from pfam HMM](image)
In a similar study by Liu et al. 2006 used computational methods to predict pathogenesis-related proteins (PRs) type proteins in rice. They also determined the putative protein in rice using PR1 sequence. They 3D structure was also conserved. Knowing the gene structure and the complete evidence about the gene structure and the gene in the chromosome which will give us the resistance against Fusarium wilt.

For better understanding and for obtaining multiple resistances against pathogens, R-genes related to the resistance against pathogens. R-genes could also determine the putative protein in the region shown here is the part of the alignment showing significant homology of the predicted protein with other NBS-LRR Proteins.

Figure 5: The multiple sequence alignment. The region shown here is the part of the alignment showing significant homology of the predicted protein with other NBS-LRR Proteins.
functional copy becomes difficult. In silico studies may help in actually finding the functional copies and enhance their introgression into other cultivars. R-genes can be transformed to commercially suitable varieties to obtain resistance against diseases. The application of the present study will be to clone the full length cDNA of the genes for the gene giving resistance against Fusarium and transform it into commercial variety.

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