ANALYSIS OF RESISTANT GENE ANALOGUES IN MUNGBEAN \([Vigna radiata \text{ (L.)} \text{ Wilczek}]\) AND RICEBEAN \([Vigna umbellata \text{ (Thunb.)} \text{ Ohwi and Ohashi}]\)

By

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Abstract:

Pulses are important food crops due to their high protein and essential amino acid content. In addition, they are also used as animal feed. Pulses play a key role in crop rotation due to their ability to fix atmospheric nitrogen. Mungbean (*Vigna radiata* (L.) Wilczek) is one of the major pulse crops which is being grown since ancient times for its use as dry bean or fresh sprout, as a green manure crop and use of its haystacks as forage for livestock. *Mungbean Yellow Mosaic Virus* (MYMV), a whitefly-transmitted geminivirus is one of the major diseases affecting blackgram, mungbean and soybean. Manipulation of host-plant resistance is an efficient method of controlling this devastating disease (Gupta *et al.*, 2005). Wild relatives of mungbean are important sources of resistance for many biotic and abiotic stresses including MYMV. Ricebean [*Vigna umbellata* (Thunb.) Ohwi and Ohashi] a wild relative of mungbean belonging to the subgenus *Ceratotropis* in the genus *Vigna* has been reported to possess resistance against MYMV (Monika *et al*., 2001). In this study, an attempt was made to understand the genetic difference between mungbean and ricebean for the presence/absence and expression pattern of the homologue of a resistant gene namely *N*-gene of tobacco. Nucleotide and protein sequence of *N*-gene of tobacco and protein sequences similar to *N*-gene, reported from various crops were retrieved from NCBI-Genbank database. Conserved regions were predicted by multiple sequence alignment and degenerate primers were designed to amplify the homologue of *N*-gene of tobacco in mungbean and ricebean. PCR analysis revealed the presence of *N*-gene homologue in all the accessions of both mungbean and ricebean. Agroinoculation studies confirmed the resistance of ricebean accession TNAU-red and susceptibility of mungbean variety VRM 1 against MYMV. Semi-quantitative RT-PCR analysis revealed the down-regulation of the *N*-gene homologue in mungbean and up-regulation in ricebean upon MYMV infection. This differential expression of the homologue of *N*-gene of tobacco upon MYMV infection may play a crucial role in conferring resistance against MYMV.

INTRODUCTION

Pulses, due to their high protein value are sometimes referred to as the poor man's meat and rich man's vegetable. They contain certain essential amino acids like lysine and tryptophan which are not present in cereals. Mungbean [*Vigna radiata* (L.) Wilczek], also known as green gram, green bean, moong, mash bean, golden gram and green soy is an excellent source of easily digestible proteins.
with low flatulence which complements the staple rice diet in Asia (Khattak et al., 2002). Yellow mosaic disease (YMD) is one of the major constraints limiting mungbean productivity in South-East Asia. Introduction of MYMV-resistant mungbean may be helpful in reducing environmental hazards resulting due to excessive use of pesticides. Wild relatives of mungbean are suggested to possess resistance against MYMV, drought, salinity and temperature stresses. Ricebean [Vigna umbellata (Thumb.) Ohwi and Ohashi], a wild relative of mungbean belonging to the subgenus Ceratotropis in the genus Vigna (Ohashi et al., 1988) has shown its resistance against MYMV under field conditions (Monika et al., 2001).

Plant disease resistance genes (R genes) and Resistant Gene Analogs (RGAs) are directly involved in plant defense mechanism. Resistant Gene Analogues (RGAs) are potential gene fragments that contain structural motifs, such as the NBS and LRR regions that are common to most cloned R genes (Hulbert et al., 2001). They may function in resistance against similar or different pathogens, or other function(s) unrelated to resistance. RGAs have the potential to serve as closely linked markers for marker assisted breeding or even as resistant gene candidates (Hammond and Jones, 2000; Taler et al., 2004). Conservation of some protein motifs among disease resistance genes of different plant species provides a method for isolating additional homologous disease resistance genes in these or other species.

Through PCR based amplification using degenerate primers designed from conserved motifs in the NBS domain, several disease resistance gene homologues have been cloned in monocot and dicot species (Leister et al., 1996; Leister et al., 1998; Kanazin et al., 1996; Yu et al., 1996). Over forty R genes from different plant species (E.g., flax, Arabidopsis, tomato, potato, tobacco, rice maize, pepper, barley, lettuce) involved in specific resistance to different types of pathogens (viruses, bacteria, fungi, insects, nematodes, oomycetes) have been isolated to-date (Martin et al., 2003). The NBS domain is believed to participate in signal transduction, while the LRR domain is thought to be involved in ligand binding and pathogen recognition (Young, 2000).

Among the R-genes, N-gene of tobacco confers resistance to Tobacco Mosaic Virus (Whitham et al., 1994). The N-gene protein has three signature motifs common to many other plant R genes (TIR-NBD-LRR) (Fluhr, 2001). The interaction between the resistance gene N of tobacco and Tobacco Mosaic Virus (TMV) has long served as a classical model for understanding the host plant's response against pathogens (Dinesh kumar et al., 1995). Many of the identified
RGAs are probably pseudogenes or non-functional genes (Pan et al., 2000). This situation warrants confirmation of expression of RGAs in the host plant and its response against pathogen. Based on these facts, the present study was undertaken with the following objectives:

1. Identifying the conserved domain(s) in \( N \)-gene of tobacco using bioinformatics tools.
2. Designing degenerate primers to amplify the homologue of \( N \)-gene of tobacco in mungbean and ricebean.
3. PCR and RT-PCR analysis, to study the relationship between tobacco \( N \)-gene homologue in mungbean and ricebean germplasm lines and MYMV resistance.

**REVIEW OF LITERATURE**

2.1. Pulses and Mungbean

India is the world's largest pulse producer accounting for 27-28 per cent of global pulse production. A report in the commodity online 2007, states that India harvests between 12- 15 million tonnes of pulses each year but the yield has been pretty much static for the last 30 years averaging between 500-600 lb/ acre. A sizeable population in the country still depends on vegetarian diets to meet its protein requirement. Mungbean, \((Vigna radiata \text{ (L.) wilczek})\) also known as greengram, green bean, moong, mash bean, golden gram, and green soy is one of the important legume crops grown in India. It also plays an important role in sustaining soil fertility by improving soil physical properties and fixing atmospheric nitrogen. It is a drought resistant crop and suitable for dryland farming and predominantly used as an intercrop with other crops. Third Advance Estimates of green gram (2007-2008) by Agricultural statistics division, Government of India (Economic Survey) projected 1.56 million tonnes production in the current year.

2.1.1. Viruses as a major constrain in pulse production

One of the major constrains in pulse production are pathogens and viruses are among the most important groups of plant pathogens affecting pulse production worldwide. Plant viral diseases inflict serious economic losses in major crops by reducing yield and compromising quality (Kang et al., 2005).
Mungbean Yellow Mosaic India Virus (MYMIV) (Mandal et al., 1997) and Mungbean Yellow Mosaic Virus (MYMV) (Morinaga et al., 1990) were suggested to be associated in the etiology of Yellow Mosaic Diseases (YMD) of legumes in India and South Asia. MYMV and MYMIV occur across the Indian subcontinent. MYMV affect the majority of legume crops including mungbean (Vigna radiata), black gram (Vigna mungo), pigeonpea (Cajanus cajan), soybean (Glycine max), mothbean (Vigna aconitifolia) and common bean (Phaseolus vulgaris) (Javaria et al., 2007).

Evolution of new viruses through recombination challenges the efforts to confer resistance to these pathogens. Recombination can provide selective advantage in the evolution of viruses within strains, species, genera and family (Keese and Gibbs 1993; Holland, 1998). It has now been accepted that recombination contributed to the diversity of Gemini viruses and therefore, to the emergence of new variants and species reported worldwide. In India, recombination between strains of Tomato leaf curl virus has been detected from Bangalore (Kirthi et al., 2002) and analysed the recombination events (Girish and Usha, 2005).

2.1.2. Importance of wild relative species in pulse breeding.

Intensive modern breeding has contributed to a narrowing of crop gene pools as a few improved cultivars dominate large areas (Ladizinsky, 1985). The wild relatives of crop species are sources of important gene(s) for agriculture and genetic diversity measured at the biochemical and DNA level is generally greater in wild species than their related cultigen (Xu et al., 2000).

2.1.3. Ricebean

Ricebean (V. umbellata (Thunb.) Ohwi and Ohashi) is an annual food legume belonging to the subgenus Ceratotropis in the genus Vigna. It is occasionally cultivated in different parts of South-east and East Asia. It is a small, hairy annual vine which bears yellow flowers and small edible beans. It grows prolifically and is a minor food (for its green pods, seeds) and forage crop and occasionally a weed (Somta et al., 2006).
2.1.4. Importance of rice bean in MYMV resistance

In an attempt to transfer *Mung Bean Yellow Mosaic Virus* (MYMV) resistance from ricebean to mungbean, interspecific hybrids were produced from ricebean and mungbean. The hybrids exhibited twining habit like ricebean, possessed intermediate leaves and pods and were resistant to MYMV. In respect of yield components, they were quite promising (Monika et al., 2001).

2.2. Yellow Vein Mosaic Disease (YMD)

The viruses causing yellow mosaic diseases of legumes across southern Asia, four of which have been identified so far, are bipartite begomoviruses (Genus: Begomovirus, family: Geminiviridae). They are *Mungbean Yellow Mosaic Virus, Mungbean Yellow Mosaic India Virus, Horsegram Yellow Mosaic Virus and Dolichos Yellow Mosaic Virus* (Javaria et al., 2007). The genome of bipartite begomoviruses is composed of DNA A, which encodes proteins required for replication, transcription and encapsidation, and DNA B, which encodes proteins required for movement functions. Both components are organized into divergent transcription units separated by an intergenic region (IR) that contains sequences conserved between the two DNA components and are referred to as the common region (CR) (Girish and Usha, 2005).

2.2.1. Mung Bean Yellow Mosaic disease

Among the white fly transmitted Gemini viruses or Begomo viruses, *Mungbean Yellow Mosaic Virus* (MYMV) is important as it infects five major leguminous species, Black gram, Mungbean, French bean, Pigeon pea and Soybean, causing an annual loss of yield of about $300 million (Varma et al., 1992). It produces typical yellow mosaic symptoms and is easily transmitted by the whitefly vector, *Bemisia tabaci* (Nene., 1973).

2.2.2. Symptoms

In black gram and mungbean, faint yellow specks or spots and irregular blotching of tip leaves; slight leaf malformation (Nariani, 1960). In black gram, MYMV causes irregular yellow green patches on older leaves and completes yellowing of young leaves of susceptible varieties. In black gram, there are two types of yellow mosaic symptoms ‘yellow mottle’ and ‘necrotic mottle’ that can be distinguished (Nair and Nene, 1974).
Other crops, including cowpea, develop similar symptoms as a result of infection with MYMV. Plants exhibiting necrotic mottle symptoms are not as severely affected as the plants exhibiting typical yellow mosaic; this suggests that the necrotic mottle is a type of resistant reaction to the disease (Biswas and Varma, 2001).

2.3. Mechanisms of virus resistance

The mechanisms of virus resistance include, cellular resistance, Coat Protein Mediated Resistance (CPMR), Replicase mediated resistance, Movement protein mediated resistance, Satellite RNA, Post Trancriptional Gene Silencing and Plant disease resistance genes.

Resistance at the single cell level may be characterized as a state where virus replication does not occur, or occurs at essentially undetectable levels in inoculated cells. This type of resistance has been termed “extreme resistance” (ER), “cellular resistance,” or “immunity” (Fraser, 1990; Fraser, 1986). CPMR results from the propensity of the transgenically expressed coat protein to form aggregates (Dasgupta et al., 2003). Several important crops have been engineered for virus resistance using CPMR approach and released for commercial cultivation. Replicase (Rep) protein-mediated resistance against a virus in transgenic plants was first shown in tobacco against TMV in plants containing the 54 kDa putative Rep gene (Golemboski et al., 1990). The resistance generated by the use of Rep sequences is very tight; a high dosage of input virus can be resisted easily by the transgenic plant. Movement proteins (MP) are essential for cell-to-cell movement of plant viruses. Resistance conferred by transgenic expression of a dysfunctional TMV MP is likely due to competition for plasmodesmatal binding sites between the mutant MP and the wild-type MP of the inoculated virus. The above resistance was moreover seen to be effective against distantly related or unrelated viruses (Cooper et al., 1995). The mechanism behind sat-RNA mediated resistance may be attributed to the reduction in accumulation of the helper virus and its long distance movement and down-regulation of replication. However, as sat-RNA spreads epidemically, sufficient caution will have to be exercised in adopting this technology (Dasgupta et al., 2003). In Post Trancriptional Gene Silencing, a complex of cellular factors, namely RNA-dependent RNA polymerase (RdRp) (Mourrain et al., 2000), RNA-helicase (Dalmay et al., 2001), translation elongation factor (Zou et al., 1998), RNAse (Ketting et al., 1999) etc along with the small 21–25 nucleotide RNA (of the elicitor RNA) acting as the guide RNA (Hammond et al., 2001), supposedly degrade viral RNA molecules bearing homology with the elicitor RNA. This degradation process,
initiating from a concerned cell having the elicitor RNA, spreads later within the entire organism in a systemic fashion. The mechanism of resistance offered by plant disease resistant genes are described below in detail.

2.4. Plant disease resistant genes (R genes) and resistant gene analogues (RGAs)

Resistant genes (R genes) in plants are defined by the classical gene-for-gene hypothesis (Flor, 1971), which states that for every incompatible host pathogen interaction, there exist matching R genes in the host and Avr genes in the pathogen. The interaction between R genes and Avr genes, in many cases, results in a resistance reaction, known as Hypersensitive Reaction (HR), which can be defined as a specific response of a host towards a pathogen. HR results in localized cell death, appearing as necrotic lesions at the site of pathogen entry. HR results in the arrest of pathogen spread, thereby effectively restricting it to the dead cells (Dasgupta et al., 2003). These local responses can in turn trigger long lasting systemic response (Systemic Acquired Resistance, SAR) that primes the plant for resistance against a broad spectrum of pathogens (Dong, 2001 and Metraux, 2001).

R genes contain several structural domains. They are Nucleotide Binding Site (NBS) domain, Leucine Rich Repeat (LRR) domain, Serine/Threonine Kinases (STK) domain, TIR domain, RPW8 domain and Coiled Coil (CC) domain etc. (Dangl and Jones, 2001).

Sequences with unknown function, but encoding these conserved domains have been defined as resistant gene analogues (RGAs). In other way, resistant gene analogues are potential gene fragments that contain structural motifs, such as the NBS and LRR regions that are common to most cloned R gens. They may function in resistance against similar or different pathogens, or other function(s) unrelated to resistance. The conserved motifs within plant NBS domains make it possible to use degenerate primers and PCR to isolate RGAs or resistant gene like (RGLs) sequences. RGAs have the potential to serve as closely linked markers for marker assisted breeding or even as resistant gene candidates (Hammond and Jones, 2000; Taler et al., 2004).

2.5. Classes of R genes.

R genes can be divided into five classes based on the common structural domains of the proteins they encode: proteins containing a Nucleotide Binding Site (NBS) and a Leucine Rich
Repeat (LRR) region, extracellular LRR proteins with a single TransMembrane (TM) domain and a short cytoplasmic domain, intracellular Serine/Threonine Kinases (STKs), extracellular LRR proteins with a single TM domain and an intracellular protein kinase domain, and RPW8 class with an amino terminal TM domain and an intracellular coiled coil (CC) domain (Dangl and Jones, 2001). The majority of functionally described R genes are the NBS-LRR type. The NBS domains are characteristic of various proteins with ATP/GTP binding activity and comprise the P-loop, kinase 2a, kinase 3a and GLPL motifs (Traut, 1994), while LRR domains play roles in the protein–protein interaction (Kobe and Deisenhofer, 1994).

2.5.1. Structural domains of resistant gene products.

2.5.1.1. Leucine Rich Repeat (LRR) domain

LRRs are multiple, serial repeats of a motif, 24 amino acids in length (Kobe and Deisenhofer, 1994). LRRs contain leucines or other hydrophobic residues at regular intervals and can also contain regularly spaced prolines and asparagines. Functional specificity in LRRs resides less in the conserved hydrophobic residues (which are oriented internally to provide the characteristic structure) than in the intervening, exposed amino acids. Many studies illustrate its importance in recognition specificity (Dodds et al., 2001). Some studies also revealed a signaling role (Hwang et al., 2000).

2.5.1.2. NBS domain

NBS domains occur in diverse proteins with ATP or GTP binding activity, such as ATP synthase p subunits, ras proteins, ribosomal elongation factors, and adenylate kinases (Saraste et al., 1990; Traut, 1994). Even more than LRRs, NBS domains in other proteins have been the subject of thorough structure-function analyses. The presence of the highly conserved NBS domain in some R gene products suggests that nucleotide triphosphate binding is essential for the functioning of these proteins. The NBS found in R proteins is part of a larger domain that has homologues regions to the Caenorhabditis elegans caspase regulator CED-4 and its human homologue APAF-1 both of which are proteins involved in apoptosis. This shared domain in APAF-1, R gene products and CED-4, known as NB-ARC domain, contains a kinase 1a (p-loop), kinase 2 and kinase 3a motifs, and five small motifs of unknown function. Thus the NB-ARC is a larger
domain in the NBS-LRR class of proteins comprising several conserved motifs (Van der Biezen and Jones, 1998b).

2.5.1.3. Serine Threonine Kinases

The function of STK domain in R genes is assigned to be phosphorylation. Both Pto domain in tomato (Sessa et al., 2000) and Xa21 domain in rice (Liu et al., 2002) are functional STKs. Pto interacts directly with Avr-Pto. But resistance is dependent on the presence of Prf (a CNL-type R protein) since Pto has no receptor domain.

2.5.1.4. TIR Domain

N and L6 genes form a second subgroup of NBS-LRR R genes in that they encode a large N-terminal domain with similarity to the cytoplasmic signaling domain of the Drosophila Tol1 protein and mammalian interleukin-1 receptors (IL-1R) (Whitham et al., 1994; Lawrence et al., 1995). The RPf5 R gene from Arabidopsis has also been cloned and placed in this class of R genes. This domain is said to function in signal transduction. But some studies suggest that it may also involve in pathogen recognition (Luck et al., 2000).

2.5.1.5. CC Domain

It comprises two to five α-helices that pack together to form a supercoiled helical bundle. The amino acid sequences of CC domains show heptad periodicity, i.e. every seventh residue occupies an equivalent position on the helix surface (Lupas, 1996). Leucine zippers constitute a subset of CCs in which there is a repeating pattern of leucine residues. The function of CC domain is not clear but it seems to be involved in signaling rather than recognition (Martin et al., 2003).

2.6. Origin and evolution of R genes

Several approaches may be taken while studying the evolution of R genes. R genes as members of a plant gene family and their evolution within this family, co-evolution of host and pathogen genes in R gene mediated resistance, and evolution of resistance/immunity-related genes from a common ancestor of plants and animals, as suggested by the homologous regions of R genes and members of animal innate immunity systems. These approaches are presented separately below, although they are often inter-related.
2.6.1. **R genes as members of a plant gene family**

Studies have focused on NBS-LRR family of proteins because it’s only known function is in disease resistance. Classification based on protein domains, intron positions, sequence conservation, and genome distribution has been used to define specific subgroups of CNL (CC-NBS-LRR) and TNL (TIR-NBS-LRR) proteins. Although the TNL family is nearly twice the size of the CNL family in *Arabidopsis*, the data suggest that TNLs are more homogeneous and have amplified more recently in this genome than have CNLs (Meyers *et al*., 2003). No TNL encoding genes have been identified in cereal genomes, although they are found in gymnosperms, suggesting that the grasses might have lost this type of gene (Bai *et al*., 2002; Zhou *et al*., 2004; Meyers *et al*., 2002). Intriguingly, the *Arabidopsis* R gene *RPP2A* has recently been demonstrated to encode a protein that is similar to a fusion of TN and TNL proteins (Sinapidou *et al*., 2004).

Genetically defined clusters of R genes are well known, and molecular studies have demonstrated that this clustering usually results from tandem duplications of paralogous sequences. Clusters of closely related and co-localized R genes frequently exchange sequences, but there is no evidence of sequence exchange among related genes that are located in separate clusters (Baumgarten *et al*., 2003). However, sequence analysis of *Arabidopsis* genome also suggests that some NBS-LRR genes have been translocated from their clusters to distal and probably random locations of the genome as a result of some small scale genomic duplications termed as “ectopic duplications” (Leister, 2004).

2.6.2. **Host-plant co-evolution**

Host-plant co-evolution is the widely accepted theory about the evolution of Avr genes in pathogens. It suggests that the Avr genes have initially evolved to function as virulence factors (Vivian and Gibbon, 1997). *R* genes have evolved as a plant surveillance system to recognize the virulent pathogens and induce host defense responses.

‘Arms race’ hypothesis suggests that plants and their pathogens continually improve the effectiveness of their defensive and offensive proteins to counteract the changes on their ligand (Holub., 2001). This hypothesis would have been adequate to describe the co evolution of *R* and Avr genes if recognition in *R* gene mediated resistance is a simple receptor ligand interaction
involving only one R protein and one Avr protein. However, plant pathogen interactions are much more complex involving the expression of various defense and disease related proteins by naturally variable host and pathogen populations.

The overall interaction of the defense and disease related proteins is the likely determinant of compatibility or on compatibility of hosts and pathogens. Such a complex interaction requires a high level polymorphism in pathogen Avr proteins and the host R proteins that recognize them. Sequence analysis and population studies suggest that balancing or frequency dependent selection maintains this high level of polymorphism at R gene loci (Meyers et al., 2005). Balancing selection occurs as the interplay of the increase in fitness of the host brought about by an R gene in the presence of the pathogen and fitness cost associated with that R gene in the absence of the pathogen (Tian et al., 2003).

The LRR domain which is found in most R proteins seems to be involved in ligand contact either directly or indirectly (Dangl and Jones, 2001) and many studies illustrate its importance in recognition specificity (Ellis et al., 1999; He et al., 2000; Dodds et al., 2001). Therefore, co-evolution studies usually concentrate on the LRR domain and its evolution to recognize the Avr proteins produced by the pathogens. The clustering of NBS-LRR genes creates a large pool of LRR domains for evolutionary selection to act on at the same time protecting the polymorphism at this domain.

### 2.6.3. Similarity to animal proteins

The NB-ARC domain found in all plant NBS-LRR proteins is structurally related to regulators of animal apoptosis, including human APAF-1 and nematode CED-4, this region functions as a module for protein-protein interactions (Van der Biezen and Jones, 1998b). In addition to the NB-ARC domain TNLs contain a TIR domain that is homologues to the signaling domain in the drosophila toll and mammalian Interleukin-I receptor proteins which is involved in protein-protein interaction involved in innate immunity.

Drosophila toll and mammalian Toll-like receptors (TLRs) contain an LRR domain in addition to the TIR domain. These innate immune receptors recognize conserved pathogen encoded structures with their extracellular LRR domain and couple to internal cell-death signals,
kinase cascades and transcriptionally-activated effector arms with their intracellular TIR domain (Aderem and Ulevitch, 2000). Human genome contains about 15-20 TLRs (Dangl and Jones, 2001).

Members of the mammalian immunity related NOD family (Inohara et al., 2002) are NBS-LRR proteins that also carry an amino terminal Caspase-Activating Recruitment Domain (CARD). These intracellular proteins recognize pathogen ligands and induce host defence responses in a similar manner to R proteins.

2.7. Resistant genes and defense signal transduction.

The studies that have been conducted to understand the recognition of R proteins and their effectors and how they are involved in signal transduction in disease resistance mechanism reveals that it is a slow process because plant cells have very slow abundance of R proteins, thus making the biochemical study of these proteins difficult (Martin et al., 2003).

2.7.1. Effector recognition

One mechanism for effector recognition is the receptor-ligand model, in which the R gene product interacts directly with the Avr protein to initiate host defense response. But a direct interaction with the Avr protein has not been demonstrated for most R proteins. So, it is more probable that the recognition takes place as a result of the formation of a complex containing both host and pathogen proteins.

‘Guard hypothesis’ is the most influential model for the indirect effector recognition of R proteins (Van der Biezen and Jones, 1998a). It suggests that the Avr protein of the pathogen (eg. AvrPto) interacts with a target plant protein (eg. Pto) to promote disease and that R protein (eg. Prf) recognizes the effector–target complex to induce host defense responses, thus guarding the plant against effector attacks. Following the guard hypothesis several mechanistic variations of this model have been proposed (Dangl and Jones, 2001; Mackey et al., 2002; Shao et al., 2002), all sharing the main concept that effector recognition by the R protein takes place indirectly as the R protein recognizes the interaction between the effector and the target of its virulence function. Although experimental evidence indicates that the guard hypothesis does not apply to Pto
mediated resistance (Bogdanove, 2002), this model or its variations may apply to other R protein dependent disease resistance mechanism in plants.

2.7.2. Signal transduction

The cellular processes following effector recognition in R gene-mediated resistance in several plants have some essential similarities (Piedras et al., 1998; Felix et al., 1999; Grant et al., 2000). Changes in ion flux, including calcium influx, occurs within minutes of pathogen attack, and then the production of Reactive Oxygen Intermediates (ROI s), including H$_2$O$_2$ and /or O$_2$ and the activation of mitogen activated protein kinases and other protein kinase pathways (Romeis et al., 1999). ROI s may take part in pathogen elimination, downstream signalling, or both. Nitric Oxide (NO) accumulation also reported in some studies (Delledonne et al., 1998).

Signaling molecules like protein kinases and transcription factors are expressed within fifteen minutes of pathogen attack. The new set of genes transcribed in response to pathogen makes up about 1% of the total mRNA in the plant cell (Durrant et al., 2000). In addition to the protein kinases, NO and ROIs may also contribute to the transcriptional activation of defense genes in the infected and neighboring cells. These defense genes are involved in salicylic acid biosynthesis, induction of ethylene biosynthesis, strengthening of the cell wall, production of various antimicrobial compounds and finally HR (Scheel, 1998). It is not yet clear which of these events in host pathogen response are mediated directly by the R protein.

2.8. Importance of N gene of tobacco

N-gene fall under the TIR-NBS-LRR class of R genes which are among the most extensively studied family of R genes. The majority of the R genes cloned and sequenced until now are part of the nucleotide binding site-leucine-rich repeat (NBS-LRR) gene family (Rommens and Kishore, 2000). The interaction between the resistance gene N of tobacco and Tobacco Mosaic Virus (TMV) has long served as a classical model for the study of plant-resistance responses to pathogens and provides an excellent system to isolate R genes and gain insight to its functions (Dineshkumar et al., 1995).
2.9. **PCR based identification of RGAs.**

Leister *et al* (1996) developed a PCR based method to easily isolate *R* gene analogues (RGAs), from a wide variety of plant species, in which they used degenerate primers that amplify between the kinase 1a motif of the NB-ARC domain and the GLPL motif that lies about 160 amino acids further downstream.

RGAs have been successfully isolated using the PCR based approach from a wide range of plants including Potato (Leister *et al*., 1996), Soybean (Kanazin *et al*., 1996), Arabidopsis (Speulman *et al*., 1998), Maize (Collins *et al*.,1998), Rice (Leister *et al*., 1998), Wheat and Barley (Seah *et al*., 1998), Tomato (Ohmori *et al*., 1998), Lettuce (Shen *et al*., 1998, Bean (Rivkin *et al*., 1999), Citrus (Deng *et al*., 2000), Coffee (Noir *et al*., 2001), Chick pea (Huettel *et al*., 2002), Barrel medic (Zhu *et al*.,2002), Grapevine (Di Gaspero *et al*., 2002), Peanut (Bertioli *et al*., 2003), Cotton (Tan *et al*., 2003), Cassava (Lopez *et al*., 2003), Pine( Liu and Ekramoddoullah, 2003), Strawberry (Martinez *et al*., 2004), Oat (Irigoyen *et al*., 2006), Buffalo grass and *Argostis* species (Budak *et al*., 2006a).

2.10 **Expression analysis and RT-PCR**

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) is a highly sensitive and specific method useful for the detection of rare transcripts or for the analysis of samples available in limiting amounts (Carding *et al*., 1992). By the RT-PCR method, many studies have been conducted for the expression analysis of disease resistant genes in ground nut (Coelho *et al*., 2007), rice (Bhatti *et al*., 2008), sunflower (Radwan *et al*., 2004) etc.

2.11. **Agroinoculation**

Agroinoculation is a less-expensive and more reproducible strategy for infecting plants with RNA viruses. Agroinoculation with a Gemini virus was first reported for *Tomato golden mosaic virus* (TGMV). Grimsley *et al*. (1986) defined agroinoculation as a method by which infectious viral clones could be introduced into plants using *Agrobacterium tumefaciens*. Agroinoculation could also be used for understanding the complexity of plant–virus interactions, to study viral replication and for assays evaluating the resistance of transgenic plants to Gemini viruses (Bowdoin *et al*., 1999 and 2004; Shepherd *et al*., 2005). Karthikeyan *et al*.(2004) done agroinoculation of cloned
genomic components of MYMV-Vig was done to check whether each of the five DNA B components can cause the yellow mosaic disease along with one DNA A component. Jacob et al. (2003) has also reported agroinoculation with an isolate of MYMV that infects blackgram from Madurai, southern India (MYMV-Vig). Malathi et al. (2005) found out that MYMIV-[Cp] did not infect blackgram, mungbean, or soybean by whitefly transmission, but through agroinoculation it was able to infect blackgram, mungbean, and soybean, thereby showing new host adaptation by agroinoculation.

2.12. Utilization of RGAs

2.12.1. Molecular isolation of resistant genes.

The isolation of $R$ genes was achieved with the development of technologies for cloning plant genes of unknown structure or molecular function. Several methods can be used for identifying and cloning genes that are differentially regulated including cDNA –RFLP (Durrant et al., 2000), differential display PCR, and microarray and gene chip technologies (Maleck et al., 2000; Seo et al., 2007).

Several strategies to search for candidate genes for disease resistance could be developed. One of these consists of isolating genes that are expressed differentially in resistant and susceptible plants during the infection process. EST databases can be explored in search of orthologous resistance and defence genes (Lanaud et al., 2003).

2.12.2. Engineering of disease resistance by using RGAs.

For nearly a century breeders have been studying the inheritance of $R$ genes and using classical breeding techniques for the intergression of $R$ genes from wild populations into elite cultivars. Traditional breeding methods are very time consuming, development of resistant cultivars may take up to 15-20 years. RGAs can be utilized as a marker to be applied in marker assisted selection for early release of disease resistant varieties.

Many case studies have been reported on transfer of $R$ genes within and across species to confer disease resistance. For e.g. Bs2 gene in pepper which confers resistance to Xanthomonas campestris was transferred to tomato (Tai et al., 1999) and N gene of tobacco resistant to Tobacco Mosaic Virus was transferred to tomato (Levy et al., 2004).
Many R genes have a narrow range of resistance, often to only one or a few strains of a single pathogen species. Many R genes lack durability because they can be defeated by a single loss-of-function mutation in the corresponding Avr gene (thereby rendering the pathogen ‘invisible’). Because individual Avr genes often make only incremental contributions to virulence, pathogens can afford to alter or discard an Avr gene with little or no fitness penalty. Unfortunately, R genes are often quickly defeated by co-evolving pathogens (Pink, 2002). Many R genes recognize only a limited number of pathogen strains and therefore do not provide broad-spectrum resistance. Pyramiding and multiline deployment have not been widely used, owing to the time required for breeding assortments of R genes into elite cultivars. However, recent molecular-level insights into the function of R proteins and downstream signal transduction pathways might provide strategies to remedy these deficiencies (McDowell and Woffenden, 2003).

Broad-spectrum resistance can be achieved by coordinate expression of an R gene and a corresponding Avr transgene, controlled by a pathogen-inducible promoter. This tactic enables induction of defense by multiple pathogens without pyramiding numerous R transgenes. Furthermore, this system might circumvent the loss of durability commonly observed with R genes because the Avr gene is not under selective pressure for mutation (De Wit, 1992).

The most exciting approach towards engineering improved resistance to multiple diseases may be the development of new R genes having multiple specificities. The Fen (resistance to the insecticide Fenthion) and Pto genes are located in the same R gene cluster in the tomato genome and they are 86% identical in nucleotide sequence. A functional gene was made by domain swapping of the two genes, thus raising the possibility of creating a hybrid gene containing multiple specificities. Another novel strategy, termed two-component approach, has been developed lately and holds lot of promise for introducing broad-spectrum resistance. This strategy involves generation of transgenic plants that express a pathogen Avr gene under the control of a heterologous infection-inducible promoter. If the plant carries the matching R gene, it will respond with an HR at the site of infection thus limiting the pathogen. The key to this approach is the identification of suitable promoters that respond or are induced only following infection by broad range pathogens.
MATERIALS AND METHODS

The present study was conducted at Department of Plant Molecular Biology and Biotechnology, Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore during 2006-2008.

3.1. R-gene and retrieval of its gene sequence

Nucleotide and amino acid (1144 amino acids) sequences encoding for N-gene of tobacco (Accession no. sp|Q40392|TMVRN NICGU) reported from Nicotiana glutinosa were retrieved from NCBI-Genbank database (http://www.ncbi.nlm.nih.gov).

3.2 Sequence comparison, multiple sequence alignment and designing of degenerate oligonucleotide primers

Homologous proteins of N-gene of tobacco were identified by Protein-protein BLAST (blastp) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search using the N-gene sequence as a query. Homologous protein sequences reported from six different plant species were retrieved and used for multiple sequence alignment and conserved domain search.

Multiple sequence alignment of the selected protein sequences was done by T-coffee (http://igs-server.cnrs-mrs.fr/Tcoffee) online tool. Conserved domains were identified by using blastp search tool. Three oligonucleotide primers were designed from the highly conserved region (NBS-LRR domain) among the six different species of crops (VFLSFR, GMGGVGK and LPLALKV). The degeneracy of the primer is assured by choosing the alternate nucleotides for the varying amino acids from the codon table (Annexure 1). The primers were checked for their quality (GC content, annealing temperature and presence of dimers) using the software, Fast PCR.

3.4. Plant materials taken for the study

Mungbean (Vigna radiata (L.) wilczek) and Ricebean [(V. umbellata (Thunb.) Ohwi and Ohashi)] accessions (Table 1) were obtained from National Pulse Research Centre, Vamban, Tamil Nadu Agricultural University.
3.5. Isolation of genomic DNA from ricebean and mungbean

3.5.1. Isolation of genomic DNA from ricebean leaves

Leaf samples were collected from 15 day old seedlings and utilized for genomic DNA extraction.

3.5.1.1. Reagents required

a. Cetyl Trimethyl Ammonium Bromide (CTAB) Extraction Buffer:
   
i. CTAB 2% W/V
   ii. Tris HCl pH 8.0 100mM
   iii. Sodium Chloride 1.4M
   iv. EDTA 20mM

b. Tris EDTA (TE) Buffer:
   
i. TRIS HCl (pH 8.0) 10 mM
   ii. EDTA 1 mM

c. Ice-cold Isopropanol

d. Chloroform: Isoamylalcohol (24:1 V/V)

e. Sodium acetate (3.0 M) pH 5.2 (pH adjusted using glacial acetic acid)

f. Ethanol (100% and 70%)

g. Proteinase k (1µL/ 10ml buffer)

Prior to DNA isolation, pestle and mortar, spatula, eppendorf tubes and tips, were autoclaved at 121°C for 30 min in TOMY High-pressure steam sterilizer ES-315.

Protocol

- Approximately 100–200 mg of leaf tissue was taken and ground in a pre-chilled pestle and mortar using liquid nitrogen and mixed with 1.5 ml of Cetyl Trimethyl Ammonium Bromide (CTAB) extraction buffer.
• 750 µl of the sample was taken in a 1.5 ml eppendorf tube and kept at 65°C for 10 min.

• To this, equal volume (750 µl) of chloroform: isoamylalcohol (24:1) was added and centrifuged at 10,000 rpm for 10 min.

• The top aqueous phase was transferred into a new 1.5 ml eppendorf tube and equal volume of chloroform: isoamylalcohol (24:1) was added, mixed and centrifuged at 10,000 rpm for 10 min.

• Aqueous phase was transferred into a new eppendorf tube and to this 2 volumes of ice cold ethanol and ½ volume of 5M NaCl were added.

• The mixture was incubated at −20°C for 1 h and centrifuged at 12,000 rpm at 4°C for 10 min.

• The pellet was washed with 70% ethanol and centrifuged for 5 min at 12,000 rpm.

• The pellet was air dried for 5 min.

• Depending upon the size of the pellet, DNA was dissolved in 250-500 µl of 1X TE (pH 8.0) and stored at −20 °C.

3.5.1.4. Isolation of mungbean genomic DNA by using DNA isolation kit

Mungbean genomic DNA was isolated by Sigma Miniprep DNA Isolation Kit as per the manufacturer’s protocol.

3.5.2. Quality and quantity check of genomic DNA of mungbean and ricebean

Isolated genomic DNA was checked for its purity and intactness and then quantified. The crude genomic DNA was resolved by agarose gel electrophoresis (0.8 % agarose gel) stained with ethidium bromide and was visualized in a gel documentation system (Alpha Imager™1200, Alpha Innotech Corp., CA, USA). DNA was quantified by using Spectrophotometer by reading the absorbance at 260 nm. Based on the quantification data; DNA dilutions were made in 1X TE buffer to a final concentration of 50ng/µl and stored in -20°C for further use.

3.6. PCR amplification of N-gene homologue in mungbean and ricebean

To standardize the annealing temperature, gradient PCR was performed using Eppendorf Master Cycler Gradient. PCR amplification was performed in 15 µl reaction volume using the following temperature conditions.
Profile 1: 94˚C for 5 minutes Initial denaturation
Profile 2: 94˚C for 1 minute Denaturation
Profile 3: 55˚C for 1 minute Annealing
Profile 4: 72˚C for 1 minute Extension
Profile 5: 72˚C for 5 minutes Final extension
Profile 6: 4˚C Hold the samples

Profiles 2, 3 and 4 were programmed to run for 48 cycles

**PCR mixture**

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<th>Volume</th>
<th>Stock</th>
<th>Final concentration</th>
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<tr>
<td>2.</td>
<td>DNA</td>
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<td>50 ng</td>
<td>6.6 ng/ µl</td>
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<tr>
<td>3.</td>
<td>Forward primer</td>
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<tr>
<td>4.</td>
<td>Reverse primer</td>
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<td>5 mM/ µl</td>
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<tr>
<td>5.</td>
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<td>2.5 mM</td>
<td>0.25 mM/ µl</td>
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<tr>
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<td>Taq buffer</td>
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<td>1.33X</td>
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<tr>
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<td>Taq</td>
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</tbody>
</table>

3.7. Agarose gel electrophoresis

3.7.1. Materials required

i. Loading Dye

ii. Glycerol 50% (v/v)

iii. Bromophenol blue 0.5% (w/v)
3.7.2. Reagents required

10X TBE (Tris Borate EDTA buffer)

a) Tris Base 107.8 g  
b) Boric acid 55.03 g  
c) EDTA(Na₂.2H₂O) 8.19 g  

(Dissolved in 800 ml of sterile water and made up to 1000 ml)

Protocol

- The Pyrex gel casting plate’s open ends were sealed with cello tape and the comb was placed properly in casting plate kept on a perfectly horizontal platform.
- 0.8 % (0.8 g/100 ml) agarose was added to 1x TBE, boiled until the agarose dissolved completely and then allowed to cool.
- Ethidium bromide (DNA intercalating agent) was added when temperature reached 55-60°C, as a staining agent.
- Then it was poured into the gel casting plate and allowed to solidify.
- The comb and the cello tape were removed carefully after solidification of the agarose.
- The casted gel was placed in the electrophoresis unit with wells towards the cathode and submerged with 1X TBE to a depth of about 1cm.

Loading the DNA samples

- 1 μl of DNA sample dissolved in TE was pipetted onto a parafilm and mixed well with 3 μl of 10X loading dye by pipetting up and down 1 or 2 times.
- The gel was run at 80 V, 400 Amp for 1-1.5 hours and bands were visualized and documented using a gel documentation system (Model Alpha Imager 1200, Alpha Innotech Corp., USA).
3.8. Expression analysis of N-gene homologue in mungbean and ricebean

3.8.1. Selection of varieties

Mungbean (Variety: VRM1) and ricebean (TNAU red) were selected for agroinoculation and subsequent expression analysis.

3.8.2. Agroinoculation

The tandem viral constructs of MYMV (VA 221 and VA 239) mobilized in Agrobacterium tumefaciens strains Ach 5 and C 58 collected from M. K. U, Madurai were used. Agroinoculation was done on surface sterilized overnight sprouted seeds of green gram (VRM 1) and ricebean (TNAU red). Agrobacterium tumefaciens strains harbouring the appropriate partial tandem repeat clone were grown to 1 OD at 600 nm in 2 ml AB minimal medium pH 7.0 (Annexure II) containing antibiotics streptomycin (150 mg/L), spectinomycin (50 mg/L) and tetracycline (5 mg/L) at 28º C at 220 rpm. From this 1 ml culture was taken to inoculate another 50 ml AB minimal medium pH 7.0 containing above mentioned antibiotics and grown to 1 OD at 600 nm at 28º C at 220 rpm. The culture was spinned at 4000 rpm for 10 min at 25º C. Cells obtained were resuspended in 50 ml AB minimal medium pH 5.6 with 100 µl acetylsyringone (100 µm). Seed coat of the sprouted seeds was removed by using forceps and pricked around the hypocotyl region and were immediately immersed in appropriate Agrobacterium tumefaciens culture. It was Left for overnight, next day after washing the seeds with distilled water were sown in pots containing (1:1) ratio of autoclaved sand and vermiculate. Agroinoculated plants were maintained in a growth chamber set at 25° C temperature, 60%–70% relative humidity and 16/18 hr photoperiod. Hoagland’s solution was applied twice in a week for proper growth of the plants (Annexure III).

3.8.3. Collection of samples

Leaf samples were collected 25 days after agroinoculation, when the mungbean yellow mosaic symptoms were clearly seen in the leaves of mungbean plants.

3.8.3. Isolation of RNA (TRI reagent method)

Prior to extraction, pestle and mortar, spatula, eppendorf tubes and tips, were soaked in Diethyl pyrocarbomate (DEPC) for 12 hrs in 0.02% solution. Materials were completely air dried in shadow and autoclaved two times at temperature 121°C for 30 min (TOMY High-pressure steam sterilizer ES-315). Total RNA was extracted by TRIZOL method using TRI Reagent (Sigma-aldrich, inc., USA) (Chomczynski and Sacchi, 1987).
Protocol

- 200 mg of frozen plant tissue was ground in a pre-chilled mortar and pestle with liquid Nitrogen.

- Sample was added to the DEPC-treated (200 µl of DEPC (Di Ethyl Pyro Carbamate) in one litre of distilled water) eppendorf tube and 1 ml of TRI Reagent was added and homogenized thoroughly by vortexing for 30 sec.

- The mixture was centrifuged at 12000 rpm for 10 min in cold.

- Supernatant was transferred to a new DEPC-treated tube and 0.3 ml chloroform was added and vortexed for 15 sec and incubated at room temperature (RT) for 2-3 min.

- After centrifugation at 12000 rpm for 15 min in cold, the supernatant was transferred into a new tube (~ 60% of initial volume) and 0.3 ml of 2-propanol and 0.3 ml of 2M NaCl was added, mixed by inversion for 2 min and incubated at RT for 1 hr.

- Centrifugation was carried out at 12000 rpm for 10 min in cold.

- The supernatant was removed and the pellet was washed with 1 ml 75% ethanol with gentle mixing.

- Again Centrifugation was carried out at 10000 rpm for 5 min in cold to settle down the RNA pellet.

- Supernatant was discarded and the pellet was air dried.

- The RNA pellet was dissolved in 20-30 µl of DEPC-treated water depending on pellet size and stored in -80°C for future use.

3.8.4. RNA quality check

The quality of total RNA isolated from both treated and control plants were analyzed on a 1.2% agarose gel and visualizing the intactness of 23s and 18s rRNA bands.

3.8.5. Quantification of RNA

The RNA was quantified spectrophotometrically by using Nanodrop spectrophotometer Nucleic Acid and Protein Quantification instrument, ND-2000 (Nanodrop technologies, USA), according to manufacturer’s instructions.
3.8.6. Normalisation of RNA

The RNA obtained was normalized by diluting from the stock, so as to obtain 500 ng of RNA in each sample. The diluted RNA was electrophoresed on a 1.2% agarose gel, for checking the uniform brightness and the intactness of the 23s and 18s rRNA bands.

3.8.7. Semi-quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR expression analysis was carried out using One-step Superscript-II RT-PCR system (Invitrogen Inc., USA) as per the manufacturer's instruction. RT-PCR was performed in a 25 µl volume reaction mixture containing 12.5 µl of Superscript II buffer and 0.5 µl Superscript II reverse transcriptase-Taq polymerase enzyme mix with 0.5 µg of total RNA and gene-specific primer pair (TNMVRP 1 and 2). The cycle conditions were as follows: Reverse transcription at 50 °C for 40 min; pre-amplification denaturation at 95 °C for 5 min, 40 cycles of [denaturation at 95 °C for 1 min, primer annealing at 55 °C for 1 min, and primer extension at 72 °C for 1 min], and a final extension of RT-PCR products at 72°C for 5 min. RT-PCR products were separated by electrophoresis on 1.2% agarose gels, (60 Volts, 400 Amp, 3 hrs) and stained with ethidium bromide. Bands were visualized using UV-light in Alpha Imager 2200 (Alpha Innotech Corporation, San Leandro, CA).

Reaction mix details

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</tr>
<tr>
<td>2</td>
<td>RT-PCR buffer (2X)</td>
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</tr>
<tr>
<td>3</td>
<td>Forward primer (10µM)</td>
<td>1.5</td>
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<td>4</td>
<td>Reverse primer</td>
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<td>5</td>
<td>MgCl₂ (25mM)</td>
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</tr>
<tr>
<td>6</td>
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</tr>
<tr>
<td>7</td>
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<td>6.8</td>
</tr>
<tr>
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EXPERIMENTAL RESULTS

The results of the studies on “Analysis of resistant gene analogues in mungbean [Vigna radiata (L.) Wilczek] and ricebean [Vigna umbellata (Thunb.) Ohwi and Ohashi]” are presented hereunder.

4.1. Selection of tobacco N-gene sequence for RGA analysis

Based on the previous reports, a resistant gene namely, "N-gene of tobacco" was selected for this study. Genes homologous to N-gene of tobacco have been reported in a variety of crops. A nucleotide sequence of N-gene (Accession # Q40392|TMVRN NICGU) of *Nicotiana glutinosa* was retrieved from NCBI-Genbank database and used for further analysis to understand the role of N-gene homologue in MYMV resistance.

4.2. Identifying genes homologous to N-gene of tobacco

The retrieved nucleotide and protein sequence of N-gene of tobacco (Q40392) was used for identifying reported homologous genes in various crops using NCBI-BLAST analysis. Amino acid sequence representing the N-gene of tobacco was blasted against Protein database (Organism: Viridiplantae) using NCBI-blastp programme. Blast analysis resulted in the identification of six different homologous genes reported in various crops viz., *Solanum tuberosum* (72% similarity), *Lycopersicum esculentum* (70%), *Medicago truncatula* (57%), *Phaseolus vulgaris* (56%), *Cucumis melo* (55%) and *Populus trichocarpa* (53%). The details of the homologous genes are listed in (Table 2).

4.3. Prediction of conserved domain and designing degenerate primers

Analysis of the protein sequence using BLAST program revealed the presence of two conserved domains namely TIR domain and NB-ARC domain (Fig. 1).

In addition, T-coffee multiple sequence alignment showed the presence of three highly conserved amino acid regions in the NBS-LRR domain (VFLSFR, MGGVGK and LPLALKV) which were selected for designing oligonucleotide primers. Three degenerate primers were designed within the boundary of NBS-LRR domain of N-gene of tobacco (Table 3; Fig 2). The position of the primers in N-gene sequence of *Nicotiana glutinosa* (gi|46577339|sp|Q40392|TMVRN_NI) is shown in (Fig. 3).
4.4. PCR amplification of *N*-gene homologue in mungbean and ricebean

The oligonucleotide primer pair TNMVRP 1 and 2 meant to amplify a 627 basepair region and the primer pair TNMVRP 2 and 3 meant to amplify a 528 bp region of the NBS-LRR domain of tobacco *N*-gene, were used for PCR analysis of RGA present in mungbean and ricebean accessions. In the PCR analysis using genomic DNA isolated from 16 mungbean and 24 ricebean accessions, it was noticed that all the accessions of both mungbean and ricebean were found to contain the genomic fragments of expected size and thereby it was confirmed that homologue of *N*-gene of tobacco is present in all the accessions (Plates 1, 2, 3, 4).

4.5. Expression analysis of *N*-gene homologue in mungbean and ricebean

4.5.1. Agroinoculation

Mungbean variety VRM 1 (known for high yield in farmers’ field) and rice bean accession TNAU red were selected for agroinoculation and subsequent expression analysis. The two *Agrobacterium* strains harbouring the tandem viral constructs of MYMV (VA 221 and VA 239) were used for agroinoculation.

The agroinoculated mungbean (VRM 1) plants showed symptoms on the 13th day (those agroinoculated with VA 239) and on the 15th day (those agroinoculated with VA 221). But the control plants and the agroinoculated ricebean (TNAU Red) did not show any symptoms at all, even after 20 days of inoculation in the growth chamber (Plate 5). At the 25th day, when the symptoms were clearly seen on the leaves, the leaf samples were collected for RNA isolation.

4.5.2. Isolation of RNA (TRI reagent)

Total RNA was isolated from all the samples (control and agroinoculated leaf samples of mungbean (VRM 1) and ricebean (TNAU Red)).

4.5.3. RNA quality Check and quantification

RNA quality was checked by resolving the total RNA by agarose gel electrophoresis (0.8% agarose gel). Integrity of RNA was assessed by the presence of intact bands of 18s and 23s rRNA. Total RNA was quantified by measuring the absorbance at 260/280 nm and 260/230 nm using a Nanodrop spectrophotometer Nucleic Acid and Protein Quantification instrument, ND-2000 (Nanodrop technologies, USA).
4.5.4. Normalisation of RNA

RNA was normalized by adding sterile nuclease free water so as to get the final concentration of 500ng RNA/ µl. It was also visualised by running 0.8 % agarose gel, staining with ethidium bromide and visualized under UV-light in Alpha imager [Alpha Imager™1200, Alpha Innotech Corp., CA, USA] (Plate 6). Equal intensity of rRNA bands reflected the equal amount of total RNA.

4.5.6. Semi-quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Differential expression of \( N \)-gene of tobacco in mungbean and ricebean accessions during MYMV infection was analysed by semi-quantitative RT-PCR method. Primer pair TNMVRP1 and 2 was used for amplifying the transcript of the tobacco \( N \)-gene homologue present in mungbean and ricebean. Agarose gel electrophoresis of RT-PCR amplification revealed that the tobacco \( N \)-gene homologue was found to be down-regulated in mungbean VRM 1 during MYMV infection whereas it was found to be up-regulated in the ricebean accession TNAU Red (Plate 7).
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Fig.1. BLAST search results showing the presence of TIR and NB-ARC domains
Fig.2. T-coffee multiple sequence alignment showing conserved domains
(The conserved domains are given in box)
Fig 3. Nucleotide sequence of N-gene of *Nicotiana glutinosa* showing the position of primers used for PCR amplification

```
ATGGCATCTTCTCTTCTTTCTTAGATGGAGCTATGATGTTTTCTTAAAGTTTTAGAGGGAAG
ATACTC

(TNMVRP 1)
GAAAAACGTCTTACAAGTCACTTTATACGAAGTTCTGATGATAAGGGGATAAAAAACCTTTCAAGA
TGATAA
AAGGCTAGAGTACGCGCAACCATCCAGGCTGAATCTGTAAAGCTATAGAAGAGGTCTCAATT
TGCCATT
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AATGCA
AAACTCGATTTTAAGGAAAACGTGTATTTTCTGTATGTGATGATCCCATCATGTTCCGAA
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ACTCTTT

(TNMVRP 1)
```
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CCCGAT
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GCTGCA

(TNMVRP 3)
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ATGAG
GCATGACATCTCTGCTTCAGATTCTTGTCACTAACAAGTATTTACCAGGTCAACCGGTATCCTGAA
AAGATC
CCGAGTTTGGTCCACCACCATCAGGGTTGGGATAGTAGTAGTCTACGTCAAATTTGCTGAAATTTG
TATATA
CTGATAAAATTCTTGGGATTTGCTGTATGTTACTCTCGTAGCTTAATTGACACAACAGCTCATT
GATTCC
CGTATGTGATGACAAGATGTCGCATGACCCAGAAACTTGCCTTATCAGAATGTCGAGAAGATCATCC
AACTATTCAGAATGGGATATACCTTTTCTTTGTACCTTTTTGCTTTGGGATCATGCTCAGAATATC
GGCAA
ATGGAAAAACACCAAATGATTATGGGATTATTAGGCTATCTTTTTCTGAGAAGAGAAGATGTA
TTGACT
TCGTTTGCTATAAGAAGGACCAGAGGTTATGTGCTTTCATGAAATGAGGGAAAATAGCAAT
GAACCA
ACAGAACCCTCCACTGGGATAAAGGAGGACTCAATAAACAGAAGACTCTTTTTATAGGCTCA
TCATG
GGTGA
DISCUSSION

*Vigna radiata* (L.) Wilczek, commonly known as mungbean or green gram is the most widely distributed species among the six Asiatic *Vigna* species. It is relatively drought tolerant and well adapted to a range of soil conditions including light soils and can thrive even under limited irrigation, more over; it is suited for crop rotation and crop mixtures (Baldev, 1988 and Sadapal, 1988). Each year, up to 10% of world agricultural production is lost to pests and diseases caused by a variety of pathogens including bacteria, fungi, nematodes, and viruses (Burch-Smith *et al*., 2007). *Mungbean Yellow Mosaic Virus* (MYMV) is one of the most devastating diseases in mungbean. MYMV is caused by a Gemini virus and viral disease could not be effectively controlled with chemicals. Host resistance to the virus and /or the vector has therefore been considered as the only solution to control this important disease. The aim of this study was to identify RGAs that are likely to be functional *R* genes and to study their expression patterns in control and infected ricebean and mungbean accessions.

5.1. Designing of degenerate oligonucleotide primers in NBS-LRR region of *N*-gene of tobacco

The interaction between the resistance gene *N* of tobacco and *Tobacco Mosaic Virus* (TMV) has long served as a classical model for the study of plant-resistance responses to pathogens and provides an excellent system to isolate *R* genes and gain insight to its functions (Dineshkumar *et al*., 1995). Most *R* proteins have a centrally located NB-ARC (nucleotide-binding adaptor shared by APAF-1, *R* proteins, and CED-4) domain. *N*-gene is a typical *R* gene, introduced into tobacco (*Nicotiana tabacum*) from *Nicotiana glutinosa* (Holmes, 1938). The *N* gene is a member of the Toll-IL-1 homology region–Nucleotide Binding Site–Leucine-Rich Repeat region (TIR-NBS-LRR) class of plant resistance genes (Dineshkumar and Barbara, 1999). TIR domain is a sub family of the NB-ARC domain in *R* genes (Pal *et al*., 2007). The primers were designed in the highly conserved regions (VFLSFR, MGGVGK, and LPLALKV) as revealed by the T-coffee online multiple sequence alignment software results, comprising six different crops having genes homologous to *N*-gene of tobacco.
Putative conserved domains; TIR Domain (within first 150 amino acids) and NB-ARC Domain (from 200 to 450 amino acids) were found out in the BLAST analysis. Motif discovery analysis done by Gorodkin et al., (1997) revealed that the motif VFXSFRGXDVRXXFLSH determined as consensus sequence for TIR domain (X indicates that in a given position none of the amino acid is more than 50% or frequency of two amino acids together not more than 0.8), consensus sequence GMGGXGKTTL is for Kinase1a and consensus sequence CXGLPLA for GLPL motifs (Gunduz et al., 2003). The primers were designed in these three highly conserved regions.

5.2. PCR amplification of N-gene homologue in mungbean and ricebean

Amplification of N-gene homologue in 24 ricebean and 16 mungbean accessions with the three primers (TNMVRP 1, 2 and 3) showed no polymorphism in the expected band size. All the accessions showed the presence of bands though there were multiple bands in some of the accessions in both ricebean and mungbean. In previous studies, the amplification of genomic DNA and cDNA of various plants with primers targeting the kinase 1a and the GLPL motifs has always resulted in clear bands of the expected size (500 bp) in gel electrophoresis analyses, usually along with clear bands of different sizes and the fragments that did not have a size of 500 bp were shown to be non-specific by studies on various plants (Leister et al., 1996; Deng et al., 2000; He et al., 2004; Noir et al., 2001). The NB-ARC domain is found to be a multidomain (refer fig. 1). This may account for the multiple bands present in the gel electrophoresis. All the bands which are having size above 500bp need to be eluted, cloned and sequenced to find out the difference in nucleotide or amino acid sequences to further explain the obtained result.

5.4. Expression analysis of N-gene homologue in mungbean and ricebean

Expression analysis of N-gene homologue in the ricebean (TNAU Red) and a mungbean (VRM 1) accession was conducted because many of the identified R gene homologues/analogues can be probably pseudogenes (Pan et al., 2000) or non-functional genes. Liu and Ekramoddoullah (2003) reported the amplification of RGAs from cDNA of western white pines that have been inoculated with and are resistant to white pine blister rust (Cronarium ribicola). This method is very valuable for the purpose of identifying R genes conferring resistance to a specific pathogen. RGAs amplified from cDNA are more likely to be functional R genes than those amplified from genomic DNA, since the transcription of non-functional genes would be associated with an overall fitness
cost to the plant and would be thus eliminated through evolution (Liu and Ekramoddoullah, 2003; Budak et al., 2006a; Budak et al., 2006b; Ergen et al., 2007).

Semi-quantitative RT-PCR analysis showed that the tobacco N-gene homologue present in the ricebean, TNAU red is up-regulated or over expressed after agroinoculation whereas in mungbean VRM 1 it was found to be down-regulated or under expressed after agroinoculation. Similar observations were made in case of XaI, a bacterial resistance gene in rice (Yoshimura et al., 1998), the pib rice blast resistance gene (Wang et al., 1999) and Hs1 pro-1, a nematode-resistant gene in sugar beet (Thurau et al., 2003).

Seo et al. (2007) found out that PvVTT1 gene (Phaseolus vulgaris Virus response TIR-TIR Gene), which has homology with the tobacco N-gene was found to be up regulated in BDMV (Bean Dwarf Mosaic Virus) infected tissues. The results also indicate constitutive expression of the N-gene homologue, as the transcript of N-gene homologue is amplified in control plants also.

The RT-PCR results showed that the mungbean plants treated with VA 221 showed significant down regulation of transcript of N-gene homologue when compared to the mungbean plants treated with VA 239. Among the two rice bean treatments, the plants treated with VA239 showed highly significant up-regulation of transcript of N-gene homologue than the ones treated with VA221. This may be attributed to the fact that VA 239 is a more virulent viral construct than VA 221. The viral construct VA 239 consists of DNA A and KA27 DNA B and VA 221 consists of DNA A and KA22 DNA B. Agroinoculation of mungbean with partial dimers of KA27 and KA22 DNA Bs along with DNA A revealed that KA27 DNA B caused more intense yellow mosaic symptoms with high viral DNA titre than KA22 DNA B. (Balaji et al., 2004). Even if the N gene homologue is present in the mungbean accessions, it is not showing resistance to MYMV. It may probably because of some mutations or need of activation of signaling factors and/or transcription factors or need of splicing.

The N gene, a member of TIR-NBS-LRR class of plant resistance genes, encodes two transcripts, NS and NL, via alternative splicing of the alternative exon present in the intron III. Plants harboring a cDNA-NS transgene, capable of encoding an N protein but not an Ntr protein, fail to exhibit complete resistance to TMV. Transgenic plants containing a cDNA-NS bearing intron III and containing three N genomic sequences, encoding both NS and NL transcripts, exhibit complete resistance to TMV. These results suggest that both N transcripts and presumably their encoded protein products are necessary to confer complete resistance to TMV (Dineshkumar and Barbara, 1999).
SUMMARY

The summary of present study carried out with an objective of finding out the Resistant Gene Analogues (RGAs) related to MYMV resistance in mungbean and ricebean are described below:

1. The protein sequence of a known virus resistant gene namely “N-gene of tobacco” was retrieved from NCBI-Genbank database to study the relationship between N-gene homologue present in mungbean and ricebean germplasm lines and MYMV resistance.

2. Genes homologous to N-gene of tobacco were identified from six different crops using BLAST search.

3. Multiple sequence alignment of the selected protein sequences was done with T-coffee online software and conserved domains were identified.

4. Three degenerate oligonucleotide primers were designed from the highly conserved regions (VFLSFR, MGGVGK, and LPLALKV) of the NBS-LRR domain, to amplify the N-gene homologue present in mungbean and ricebean accessions.

5. PCR amplification showed the presence of N-gene homologue (500-600 bp) in all the mungbean and ricebean accessions taken for the study.

6. Agroinoculation experiment conducted in mungbean (VRM 1) and ricebean (TNAU Red) accessions revealed the resistance of ricebean against MYMV.

7. Expression analysis of mungbean (VRM 1) and ricebean (TNAU Red) revealed that the transcript of N-gene homologue is up-regulated in ricebean and down-regulated in mungbean during MYMV infection.

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