IDENTIFICATION OF RESISTANCE GENE ANALOGS EXPRESSED IN THE ABSENCE OF PATHOGEN IN TALL FESCUE AND BERMUDAGRASS

by

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IN TALL FESCUE AND BERMUDAGRASS

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To my dear grandfather Prof. Hasan Önal,

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Kinase 1a and GLPL motifs are two of the most highly-conserved regions in the nucleotide-binding domain found in most resistance (R) proteins. In this study, degenerate primers that target these two conserved motifs were used to isolate *R* gene analogs (RGAs) from root and shoot cDNA of tall fescue (*Festuca arundinacea*) and bermudagrass (*Cynodon dactylon*) grown in a pathogen-free environment. A total of 35 clones were sequenced; four of them were found to be homologous to known *R* genes and RGAs. Three of these four sequences were obtained from bermudagrass root cDNA and are identical to one another. The fourth one was obtained from tall fescue shoot cDNA and is highly similar to the three bermudagrass sequences. The high similarity of these four sequences suggests that they may represent two alleles of the same RGA. The deduced amino acid sequence of this RGA was compared to the sequence of 27 R proteins. The five proteins that gave the closest match were MLA13 from barley, Pi-ta from rice, GPA2 from potato, RPP13 from *Arabidopsis*, and SW5 from tomato. Comparison of the secondary structure prediction for the RGA identified in this study and that for MLA13 of barley revealed similarities in protein structure at the sites of some conserved motifs. Determining the complete mRNA sequence of the RGA and cloning it in an expression system to isolate the protein for structural analyses should provide further insight into the nature of this recently-identified RGA.

UZUN YUMAK OTU VE BERMUDA ÇİMİNDE
PATOJEN YOKLUĞUNDA EKSPRES EDİLEN
DAYANIKLILIK GENİ ANALOGLARININ SAPTANMASI

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Anahtar sözcükler: dayanıklılık genleri, dayanıklılık geni analogları, dejenere primerler, uzun yumak otu, bermuda çimi

Kinaz 1a ve GLPL motifleri, dayanıklılık (R) proteinlerinin çoğunda bulunan nükleotid-bağlayıcı bölgedeki yüksek seviyede korunmuş motifler arasındadır. Bu çalışmada, patojensiz ortamda yetiştirilmiş uzun yumak otu (*Festuca arundinacea*) ve bermuda çimi (*Cynodon dactylon*) kök ve yeşil aksam cDNA örneklerinden *R* geni analogları (RGAlar) izole etmek amacıyla, bu iki korunmuş motifi hedef alan dejenere primerlerden faydalanılmıştır. Toplam 35 klonun DNA dizilemesi yapılmış, bunlardan dört tanesinin bilinen *R* genlerine ve RGAlara homolog olduğu tespit edilmiştir. Bu dört DNA dizisinden üç tanesi bermuda çimi kökünden elde edilmiş olup birbirleriyle aynıdır. Dördüncü dizi ise uzun yumak otu yeşil aksamından elde edilmiş olup diğer üç bermuda çimi dizisine yüksek derecede yakınlık göstermektedir. Gözlenen yüksek derecedeki benzerlik, bu dört DNA dizisinin aynı RGA'nın iki farklı alelini temsil ediyor olabileceğine işaret etmektedir. Bulunan RGA'nın DNA dizisinden öngörülen amino asit dizisinin 27 R proteininin dizileriyle karşılaştırılması sonucu bu RGA'ya en çok yakınlık gösteren beş proteinin arpadan MLA13, çeltikten Pi-ta, patatesten GPA2, *Arabidopsis*'ten RPP13 ve domatesten SW5 proteinleri olduğu saptanmıştır. Bu RGA'nın ve arpadan MLA13 proteinin ikincil yapı tahminlerinin karşılaştırılması sonucunda, korunmuş motiflerin olduğu bazı bölgelerde protein yapısının da benzerlik gösterdiği görülmüştür. Saptanan RGA'nın tam mRNA dizininin belirlenmesi ve protein yapı analizleri için bu genin bir ekspresyon sistemine klonlanması, ilk defa bulunan bu RGA hakkında daha detaylı bilgi sahibi olabilmemizi sağlayacaktır.

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ABBREVIATIONS

Avr	Avirulence
CARD	Caspase-activating recruitment domain
CC	Coiled-coil
CNL	CC-NBS-LRR
DEPC	Diethyl pyrocarbonate
EST	Expressed sequence tag
HR	Hypersensitive response
hrp	Hypersensitive response and pathogenicity
IL-1R	Interleukin-1 receptor
LRR	Leucine-rich repeat
MS	Murashige and Skoog
NB-ARC	Nucleotide-binding adaptor sequence shared by APAF-1, *R* gene products, and CED-4
NBS	Nucleotide-binding site
NO	Nitric oxide
PCR	Polymerase chain reaction
PPM	Plant Preservative Mixture™
QTL	Quantitative trait locus
R	Resistance
RACE	Rapid amplification of cDNA ends
RGA	Resistance gene analog
RGC	Resistance gene candidate
RGH	Resistance gene homolog
RGL	Resistance-gene-like
RLK	Receptor-like kinase
RLP	Receptor-like protein

ROI	Reactive oxygen intermediate
STK	Serine/threonine kinase
TIR	Sequence homologous to the intracellular signaling domain of the *Drosophila* Toll and mammalian IL-1R proteins
TLR	Toll-like receptor
TM	Transmembrane
TN	TIR-NBS
TNL	TIR-NBS-LRR
TX	TIR-X

1 INTRODUCTION

Plants, like animals, need to defend themselves from a wide range of potential pathogens, including viruses, bacteria, fungi, and nematodes. However, since they lack a circulatory system, plants cannot depend on a specialized proliferative immune system such as the one in vertebrates. Instead they have evolved a large variety of defense mechanisms. Some of these are general mechanisms, like structural defenses (e.g. thick waxy cuticle layer, leaf hairs) and production of phenolic compounds (Nicholson and Hammerschmidt, 1992) or enzymes such as chitinases and glucanases (Bowles, 1990). On the other hand, plants may also induce defense mechanisms upon specific recognition of pathogens.

Specific recognition of pathogens by plants usually involves the presence of a resistance (*R*) gene in the plant and the corresponding avirulence (*Avr*) gene in the pathogen. Presence of both of these genes results in plant disease resistance, whereas the absence or inactivation of either of these genes results in disease (Flor, 1971). The recognition of the Avr protein of the pathogen by the R protein of the host plant triggers a series of signal transduction events, which generally elicit a hypersensitive response (HR) followed by cell death, thus preventing the spread of infection (Cullis, 2004).

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). Despite this great diversity of host and pathogen species, many R proteins share common structural motifs containing highly-conserved sequences. Identification of the conserved regions in *R* genes has given molecular biologists an invaluable opportunity to rapidly isolate putative *R* genes from a great variety of plant species. These putative *R* genes are referred to as *R* gene analogs

(RGAs), *R* gene homologs (RGHs), *R* gene candidates (RGCs) or *R*-gene-like (RGL) sequences in literature. After the isolation of RGAs, their function may be determined by the use of expression analyses and other reverse genetics methods.

Leister et al (1996) developed a polymerase chain reaction (PCR)-based method to isolate RGAs. This method makes use of degenerate primers that target the conserved motifs of some *R* genes. In the past decade, the PCR-based method has been widely used to successfully isolate RGAs from a large number of plant species. These studies have utilized either the original primers developed by Leister et al or other primers targeting different motifs in *R* genes.

In this study, the PCR-based method was used to identify RGAs expressed in the roots and shoots of tall fescue (*Festuca arundinacea*) and bermudagrass (*Cynodon dactylon*). In order to isolate constitutively-expressed RGAs, the plants were grown in pathogen-free environment. The sequences obtained were compared to sequences available at nucleotide and protein databases and the ones homologous to *R* genes were further analyzed. To our knowledge, no RGAs have been previously isolated from these two grass species.

2 OVERVIEW

2.1 Plant-pathogen interactions and plant disease resistance

Plant pathogens are generally divided into three classes: necrotrophs, biotrophs, and hemibiotrophs. Necrotrophs kill the host cells and feed on their contents. Some necrotrophs produce toxins and/or enzymes that are effective in a wide range of host species, while the toxins produced by others are host-selective and lead to cell death in only a narrow range of hosts (Walton, 1996). Due to the need for production of a functional toxin and/or enzyme, virulence of necrotrophs is usually a dominant trait, while avirulence is recessive. Plants acquire resistance to necrotrophs either by losing or altering the target of the toxin or by detoxification (Hammond-Kosack and Jones, 1997). The first isolated *R* gene coding for resistance against a necrotroph was the maize gene *Hm1*. *Hm1* codes for a reductase enzyme that is thought to inactivate the leaf spot fungus (*Cochliobolus carbonum*) HC-toxin, which normally inhibits histone deacetylase activity (Johal and Briggs, 1992).

Biotrophs and hemibiotrophs invade host cells and alter the plant metabolism to promote their own growth and reproduction without killing the plant cells. Biotrophs require the host cells to be alive throughout their interaction with the plant, whereas hemibiotrophs may lead to cell death in the later infectious stages. Both biotrophs and hemibiotrophs tend to invade only a specific narrow range of plants. Incompatibility of host and pathogen leads to the activation of host defense mechanisms, generally eliciting HR and leading to localized cell death to prevent the spread of infection (Hammond-Kosack and Jones, 1996). The vast majority of *R* genes isolated to-date is involved in plant resistance against biotrophic or hemibiotrophic pathogens.

After decades of work on the interaction between flax (*Linum usitatissimum*) and its fungal rust pathogen (*Melampsora lini*), Flor (1971) suggested the "gene-for-gene" model for plant disease resistance mechanism. This model proposes the necessity of both an *R* gene in the host plant and the corresponding *Avr* gene in the pathogen for disease resistance, also called incompatibility. The absence or inactivation of either of these genes results in disease, or host-pathogen compatibility (Keen, 1990). Specific plant disease resistance against most biotrophic pathogens follows this simple gene-for-gene model, although it does not necessarily involve a direct interaction between R and Avr proteins.

2.1.1 Classes of *R* genes

Despite the great diversity of host and pathogen species, *R* genes can be mainly 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; Figure 2.1).

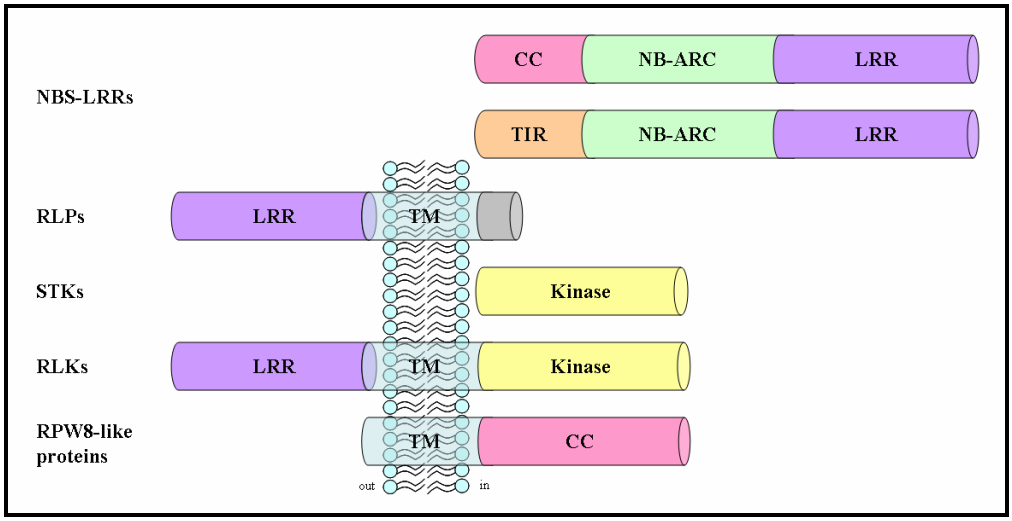

Figure 2.1 Schematic representation of R protein classes. Not drawn to scale. See text for abbreviations. (adapted from Dangl and Jones, 2001)

NBS-LRR genes constitute by far the largest class of *R* genes. They are thought to be highly abundant in plant genomes; sequence analyses of *Arabidopsis* (Arabidopsis Genome Initiative, 2000) and rice (Goff et al, 2002) genomes reveal the presence of over 150 and 600 putative NBS-LRR genes, respectively. NBS-LRR genes usually reside in clusters, in which both different *R* genes and the paralogs of the same *R* gene can be found (Young, 2000). Although no cellular localization of the protein products can be predicted via sequence analyses of these genes and the proteins are generally thought to be cytoplasmic, the protein product of at least one member of this class (RPM1) is known to be associated with the plasma membrane (Boyes et al, 1998). NBS-LRR proteins can be subdivided into two groups based on their amino-terminal sequence (Figure 2.1). Amino-terminus of the first group (e.g. *N* gene in tobacco; Whitham et al, 1994), known as the TIR domain, is homologous to the intracellular signaling domain of the *Drosophila* Toll and mammalian interleukin-1 receptor (IL-1R). These proteins are known as TIR-NBS-LRRs or TNLs. On the other hand, members of the second group (e.g. *RPS2* gene in *Arabidopsis*; Bent et al, 1994), known as CC-NBS-LRRs or CNLs, carry a putative CC domain at the amino-terminus (Dangl and Jones, 2001).

Members of the second class of *R* genes encode receptor-like proteins (RLPs) with an amino-terminal extracellular LRR domain, a single TM domain, and a short carboxy-terminal cytoplasmic domain (Meyers et al, 2005). The *Cf* genes in tomato, which confer resistance to the fungus *Cladosporium fulvum*, are the best-studied examples of this class (Jones et al, 1994). The recently-isolated *RPP27* gene of *Arabidopsis* (Tör et al, 2004) and *HcrVf2* gene of apple (Belfanti et al, 2004) also belong to this class.

The typical example of intracellular STK class of *R* genes is the *Pto* gene from tomato, which codes for a protein that confers resistance to *Pseudomonas syringae* strains that carry the avirulence gene *AvrPto*. Although the protein product of *Pto* has no distinct receptor domain, it appears to be directly interacting with the protein product of *AvrPto* (Tang et al, 1996). However, the function of the Pto kinase requires the presence of the CNL protein Prf (Salmeron et al, 1996).

Genes encoding TM receptor proteins with an extracellular LRR domain and an intracellular protein kinase domain constitute the fourth class of *R* genes. The protein products of these genes are known as receptor-like kinases (RLKs). *Xa21* (Song et al, 1995) and *Xa26* (Sun et al, 2004) genes of rice encode RLKs that confer resistance against the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae*.

The RPW8 class is the most recently discovered class of *R* genes. *RPW8* genes of *Arabidopsis* code for small proteins with an amino-terminal TM domain and an intracellular CC domain, which confer resistance against powdery mildew. Unlike most other *R* genes, *RPW8* has a broad resistance spectrum. However, the defense response induced by the *RPW8* genes is very similar to that triggered in pathogen-specific resistance (Xiao et al, 2001).

Some *R* genes do not fit into any of these five classes listed. One of them is *Hm1*, which codes for a toxin reductase, as mentioned above. Another is the barley *Mlo* gene, the recessive mutant allele of which confers resistance to the powdery mildew pathogen, *Blumeria graminis*; the protein product of *Mlo* is thought to be a negative regulator of plant defense responses (Büschges et al, 1997). The protein product of the $Hs1^{pro-1}$ gene of sugar beet, which confers resistance to the nematode *Heterodera schachtii*, lacks any obvious protein interaction domain (Cai et al, 1997). On the other hand, the *Ve* genes in tomato, which confer resistance to the fungus *Verticillium alboatrum*, code for putative cell-surface glycoproteins with receptor-mediated endocytosis-like signals (Kawchuk et al, 2001).

2.1.2 Structural domains of R proteins

Many *R* genes encode proteins that share some common structural domains, as mentioned above. Although, in many cases, the exact functions of these domains in R proteins are not known, the functional mechanisms of similar domains in other proteins, as well as mutational studies on R proteins provide important insight into the functional significance of these domains in R proteins.

2.1.2.1 The LRR domain

Most R proteins possess an LRR domain. This domain consists of a variable number of repeated motifs with leucines or other hydrophilic amino acids at regular distances, predicted to form flexible and parallel β-sheets (Jones and Jones, 1997; Martin et al, 2003). LRR domains are found in various proteins of diverse function and are known to be involved in protein-protein or protein-carbohydrate interactions, as well as peptide-ligand binding (Kajava, 1998). Experimental data for various R proteins suggest that LRR domains have an important role in recognition specificity (Ellis et al, 1999; He et al, 2000; Dodds et al, 2001). The LRR domain sequences in *R* genes have an overall high degree of tolerance for base substitutions, which would be necessary for the evolution of new specificities if the LRR domain is in fact involved in pathogen recognition (Dinesh-Kumar et al, 2000; Axtell et al, 2001; Tornero et al, 2002). On the other hand, some studies also suggest a signaling role for the LRR domain (Warren et al, 1998; Hwang et al, 2000).

2.1.2.2 The NBS

The NBS is essential for nucleotide-binding in ATP- and GTP-binding proteins (Saraste et al, 1990), although such a nucleotide-binding mechanism has not yet been clarified for R proteins (Dangl and Jones, 2001). The NBS found in R proteins is part of a larger domain that has homologous regions to the *Caenorhabditis elegans* caspase regulator CED-4 and its human homolog APAF-1, both of which are proteins of the apoptotic cell death mechanism. This shared domain in APAF-1, *R* gene products, and CED-4, known as NB-ARC, contains the kinase 1a (P-loop), kinase 2, and kinase 3a motifs, as well as five other short motifs of unknown function (van der Biezen and Jones, 1998b).

In *C. elegans* and humans, proteins attached to the mitochondrial membrane bind CED-4 and APAF-1, respectively, which in turn bind caspases, together

forming the apoptosome complex. When cell death is triggered, this complex dissociates, leading to the activation of caspases and further downstream signaling, followed by apoptosis. The functional significance of the sequence similarity of NBS-LRR-type R proteins to CED-4 and APAF-1 has not yet been clearly established. However, it has been proposed that the NBS of R proteins may be involved in ATP/GTP hydrolysis, leading to the activation of downstream effectors (van der Biezen and Jones, 1998b).

2.1.2.3 The CC domain

The CC structure 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 CC structure is found in a variety of proteins with diverse function and is thought to be involved in protein-protein interactions. The function of the CC domain in R proteins is not clear, but it seems to be involved in signaling rather than recognition (Martin et al, 2003).

2.1.2.4 The TIR domain

Since the TIR domain in animal proteins mediates protein-protein interactions important for the innate immune system (Horng and Medzhitov, 2001), the sequence similarity of the TIR domain of TNLs to the signaling domain of the *Drosophila* Toll and mammalian IL-1R proteins suggests that this domain may also be involved in signaling in plant disease resistance. It has been reported that the amino acid residues necessary for Toll and IL-1R signaling are also essential for the function of the tobacco *N* gene (Dinesh-Kumar et al, 2000), which further supports this view. Although usually thought to function in signal

transduction, there are studies suggesting that the TIR domain may also be involved in pathogen recognition, perhaps through interactions with the LRR domain (Luck et al, 2000).

Beside TNLs, more than 50 genes in the *Arabidopsis* genome encode proteins that carry a TIR domain, but lack either only the LRR domain or both the NBS and the LRR domains (Meyers et al, 2003). Members of these two gene families, referred to as TIR-NBS (TN) and TIR-X (TX), respectively, are also found in grass genomes in low numbers (Meyers et al, 2002).

2.1.2.5 The STK domain

Since phosphorylation of proteins is one of the most common ways in which living organisms regulate their biochemical activities, it is not surprising to find STK domains in some R proteins. Both the Pto protein in tomato (Sessa et al, 2000) and the kinase domain of Xa21 in rice (Liu et al, 2002) are functional STKs. Autophosphorylation of Pto is required for the AvrPto-dependent *P. syringae* resistance in tomato (Sessa et al, 2000). Although Pto interacts directly with AvrPto, it has no distinct receptor domain and resistance is dependent on the presence of Prf, a CNL-type R protein. Constitutive expression of Pto, on the other hand, leads to resistance that is still Prf-dependent, but Avr-Pto-independent (Rathjen et al, 1999).

2.1.3 *R*-gene-mediated defense response

Although many studies have been conducted to understand how R proteins recognize their effectors and how they are involved in signal transduction to promote disease resistance, this process has been slower than might have been expected. The main reason for this is that plant cells have very low abundance of R proteins, thus making the biochemical study of these proteins difficult (Martin et al, 2003). Therefore,

our understanding of R protein function, especially its role in effector recognition, depends largely on models that are based on relatively small amount of biochemical information available and the partial sequence homology of R proteins to known functional domains.

2.1.3.1 Effector recognition

The simplest mechanism for effector recognition follows the receptor-ligand model, where the *R* gene product interacts directly with the Avr protein to initiate host defense response. However, this model is an oversimplification, since a direct interaction with the Avr protein has not been demonstrated for most R proteins. It is more probable that the recognition takes place as a result of the formation of a complex containing both host and pathogen proteins.

The most influential model for the indirect effector recognition of R proteins is the "guard" hypothesis (van der Biezen and Jones, 1998a). This model is based on the dual requirement of Prf and Pto for AvrPto-triggered resistance in tomato. It suggests that the Avr protein of the pathogen (e.g. AvrPto) interacts with a target plant protein (e.g. Pto) to promote disease and that the R protein (e.g. 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 mechanisms in plants.

2.1.3.2 Signal transduction

The studies on cellular processes following effector recognition in *R*-gene-mediated resistance in several different plants have revealed essential similarities (Jabs et al, 1997; Piedras et al, 1998; Felix et al, 1999; Grant et al, 2000). Within minutes of pathogen attack, changes in ion flux, including calcium influx, occurs, followed by the production of reactive oxygen intermediates (ROIs), including H_2O_2 and/or O_2^-, and the activation of mitogen-activated protein kinase and other protein kinase pathways (Ligterink et al, 1997; Romeis et al, 1999). The role of ROIs in host response may be pathogen elimination, downstream signaling, or both. In addition to these responses, some studies have shown that nitric oxide (NO) accumulation occurs via an unidentified pathway (Delledonne et al, 1998).

Signaling molecules like protein kinases and transcription factors are expressed within 15 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). The protein kinases are either upstream or independent of the oxidative response, and may be involved in the activation of latent transcription factors that are required for the activation of defense genes (Dröge-Laser et al, 1997). 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.

2.1.4 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.

2.1.4.1 *R* genes as members of a plant gene family

Evolutionary studies on plant *R* genes focus on NBS-LRR genes since they comprise the majority of the identified *R* genes, are highly abundant in plant genomes, and are known to function only as resistance factors. Since *Arabidopsis* and rice genomes have been sequenced, most sequence information available on *R* genes and RGAs comes from these two species, usually taken as models for dicots and grasses, respectively.

Rice genome contains over 600 putative NBS-LRR genes, none of which are of the TNL-type. On the other hand, the majority of more than 150 putative NBS-LRR genes in *Arabidopsis* are TNLs. Sequence analyses of the NBS-LRR genes in *Arabidopsis* predict that TNLs are more homogenous and that they have amplified more recently than CNLs (Meyers et al, 2003). These finding together suggest that the amplification of TNL sequences has occurred after the divergence of monocots and dicots.

NBS-LRR genes are often found in clusters composed of tandemly-duplicated paralogs (Michelmore and Meyers, 1998). These extended clusters are predicted to have resulted from unequal crossing-over. Although there is frequent exchange of sequences within NBS-LRR clusters in *Arabidopsis*, there is no

evidence in support of sequence exchange between related NBS-LRR genes in different clusters (Baumgarten et al, 2003). However, sequence analyses of *Arabidopsis* genome also suggest 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 "ectopic duplications" (Leister, 2004).

2.1.4.2 Host-pathogen co-evolution

Since functional Avr proteins are required for *R*-gene-mediated resistance, why the *Avr* genes have evolved in pathogens is a big question. The widely-accepted theory suggests that the *Avr* genes of pathogens have initially evolved to function as virulence factors (Vivian and Gibbon, 1997). *R* genes, in turn, have evolved as a plant surveillance system to recognize the virulent pathogens and induce host defense responses.

The "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 could be defined as a simple receptor-ligand interaction involving only one R protein and one Avr protein. However, plant-pathogen interactions are generally 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, rather than a simple interaction between single R and Avr proteins, is the likely determinant of compatibility or incompatibility 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 analyses 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 the 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 Avr proteins produced by 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.

All plant-pathogenic bacteria have HR and pathogenicity (*hrp*) genes, which are involved in directing the ability of the bacteria for pathogenicity in host and HR in non-host plants (Lindgren, 1997). These *hrp* genes encode a type-III protein secretion system that is capable of delivering Avr proteins into the cytoplasm of host plant cells (Alfano and Collmer, 1997). This finding helps to explain the lack of evidence for cellular localization of NBS-LRR proteins, especially for those involved in resistance to bacterial pathogens.

2.1.4.3 Comparison of plant R proteins to animal proteins

As discussed above, the NB-ARC domain found in all plant NBS-LRR proteins is shared by the *C. elegans* caspase regulator CED-4 and its human homolog APAF-1, both of which are proteins of the apoptotic cell death mechanism. In addition to the NB-ARC domain, TNLs contain a TIR domain that is homologous to the signaling domain in the *Drosophila* Toll and mammalian IL-1R proteins, which is involved in protein-protein interactions important for innate immunity.

The *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 defense responses, in a similar manner to R proteins.

Figure 2.2 presents a comparison of R protein structures to homologous animal proteins. The high level of sequence and structural similarities between animal and plant defense-related proteins suggest that these proteins may have evolved from a common ancestor.

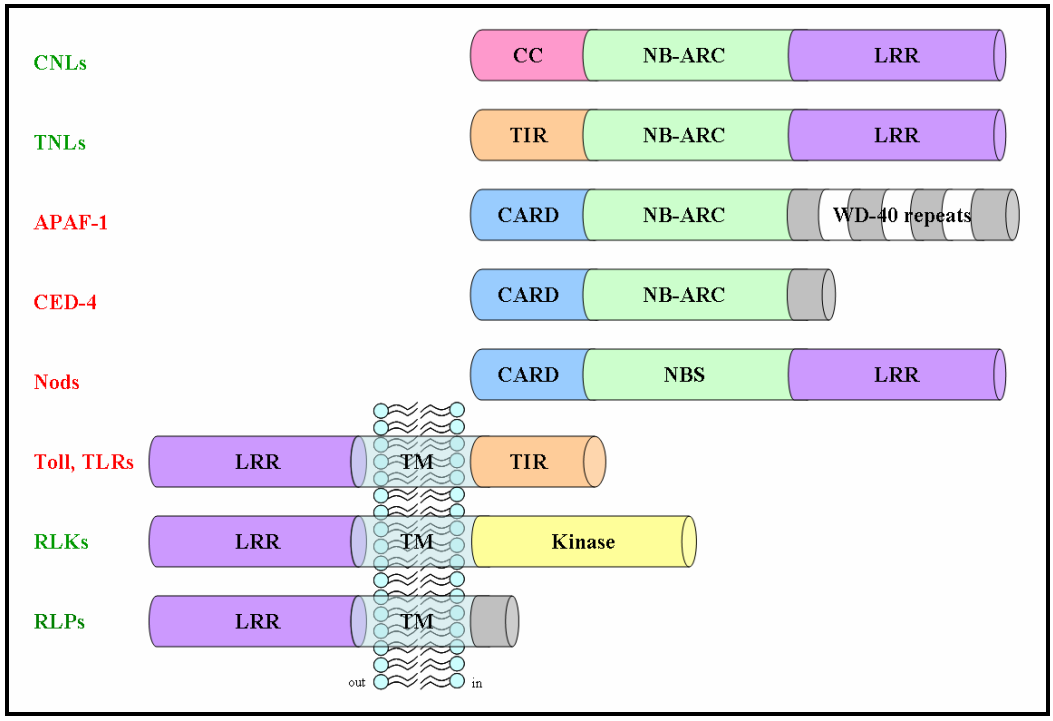

Figure 2.2 Comparison of R protein structures to homologous animal proteins. Not drawn to scale. Plant proteins are shown in green; animal proteins are shown in red. See text for abbreviations. (adapted from Dangl and Jones, 2001)

2.1.5 Utilization of *R* genes

For nearly a century, breeders have been studying the inheritance of *R* genes and using classical breeding techniques for introgression of *R* genes from wild populations into elite cultivars (Biffen, 1912). Traditional breeding methods are very time-consuming; development of resistant cultivars may take up to 15-20 years. Even after such a long and laborious process, the breeders are faced with various problems. Many *R* genes do not confer durable disease resistance in the host cultivar, some are genetically linked to undesirable traits, and in some cases the pathogens rapidly evolve to overcome the resistance conferred by the selected *R* genes (Rommens and Kishore, 2000).

As molecular data for *R* genes started to become available, markers were developed to aid selection. In marker-assisted breeding programs, the progeny is screened at the molecular-level, without the need to grow the plants for observation of their resistance phenotypes. Therefore, the time needed to develop resistant cultivars using molecular markers may be 50-70 % less than the time needed in traditional breeding programs (Schneider et al, 1997).

Further research on *R* genes has allowed the successful transfer of these genes within and, in some cases, across plant species to confer disease resistance in the host. For example, the pepper *Bs2* gene, which confers resistance to the bacterial pathogen *Xanthomonas campestris*, was transferred to tomato (Tai et al, 1999), and the tomato *Pto* gene, which confers resistance to *P. syringae*, was transferred to tobacco (Rommens et al, 1995). Both genes were shown to maintain their resistance phenotypes in the host plants. Since transgenic studies allow the isolation and transfer of single genes, retention of unwanted and genetically-linked traits is no more a problem. Recombinant DNA techniques also allow the simultaneous transfer of multiple *R* genes to a host, which might enhance the chances for durable resistance since tightly-linked *R* genes can act synergistically (Parniske et al, 1997).

2.2 PCR-based identification of RGAs

The NB-ARC domain of NBS-LRR proteins contains several highly-conserved motifs (Figure 2.3). For plants like *Arabidopsis* and rice, RGAs may be identified simply by searching the genome for regions homologous to these conserved motifs. However, extensive genomic sequences are not available for most plants. Therefore, Leister et al (1996) developed a PCR-based method to easily isolate RGAs from a large variety of plant species. 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. This PCR-based method made it easier to isolate putative *R* genes, or RGAs, from plants. Although other PCR primers that target different conserved motifs of *R* genes have been developed in the past decade (Yu et al, 1996; Peñuela et al, 2002; Yuksel et al, 2005), primers targeting the kinase 1a and GLPL motifs are still the most commonly-used primers in PCR-based identification of RGAs.

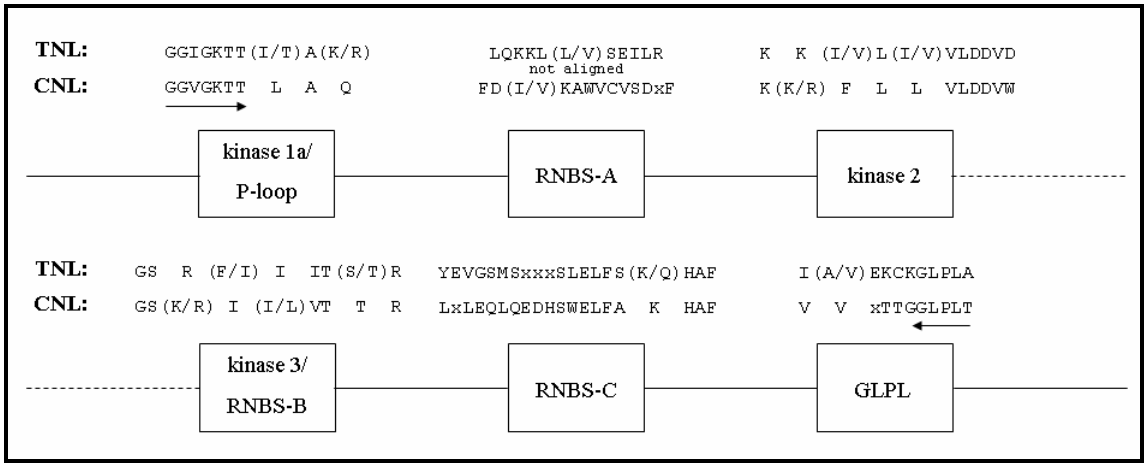

Figure 2.3 Some conserved motifs of the NB-ARC domain. Not drawn to scale. Sequences above the boxes are the consensus sequences of the motifs for TNLs and CNLs. Arrows indicate the target regions of the primers used in this study. (adapted from Peñuela et al, 2002)

RGAs have been 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 (Seah et al, 1998), 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 tree (Noir et al, 2001), chickpea (Huettel et al, 2002), barrel medic (Zhu et al, 2002), grapevine (Di

Gaspero and Cipriani, 2002), peanut (Bertioli et al, 2003), cotton (Tan et al, 2003), cassava (López et al, 2003), pine (Liu and Ekramoddoullah, 2003), strawberry (Martínez Zamora et al, 2004), oat (Irigoyen et al, 2006), and buffalograss (Budak et al, 2006).

The majority of the studies that utilize the PCR-based method use genomic DNA as the template. Although a large number of RGAs are amplified in such studies, many of the identified RGAs are probably pseudogenes (Pan et al, 2000a) or non-functional genes. RGAs amplified from cDNA are, on average, 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. However, only very few reports of PCR-based amplification of RGAs from cDNA template were found (Liu and Ekramoddoullah, 2003; Budak et al, 2006).

2.3 Tall fescue (*Festuca arundinacea*)

Tall fescue is an important cool-season perennial forage grass species that belongs to the family Poaceae and the subfamily Festucoideae. It is widely grown in the east central and southeast USA, southern Europe, and many other parts of the world. Tall fescue is commonly used in pastures, lawns, sports fields, highway medians and roadsides (Barnes, 1990). It is a wind-pollinated allohexaploid species with a high degree of self-incompatibility, which makes the breeding of tall fescue difficult and complex (Stadelmann et al, 1999). Genetic transformation of tall fescue plants has been achieved both by direct gene transfer to protoplasts (Wang et al, 1992; Dalton et al, 1995) and by particle bombardment (Spangenberg et al, 1995; Cho et al, 2000). *F. arundinacea* has also been utilized as a model system for large-scale sequencing of expressed sequence tags (ESTs), gene discovery, and genetic engineering (Wang et al 2001). To date, no *R* genes have been isolated from tall fescue, although a quantitative trait locus (QTL) conferring resistance to the crown rust pathogen *Puccinia coronata* Corda has been mapped in meadow fescue (*F. pratensis*) and this crown rust resistance has been transferred to Italian ryegrass (*Lolium multiflorum*; Roderick et al, 2003). To our knowledge, this study reports the first attempt to identify RGAs in *Festuca* species.

2.4 Bermudagrass (*Cynodon dactylon*)

Bermudagrass is a warm-season perennial turf grass species of the family Poaceae and the subfamily Chloridoideae. Although it is native to North Africa and southern Europe, bermudagrass can be found in many temperate to tropical regions of the world today. Since it stays green during hot weather, *C. dactylon* is a valuable pasture and excellent fodder grass (Hu et al, 2005). Molecular biology and genetics-based studies on bermudagrass are mostly limited to genetic transformation studies using *Agrobacterium*-mediated (Li et al, 2005) and biolistic transformation methods (Li and Qu, 2004), assessment of genetic variation in bermudagrass cultivars utilizing molecular markers (Wu et al, 2004), and a recent linkage-mapping study (Bethel et al, 2006). To date, no *R* genes have been isolated from *Cynodon* species and this study reports the first attempt to identify RGAs in bermudagrass.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

Festuca arundinacea 'Apache' and *Cynodon dactylon* seeds used in this study were obtained from a commercial seed company.

3.1.2 Equipment and supplies

The equipment and supplies used in this study are listed in Appendix A and Appendix B, respectively.

3.1.3 Growth media, buffers, and solutions

Enzymes were only used with the buffers supplied by the manufacturers. All other growth media, buffers, and solutions used in this study were prepared following the protocols outlined by Sambrook and Russell (2001).

3.2 Methods

3.2.1 Plant growth

Seeds surface-sterilized in 5 % Plant Preservative Mixture™ (PPM) for 7 hours were planted in plant tissue culture medium containing 4.4 g/L Murashige and Skoog (MS) medium basal salt mixture including vitamins, 30 g/L sucrose, 0.1 % PPM, and 7 g/L agar (pH 5.7). Plants were grown in a 26-ºC growth room with a photoperiodicity of 16:8 (light:dark).

3.2.2 Total RNA isolation

200 mg shoot and 300 mg root samples were excised from 3-week-old *F. arundinacea* and 6-week-old *C. dactylon* seedlings. Root samples were swirled in warm diethyl pyrocarbonate (DEPC)-treated H_2O and briefly blot-dried on sterile filter paper to remove agar contamination. Samples were homogenized in 2 mL of TRIzol® reagent using mortars and pestles previously treated with 70 % ethanol for 5 hours and autoclaved. The homogenized samples were transferred to microtubes and incubated for 5 minutes at room temperature. 0.4 mL of chloroform was added to each sample. The tubes were shaken vigorously for 15 seconds and incubated for 3 minutes at room temperature. The samples were centrifuged at 11,000 x *g* for 15 minutes at 4 ºC. The upper aqueous phase from each sample was transferred to a clean microtube. RNA was precipitated by addition of 1 mL of 2-propanol and mixing. The samples were incubated for 10 minutes at room temperature and centrifuged at 11,000 x *g* for 10 minutes at 4 ºC. The supernatant was discarded and the pellets were washed with 2 mL of 75 % ethanol. The samples were centrifuged at 7,500 x *g* for 5 minutes at 4º C. The supernatant was discarded and the pellets were briefly air-dried. The pellets were dissolved in 50 µL of DEPC-treated H_2O and incubated for 10 minutes at 55 ºC. RNA was quantified by measuring the absorbance of samples at 260 nm using a NanoDrop spectrophotometer. The samples were stored at -20 ºC.

3.2.3 DNase I treatment

DNase treatment of the RNA samples was performed in 50-µL reactions containing 1X Reaction Buffer with $MgCl_2$, 5 µg of RNA sample, and 5 u of RNase-free DNase I. The reactions were incubated for 30 minutes at 37 ºC in a water bath. For DNase I clean-up, 5 µL of 25 mM EDTA was added and the samples were incubated for 10 minutes at 65 ºC. RNA was quantified by measuring the absorbance of samples at 260 nm using a NanoDrop spectrophotometer. The samples were stored at -20 ºC.

3.2.4 First strand cDNA synthesis

First strand cDNA synthesis was done using the Omniscript RT Kit in 20-µL reactions containing 1X Buffer RT, 0.5 mM dNTP mix, 0.5 µg of oligo$(dT)_{12-18}$ primer, 10 u of RNaseOUT™ Recombinant Ribonuclease Inhibitor, 1 µg of DNase I-treated RNA sample, and 4 u of Omniscript Reverse Transcriptase. The samples were incubated for 60 minutes at 37 ºC and stored at -20 ºC.

3.2.5 Initial amplification

Degenerate primers designed by Budak et al (2006), which target the conserved kinase 1a and GLPL motifs of the NB-ARC domain, were used for the amplification of RGAs (Table 3.1). 75-µL PCR reactions containing 1X *Pfu* Buffer with $MgSO_4$, 0.2 mM dNTP mix, 0.2 µM primers, 2.5 µL of first strand cDNA, and 2.5 u of *Pfu* DNA Polymerase were carried out at 2 minutes of initial denaturation at 95 ºC, 35 cycles of 95 ºC for 1 minute, 45 ºC for 1 minute and 72 ºC for 1 minute and 30 seconds, and a final elongation at 72 ºC for 5 minutes. 75 µL of PCR products were run on 1 % agarose gel in 0.5X TBE buffer stained with ethidium bromide at 100 V for 1 hour. The bands were visualized under UV light.

Table 3.1 Primers used for RGA amplification. (adapted from Budak et al, 2006)

Name	Orientation	Sequence	Target motif
RGL1	Forward	5'-GGAGGGGTTGGGAARACAAC-3'	kinase 1a
	Reverse	5'-CCHACGCCRATGGAWGACC-3'	GLPL
RGL2	Forward	5'-GGGGGGGTGGGGAAGACGAC-3'	kinase 1a
	Reverse	5'-AGGGCTAAGGGGAGGCCACGAC-3'	GLPL
RGL5	Forward	5'-GGGGGGGTGGGGAAGACGAC-3'	kinase 1a
	Reverse	5'-AGGGCTAGGGGGAGGCCCGCC-3'	GLPL

3.2.6 Gel extraction

The agarose gel fragments of molecular weight 400-600 bp, as indicated by the molecular weight marker, were excised with a clean scalpel. QIAquick Gel Extraction Kit was used following the manufacturer's protocol to obtain the cDNA fragments of desired molecular weight. cDNA was eluted in 30 μL of Elution Buffer and quantified by measuring the absorbance of samples at 260 nm using a NanoDrop spectrophotometer. The samples were stored at -20 ºC.

3.2.7 Amplification of gel extraction products

The original primers used for the initial amplification (Table 3.1) were also used in the amplification of gel extraction products. 100-μL PCR reactions containing 1X *Pfu* Buffer with $MgSO_4$, 0.2 mM dNTP mix, 0.2 μM primers, 15 μL of gel extraction product and 3 u of *Pfu* DNA Polymerase were carried out at 2 minutes of initial denaturation at 95 ºC, 35 cycles of 95 ºC for 1 minute, 55 ºC for 1 minute and 72 ºC for 1 minute and 30 seconds, and a final elongation at 72 ºC for 5 minutes. 10 μL of PCR products were run on 1 % agarose gel in 0.5X TBE buffer stained with ethidium bromide at 100 V for 1 hour. The bands were visualized under UV light.

3.2.8 PCR purification

90 μL of PCR products were purified using the QIAquick PCR Purification Kit. The manufacturer's protocol was followed directly. cDNA was eluted in 30 μL of Elution Buffer and quantified by measuring the absorbance of samples at 260 nm using a NanoDrop spectrophotometer. The samples were stored at -20 ºC.

3.2.9 A-tailing

Adenosine tails were added to the blunt-ended PCR products in 10-μL reactions containing 1X Mg-free Taq Buffer, 2 mM $MgCl_2$, 0.2 mM dATP, 7 μL of purified PCR product, and 5 u of aTaq DNA Polymerase. The reactions were incubated at 70 ºC for 30 minutes. The samples were stored at -20 ºC.

3.2.10 Ligation to vector

The PCR products were ligated to the pGEM®-T Vector (Appendix C) in 10-μL reactions containing 1X Rapid Ligation Buffer, 50 ng of pGEM®-T Vector, 3 μL of A-tailed PCR product, and 3 u of T4 DNA Ligase. The ligation reactions were incubated at 4 ºC for 12 hours.

3.2.11 Transformation

2 μL of each ligation reaction was added to a sterile 1.5-mL microtube on ice. 50 μL of JM109 High Efficiency Competent Cells (*Escherichia coli*) were transferred to the microtubes containing the ligation reactions. The tubes were gently mixed by

flicking and incubated on ice for 20 minutes. The cells were heat-shocked for 50 seconds in a water bath at 42 ºC and were immediately returned to ice for 2 minutes. 950 µL of SOC medium was added to each tube. The transformation reactions were incubated at 37 ºC for 1.5 hours with shaking at 150 rpm. 100- and 200-µL aliquots of transformation reactions were plated on LA plates containing 100 µg/mL ampicillin, 10 nmol of IPTG, and 1 µg of X-Gal. The plates were incubated at 37 ºC for 12 hours.

3.2.12 Plasmid isolation

Selected white colonies were inoculated in 2 mL of LB medium containing 100 µg/mL ampicillin and incubated at 37 ºC for 10 hours with shaking at 270 rpm. The bacterial cells were harvested by centrifugation at 8,500 x *g* for 3 minutes at room temperature. Plasmids were isolated using QIAprep Spin Miniprep Kit. The manufacturer's protocol was followed directly. DNA was eluted in 30 µL of Elution Buffer and quantified by measuring the absorbance of samples at 260 nm using a NanoDrop spectrophotometer. The samples were stored at -20 ºC.

3.2.13 Plasmid screening

Two PCR reactions were done for each plasmid, one using the primers that the inserts were originally amplified with, and the other using the universal primers SP6 (5'-TATTTAGGTGACACTATAG-3') and T7 (5'-TAATACGACTCACTATAGGG-3'). 20-µL PCR reactions containing 1X Mg-free Taq Buffer, 2 mM $MgCl_2$, 0.2 mM dNTP mix, 0.2 µM primers, 0.5 µL of plasmid DNA, and 1.0 u of aTaq DNA Polymerase were carried out at 2 minutes of initial denaturation at 95 ºC, 35 cycles of 95 ºC for 1 minute, 45 ºC for 1 minute and 72 ºC for 1 minute and 30 seconds, and a final elongation at 72 ºC for 5 minutes. 3.5 µL of PCR products were run on 1 % agarose gel in 0.5X TBE buffer stained with ethidium bromide at 135 V for 30-45 minutes. The bands were visualized under UV light.

3.2.14 Sequencing

Inserts of selected plasmids were sequenced commercially by İontek, Turkey (http://www.iontek.com.tr). Sequencing reactions were performed using the SP6 universal primer.

3.2.15 Sequence analysis

The sequences obtained were first exposed to the VecScreen algorithm (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) in order to remove the contaminating vector sequences. BLASTN, BLASTX, and TBLASTX algorithms (Altschul et al, 1997) were used to compare the insert sequences to sequences available at the Entrez nucleotide and protein databases (http://www.ncbi.nlm.nih.gov/BLAST). The CLUSTALW algorithm (Thompson et al, 1994) was used for the multiple sequence alignments and the BL2SEQ algorithm (Altschul et al, 1997) was used for comparing two amino acid sequences to each other.

Protein secondary structure prediction was done using the PELE Protein Structure Prediction Tool available at Biology WorkBench (http://workbench.sdsc.edu). This tool utilizes seven different algorithms, and each algorithm assigns a structure to each amino acid as α-helix, β-strand or coil. This tool also provides a "joint prediction," which incorporates the predictions made by the other algorithms and uses a "winner takes all" procedure for each amino acid prediction to assign the structure.

4 RESULTS

4.1 Plant growth

The plants were grown under sterile conditions in tissue culture environment. No contamination was observed on the growth medium or on the plants. The seedlings were free of chlorotic or necrotic signs at the time of RNA isolation.

4.2 Initial amplification and gel extraction

Results of the initial PCR amplification are presented in Figure 4.1. None of the samples displayed a clear and specific band at the expected size of 500 bp, although FS1, FR1, FR2, FR5, BR1, BS2, BR2, and BS5 (see Appendix D for a list of the abbreviated names of the samples) displayed a faint band around that size, along with various bands of different sizes. The agarose gel pictures of FS2, FS5, BS1, and BR5 showed either a light smear or no visible presence of DNA at all, around the 500-bp region. However, DNA was extracted from agarose gel pieces corresponding to molecular weight between 400 bp and 600 bp for all samples, regardless of the presence or absence of a clear band in this molecular weight range (Figure 4.1). The concentrations of the gel extraction products were between 1.52 and 16.45 ng/μL. To increase the concentrations, the PCR amplifications were repeated using the gel extraction products as the template.

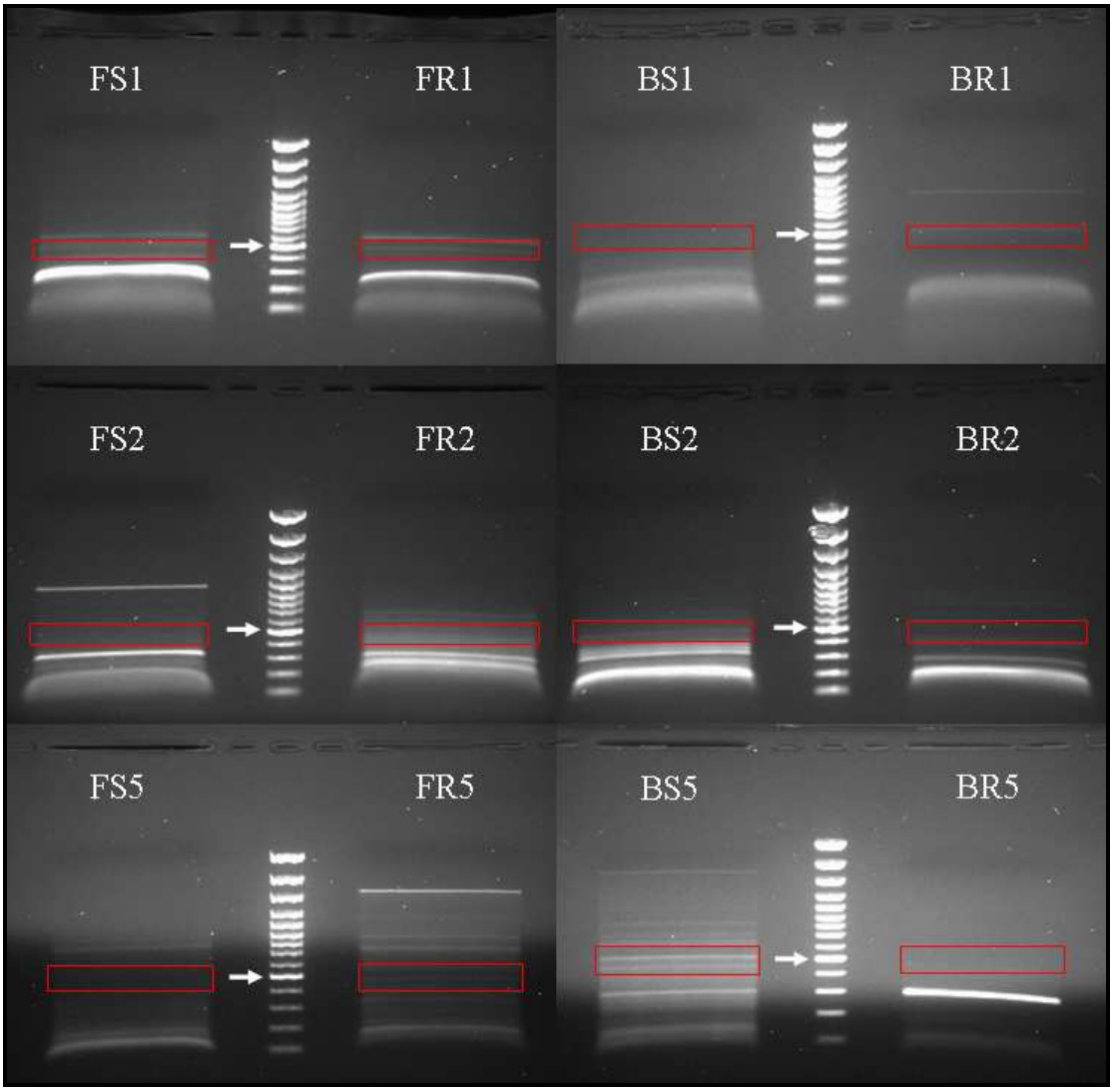

Figure 4.1 Agarose gel electrophoresis results of the initial amplification products.
The arrows point to the 500-bp band of the molecular weight marker.
The red boxes indicate the regions that were excised and used for gel extraction.
See Appendix D for a list of the abbreviated names of the samples.

4.3 Amplification of gel extraction products and PCR purification

The re-amplified gel extraction products were run on 1 % agarose gel (Figure 4.2). FR2, FS5, BS1, BR1, BS2, BR2, BS5, and BR5 displayed bands at around 500 bp, while the other samples showed either a light smear or no visible presence of DNA at all, around that size. All amplified gel extraction products were purified. The concentrations of the PCR purification products were between 10.51 and 61.31 ng/μL; all samples showed an increase in concentration from the gel extraction products.

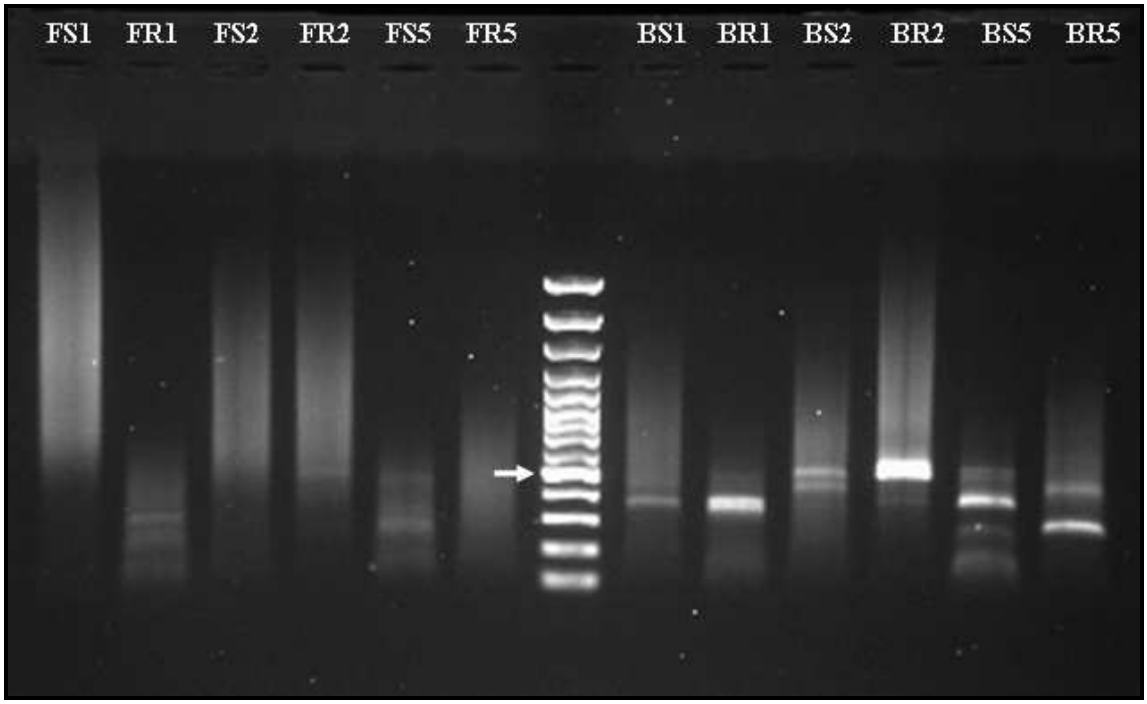

Figure 4.2 Agarose gel electrophoresis results of the PCR purification products. The arrow points to the 500-bp band of the molecular weight marker. See Appendix D for a list of the abbreviated names of the samples.

4.4 Transformation and plasmid screening

JM109 High Efficiency Competent Cells (*E. coli*) were transformed with the PCR purification products A-tailed and ligated into pGEM®-T Vector. 10 white colonies were randomly chosen for each of the twelve samples for plasmid isolation. Isolated plasmids were screened by PCR using the primer pair that the inserts were originally amplified with or the universal primers SP6 and T7, which have binding sites on the pGEM®-T Vector (Appendix C). The PCR products were run on 1 % agarose gel. Figure 4.3 is a representative agarose gel picture of the PCR products; electrophoresis results of the other samples were very similar. For most samples, no band of the expected size was visible for the PCR amplification done with the RGL primers, and the bands that were visible were usually very faint. Also, almost all of the plasmids amplified with the RGL primers showed a bright smear at the regions corresponding to molecular weight over 3,000 bp. Therefore, the plasmids to be sequenced were selected based on the result of their amplification with the universal primers. A total of 35 plasmids that showed a SP6/T7 band at around 650 bp were chosen for sequencing.

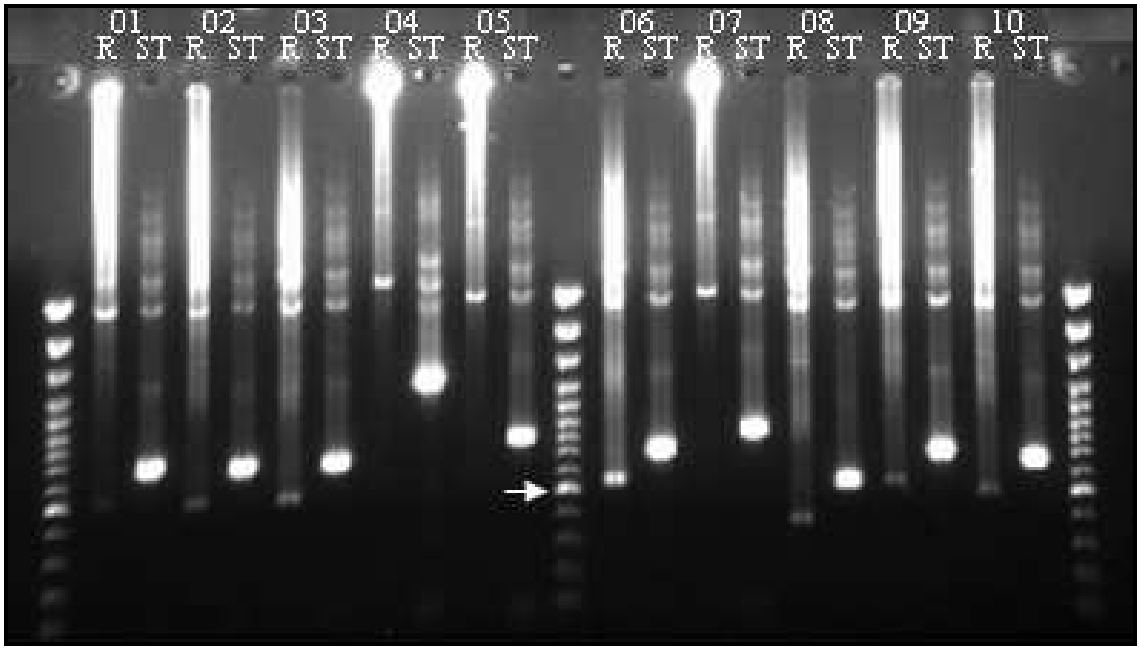

Figure 4.3 Plasmid PCR results of the BR1 clones.
R: plasmids amplified with RGL1 primers; ST: plasmids amplified with SP6/T7 primers.
The arrow points to the 500-bp band of the molecular weight marker.

4.5 Sequence analysis

The obtained sequences were first exposed to the VecScreen algorithm in order to remove the vector contamination. The insert sequences were then compared to the nucleotide and protein sequences available at the Entrez nucleotide and protein databases using the BLASTN, BLASTX, and TBLASTX algorithms. Alignments with an E Value < 0.0001 were considered to be significant. Out of the 35 sequences, 4 did not show significant similarity to any known sequence, 6 aligned with sequences of unknown function, 21 were significantly similar to genes not related to disease resistance, and 4 were homologous to known NBS-LRR-type *R* genes and RGAs in various plant species. Table 4.1 shows a list of the 25 clones that were homologous to known genes. All information given in Table 4.1 was obtained by comparing the deduced amino acid sequence of the inserts to the Entrez protein database, with the exception of FS1-05, FS1-08, FR5-01, and BS1-07, the nucleotide sequence of which were compared directly to the Entrez nucleotide database to obtain the homologs presented in this table.

Table 4.1 Homology of the clone inserts to known genes or proteins.

Sample	Homolog(s)
FS1-03	NBS-LRR-type R protein (*Oryza sativa*, *Hordeum vulgare*, *Triticum aestivum*, *Triticum monococcum*, *Vitis bashanica*,…)
FS1-05	NADH dehydrogenase (*Zea mays*), 23s rRNA (*Festuca arundinacea*, *Saccharum* hybrid cultivar)
FS1-08	NADH dehydrogenase (*Zea mays*), 23s rRNA (*Festuca arundinacea*, *Saccharum* hybrid cultivar), small subunit rRNA (*Anthoceros punctatus*), large subunit rRNA (*Dioscorea* sp., *Sphagnum rubellum*),…
FS5-01	esterase (*Oryza sativa*, *Alopecurus myosuroides*, *Avena sativa*, *Arabidopsis thaliana*, *Brassica napus*,…)
FR1-05	myosin heavy chain (*Oryza sativa*, *Zea mays*, *Nicotiana tabacum*, *Arabidopsis thaliana*, *Helianthus annus*,….)
FR1-09	methionine synthase (*Oryza sativa*, *Hordeum vulgare*, *Sorghum bicolor*, *Zea mays*, *Catharanthus roseus*,…)
FR5-01	NADH dehydrogenase (*Zea mays*), retrotransposon protein (*Oryza sativa.*), 18s rRNA (*Cucumis melo*, *Triticum aestivum*, *Plantago sericea*),…
BS1-06	chaperonin-60 α subunit (*Oryza sativa*, *Triticum aestivum*, *Arabidopsis thaliana*, *Pisum sativum*, *Trifolium pratense*,…)
BS1-07	18s rRNA (*Zea mays*, *Hordeum vulgare*, *Sorghum bicolor*, *Tripsacum dactyloides*, *Hevea brasiliensis*,…)
BS2-02	Cytochrome p450 monooxygenase (*Oryza sativa*, *Medicago truncatula*, *Gossypium arboreum*, *Arabidopsis thaliana*, *Sorghum bicolor*…)
BS2-04	DNA-binding protein (*Arabidopsis thaliana*, *Medicago truncatula*, *Oryza sativa*, *Pisum sativum*)
BS2-08	DNA-binding protein (*Arabidopsis thaliana*, *Medicago truncatula*, *Oryza sativa*, *Pisum sativum*)
BS2-09	Cytochrome p450 monooxygenase (*Oryza sativa*, *Medicago truncatula*, *Gossypium arboreum*, *Arabidopsis thaliana*, *Sorghum bicolor*…)
BS2-10	Cytochrome p450 monooxygenase (*Oryza sativa*, *Medicago truncatula*, *Gossypium arboreum*, *Arabidopsis thaliana*, *Sorghum bicolor*…)
BS5-01	Chlorophyll a/b-binding protein (*Zea mays*, *Oryza sativa*, *Triticum aestivum*, *Musa balbisiana*, *Pinus roxburghii*,…)

Table 4.1 (continued)

BS5-02	glutathione S-transferase (*Cynodon dactylon*, *Zea mays*, *Pennisetum glaucum*, *Oryza sativa*, *Triticum aestivum*,…)
BS5-03	DNA-binding protein (*Arabidopsis thaliana*, *Medicago truncatula*, *Oryza sativa*, *Pisum sativum*)
BS5-04	DNA-binding protein (*Arabidopsis thaliana*, *Medicago truncatula*, *Oryza sativa*, *Pisum sativum*)
BS5-09	oxygen-evolving protein (*Oryza sativa*, *Fritillaria agrestis*, *Nicotiana tabacum*, *Lycopersicon esculentum*, *Spinacia oleracea*,...)
BR1-02	NBS-LRR-type R protein (*Oryza sativa*, *Hordeum vulgare*, *Triticum aestivum*, *Vitis bashanica*, *Zea mays*,…)
BR1-03	NBS-LRR-type R protein (*Oryza sativa*, *Hordeum vulgare*, *Triticum aestivum*, *Vitis bashanica*, *Zea mays*,…)
BR1-10	NBS-LRR-type R protein (*Oryza sativa*, *Hordeum vulgare*, *Triticum aestivum*, *Vitis bashanica*, *Zea mays*,…)
BR2-06	aquaporin (*Hordeum vulgare*, *Zea mays*, *Vitis vinifera*, *Raphanus sativus*, *Oryza sativa*,…)
BR5-03	oxygen-evolving protein (*Oryza sativa*, *Fritillaria agrestis*, *Nicotiana tabacum*, *Lycopersicon esculentum*, *Spinacia oleracea*,...)
BR5-07	oxygen-evolving protein (*Oryza sativa*, *Fritillaria agrestis*, *Nicotiana tabacum*, *Lycopersicon esculentum*, *Spinacia oleracea*,...)

The insert sequences of the FS1-03, BR1-02, BR1-03, and BR1-10 clones showed great homology to known NBS-LRR-type *R* genes, as well as to numerous RGAs and NB-ARC domain-containing proteins. All of these four sequences represent fragments amplified with the RGL1 primers. One of these fragments (FS1-03) was amplified from tall fescue shoot cDNA, whereas the other three (BR1-02, BR1-03, BR1-10) were amplified from bermudagrass root cDNA. The amplified regions (excluding the primer sequences) of BR1-02, BR1-03, and BR1-10 were identical to one another and they differed only in 2 out of 463 nucleotides from FS1-03 (Figure 4.4).

```
FS1-03    1 GGAGGGGTTGGGGAGACAACCATTGCGTTGGCGCTCTACCGGAACAGCGG 50
BR1-02    1 GGAGGGGTTGGGAAAACAACCATTGCGTTGGCGCTCTACCGGAACAGCGG 50
BR1-03    1 GGAGGGGTTGGGAAGACAACCATTGCGTTGGCGCTCTACCGGAACAGCGG 50
BR1-10    1 GGAGGGGTTGGGAAAACAACCATTGCGTTGGCGCTCTACCGGAACAGCGG 50

FS1-03   51 GGATCAGTTTGATTTCCGGGCGATGGTCACGTTATCTCGCTCCTCCGACA 100
BR1-02   51 GGATCAGTTTGATTTCCGGGCGATGGTCACGTTATCTCGCTCCTCCGACA 100
BR1-03   51 GGATCAGTTTGATTTCCGGGCGATGGTCACGTTATCTCGCTCCTCCGACA 100
BR1-10   51 GGATCAGTTTGATTTCCGGGCGATGGTCACGTTATCTCGCTCCTCCGACA 100

FS1-03  101 TTGAGACGGTCCTCGTCGATATACTGAGCCAAGTCAAGCTGCAAGGACAG 150
BR1-02  101 TTGAGACGGTCCTCGTCGATATACTGAGCCGAGTCAAGCTGCAAGGACAG 150
BR1-03  101 TTGAGACGGTCCTCGTCGATATACTGAGCCGAGTCAAGCTGCAAGGACAG 150
BR1-10  101 TTGAGACGGTCCTCGTCGATATACTGAGCCGAGTCAAGCTGCAAGGACAG 150

FS1-03  151 CAAGGCCACCATGGAGGTATTTCTTACCTTACACGAGAGCTGGCGATGCA 200
BR1-02  151 CAAGGCCACCATGGAGGTATTTCTTACCTTACACGAGAGCTGGCGATGCA 200
BR1-03  151 CAAGGCCACCATGGAGGTATTTCTTACCTTACACGAGAGCTGGCGATGCA 200
BR1-10  151 CAAGGCCACCATGGAGGTATTTCTTACCTTACACGAGAGCTGGCGATGCA 200

FS1-03  201 CCTCGAAGGACGAAGCTACTTGATCTTAGTTGATGACGTATGGTCTCCAT 250
BR1-02  201 CCTCGAAGGACGAAGCTACTTGATCTTAGTTGATGACGTATGGTCTCCAT 250
BR1-03  201 CCTCGAAGGACGAAGCTACTTGATCTTAGTTGATGACGTATGGTCTCCAT 250
BR1-10  201 CCTCGAAGGACGAAGCTACTTGATCTTAGTTGATGACGTATGGTCTCCAT 250

FS1-03  251 ATATGTGGGAGAAGATTAAATACTCACTGCCTAGAACTAACAGAGGCAGT 300
BR1-02  251 ATATGTGGGAGAAGATTAGATACTCACTGCCTAGAACTAACAGAGGCAGT 300
BR1-03  251 ATATGTGGGAGAAGATTAGATACTCACTGCCTAGAACTAACAGAGGCAGT 300
BR1-10  251 ATATGTGGGAGAAGATTAGATACTCACTGCCTAGAACTAACAGAGGCAGT 300

FS1-03  301 AGAATAATAGTCACCACACGGTTTCAAGCTGTTGCCAGTGCCTGCAAGAG 350
BR1-02  301 AGAATAATAGTCACCACACGGTTTCAAGCTGTTGCCAGTGCCTGCAAGAG 350
BR1-03  301 AGAATAATAGTCACCACACGGTTTCAAGCTGTTGCCAGTGCCTGCAAGAG 350
BR1-10  301 AGAATAATAGTCACCACACGGTTTCAAGCTGTTGCCAGTGCCTGCAAGAG 350

FS1-03  351 AGGTAAAGGAGATCGTGTTCATACGGTGGGTGTACTTACCGATGAAAAGC 400
BR1-02  351 AGGTAAAGGAGATCGTGTTCATACGGTGGGTGTACTTACCGATGAAAAGC 400
BR1-03  351 AGGTAAAGGAGATCGTGTTCATACGGTGGGTGTACTTACCGATGAAAAGC 400
BR1-10  351 AGGTAAAGGAGATCGTGTTCATACGGTGGGTGTACTTACCGATGAAAAGC 400

FS1-03  401 CTAGAGAACTATTCATGGCCGAATCGAAAATGGGCAATGAAAACCATAAC 450
BR1-02  401 CTAGAGAACTATTCATGGCCGAATCGAAAATGGGCAATGAAAACCATAAC 450
BR1-03  401 CTAGAGAACTATTCATGGCCGAATCGAAAATGGGCAATGAAAACCATAAC 450
BR1-10  401 CTAGAGAACTATTCATGGCCGAATCGAAAATGGGCAATGAAAACCATAAC 450

FS1-03  451 AAAGTTCCACCCAGACTCTGGGAAATGTGTGGGGGTCTTCCATTGGCGTA 500
BR1-02  451 AAAGTTCCACCCAGACTCTGGGAAATGTGTGGGGGTCATCCATTGGCGTT 500
BR1-03  451 AAAGTTCCACCCAGACTCTGGGAAATGTGTGGGGGTCTTCCATTGGCGTT 500
BR1-10  451 AAAGTTCCACCCAGACTCTGGGAAATGTGTGGGGGTCATCCATCGGCGTT 500

FS1-03  501 GG 502
BR1-02  501 GG 502
BR1-03  501 GG 502
BR1-10  501 GG 502
```

Figure 4.4 Nucleotide sequence alignment of the inserts homologous to *R* genes. The primer sequences are underlined; the variable nucleotides are shown in bold.

When the deduced amino acid sequences of these four inserts were examined, it was seen that the kinase 1a and GLPL motif sequences, which were targeted by the RGL primers used, were in the same reading frame. The deduced amino acid sequences of the inserts (excluding the primer sequences) did not contain a stop codon in this frame. The alignment of these deduced amino acid sequences are presented in Figure 4.10. The two nucleotides that differ between FS1-03 and the other three inserts both result in amino acid changes. Although the deduced amino acid sequences align with conserved motifs of both TNLs and CNLs, closer homology to CNL motifs is observed

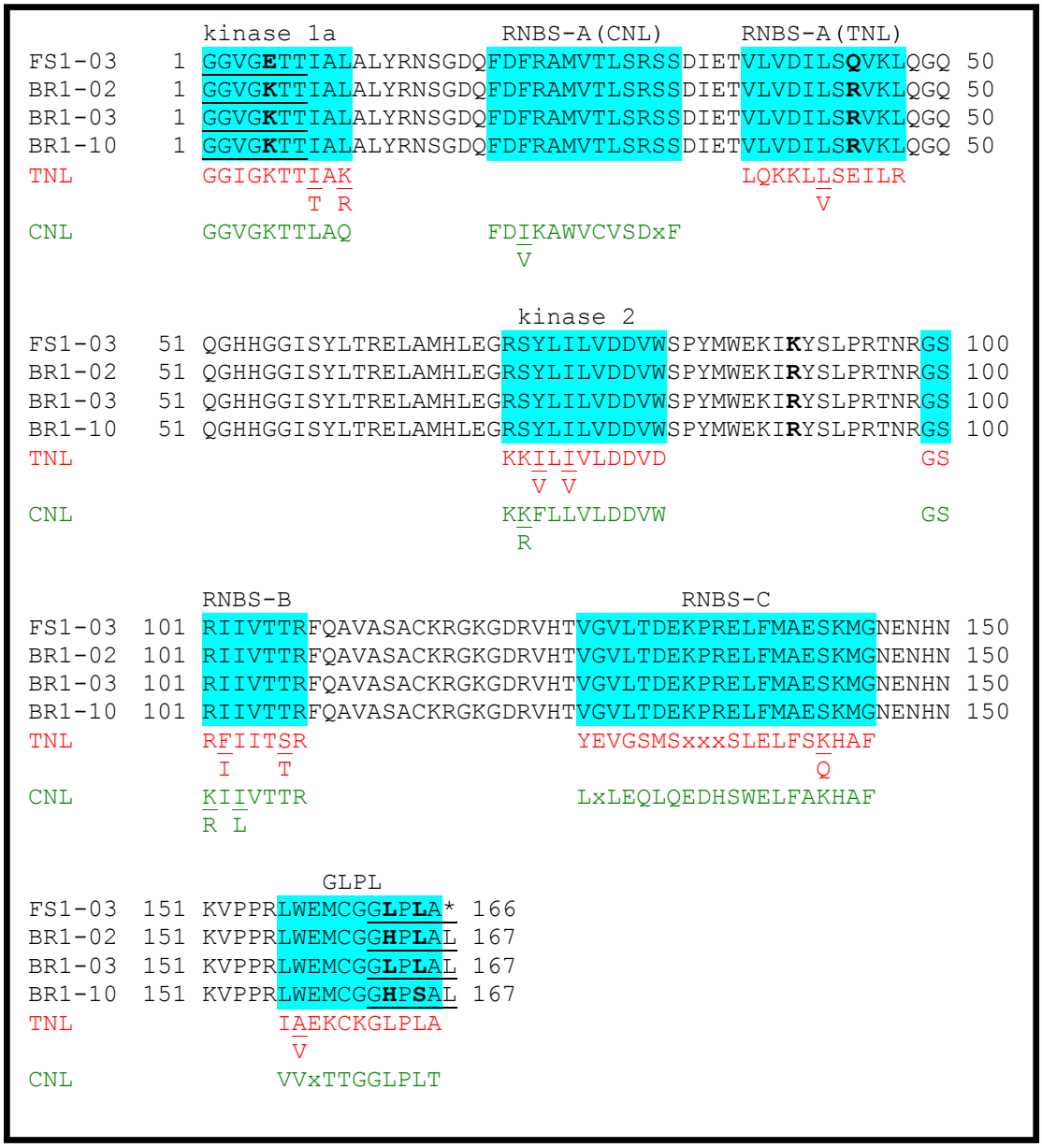

Figure 4.5 Amino acid sequence alignment of the inserts homologous to *R* genes. Sequences deduced from the primers are underlined. The variable amino acids are shown in bold. *: stop codon. The regions that align with the conserved NB-ARC domain motifs are high-lighted, and the consensus sequences for these conserved motifs for CNLs and TNLs are given.

Due to the high amount of similarity between the four insert sequences that showed homology to *R* genes, only the BR1-03 sequence was chosen for further analysis. The BL2SEQ algorithm at Biology WorkBench was utilized to compare the deduced amino acid sequence of BR1-03 to 27 NBS-LRR-type R proteins (8 TNLs and 19 CNLs). The deduced amino acid sequence of BR1-03 including and excluding the primer sequences were used in the comparison (Table 4.2). Overall, BR1-03 showed greater homology to CNL-type proteins than to TNLs. All of the CNLs used in the comparison were significantly similar to the deduced amino acid sequence of BR1-03 both when the primer sequences were included and when they were excluded in the comparison. On the other hand, the TNL-type proteins P2 and RPS5 of flax and *Arabidopsis*, respectively, did not show significant homology to BR1-03 even when the primer sequences were included in the comparison. Excluding the primer sequences resulted in an increase of the E-value for all cases, even increasing it above the significant similarity threshold for most of the TNLs studied. The only two TNL-type proteins that were significantly similar to the deduced amino acid sequence of BR1-03 when the primer sequences were excluded in the comparison were N and M proteins of flax and tobacco, respectively.

Table 4.2 Homology of BR1-03 to some NBS-LRR-type R proteins.

	Name	Source plant	Accession no.	E-Value	
				primers included	primers excluded
TNLs	L	flax (*Linum usitatissimum*)	AAD25976	2e-06	0.006
	M	flax (*Linum usitatissimum*)	AAB47618	2e-10	5e-05
	N	tobacco (*Nicotiana tabacum*)	BAD12594	1e-11	4e-06
	P2	flax (*Linum usitatissimum*)	AAK28806	0.002	0.061
	RPP1	thale cress (*Arabidopsis thaliana*)	NP_190034	4e-06	0.18
	RPP4	thale cress (*Arabidopsis thaliana*)	NP_193420	2e-06	0.002
	RPP5	thale cress (*Arabidopsis thaliana*)	NP_849398	9e-06	0.008
	RPS5	thale cress (*Arabidopsis thaliana*)	NP_199338	2e-04	2.6

Table 4.2 (continued)

CNLs	BS2	pepper (*Capsicum chacoense*)	AAF09256	3e-16	1e-11
	GPA2	potato (*Solanum tuberosum*)	CAB55838	1e-21	1e-15
	Hero	tomato (*Lycopersicon esculentum*)	CAD29728	5e-17	7e-12
	HRT	thale cress (*Arabidopsis thaliana*)	AAF36987	6e-11	3e-08
	I2	tomato (*Lycopersicon esculentum*)	AAD27815	7e-18	3e-11
	Mi-1.1	tomato (*Lycopersicon esculentum*)	AAC67237	2e-15	2e-12
	Mi-1.2	tomato (*Lycopersicon esculentum*)	AAC67238	4e-14	3e-11
	MLA13	barley (*Hordeum vulgare*)	AAO16000	8e-25	1e-18
	Pib	rice (*Oryza sativa*)	BAA76282	2e-14	8e-10
	Pi-ta	rice (*Oryza sativa*)	AAO45178	7e-25	8e-18
	R1	potato (*Solanum tuberosum*)	CAD88974	1e-16	1e-11
	RP1	maize (*Zea mays*)	AAS89974	9e-10	7e-07
	RPM1	thale cress (*Arabidopsis thaliana*)	Q39214	1e-18	5e-13
	RPP8	thale cress (*Arabidopsis thaliana*)	Q8W4J9	5e-11	3e-08
	RPP13	thale cress (*Arabidopsis thaliana*)	Q9M667	3e-20	3e-15
	RPS2	thale cress (*Arabidopsis thaliana*)	Q42484	2e-10	2e-05
	RPS5	thale cress (*Arabidopsis thaliana*)	O64973	6e-16	4e-11
	SW5	tomato (*Lycopersicon esculentum*)	AAG31013	2e-18	7e-14
	XA1	rice (*Oryza sativa*)	BAA25068	4e-12	2e-09

The amino acid sequences of the five CNLs that gave the lowest E-values in comparison to BR1-03 (MLA13, Pi-ta, GPA2, RPP13, and SW5) were aligned with the deduced amino acid sequence of BR1-03 using the CLUSTALW algorithm available at Biology WorkBench (Figure 4.6). Sequences of the TNL-type proteins N and M were also included in the alignment for the sake of comparison. The primer sequences of BR1-03 were retained to facilitate alignment. The sequences aligned most strongly at the sites of conserved motifs.

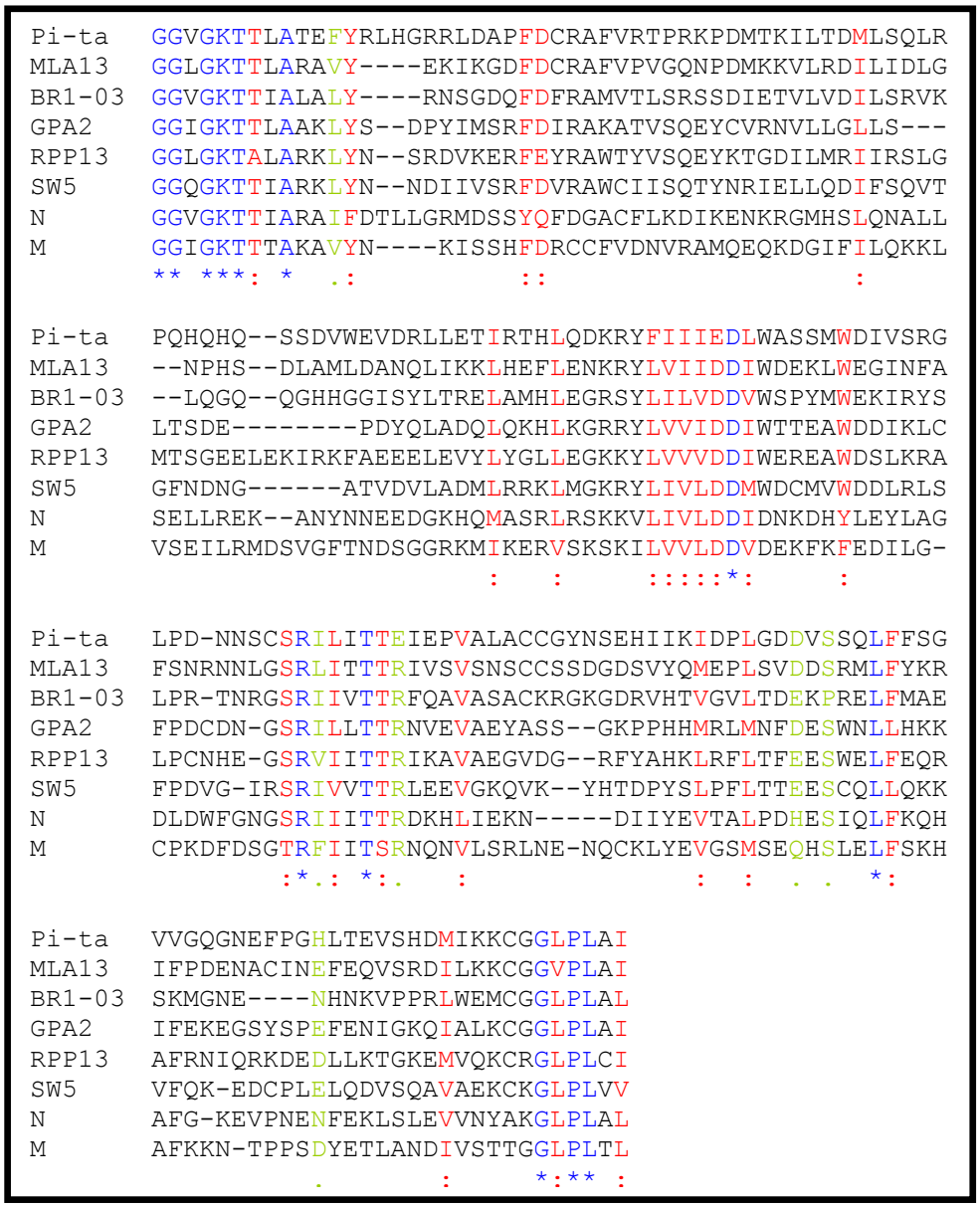

Figure 4.6 Alignment of BR1-03 with select NBS-LRR-type R proteins.
*: single, fully conserved residue, :: conservation of strong groups, .: conservation of weak groups.

The secondary structure predictions of BR1-03 and MLA13 were done using the PELE Protein Structure Prediction Tool. Figure 4.7 shows the secondary structure prediction for BR1-03 by the seven different algorithms used in this tool, as well as the joint prediction. The comparison of the secondary structure predicted for BR1-03 and the homologous regions of MLA13 is presented in Figure 4.8. This figure illustrates that BR1-03 and MLA13 may have similar protein secondary structures within the compared regions, especially at the sites of the conserved motifs.

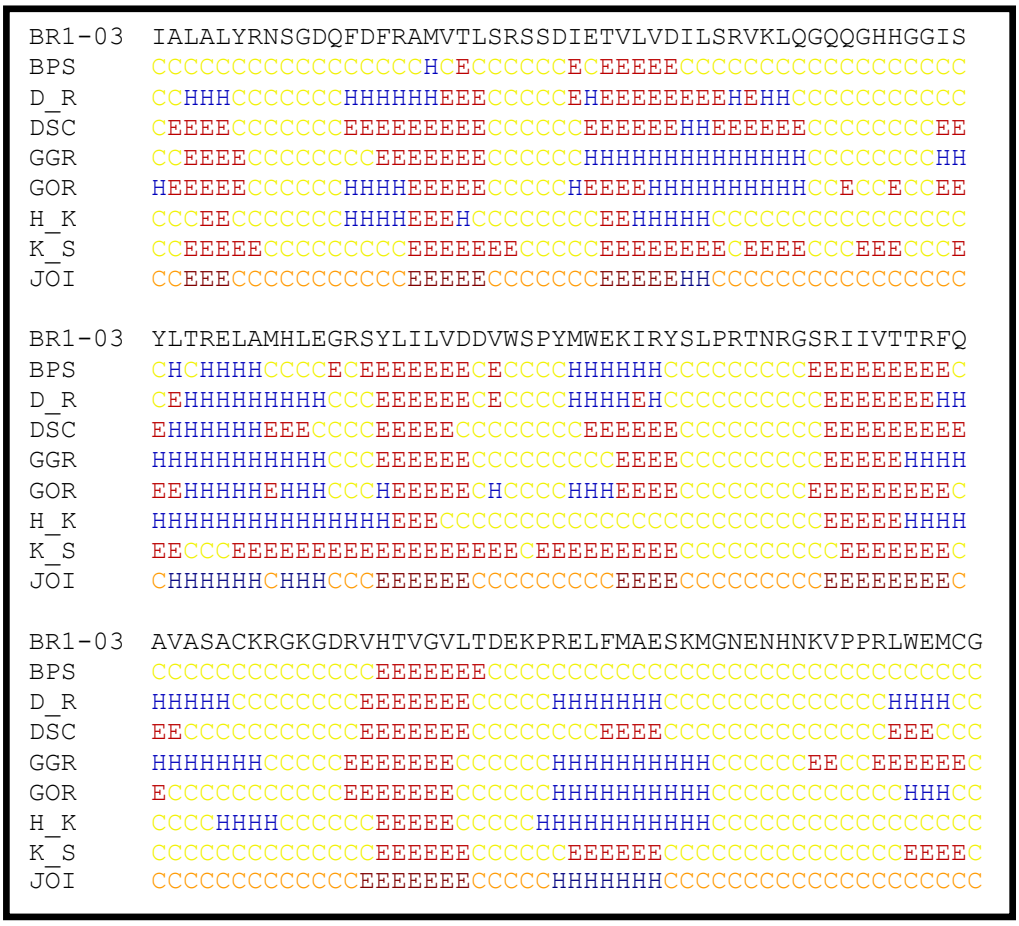

Figure 4.7 Secondary structure prediction for BR1-03. C: coil; E: β-strand; H: α-helix. BPS: Burgess et al, 1974; D_R: Deleage and Roux, 1987; DSC: King and Sternberg, 1996; GGR: Garnier et al, 1996; GOR: Garnier et al, 1978; H_K: Holley and Karplus, 1989; K_S: King and Sternberg, 1990; JOI: Joint prediction (see Section 3.2.15).

Figure 4.8 Secondary structure prediction alignment of BR1-03 and MLA13. C: coil; E: β-strand; H: α-helix. Green sequences represent the conserved motifs RNBS-A, kinase 2, RNBS-B, and RNBS-C, respectively. JOI: Joint prediction (see Section 3.2.15).

5 DISCUSSION

The aim of this study was to identify RGAs that are likely to be functional *R* genes. Since the expression of non-functional genes would be associated with an overall fitness cost to the plant and would thus be eliminated through evolution, cDNA was used as the template for the PCR-based amplification of RGAs in this study. In the literature, only two studies were found that directly aimed to amplify RGAs from a cDNA template. Liu and Ekramoddoullah (2003) reported the amplification of RGAs from cDNA of western white pines that have been inoculated with and are resistant to the white pine blister rust (*Cronartium ribicola*). This method is very valuable for the purpose of identifying *R* genes conferring resistance to a specific pathogen. However, in this study no such specific pathogen was in question. Therefore, the plants were not inoculated with a certain pathogen. Budak et al (2006) reported the amplification of RGAs from cDNA of buffalograss grown in soil, without any pathogen application. Soil and open air always contain a wide spectrum of microorganisms, so the RGAs they have reported may be *R* genes expressed in response to these pathogens or those that are constitutively-expressed. In this study, constitutively-expressed RGAs were targeted, since they are likely to be functional *R* genes. Therefore, the plants were grown in a pathogen-free environment. To our knowledge, this study is the first to isolate RGAs from cDNA of plants grown under pathogen-free tissue culture environment.

It is known that some *R* genes are constitutively expressed in plants, but at very low levels (Michelmore and Meyers, 1998). In previous studies, the amplification of genomic DNA or cDNA of various plants with primers targeting the kinase 1a and GLPL motifs has almost always resulted in clear bands of the expected size (500 bp) in gel electrophoresis analyses, usually along with clear bands of different sizes (Leister et al, 1996; Deng et al, 2000; Di Gaspero and Cipriani, 2002; He et al, 2004; Budak et al, 2006). However, in this study no such clear and bright band of 500 bp was visible,

although all of the amplified samples had bands of different sizes (Figure 4.1). This was most likely due to the very low levels of *R* gene mRNA present in the templates used.

Since fragments that did not have a molecular weight of about 500 bp were shown to be non-specific by studies on various plants (Leister et al, 1996; Deng et al, 2000; Noir et al, 2001; He et al, 2004) and since no clear bands of the expected size were observed in this study, the gel fragment between 400 and 600 bp for each sample, as shown in Figure 4.1, was excised and used in gel extraction. The concentrations of the gel extraction products were very low as expected from the low visibility of the amplification products on the agarose gel. To increase the concentrations of the amplified fragments of molecular weight between 400 and 600 bp, the gel extraction products were used as the template for a second PCR amplification using the same primers as before. These PCR products were purified and all of the PCR purification products showed an increase in concentration, as anticipated, from the gel extraction products used as their template. However, when the PCR purification products were run on agarose gel, it was seen that only some of the samples gave bands of molecular weight between 400 and 600 bp, and that almost all of the samples had smears and some even had clear bands outside this range (Figure 4.2). This was an unexpected result since the template used for the second amplification contained only fragments between 400 and 600 bp. The reason for this unexpected result is not known. Nevertheless, all of the PCR purification products were inserted in plasmid vectors and cloned in *E. coli*.

A total of 120 colonies for the 12 samples were randomly selected for plasmid purification. The plasmids were used as the template in PCR reactions with either the original RGL primers or the universal primers SP6 and T7. The agarose gel electrophoresis results of the plasmid PCR products of all samples looked very similar. As can be seen in Figure 4.3, all screening done with SP6 and T7 resulted in amplified inserts represented by clear bands, although not always of the expected size. The distance between these two primers in a circularized pGEM®-T Vector without an insert is about 150 bp (Appendix C), so the fragments amplified with the RGL primers were expected to be about 150 bp smaller than those amplified with the SP6/T7 primers. However, most of the plasmid PCR products amplified with the RGL primers had very faint bands and some had no visible bands at all around that size. On the other hand, all the PCR products amplified with the RGL primers displayed large and bright smears

over 3,000 bp. The reason for this could not be explained since all of the plasmid PCR reactions were prepared from a master mix and differed only in primers and DNA templates. It is unlikely to have resulted from a contamination in the RGL primers because that would require the contamination of all three RGL primer pairs in the same manner. All of the plasmid PCR products, regardless of the primers they were amplified with, displayed several bands of molecular weight over 3,000 bp for super-coiled, circular, and linearized plasmids. Due to the problems with the fragments amplified with the RGL primers, only the agarose gel electrophoresis results of those amplified with the SP6/T7 primers were taken as the basis for plasmid selection. The plasmids giving bands of around 650 bp after amplification with the SP6/T7 primers were chosen for sequencing.

In this study, the yield of RGAs recovered was very low. Out of 120 colonies screened, 35 were sequenced, only four of which gave similarity hits to known *R* genes. This might be a result of the low success of the RGL primers used to amplify specific fragments of 500 bp (Figures 4.1 and 4.2). Most of the clones sequenced were homologous to unknown sequences or genes unrelated to disease resistance. Although these sequences are still valuable as ESTs and putative genes (Table 4.1), they were not the desired results. One aim of this study was to compare the RGAs expressed in the shoots to the ones expressed in the roots. However, due to the low yield of RGAs recovered, it was not possible to do such a comparison.

An interesting data not reported is that almost half of the clones sequenced contained several repeats of the primer sequences. This may have arisen as a result of a complication during the PCR or the ligation steps, where several amplified fragments may have ligated onto one another. Although some of these samples containing several primer sequences gave similarity hits to known proteins, none of the four clones with sequences homologous to *R* genes were recovered from them. So, these clones, which were the focus of further sequence analyses, were not affected by the mentioned complication.

Out of the four sequences that showed significant homology to *R* genes, three were obtained from bermudagrass root cDNA and the fourth was obtained from tall fescue shoot cDNA. The three from bermudagrass were identical to one another

(excluding the primer sequences) and were highly similar to the one from tall fescue (Figures 4.4 and 4.5). The great homology between RGAs from two different species was not expected and will be further investigated.

The four sequences identified to be homologous to *R* genes possibly represent two alleles of the same RGA. Since they are almost identical in sequence, only one of them was selected for further analyses. Because no TNLs have been isolated from grasses before (Pan et al, 2000b), the RGAs identified in this study were expected to be of the CNL-type. The C-terminal amino acid of the kinase 2 motif indicates, with 95 % accuracy, whether the RGA is of CNL- or TNL-type: CNL kinase 2 domains end with a tryptophan (W) residue, whereas TNL kinase domains end with an aspartic acid (D) residue (Meyers et al, 1999). The predicted kinase 2 motif of BR1-03 ends with a W (Figure 4.5), suggesting that this sequence represents a CNL-type RGA. Also, comparing the deduced amino acid sequence of BR1-03 to 27 NBS-LRR proteins of both CNL and TNL types has given closer similarity hits to CNLs (Table 4.2). The five proteins studied that gave the closest match to BR1-03 were MLA13 from barley, Pi-ta from rice, GPA2 from potato, RPP13 from *Arabidopsis*, and SW5 from tomato, all of which are CNLs.

MLA13, Pi-ta, GPA2, RPP13, and SW5 confer resistance to *B. graminis* (fungus), *Magnaporthe grisea* (fungus), *Globodera pallida* (nematode), *Peronospora parasitica* (oomycete), and Tomato Spotted Wilt Virus, respectively (Martin et al, 2003). The wide range of pathogen specificity suggests that the portion of the NB-ARC domain studied is not involved in effector recognition. This finding is concordant with the hypothesis that the LRR domain is the major determinant of recognition specificity. On the other hand, the two proteins that showed the greatest homology to BR1-03 were MLA13 and Pi-ta, both of which confer resistance to fungal pathogens. Since they were both isolated from members of the family Poaceae, just like tall fescue and bermudagrass, it may be suggested that the RGA identified in this study might be also be involved in resistance to fungal pathogens.

Figure 4.6 shows that the regions of the deduced amino acid sequence of BR1-03 that show the greatest homology to the five CNL proteins mentioned, as well as the TNLs N and M, are the sites of conserved motifs, especially kinase 2, RNBS-B, and

RNBS-C. Figure 4.8 illustrates that the predicted protein secondary structures of these sites are conserved, as well, between MLA13 and BR1-03. This finding suggests that the regions of conserved motifs may be involved in R-protein function.

6 CONCLUSION

As a result of this study, putative CNL-type *R* genes were identified from tall fescue and bermudagrass. After verifying the source of these RGAs with the use of gene-specific primers, these genes will be further investigated. The rapid amplification of cDNA ends (RACE) method will be employed to obtain the complete mRNA sequence, following which the complete cDNA will be cloned into an expression vector for protein structure studies.

This study has demonstrated that isolation of constitutively-expressed RGAs is very low-yielding. Therefore, the study may be repeated for genomic DNA of tall fescue and bermudagrass or for cDNA of these plants exposed to pathogens. Also, only one of the three primers used in this study was successful in amplifying RGA fragments. Therefore, different primer pairs will also be used in further studies to increase the number of RGAs that can be identified in tall fescue and bermudagrass.

Upon isolation of RGAs, their function as *R* genes may be determined via functional analyses and other reverse genetics methods. Characterization of an *R* gene with resistance activity against a specific pathogen is very beneficial. The data obtained from such a study would facilitate the design of better molecular markers to be used in marker-assisted selection of disease resistant plants, as well as being an invaluable tool for transferring this disease resistance to other plant species. The *R* gene characterization data would also be useful for evolutionary studies investigating both plant evolution and diversity, and host-pathogen co-evolution.

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APPENDIX A

Equipment

Autoclave sterilizers:	Hirayama, Japan (HiClave HV-110)
	Nüve, Turkey (OT 032)
Centrifuges:	Eppendorf, Germany (Centrifuge 5415C, 5415D)
Cold room:	Alarko Carrier, Turkey
Electronic balances:	Schimadzu, Japan (Libror EB-3200HU)
	Sartorius, Germany (BP221S)
Electrophoresis equipment:	Labnet International, USA (Gel XL Ultra V-2™)
Freezers:	Bosch, Turkey
	Thermo Electron Corporation, USA (Thermo Forma 917)
Gel documentation system:	Bio-Rad Laboratories, USA (Universal Hood II)
Heating block:	Fisher, France (Bioblock Scientific™)
Heating magnetic stirrer:	VELP Scientifica, Italy (ARE)
Incubator:	Memmert®, Germany (D06059 Modell 300)
Incubator shaker:	New Brunswick Scientific, USA (Innova® 4330)
Laboratory bottles:	SCHOTT DURAN®, Germany
Laminar flow cabinets:	Heraeus Instruments, Germany (HERAsafe® HS12)
Micropipettes:	Gilson, USA (Pipetman® P)
	Eppendorf, Germany (Research®)

Microwave:	Vestel, Turkey
pH meter:	Windaus Labortechnik, Germany (TitroLine alpha)
Plant growth room:	DigiTech, Turkey
Refrigerators:	Bosch, Turkey
Software:	Invitrogen Corporation, USA (Vector NTI 9.1.0)
	Technelysium Pty. Ltd., Australia (Chromas 2.31)
Spectrophotometer:	NanoDrop Technologies, USA (ND-1000)
Thermal cycler:	GMI, USA (MJ Research PTC-100)
Vortex mixer:	VELP Scientifica, Italy (ZX3)
Water bath:	Techne, UK (Refrigerated Bath RB-5A)
Water purification system:	Millipore, USA (Milli-Q Academic)

APPENDIX B

Supplies

Disposable Labware:

3-part syringes:

Ayset, Turkey (5 mL, 50 mL)

Centrifuge tubes:

Techno Plastic Products AG, Switzerland (91015, 91050)

ClickFit Cap microtubes:

TreffLab, Switzerland (96.8185.9.03, 96.7811.9.03, 96.9329.9.01)

Diamond® Tips:

Gilson, USA (D10, D200, D1000)

PCR-tubes:

TreffLab, Switzerland (96.9852.9.01)

Petri dishes:

ISOLAB Laborgeräte GmbH, Germany (113.02.002)

Polystyrene round-bottom test tubes:

Becton Dickinson Falcon™, USA (352001)

Puradisc™ FP 30 syringe filters:

Whatman® Schleicher & Schuell, UK (10462200)

Quantitative filter paper:

Whatman® Schleicher & Schuell, UK (10300109)

Tips for pipettes:

TreffLab, Switzerland (96.9515, 96.8700, 96.8276)

Vitro Vent containers:

Duchefa Biochemie B.V., the Netherlands (V1601)

Chemical Supplies:

2-Propanol extra pure:

Merck KGaA, Germany (1.00995)

2-Propanol puriss., ≥99.5% (GC):

Riedel-de Haën®, Germany (24137)

6X Loading Dye Solution:

Fermentas, Canada (R0611)

Agar Type A, plant cell culture tested:

Sigma-Aldrich Co., USA (A4550)

Agarose low EEO (Agarose Standard):

AppliChem GmbH, Germany (A2114)

Ampicillin sodium salt *BioChemica*:

AppliChem GmbH, Germany (A0839)

aTaq DNA Polymerase:

Promega, USA (M1245)

Boric acid for molecular biology, ~99%:

Sigma-Aldrich Co., USA (B6768)

Chloroform Biotechnology Grade:

Amresco® Inc., USA (0757)

D-(+)-Glucose monohydrate *BioChemika Ultra*, ≥99.5% (HPLC):

Fluka, Switzerland (49158)

dATP, molecular biology grade:

Fermentas, Canada (R0141)

Deoxyribonuclease I (DNase I), RNase-free:

Fermentas, Canada (EN0521)

Diethyl pyrocarbonate, ≥97% (NMR):

Sigma-Aldrich Co., USA (D5758)

dNTP mix:

Promega, USA (U1515)

Ethanol absolute extra pure:

Merck KGaA, Germany (1.00986)

Ethidium Bromide Solution 1 % in water:

Merck KGaA, Germany (1.11608)

Ethylenediaminetetraacetic acid disodium salt dihydrate for molecular biology, ≥99%:

Sigma-Aldrich Co., USA (E5134)

GeneRuler™ 100bp DNA Ladder Plus:

Fermentas, Canada (SM0321)

IPTG, dioxane-free:

Fermentas, Canada (R0393)

LB Broth:

Sigma-Aldrich Co., USA (L3022)

Luria Agar:

Sigma-Aldrich Co., USA (L3147)

Magnesium chloride hexahydrate puriss., 99-101%, total impurities ≤0.0001% Al:

Riedel-de Haën®, Germany (13152)

MS medium basal salt mixture including vitamins:

Duchefa Biochemie B.V., the Netherlands (M0222)

Oligo(dT)$_{12-18}$ Primer:

Invitrogen, USA (18418-012)

Oligonucleotides:

Integrated DNA Technologies Inc. USA

İontek, Turkey

Pfu DNA Polymerase (recombinant):

Fermentas, Canada (EP0502)

pGEM®-T Vector System II:

Promega, USA (A3610)

Plant Preservative Mixture™:

Plant Cell Technology, USA

Potassium chloride *BioChemika Ultra*, ≥99.5% (AT):

Fluka, Switzerland (60129)

RNaseOUT™ Recombinant Ribonuclease Inhibitor:

Invitrogen, USA (10777-019)

Sodium chloride EMPROVE®:

Merck KGaA, Germany (1.06400)

Sodium hydroxide pellets pure:

Merck KGaA, Germany (1.06462)

Sucrose Grade I, plant cell culture tested:

Sigma-Aldrich Co., USA (S5390)

Tris Buffer Grade:

AppliChem GmbH, Germany (A1379)

Tris(hydroxymethyl)aminomethane Biotechnology Grade:

Amresco® Inc., USA (0826)

TRIzol® Reagent:

Invitrogen, USA (15596)

Tryptone:

AppliChem GmbH, Germany (A1553)

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside):

Promega, USA (V3941)

Yeast extract *BioChemica*:

AppliChem GmbH, Germany (A1552)

Commercial Kits:

Omniscript RT Kit:

Qiagen Inc., USA (205111)

QIAprep Spin Miniprep Kit:

Qiagen Inc., USA (27106)

QIAquick Gel Extraction Kit:

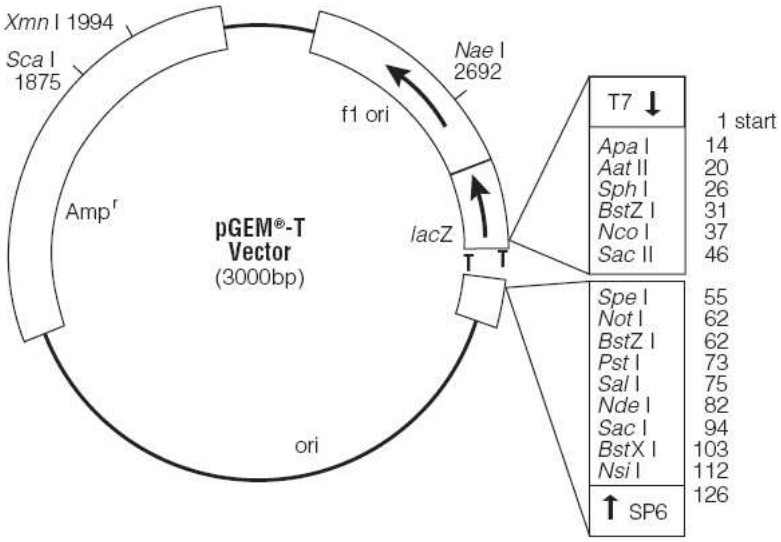
Qiagen Inc., USA (28706)

QIAquick PCR Purification Kit:

Qiagen Inc., USA (28104)

APPENDIX C

pGEM®-T Vector map and sequence reference points



T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–113
SP6 RNA polymerase promoter (–17 to +3)	124–143
SP6 RNA polymerase transcription initiation site	126
pUC/M13 Reverse Sequencing Primer binding site	161–177
*lac*Z start codon	165
lac operator	185–201
β-lactamase coding region	1322–2182
phage f1 region	2365–2820
lac operon sequences	2821–2981, 151–380
pUC/M13 Forward Sequencing Primer binding site	2941–2957
T7 RNA polymerase promoter (–17 to +3)	2984–3

APPENDIX D

Abbreviated names of the amplified cDNA samples

		Primer pair used for initial amplification		
		RGL1	**RGL2**	**RGL5**
cDNA source	**Tall fescue shoot**	FS1	FS2	FS5
	Tall fescue root	FR1	FR2	FR5
	Bermudagrass shoot	BS1	BS2	BS5
	Bermudagrass root	BR1	BR2	BR5