Theor Appl Genet (2002) 104:1283–1289
DOI 10.1007/s00122-001-0821-0

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Isolation from alfalfa of resistance gene analogues containing nucleotide binding sites

Received: 1 December 2000 / Accepted: 21 May 2001 / Published online: 19 April 2002

Abstract Cloned resistance (R) genes from a broad range of plant species are known to share similarities in DNA sequence and structural motifs. Degenerate oligonucleotide primers designed from conserved regions of the nucleotide binding site (NBS), common to many R genes, were used to amplify the NBS regions from genomic DNA from alfalfa (*Medicago sativa* L). Sequence comparisons of the amplified fragments indicated that at least 18 families of NBS-containing R genes are present in alfalfa. Comparisons to R genes from other species suggested a polyphyletic origin of these gene families. Using the same degenerate primers, PCR analysis of cDNA prepared from a plant not challenged with a pest or pathogen revealed that many of the NBS-containing gene families were transcribed actively. Amplification of NBS regions from other *Medicago* species showed the presence of some NBS-containing genes not present in alfalfa. These results indicate that the NBS-containing R genes comprise a large gene family in *Medicago*, at least some of which are transcribed in healthy plants, and that different *Medicago* species carry unique NBS genes.

Keywords Disease resistance · Degenerate oligonucleotides · Resistance gene analogues · alfalfa · *Medicago sativa* L.

Communicated by P. L. Pfahler

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Introduction

Disease resistance (R) genes in plants have been found to share significant homologies in DNA sequences, amino acid sequences, and structural motifs. The most abundant classes of R genes contain a nucleotide-binding site and a leucine-rich repeat (NBS-LRR). Additional structures, such as the toll-interleukin receptor (TIR) or leucine zipper (LZ), may also be present, resulting in TIR-NBS-LRR or LZ-NBS-LRR constructions. Evidence from *Arabidopsis* studies suggests that over 1% of a plant's genome may be composed of NBS-containing R genes (Meyers et al. 1999).

The NBS regions of well-characterized R genes are highly homologous (Staskawicz et al. 1995) and contain several highly conserved motifs (Table 1). Of those motifs, the P-loop and the kinase-2 domains have been well characterized for ATP- and GTP- binding proteins (Bourne et al. 1991; Traut 1994; Walker et al. 1982). The P-loop interacts directly with the phosphate of the bound nucleotide (Meyers et al. 1999; Saraste et al. 1990), and mutations of key residues in the P-loop of the tobacco N gene have led to partial loss of function (Baker et al. 1997). The kinase-2 domain is involved in coordinating the ion (Mg_{2+}) required for phospho-transfer reactions (Traut 1994). Additional motifs such as the kinase-3a and the GLPL, a putative membrane-spanning domain so named because of the consensus amino acid sequence, also have been found in the NBS regions of plant R genes.

Amplification of a representative sample of the NBS superfamily in plants has been possible using degenerate primers based on the NBS motifs. This approach has been successful in soybean (Kanazin et al. 1996; Yu et al. 1996), potato (Leister et al. 1996), and lettuce (Shen et al. 1998). The P-loop, the kinase-3a, and GLPL motifs have been the most commonly used. In soybean, the polymerase chain reaction (PCR) using a forward primer designed for the P-loop with a reverse primer for the GLPL motif (Kanazin et al. 1996) and a reverse primer for the kinase-3a motif (Yu et al. 1996) detected at least

Table 1 Characteristic motifs found in nucleotide binding site regions in plant genes

Motif	Synonym or specific motif type	Consensus amino acid sequence	Location[a]
P-loop	Kinase-1a, G-1	GVGKTT	1
	RNBS-A-nonTIR	FDLxAWVCVSQxF	20
	RNBSA-TIR	FLENIRExSKKHGLEHLQKKLLSKLL	23
Kinase-2	G-3	LLVLDDVW	74
Kinase-3a	RNBS-B	GSRIIITTRD	102
	RNBS-C	YEVxxLSEDEAWELFCKxAF	122
GLPL		CGGLPLA	162
	RNBS-D-NonTIR	CFLYCALFPED	223
	RNBS-D-TIR	FLHIACFF	219

[a] Approximate locations relative to the start of the NBS region, based on an average of 14 characterized R genes from Meyers et al. (1999)

11 different classes of NBS located in at least 11 of the 26 linkage groups of the soybean genetic map. At least five of these classes mapped near known resistance gene loci (Kanazin et al. 1996; Yu et al. 1996). Similar results were obtained for maize, in which 11 classes of NBS genes were found, and 6 of which mapped in close proximity to disease-resistance loci (Collins et al. 1998). Because of the difficulties of determining associations of molecular markers with disease resistance in alfalfa imposed by the autotetraploid genetic structure (Skinner et al. 2000), it is desirable to find markers comprised of the resistance genes themselves. As a first step toward this objective, this study was designed to use a degenerate PCR approach to isolate the NBS family of R gene analogs in *Medicago sativa* and related *Medicago* species and investigate their variability.

Materials and methods

Total genomic DNA was extracted from the combined leaves of ten alfalfa plants of germplasm UC123 (Lehman et al. 1983) using the method described by Dellaporta et al. (1983). UC123 carries high levels of resistance to blue alfalfa aphid and downy mildew isolates 5 and 7 (Lehman et al. 1983). The degenerate PCR primers used were those designed by Kanazin et al. (1996) from the P-loop and the GLPLA motifs of the NBS region of R genes. The primers were: LM638: 5′ -GGIGGIGTIGGIAAIACIAC-3′, and REV7: 5′ -ARIGCTARIGGIARICC-3′.

The PCRs were carried out in a total volume of 50 µl with 25 ng template DNA, 200 n*M* each primer, 200 µ*M* each dATP, dCTP, dGTP and dATP, 16 m*M* $(NH_4)_2$ SO_4, 67 m*M* Tris-HCl (pH 8.8 at 25 °C), 0.01% Tween-20, 1.5 m*M* $MgCl_2$, and 1 u of Biolase (Bioline, Springfield, N.J.). Forty cycles of PCR were conducted as: 94 °C for 1 min, 45 °C for 30 s, and 68 °C for 30 s. Amplification products were observed on a 1% agarose gel stained with ethidium bromide. These were digested with Tsp5091 (↓AATT) (New England Biolabs, Beverly, Mass.) and resolved using 6% polyacrylamide gel electrophoresis (PAGE) stained with Sybr Green (Molecular Probes, Eugene, Ore.) or ethidium bromide.

The PCR products were cloned using the Topo TA cloning kit (Invitrogen, Carlsbad, Calif.). Individual clones were digested with the Tsp509I enzyme, and digests were resolved using 6% PAGE gels. Ninety-four individual clones were PCR-amplified using primers from within the plasmid (M13 primers). Amplification products from each clone were purified using Centricon YM-100 spin columns (Millipore, Bedford, Mass.) prior to sequencing in order to remove unincorporated primers and nucleotides. Sequencing reactions were prepared using the Perkin Elmer Big Dye DNA sequencing kit (Perkin Elmer, Foster City, Calif.). Sequencing reactions were cleaned using the Centri-sep spin columns (Princeton Separations, Adelphia, N.J.). Sequences were read by the DNA Sequencing and Synthesis Facility at Iowa State University, Ames, Iowa.

Sequence analyses and alignments were carried out using MacVector 6.5 and Assemblylign (Oxford Molecular, Madison, Wis.) software. Sequence comparisons with NBS from other species were done using the BLAST algorithm on the GenBank nonredundant database. Sequence similarity and bootstrap analyses were performed using the neighbor-joining and UPGMA methods with *PAUP* software (Sinauer Assoc, Sunderland, Mass.).

To assess whether the NBS genes were transcribed, we extracted total RNA from alfalfa plant ISC 35, a plant selected from Indiana Synthetic C on the basis of resistance to potato leafhopper (Elden and Elgin 1992), using the TRIazol Reagent (GibcoBRL, Gaithersburg, Md.) according to the manufacturer's procedure. First-strand cDNA was obtained by using the RETROscript RT-PCR kit (Ambion, Austin, Tex.). The PCR then was run on the cDNA using the degenerate primers LM638 and REV7 described above and the following non-degenerate primers: FOR-NBS: 5′ -GGGGGGGTGGGGAAGACGAC-3′, and REV-NBS7: 5′ -AGGGCTAGGGGGAGGCC-3′. The PCR profile and conditions were as described above.

Amplification products were observed on a 1% agarose gel stained with ethidium bromide. The 500-bp amplification product was removed from the gel using Ultrafree-DA columns (Millipore, Bedford, Mass.) and cloned using the pGEM-T Easy vector system (Promega, Madison, Wis.). Inserts from five clones were amplified with M13 primers and sequenced to assess whether the cDNA clones were of NBS regions.

To examine resistance gene analog diversity within the genus *Medicago*, we isolated DNA using the method of Dellaporta et al. (1983) from leaves of individual plants from *M. constricta*, *M. blancheana*, *M. radiata*, *M. praecox*, *M. platycarpa*, *M. medicaginoides*, *M. ruthenica*, *M. murex*, *M. rugosa*, *M. rigidula*, *M. orbicularis*, *M. polymorpha*, and *M. lupulina*. Degenerate PCR was done using primers LM638 and REV7 and the amplification conditions described above. Variation in R gene analogs was assessed with restriction digests with Tsp5091 of the 500-bp degenerate PCR amplification product. Digestion products were resolved on 6% polyacrylamide gels and stained with Sybr Green.

Results and discussion

The PCR with the degenerate primers designed for the NBS region of R genes initially showed what appeared to be a single 500-bp product on a 1% agarose gel (Fig. 1). However, when this band was digested with Tsp509I, the resulting band sizes summed to greater than 500 bp (Fig. 2), indicating that several different sequences of about 500 bp in length had been amplified. PCR amplification of 94 of the individual clones using M13 primers yielded amplification products of the expected size of about 718 bp, which takes into account the plasmid sequence of 218 bp plus about 500 bp of NBS sequence.

We obtained 94 clones containing 51 unique DNA sequences, and these have been deposited in GenBank with

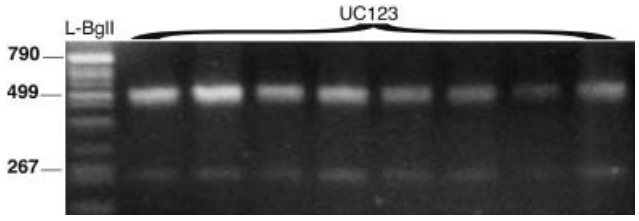

Fig. 1 Eight replications of the products from alfalfa (*Medicago sativa*) cv. UC123 resulting from PCR using degenerate primers designed for the P-loop and the GLPL motifs of the nucleotide binding site of plant genes. *L-Bgl* is Lambda phage DNA digested with *Bgl*I. Sizes are indicated in base pairs

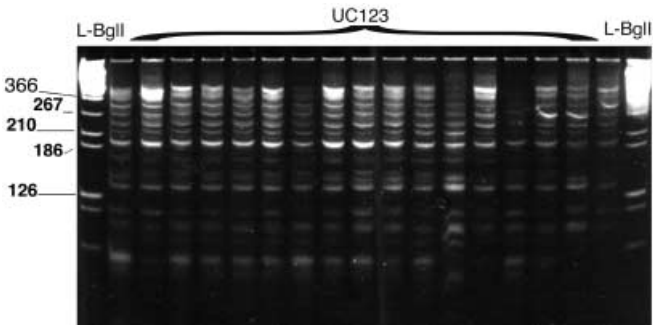

Fig. 2 Seventeen replications of the *Tsp*509I-digested products from alfalfa (*Medicago sativa*) cv UC123 resulting from PCR using degenerate primers designed for the P-loop and the GLPL motifs of the nucleotide binding site of plant genes. *L-Bgl* is Lambda phage DNA digested with *Bgl*I. Sizes are indicated in base pairs

accession numbers AF230813-AF230830 and AF230831-AF230865. These sequences were highly homologous to NBS regions from other species. Nucleotide sequence alignments revealed six major branches that were considered as to be separate clusters of MSRGA (*Medicago sativa* R gene analogue) clones (Fig. 3). Five of the six clusters (A, B, D, E, and F) contained from 8 to 13 unique MSRGAs. Although cluster C contained only one sequence, MSRGA22, it was considered as separate because of the genetic distance relative to the other five clusters (Fig. 3). Cluster C (MSRGA22) shared 73% or less homology with the other clusters (Table 2). Although many of the 51 sequences showed very high homology, the few nucleotide differences that did occur may have functional importance. For example, an NBS region in potato associated with virus resistance had only 12 nucleotide differences from homologous regions found in all of the susceptible lines (Sorri et al. 1999). For nucleotide translation analysis purposes, sequences that shared more than 95% homology

Fig. 3 Dendrogram generated from nucleotide binding site regions of *M. sativa* resistance gene analogs (MSRGA). The neighbor-joining algorithm was applied to the nucleotide sequence alignment. Genetic distance values are given *below* the branches, and bootstrap values from 1,000 cycles of resampling, neighbor-joining algorithm, are given *above* the branches

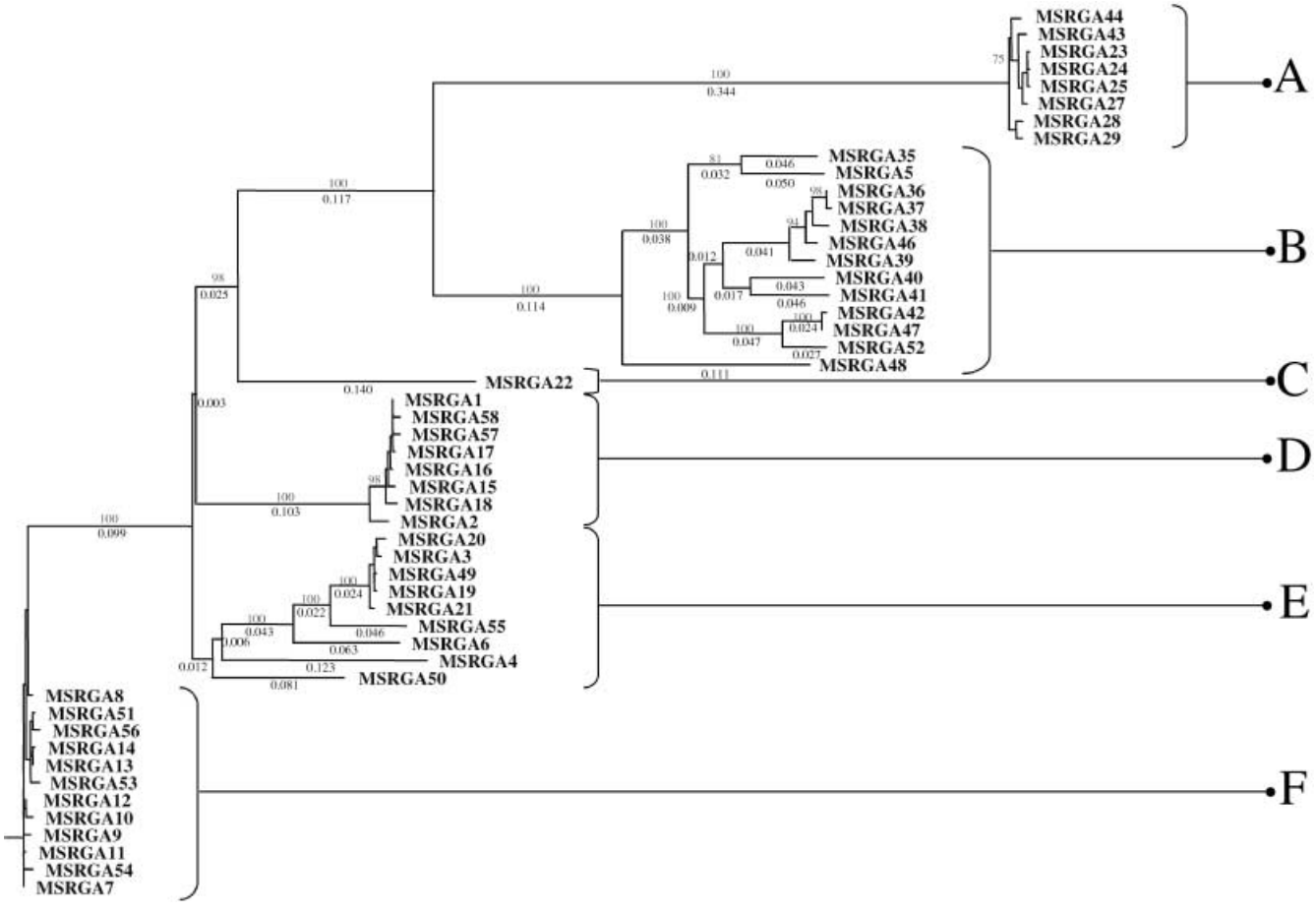

Table 2 Percent homology of nucleotide squences of *Medicago sativa* resistance gene analogue (MSRGA) nucleotide binding site regions

Cluster[a]		A		B								C	D	E			
	MSRGA	23	28	52	42	36	41	40	5	35	48	22	1	50	4	3	6
A	28	97	–														
B	52	35	36	–													
	42	37	37	94	–												
	36	36	37	87	83	–											
	41	36	37	86	87	88	–										
	40	37	38	81	82	85	91	–									
	5	35	36	79	81	81	84	89	–								
	35	36	37	86	87	80	83	87	89	–							
	48	36	37	75	76	75	78	78	77	76	–						
C	22	38	39	51	50	51	52	50	50	50	50	–					
D	1	38	38	48	48	49	49	48	48	49	49	71	–				
E	50	39	39	48	48	49	48	49	49	49	50	73	76	–			
	4	38	39	47	47	48	47	47	48	48	48	70	74	78	–		
	3	38	38	47	47	48	48	48	48	47	50	73	77	81	71	–	
	6	38	38	46	46	47	48	49	49	48	50	70	76	81	77	88	
F	7	37	38	51	51	51	51	52	52	52	53	73	77	80	75	78	77

[a] Clusters were defined by nearest neighbor search algorithm.

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          RNBS-A-nonTIR                          Kinase-2          Kinase-3a              RNBS-C
MSRGA44*  FDKVVMAVVSQNP_____________45aa_____________LIVLDDVW___17aa__CIKILFTSRD_12aa_VHVSVCXRMKLGAYFKKWQEM
MSRGA28   FDKVVMAVVSQNP_____________45aa_____________LIVLDDVW___17aa__CIKVLFTSRD_12aa_VHVSVLLEDEAWSLFQEMAGD
MSRGA23   FDKVVMAVVSQNP_____________45aa_____________LIVLDDVW___17aa__CIKILFTSRD_12aa_VHVSVLLEDEAWSLFQEMAGD

          RNBS-A-TIR
MSRGA6    IIDDLSKMYR_HDSPVSAQKQILLQTLG_27-28aa_FIILDNVD_17-18aa_GSRIIIISRD_12aa_YKVPLLNKTNSLQLFSQKAFK
MSRGA3    LIDDVSKIYR_HDGPMSAQKQILCQTLG_27-28aa_LIILDNVD_17-18aa_GSRIIITTRD_12aa_YKVPLLNNTNSLQLFSQKAFK
MSRGA50   FIDDLSKIYR_HDGPIGAQKQILHQTLG_27-28aa_LIVVDNVD_17-18aa_GSRIIIISRD_12aa_YKVPLLNGTNSLQLFCQKAFK
MSRGA7    FIDDVSKICK_HDGPVAAQKQILSQTLG_27-28aa_FIILDNVD_17-18aa_GSRIIIISRD_12aa_FKVPLLNQTNSLQLFCQQAFK
MSRGA4    FIDDVSKTFRLHDGPLGVQKQILLQTLG_27-28aa_LIILDNVD_17-18aa_GSRIVVISRD_12aa_YKVPLLDWTNSRRLLCQKAFK
MSRGA22   FIDDVSKIYG_DYGPIGVQKQLLSQTLK_27-28aa_LVVLDNVD_17-18aa_GSRIIVISRD_12aa_YKVRLLDHNNAFQLFCRKAFK
MSRGA1    LIDDVSKVFK_VDGPIGVQKQLLHQTLG_27-28aa_LIVLDNVD_17-18aa_GSRIVIVCRD_12aa_YKVQPLNQTNSLQLFCRKAFK
MSRGA36   FLHNVRENSD_KHGLIYLQEQLLSKSIG_27-28aa_LLILDDVN_17-18aa_GSRVIITTRD_12aa_YEAHGLNEEQALELLRTKAFK
MSRGA52*  SLHNVRENSD_KHGLIYLQEQPLSKSIG_27-28aa_LLILDDID_17-18aa_GSRVIITTRD_12aa_YDVDGLNEEEALELLRWKALK
MSRGA42*  FLHNVRENSV_KHGLEYLQEQLLSKSIG_27-28aa_LLILDDID_17-18aa_GSRVIITTRD_12aa_YDVDGLNEEEALELLRWKALK
MSRGA35*  FLHNVRENSI_KYGLEYLQEQLLLKSIG_27-28aa_LVILDDVD_17-18aa_GSKVIISTRD_12aa_YDVDGLNEEEALELLRWKALK
MSRGA5*   FLHNVRENSI_KYGLEYLQEQLLFKSIG_27-28aa_LLILDDVD_17-18aa_GSRVIITTRD_12aa_YELDVLNKEEALELFGXMAFK
MSRGA41   FLHNVRENSL_KHGLEYLQEQLLFKSIG_27-28aa_LLILDDID_17-18aa_GSRVIITTRD_12aa_YEAYGLNKEQALELLKTKAFK
MSRGA40   FLHNVRENSI_KYGLEYLQEQLLFKSIG_27-28aa_LLILDDID_17-18aa_GSRVIITTRD_12aa_YEAYGLNKEQALELLRTKAFK
MSRGA48   FLHNVRENSE_KHGLEHLQNDFLSKTVG_27-28aa_LLILDDVN_17-18aa_GSRVIITTRD_12aa_YEIDELNKEEALELIRSKAFK
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Fig. 4 Amino acid alignment of functional motifs of *M. sativa* resistance gene analog-(*MSRGA*) encoded sequences. * *MSRGAs* sequences containing stop codons

were considered to represent one MSRGA family. The resulting 18 families were represented by MSRGAs 1, 3, 5, 4, 6, 7, 22, 36, 40, 41, 42, 48, 50, 52, and 55.

Sequences were translated to look for motifs characteristic of plant NBS regions (Meyers et al. 1999). Figure 4 shows the amino acid alignments of the functional motifs of the most representative families of the MSRGAs. The P-loop and the GLPL motifs were included in the degenerate primer sequence and, therefore, were excluded from the sequence data analysis. The encoded amino acid sequences showed the characteristic motifs of NBS regions, namely, kinase-2 and kinase-3a.

Translations were analyzed visually for the additional motifs found by Meyers et al. (1999), which are characteristic of NBS regions and separate plant NBS regions into two major classes: TIR-NBS and nonTIR-NBS (Table 1). A region similar to the consensus sequence for the RNBS-C motif (Table 1) was found in all of the sequences obtained (Fig. 4), confirming that they belong to the NBS-LRR gene superfamily. Another motif, RNBS-A-nonTIR (Table 1), which occurs in the NBS region of R genes that do not contain the toll-interleukin receptor domain (Meyers et al. 1999), was found in MSRGAs 23, 24, 25, 27, 28, 29, 43, and 44, suggesting that these sequences correspond to NBS regions of nonTIR-NBS-LRR genes (Fig. 4). Meyers et al. (1999) also suggested that the last residue of the kinase-2 domain could be used to predict with 95% accuracy whether an NBS region would fall in the TIR-NBS or the nonTIR-NBS phylogenetic trees. A tryptophan residue (W) was found at the end of the kinase-2 motif of the eight nonTIR-MSRGAs (Fig. 4), as would be predicted from results of Meyers et al. (1999). None of the TIR-MSRGAs had that tryptophan residue, instead the kinase-2 motif had either an aspartic acid (D) or an asparagine residue (N) (Fig. 4). Analogously, motifs simi-

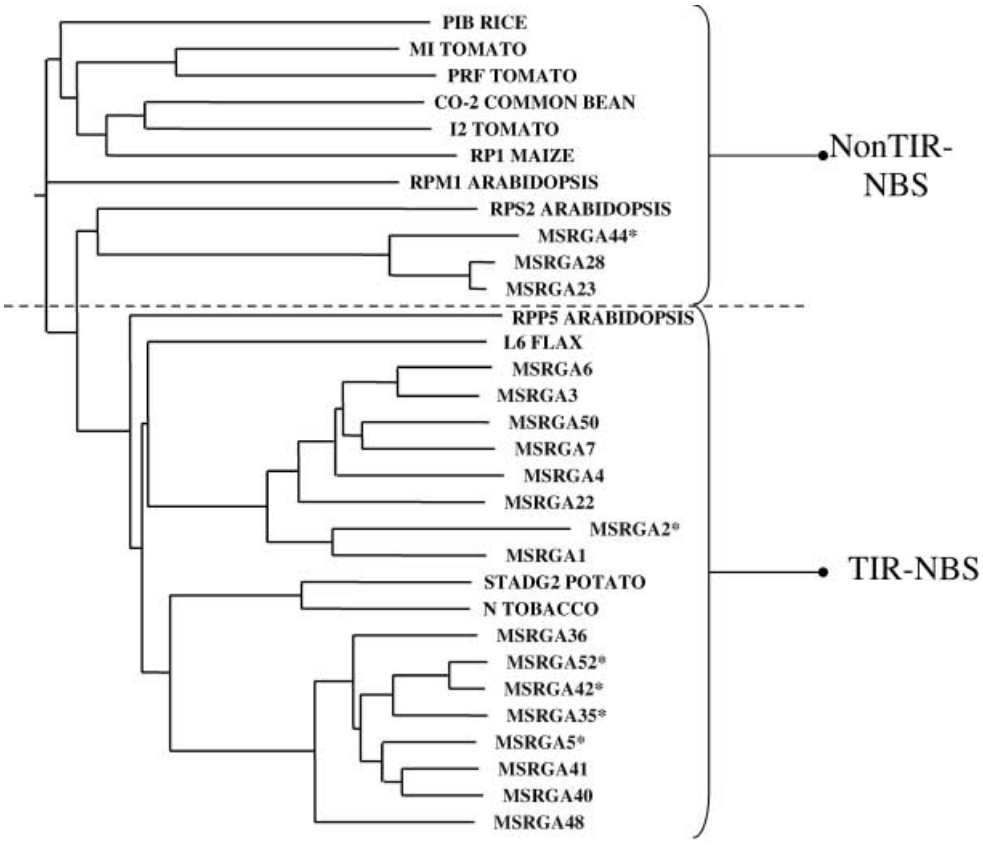

Fig. 5 Dendrogram generated from nucleotide sequence alignment of *M. sativa* resistance gene analog (*MSRGAs*) and nucleotide binding site regions of disease resistance genes from other species. * *MSRGA* sequence contain stop codons

lar to the RNBS-A-TIR (Table 1) were found in the rest of the sequences (Fig. 4), which suggests that they are NBS domains of R genes without TIR domains.

All 51 NBS regions contained the characteristic domains of plant R gene NBS. However, eight of the regions (clones MSRGA 2, 5, 35, 42, 44, 47, 52, and 55) contained stop codons that resulted in distorted amino acid sequences upon translation. MSRGA 2, 47, and 55 likely were pseudogenes, because they contained multiple stop codons and, consequently, were unable to align with the other MSRGAs at the amino acid level. Clone MSRGA2 contained a 22-bp deletion 5′ of the kinase-3a motif resulting in multiple stop codons 3′ to the deletion. Clones 2, 47 and 55 were omitted from Fig. 4.

The MSRGA5 sequence was found in 7 of the original 94 clones and encoded a stop codon between the kinase-3a and the GLPL motifs. Likewise, MSRGAs 44, 35, 42, 47, and 52 also encoded a stop codon between the kinase-3a and the GLPL motifs. The MSRGA5 sequence encoded a methionine residue after the first stop codon, suggesting the start of translation of a different product.

Amino acid alignment showed that the MSRGAs share homology with NBS regions of well-characterized R genes from other plants, including monocots and dicots: RPM1, RPS2, and RPP5 from *Arabidopsis*, L6 from flax; RP1 from maize; N from tobacco; PIB from rice; PRF, MI, and I2 from tomato (Meyers et al. 1999). The STADG2 from potato is a tightly linked marker for virus resistance, and CO-2 is a candidate resistance gene for anthracnose resistance (Meyers et al. 1999). The latter was included for comparison because it represents molecular data from likely the first verified R gene from a leguminous species.

In order to visualize the relative distance between MSRGAs and R genes from other species, we generated a dendrogram based on the amino acid sequence alignment with *Clustal W* (Fig. 5). The MSRGAs and R genes separated into two distinct branches: nonTIR-NBS and TIR-NBS (Fig. 5). Two families, MSRGA23 and MSRGA28, were placed in the nonTIR-NBS branch, where other nonTIR-NBS-LRR genes from other species were found (Fig. 5). The other 15 families were located within the TIR-NBS branch occurring with TIR-NBS-LRR genes N from tobacco, L6 from flax, and RPP5 from *Arabidopsis*. Additionally, the TIR-NBS-MSRGAs were divided in two major groups.

It is unknown whether this divergence into three major groups is functionally significant. The clones containing stop codons were found mostly in one of the three branches (Fig. 5), suggesting that those genes have evolved a mechanism in which stop codons could have some function. A recent study on the N gene from tobacco showed that stop codons may play a role in alternative splicing (Dinesh-Kumar and Baker 2000). The ratio of two altenatively spliced messages, designated Nl and Ns, played a role in N gene-mediated resistance. The Nl message was a truncated form of the N gene product caused by a UGA (opal) codon in the open reading frame. Additionally, P-loop-and UGA codon-containing genes are known to direct the incorporation of seleno-cysteine, con-

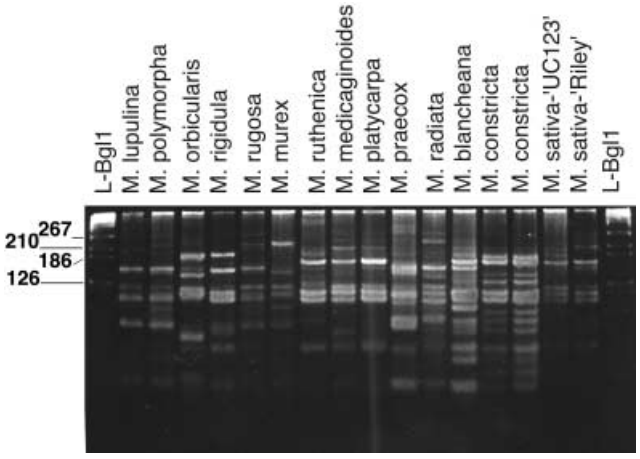

Fig. 6 Tsp509I-digested PCR fragments generated from the indicated species with degenerate nucleotide binding site region primers

sidered the 21st amino acid, further suggesting that the presence of UGA codons may not necessarily indicate nonfunctioning genes (Guimaraes et al. 1996).

To determine whether the NBS genes were transcribed, we used cDNA from plant ISC35 as a template for PCR with the NBS primers. An amplification product of about 500 bp resulted and was cloned. Sequence data were obtained from five of the cDNA clones, all of which showed high homology to previously sequenced MSRGAs from genomic DNA. The cDNA clones 4A9, 4A11, and 4B5 showed the highest homology to MSRGA1 (>99%), clone 4D1 showed the highest homology to MSRGA5 (>99%), and clone 4A12 showed the highest homology to MSRGA41 (>99%). Hence, at least those three families of MSRGAs were transcribed in *M. sativa* plant ISC35.

The cDNA clone 4D1 showed 99% homology with MSRGA5, which contained a stop codon in the region between the kinase-3a and the GLPL domains. That region is 100% homologous to the same region in cDNA clone 4D1 – 5′ -GGG *TGA* ATG GCT-3′ – which encodes NH2- G *opal* M A -COOH, confirming that the transcription product indeed has a stop codon at that position. Whether that transcription product actually is translated is unknown. However, given that *opal* codons direct the incorporation of a seleno-cysteine residue into some proteins (Guimaraes et al. 1996) and apparently are involved in the function of some R genes (Dinesh-Kumar and Baker 2000), the finding that *opal*-containing R genes are transcribed in alfalfa further suggests that UGA codons may be involved in R gene function.

To investigate the occurrence of NBS-containing genes in species related to afalfa, we used the LM638 and REV7 degenerate primers on 13 different species from the genus *Medicago*, as well as *M. sativa* cvs. UC123 and Riley. Degenerate PCR gave good amplification of a distinct 500-bp product, the expected size for NBS regions. Restriction digests with Tsp509I showed considerable variation among R gene analogs in the genus *Medicago* (Fig. 6). That variation in RGAs represents a pool of candidate R genes that might be useful in the future for introducing disease resistance from distantly related wild species.

Wild relatives in the genus *Medicago* have been suggested to be a potential gene pool for disease-and pest- resistance genes. Several species of the genus have been screened, and very high levels of resistance against races 1 and 2 of anthracnose have been reported in *M. littoralis*, *M. murex*, *M. rigidula*, *M. tenoreana*, and *M. truncatula* and high levels in *M. arborea* (Quiros and Bauchan 1988). Resistances to spring blackstem (*Phoma medicaginis*) in *M. dzhwakhetica* and *M. suffruticosa*, to bacterial wilt (*Corynebacterium insidiosum*) in *M. arborea*, and to *Stemphylium* leafspot in *M. cancellata* also have been reported (Quiros and Bauchan 1988).

Attempts to cross plants from the *M. sativa* complex with wild *Medicago* species have failed because of several barriers that make the species incompatible. Among these are ploidy level, chromosome rearrangement, pollen morphology, and poor seed development (Quiros and Bauchan 1988). The use of molecular techniques for the isolation of disease- and pest-resistance genes, along with plant transformation, may enable breeders to overcome the barriers between the wild species of the genus *Medicago* and the cultivated *M. sativa*. This would enable alfalfa breeders to make use of the R gene pool that we have found to exist within the genus *Medicago* and to incoporate it into breeding programs.

This study showed that the MSRGA family is highly diverse in alfalfa and within the genus *Medicago*. In alfalfa, we found 51 unique sequences that encoded the characteristic motifs of the NBS of disease- and pest-resistance genes. These sequences represented six major clusters according to dendrogram and bootstrap analyses. Some clusters showed little variation among the sequences (<5% nucleotide sequence divergence) and might represent alleles of homologous resistance genes. Sequences that diverged by more than 5% were considered to be members of different families. The 51 sequences were classified into 18 families of *M. sativa* R gene analogs. At least three of the 18 families were transcribed. Additionally, the three groups of NBS gene families in *M. sativa* shown by dendrogram analysis – two groups with TIR-NBS characteristics and one with nonTIR-NBS characteristics – suggested three different evolutionary origins. This gene family provides a rich source of naturally occurring genetic diversity with the potential to confer resistance to a wide range of pathogens and pests. Any strategy successful in incorporating resistance from this diverse gene pool into elite cultivars will provide a varied array of R genes, potentially contributing to sustainable forms of resistance.

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