Theor Appl Genet (2000) 100:1279–1283

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Targeted resistance gene mapping in soybean using modified AFLPs

Received: 25 September 1999 / Accepted: 3 November 1999

Abstract The soybean [*Glycine max* (Merr.) L.] linkage group F contains a vital region of clustered genes for resistance to numerous pathogens including the soybean mosaic virus resistance gene, *Rsv*1. In order to develop new genetic markers that map to this gene cluster, we employed a targeted approach that utilizes the speed and high-throughput of AFLP, but modified it to incorporate sequence information from the highly conserved nucleotide binding site (NBS) region of cloned disease resistance genes. By using a labeled degenerate primer corresponding to the p-loop portion of the NBS region of resistance genes, such as *N*, *L6*, and *Rps2*, we were able to quickly amplify numerous polymorphic bands between parents of a population segregating for resistance to *Rsv*1. Of these polymorphic bands, bulk segregant analysis revealed four markers that were closely linked to *Rsv*1. These markers were cloned and used as probes for RFLP analysis. The four clones mapped to within a 6-cM region surrounding *Rsv*1, the closest being 0.4 cM away from the gene. Sequence analysis showed that all four clones contain the p-loop sequence corresponding to the degenerate primer and that one of the four clones contains an open reading frame sequence which when translated is related to the NBS region of other cloned disease resistance genes. The rapid identification of four markers closely linked to *Rsv*1 in soybean demonstrates the utility of this method for generating markers tightly linked to important plant disease resistance genes.

Key words Disease resistance · Soybean mosaic virus · AFLP · Bulk segregant analysis · p-loop

Communicated by P.M.A Tigerstedt

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Introduction

In developing a high-resolution map surrounding a gene of interest, it is important to generate markers that specifically target that chromosomal region. We used a targeted homology-based approach to develop new markers surrounding the *Rsv*1 locus in soybean. *Rsv*1 is a valuable gene conferring resistance in soybean to one of the more important viral pathogens, soybean mosaic virus (SMV) (Irwin and Goodman 1981; Thottapilly and Rossel 1987; Ren et al. 1997). *Rsv*1 has been mapped on the soybean genome to a tight cluster of resistance genes on the F linkage group (Yu et al. 1994).

The chromosomal region surrounding *Rsv*1 has been shown to be a hot spot for disease resistance loci. Resistance genes in this region include *Rps*3, *Rpg*1 and *Rpv*1, all of which are flanked by the RFLP markers K644 and B212 (Diers et al. 1992; Ashfield et al. 1998; Saghai Maroof, unpublished). In addition, quantitative trait loci for resistance to the root knot nematode and corn earworm have also been reported to reside in this region (Tamulonis 1997; Rector et al. 1999).

Our initial focus of fine-mapping the *Rsv*1 region involved the placement of previously reported markers on a soybean map generated using an F_2 population segregating at the *Rsv*1 locus. Subsequent efforts have involved identifying new markers that map close to this gene. Yu et al. (1996) developed a targeted technique that consisted of designing degenerate primers from the conserved nucleotide binding site (NBS) region of the disease resistance genes *N* (Whitham et al. 1994) and *Rps*2 (Bent et al. 1994). These resistance genes belong to a gene family which contains NBS and leucine-rich repeat (LRR) domains. The degenerate primers were used in a polymerase chain reaction (PCR) to amplify NBS sequences in soybean which were highly similar to the NBS portion of cloned NBS-LRR disease resistance genes. These resistance gene analog (RGA) sequences were mapped to numerous chromosomal regions in soybean where disease resistance genes are known to exist. Two of these RGA markers, NBS5 and NBS61, were

mapped to the chromosomal region near *Rsv*1 (Yu et al. 1996). In a number of plant species, researchers have used similar approaches to identify RGA probes which are associated with important disease resistance genes (Kanazin et al. 1996; Leister et al. 1996; Aarts et al. 1998; Collins et al. 1998; Gentzbittel et al. 1998; Leister et al. 1998, Ohmori et al. 1998; Shen et al. 1998; Speulman et al. 1998).

The success in generating these RGA clones that map close to resistance genes of interest led us to believe we could develop a strategy that might more quickly identify clones which map to the soybean resistance gene cluster on linkage group F near *Rsv*1. To generate such clones we used degenerate primers corresponding to a motif of the conserved nbs region in an amplified fragment length polymorphism (AFLP) reaction (Vos et al. 1995). AFLP is a PCR-based marker system whereby restriction-site and size polymorphisms are detected between lines. DNA is digested with specific restriction enzymes and then oligonucleotide adaptors are ligated to the restriction ends. These adaptors serve as the recognition site for primers during the subsequent PCR reaction. Three selective nucleotides at the 3' end of the primer ensure amplification of only a small subset of the digested and ligated genomic fragments. These fragments are labeled and separated on a polyacrylamide gel in order to detect polymorphic bands.

The objective of this study is to develop and test a PCR-based strategy that combines the speed, efficiency, and high throughput of AFLP with the resistance-gene-directed degenerate primer approach. This technique was used to generate markers tightly linked to *Rsv*1.

Materials and methods

Genetic materials

The F_2 population PI96983 (*Rsv*1) × Lee68 (*rsv*1) was used for bulk segregant analysis and fine mapping of the *Rsv*1 locus (Yu et al. 1996). This population consists of 243 $F_{2:3}$ individuals and the map contains several markers surrounding the *Rsv*1 locus.

Young trifoliolate leaf tissue from greenhouse-grown plants of 12–15 $F_{2:3}$ individuals from each line was collected. DNA was isolated by freeze-drying followed by CTAB extraction as described by Saghai Maroof et al. (1984).

Marker analysis

DNA from 12 homozygous resistant and susceptible lines of the F_2 population were pooled to form bulk-resistant and bulk-susceptible samps. These DNAs, along with the parental DNAs PI96983 (resistant) and Lee68 (susceptible), were digested with the restriction enzymes *Eco*RI and *Mse*I, and adaptor sequences were ligated to the restriction ends as previously described (Vos et al. 1995; Maughan et al. 1996). An initial round of PCR was conducted using *Mse*I and *Eco*RI +1 selective primers, to amplify a subset of the digested and ligated fragments. Thirty cycles of a 25-µL PCR reaction using 1 × buffer, 0. 9 mM $MgCl_2$, 0.6 µM of each primer, 0.25 mM dNTP, 0.5 U of *Taq* polymerase and 150 ng of template was conducted with denaturation at 94°C for 60 s, primer annealing at 60°C for 30 s, and primer extension at 72°C for 30 s. An aliquot of this reaction equivalent to 25 ng of DNA was then used in a second round of amplification. The *Eco*RI +1 primer used in the first reaction and a ^{32}P end-labeled degenerate primer (corresponding to the p-loop region of resistance genes *N*, *Rps*2, and *L*6) were used in the second-round PCR reaction. The degenerate primer sequence (5' GGA ATG GGN GGN GTN GGN AAR AC 3') is the same as that used by Yu et al. (1996). PCR components were essentially the same as the previous reaction with the exception that 0.15 uM of ^{32}P end-labeled and 0.6 µM of unlabeled degenerate primers were used in combination with 0.75 µM of the *Eco*RI adapter-specific +1 primer. This second round of PCR was conducted using touchdown conditions beginning at 65°C annealing and reducing by 1°C per cycle to 56°C, followed by 26 cycles at 56°C. All other cycling conditions were as in the previous reaction.

Second-round PCR products were separated on a 7-M urea 4.5% polyacrylamide gel for 2.5 h at 60 W. The gel was then transferred to 3MM paper, covered with plastic wrap, and exposed to Kodak (New Haven, Conn.) Xomat film for 12–18 h.

PCR products which appeared to be linked to *Rsv*1, based on bulk segregant analysis, were excised from the gel by elution in 100 µl of water incubated in a boiling bath as described by Upender et al. (1995). The eluate was PCR-amplified using the same primers that generated the polymorphic product under the same or less-stringent conditions. The PCR product was then cloned into the pCNTR shuttle vector using the General Contractor Cloning Kit (5 prime, 3 prime, Boulder, Colo.) or PCR fragments were cloned into the pCR2.1-TOPO vector using the TOPO-TA cloning kit (Invitrogen, Carlsbad, Calif.). Cloned inserts were amplified with the original +1 primer and the labeled degenerate primer, again under the same second-round PCR conditions. These labeled fragments were then run on a 7 M urea, 4.5% polyacrylamide gel, along-side labeled parental samples generated under the same conditions to determine those clones that contained the proper size insert based on BSA. Tentatively confirmed positive inserts were amplified for use as RFLP markers.

The Southern-hybridization procedure was essentially as described (Yu et al. 1994). Briefly, 8 µg of parental DNA was digested individually with the enzymes *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Xba*I and *Bam*HI, according to the manufacturer's protocols (Gibco-BRL, Rockville, Md.). Digested DNA was then separated on a 1% agarose gel at 70 to 90 mA for 14–16 h. The DNA was transferred to a Hybond nylon membrane (Amersham, Piscataway, N.J.) by Southern blotting with a 0.4 N NaOH buffer. Screening blots were hybridized with $\alpha^{32}P$-dCTP, random primer-labeled probe (Ambion, Austin, Tex.). Hybridizing bands were visualized by autoradiography on Kodak Xomat film. Polymorphic bands were mapped in the *Rsv*1 segregating population. Multiple linkage analysis was determined using MAPMAKER software at LOD 3.0 (Lander et al. 1987).

Sequence analysis

Inserts were sequenced using an ABI377 DNA sequencer. Plasmid template was prepared using standard alkaline-lysis followed by purification using QiaexII (Qiagen, Valencia, Calif.). Dye-terminator cycle sequencing was done based on the manufacturer's protocols (Perkin Elmer, Foster City, Calif.). Sequence assembly and analysis was conducted using Lasergene software from DNAstar (Madison, Wis.).

Results

All 16 possible *Eco*+1/p-loop primer combinations were tested with bulk and parental DNA samples. A total of 33 polymorphisms were observed among the approximately 960 bands visualized. Four dominant markers, R11 (518 bp), R12 (171 bp), R13 (261 bp) and R14 (312 bp), were identified which appeared to be linked to *Rsv*1

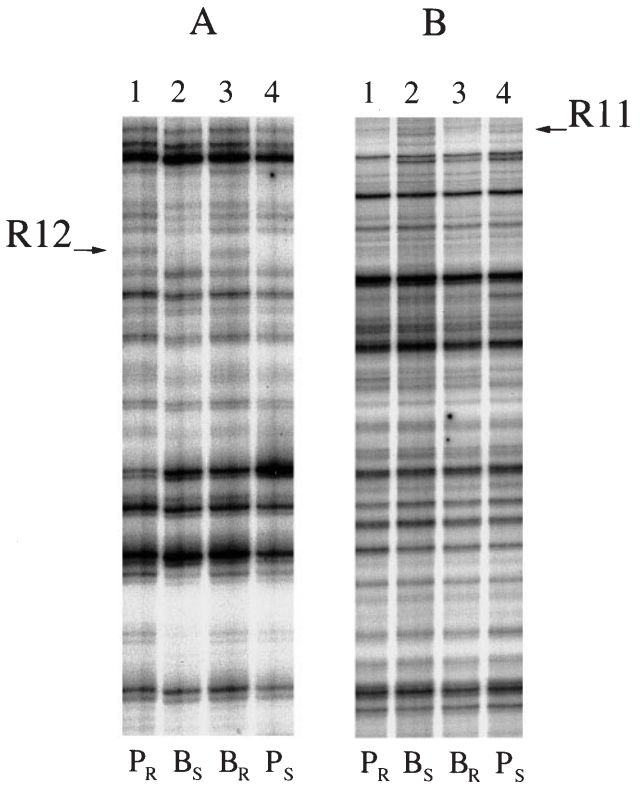

Fig. 1A, B Homology-based AFLP gel of parents and bulks of the population PI96983 × Lee68 displaying linked polymorphisms with two primer combinations. *Lanes* are as follows: *1* PI96983 (*Rsv*1), *2* bulk susceptible, *3* bulk resistant, and *4* Lee68 (*rsv*1). Primer combinations are as follows: **A** *Eco*+C/p-loop (*Eco*+C/*Mse*+T), **B** *Eco*+A/p-loop (*Eco*+A/*Mse*+G). Primer combination **A** amplifies linked marker R12, and primer combination **B** amplifies linked marker R11. *Arrows* indicate a linked polymorphism

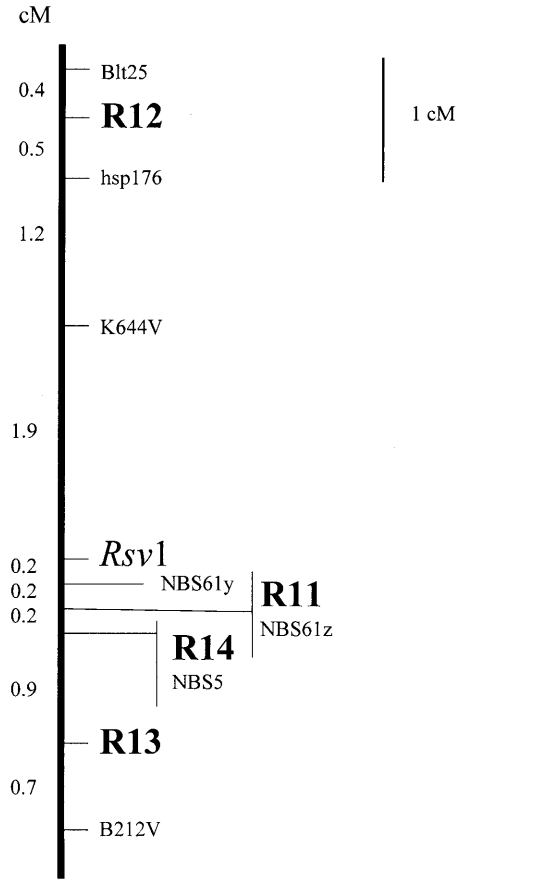

Fig. 2 Genetic map of the region surrounding *Rsv*1, showing the map location of four homology based AFLPs, *R11*, *R12*, *R13* and *R14*. All clones were mapped in the population PI96983 × Lee68 which segregates for resistance to *Rsv*1

based on BSA results. Two examples of linked bands amplified by this procedure and detected by BSA are shown in Fig. 1. Amplified products from the putatively linked bands were excised from the gel and cloned for further analysis. After confirming proper-sized inserts, each of the four positive clones was mapped by RFLP to linkage group F near *Rsv*1. All four unique positive clones mapped within 3.5 cM of *Rsv*1, and two of the four mapped within 0.6 cM of the gene (Fig. 2).

Sequence analysis of the four linked clones indicated that all contain the p-loop sequence corresponding to the degenerate primer used to amplify the sequences. Only one of the four, however, contains an additional sequence which is homologous to other RGA sequences. This clone, R14 (Genbank Accession # AF222879), co-segregates with NBS5, another RGA previously identified by Yu et al. (1996). R14 is 312 nucleotides in length, excluding the adaptor sequence which had been ligated to the fragment. This clone maps 0.6 cM from *Rsv*1 (Fig. 2). Alignment of R14 with other previously published soybean NBS sequences (Kanazin et al. 1996; Yu et al. 1996) showed that it most closely resembles NBS61, sharing 75% similarity based on Wilbur-Lipman pairwise alignment (Wilbur and Lipman 1983) (Fig. 3).

Discussion

When fine mapping a gene of agronomic importance, such as disease resistance loci, it is a useful strategy to enhance the detection of markers which specifically target the gene of interest. In addition to bulk segregant analysis and traditional AFLP, we have employed a novel homology based AFLP approach which has quickly generated four tightly linked markers to *Rsv*1, an important soybean resistance gene.

The presence of numerous p-loop-type sequences facilitated the generation of these molecular markers. In this study we used a modified AFLP approach whereby only those sequences that contain a p-loop-like region near an *Eco*RI restriction site were amplified and visualized. In the initial digestion and ligation steps, approximately 87.5% of the soybean genome is eliminated because *Mse*/*Mse* fragments are not detected in the proceeding steps. In this study we evaluated all 16 possible primer combinations using only the *Eco* +1 primer. On average we obtained 60 AFLP bands per gel lane which represents no less than 60 p-loop sequences per reaction. This does not include amplification products that migrate to the same point on the gel. Based on these observations

Fig. 3 Wilbur-Lipman alignment of the homology based AFLP-derived RGA, *R14*, which maps 0.6 cM from *Rsv*1. The alignment shows the sequence similarity of this clone to the previously reported RGA, *NBS61*. The *top line* is the NBS61 sequence. The *bottom line* is the R14 sequence. *Connecting lines* show those nucleotides which are identical between the two clones. *Dashes* demarcate shifts in the sequence to better align the two clones

we estimate there are no less than 15 000 of these type of p-loop sequences in the soybean genome. This is based on the percentage of the genome sampled, the mean number of products observed per sampling, the total number of sampling combinations, and the fact that only one of the two DNA strands is detected by labeled primer annealing per fragment. A significant percentage of these amplified sequences contain a p-loop-like sequence but do not contain additional sequences characteristic of disease resistance genes (unpublished data).

Of the four clones evaluated in this study, all of which were linked to *Rsv*1, one, R14, contains motifs identical to other cloned NBS sequences. This clone cosegregates with another RGA, NBS5. Interestingly, overall sequence similarity to NBS5 is relatively low at 27.7% (data not shown). Conversely, R14 is structurally very similar to a second RGA clone, NBS61, which represents a multigene family mapping to the *Rsv*1 region. These two clones have a similarity of 75.2% (Fig. 3). Two NBS61 loci (NBS61y and NBS61z, Fig. 2) are located, respectively, 0.4 and 0.2 cM away from this clone suggesting that all of these loci may be related sequences of a multi-gene family near *Rsv*1. Like NBS61, R14 shows multiple bands on an RFLP Southern blot. There are, however, no apparent commonalities between the banding patterns of these two clones.

The PCR-based marker system developed in this study, specifically amplifies sequences that contain motifs known to occur in a number of hypersensitive-response-type resistance genes. These NBS-LRR genes all contain p-loop, kinase-2, and putative kinase-3a motifs. Using a single restriction enzyme combination and only a few primer combinations we were able to quickly identify four markers containing p-loop sequences that tightly flank our resistance gene of interest, *Rsv*1. Identification of additional markers closely linked to other resistance genes, particularly in those regions known to contain RGA clones, or other NBS-LRR type resistance genes is certainly plausible using this technique. In addition, it is certainly possible to modify this technique by using other degenerate primers which correspond to other conserved motifs from NBS-LRR and other classes of resistance genes.

Acknowledgments This study was supported in part by the U.S. Department of Agriculture, Natural Research Initiative, Competitive Grants Program, Grant no. 96–35300–3648 and by the United Soybean Board. The authors wish to thank Randy Shoemaker for providing RFLP markers.

References

Aarts MGM, Hekkert BL, Holub EB, Beynon JL, Stiekema WJ, Pereira A (1998) Identification of R-gene homologous DNA fragments genetically linked to disease resistance loci in *Arabidopsis thaliana*. Mol Plant-Microbe Interact 11:251–258

Ashfield T, Danzer JR, Held D, Clayton K, Keim P, Saghai Maroof MA, Webb DM, Innes RW (1998) *Rpg*1, a soybean gene effective against races of bacterial blight, maps to a cluster of previously identified disease resistance genes. Theor Appl Genet 96:1013–1021

Bent AF, Kunkel BN, Dahlbeck D, Brown KL, Schmidt R, Giraudat J, Leung J, Staskawicz B (1994) *Rps*2 of *Arabidopsis thaliana*: a leucine-rich repeat class of plant disease resistance genes. Science 265:1856–1860

Chen P, Buss GR, Roane CW, Tolin SA (1991) Allelism among genes for resistance to soybean mosaic virus in strain-differential soybean cultivars. Crop Sci 31:305–309

Collins NC, Webb CA, Seah S, Ellis JG, Hulbert SH, Pryor A (1998) The isolation and mapping of disease resistance gene analogs in maize. Mol Plant-Microbe Interact 11:968–978

Diers BW, Mansur L, Imsande J, Shoemaker RC (1992) Mapping *Phytophthora* resistance loci in soybean with restriction fragment length polymorphism markers. Crop Sci 32:377–383

Gentzbittel L, Mouzeyar S, Badaoui S, Mestries E, Vear F, Labrouhe DTD, Nicolas P (1998) Cloning of molecular markers for disease resistance in sunflower, *Helianthus annuus*, L. Theor Appl Genet 96:519–525

Irwin ME, Goodman RM (1981) Ecology and control of soybean mosaic virus. In: Maramorosch K, Harris KF (eds.) Plant diseases and vectors: ecology and epidemiology. Academic Press, New York, pp 181–220

Kanazin V, Marek LF, Shoemaker RC (1996) Resistance gene analogs are conserved and clustered in soybean. Proc Natl Acad Sci USA 93:11746–11750

Lander ES, Green P, Abrahamson J, Barlow A, Daly JM, Lincoln SE, Newberg L (1987) Mapmaker: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Leister D, Ballvora A, Salamini F, Gephardt C (1996) A PCR-based approach for isolating pathogen resistance genes from potato with potential for wide application in plants. Nature Genet 14:421–428

Leister D, Kurth J, Laurie DA, Yano M, Sasaki T, Devos K, Graner A, Schulze-Lefert P (1998) Rapid reorganization of resistance gene homologues in cereal genomes. Proc Natl Acad Sci USA 95:370–375

Ma G, Chen P, Buss GR, Tolin SA (1995) Genetic characteristics of two genes for resistance to soybean mosaic virus in PI486355 soybean. Theor Appl Genet 91:907–914

Maughan PJ, Saghai Maroof MA, Buss GR, Huestis GM (1996) Amplified fragment length polymorphism (AFLP) in soybean: species diversity, inheritance, and near-isogenic line analysis. Theor Appl Genet 93:392–401

Ohmori T, Murata M, Motoyoshi F (1998) Characterization of disease resistance gene-like sequences in near-isogenic lines of tomato. Theor Appl Genet 96:331–338

Rector BG, All JN, Parrott WA, Boerma HR (1999) Quantitative trait loci for antixenous resistance to corn earworm in soybean. Crop Sci 39:531–538

Ren QT, Pfeiffer W, Ghabrial SA (1997) Soybean mosaic virus incidence level and infection time: interaction effects on soybean. Crop Sci 37:1706–1711

Saghai Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer length polymorphism in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci USA 81:8014–8018

Shen KA, Meyers BC, Faridi MNI, Chin DB, Stelly DM, Michelmore RW (1998) Resistance gene candidates identified by PCR with degenerate oligonucleotide primers map to clusters of resistnce genes in lettuce. Mol Plant-Microbe Interact 11: 815–823

Speulman E, Bouchez D, Holub EB, Beynon JL (1998) Disease resistance gene homologs correlate with disease resistance loci of *Arabidopsis thaliana*. Plant J 14:467–474

Tamulonis JP, Luzzi BM, Hussey RS, Parrott WA, Boerma HR (1997) DNA marker analysis of loci conferring resistance to peanut root-knot nematode in soybean. Theor Appl Genet 95: 664–670

Thottapilly G, Rossel HW (1987) Viruses affecting soybean. In: Singh SR, Rachie KO, Dashiell KE (eds) Soybeans for the tropics: research, production, utilization. John Wiley and Sons Ltd., Chichester, UK, pp 53–68

Upender M, Raj L, Weir M (1995) Rapid method for the elution and analysis of PCR products separated on high-resolution acrylamide gels. Biotechniques 18:33–34

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B (1994) The product of the tobacco mosaic virus resistance gene *N*: similarity to Toll and the Interleukin-1 receptor. Cell 78:1101–1115

Wilbur WJ, Lipman DJ (1983) Rapid similarity searches of nucleic acid and protein databases. Proc Natl Acad Sci USA 80:726–730

Yu YG, Saghai Maroof MA, Buss GR, Maughan PJ, Tolin SA (1994) RFLP and microsatellite mapping of a gene for soybean mosaic virus resistance. Phytopathology 84:60—64

Yu YG, Buss GR, Saghai Maroof MA (1996) Isolation of a superfamily of candidate disease resistance genes in soybean based on a conserved nucleotide-binding site. Proc Natl Acad Sci USA 93:11751–11756