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Genetics

Isolation of a superfamily of candidate disease-resistance genes in soybean based on a conserved nucleotide-binding site

(molecular cloning/degenerate oligonucleotide primer/evolution/signal transduction)

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ABSTRACT **The tobacco *N* and *Arabidopsis RPS2* genes, among several recently cloned disease-resistance genes, share a highly conserved structure, a nucleotide-binding site (NBS). Using degenerate oligonucleotide primers for the NBS region of *N* and *RPS2*, we have amplified and cloned the NBS sequences from soybean. Each of these PCR-derived NBS clones detected low- or moderate-copy soybean DNA sequences and belongs to 1 of 11 different classes. Sequence analysis showed that all PCR clones encode three motifs (P-loop, kinase-2, and kinase-3a) of NBS nearly identical to those in *N* and *RPS2*. The intervening region between P-loop and kinase-3a of the 11 classes has high (26% average) amino acid sequence similarity to the *N* gene although not as high (19% average) to *RPS2*. These 11 classes represent a superfamily of NBS-containing soybean genes that are homologous to *N* and *RPS2*. Each class or subfamily was assessed for its positional association with known soybean disease-resistance genes through near-isogenic line assays, followed by linkage analysis in F_2 populations using restriction fragment length polymorphisms. Five of the 11 subfamilies have thus far been mapped to the vicinity of known soybean genes for resistance to potyviruses (*Rsv1* and *Rpv*), *Phytophthora* root rot (*Rps1*, *Rps2*, and *Rps3*), and powdery mildew (rmd). The conserved *N*- or *RPS2*-homologous NBS sequences and their positional associations with mapped soybean-resistance genes suggest that a number of the soybean disease-resistance genes may belong to this superfamily. The candidate subfamilies of NBS-containing genes identified by genetic mapping should greatly facilitate the molecular cloning of disease-resistance genes.**

Over the past few years, we have witnessed a breakthrough in the molecular cloning of disease-resistance genes (for review, see ref. 1). The growing list of cloned resistance genes includes *HM1* (2) in maize, *Pto* (3) and *Cf9* (4) in tomato, *N* (5) in tobacco, L^6 (6) in flax, *RPS2* (7, 8) and *RPM1* (9) in *Arabidopsis*, and *Xa21* (10) in rice. The successful cloning of each of these resistance genes was facilitated by the rapid advances in the technology of transposon tagging and map-based cloning. Nonetheless, the cloning of new resistance genes using the same approaches will still be laborious and time-consuming.

A notable finding from the cloned disease-resistance genes is their similarity in amino acid sequences or conserved structure (1). All, except *HM1*, whose product directs chemical detoxification, encode proteins that contain domains involved in signal transduction or protein–protein interaction. In particular, a majority of resistance genes, including *N*, *RPS2*, L^6, and *RPM1*, have a nucleotide-binding site (NBS) and leucine-rich repeats (LRR). If these highly conserved structures are common among many resistance genes, an enormous potential exists for isolating new resistance genes based on sequence homology.

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Ellis *et al.* (11) reported that clones of L^6 (a rust-resistance gene) cross-hybridize to sequences that cosegregate with *M* (an unlinked rust-resistance gene) in flax, suggesting that homologous disease-resistance genes may be identified under low-stringency hybridization conditions using cloned genes as probes. Polymerase chain reaction (PCR) using degenerate oligonucleotide primers, however, is more sensitive in isolating conserved sequences (12, 13) and would therefore be more effective in isolating potential NBS/LRR disease-resistance genes. The use of PCR, nonetheless, could result in the identification of numerous genes that have a common conserved domain but nothing to do with disease resistance (1), especially if genes containing these domains are overabundant.

In this study, we used degenerate oligonucleotide primers to amplify NBS-containing sequences as a pool for the identification of disease-resistance genes in soybean. Our objectives were (*i*) to determine the abundance of soybean NBS sequences by isolating and cataloging them based on DNA sequence and restriction fragment length polymorphism (RFLP) pattern on Southern blots, and (*ii*) to screen for candidate genes or multigene families for a number of previously mapped soybean disease-resistance genes using near-isogenic lines and segregating populations.

MATERIALS AND METHODS

Genetic Materials. The soybean line PI 96983 was used as the source of DNA for PCR amplification of NBS-containing sequences using degenerate primers. Other sources of DNA included rice (*Oryza sativa* cv. "Teqing"), barley (*Hordeum vulgare* "TR306"), tobacco (*Nicotiana tabacum* "K326"), and potato (*Solanum tuberosum* "C234").

Fifteen soybean near-isogenic lines (NILs) for various disease-resistance genes (14), shown in Table 1, were obtained from R. L. Bernard (University of Illinois). These NILs possessing resistance genes from various sources were developed by at least six generations of backcrossing into the recurrent parents Clark, Harosoy, or Williams. F_2 mapping populations from the crosses of D26 × Lee 68 (unpublished work), PI 96983 × Lee 68 (16) and V71-370 × PI 407.162 (17) were used for the confirmation of the map positions of NBS-containing genes as indicated by NIL analysis.

Oligonucleotide Primers and PCR Conditions. A pair of degenerate oligonucleotide primers, NBS-F1 and NBS-R1, were designed based on two motifs of the NBS amino acid sequences conserved between the *N* and *RPS2* genes (Table 2).

For the amplification of NBS sequences using a pair of degenerate primers, 50 pmol of each primer and 50 ng of

Abbreviations: NBS, nucleotide-binding site; LRR, leucine-rich repeat; RFLP, restriction fragment length polymorphism; NIL, near-isogenic line; cM, centimorgan.

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Table 1. Soybean NILs that carry *Phytophthora* root rot (*Rps1, Rps2, Rps3*), potyvirus (*Rsv1* and *Rpv*), and powdery mildew (*rmd*) resistance genes from various sources

Resistance gene	Line	Parentage*
Clark NILs		
Rps1-c	L75-3901	C (6) × Arksoy
Rps1-k	L77-2015	C (6) × Kingwa
Rps2–Rj2	L76-2060	C (6) × [H (5) × D54-2437†]
Williams NILs		
Rps1-k	L77-1794	W (7) × Kingwa
Rps2–Rj2	L76-1988	W (6) × [H (5) × D54-2437]
Rps3	L83-570	W (6) × PI 86972-1
rmd	L88-8226	W (6) × PI 86972-1
rmd	L90-7978	W (6) × Jefferson
Rsv1	L78-379	W (6) × PI 96983
Rsv1-b	L83-529	W (6) × Buffalo
Rpv	L85-2308	W (6) × Dorman
Rps1-k, *Rsv1*	L81-4420	L78-379 × W82
Rps1-k, *Rpp1*	L85-2378	W82 (6) × PI 200.492
Rps1-k, *Rpp4*	L87-0482	W82 (6) × PI 459.025
Rps2–Rj2	L82-1449	H (6) × D54-2437

*The numbers in parentheses indicate the numbers of backcrosses used in the development of the NILs. Fourteen of the 15 NILs have been described in ref. 14. The remaining line, L83-529, is described in ref. 15. Abbreviations: C, Clark; W, Williams; W82, Williams 82.
†D54-2437 parentage is Roanoke, Ogden, CNS, Lincoln, and Richland. CNS is the source of *Rps2* and *Rj2*.

genomic DNA were added to 50 μl of PCR reaction mixture with 1× reaction buffer (Promega), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 2.5 units of *Taq* DNA polymerase. Thirty cycles of PCR, consisting of denaturation at 95°C for 1 min, reannealing at 55°C for 1.5 min, and extension at 72°C for 1 min, were performed in a DNA thermal cycler (Perkin–Elmer/Cetus).

Cloning of PCR Products. The PCR products were purified from an agarose gel slice using a Geneclean II Kit (Bio101). A Prime PCR cloner kit from 5 Prime-3 Prime, Inc., was used for cloning PCR products. PCR products were modified, ligated to dephosphorylated pNoTA/T7 vector, and transformed into competent cells according to the manufacturer's protocol.

RFLP Analysis. DNA extraction and RFLP analysis were as described (16). Six restriction enzymes (*Bcl*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Taq*I, and *Xba*I) were used in the digestion of 8 μg of each soybean DNA sample to detect RFLPs as described by Yu *et al.* (15). Previously mapped RFLP clones used for linkage analysis were kindly provided by R. C. Shoemaker (Iowa State University). ^{32}P-labeled DNA probes were made by the random-primer method (Ambion, Austin, TX) from the inserts of the soybean DNA clones. The post-hybridization wash conditions were: twice in 2× SSC/0.5% SDS for 10 min each at room temperature, followed by either twice in 0.5× SSC/0.1% SDS for 20 min each at 65°C (medium stringency) or twice in 0.1× SSC/0.05% SDS for 20 min each at 65°C (high stringency).

DNA Sequencing. Double-stranded plasmid DNA samples were used for DNA sequencing using an Applied Biosystems 373A automated sequencer at the Molecular Genetics Facility of the University of Georgia. Multiple-sequence alignment of the DNA sequence was made with the Lasergene software from DNAstar (Madison, WI) according to manufacturer's instructions.

RESULTS

Isolation and Classification of Soybean NBS Sequences. The PCR products amplified from soybean, rice, barley, tobacco, and potato genomic DNA using degenerate primers (NBS-F1 and NBS-R1) are shown in Fig. 1. There are two major bands amplified from soybean DNA, one at 340 bp and the other at 950 bp. The 340-bp fragment, whose size is similar to the targeted NBS fragments in the *N* and *RPS2* genes, was amplified from all five species. Only the soybean 340-bp PCR product was purified from agarose gel for further analyses.

When used as a probe to hybridize to soybean Southern blots, the 340-bp PCR product detected such a high copy number of DNA sequences that it became a smear after overnight exposure (results not shown). We reasoned that this 340-bp PCR product either belongs to a class of highly repetitive sequences or consists of different classes of sequences, at least some of which are low or moderate copy. The PCR product was cloned into the pNoTA vector, and 300 clones that had the same insert size (≈340 bp) were chosen for further characterization. Hereafter, we will designate these clones as "nbs" (NBS) and refer to them as "PCR clones."

Forty-seven randomly selected PCR clones were used as probes to hybridize at high or medium stringency to Southern blots of soybean DNA samples digested with six different restriction enzymes. In contrast to the results from hybridization with the original PCR product, each of these PCR clones hybridized to only low- or moderately low-copy DNA sequences. Many of the 47 clones detected the same multi-enzyme RFLP patterns among a set of specific soybean lines, indicating that they either are redundant clones or constitute different members of the same multigene families. Based on their distinct, non-overlapping multi-enzyme RFLP patterns on the set of soybean lines, the PCR clones were classified into 11 (*a–k*) different classes (Table 3). Thus, the original 340-bp PCR product, though of a uniform size, contains sequences of at least 11 classes or 11 multigene families.

Sequence Homology of Soybean NBS Clones to the Tobacco *N*, *Arabidopsis RPS2*, and Flax L^6 Genes. At least one clone from each of the 11 classes (*a–k*) was sequenced. The encoding amino acid sequences of the 11 classes of PCR clones are highly conserved and strikingly similar to the tobacco *N*, *Arabidopsis RPS2*, and flax L^6 genes (Fig. 2). The highest homology among all soybean entries are at the three motifs of the NBS, i.e., the P-loop (GMGGVGKTT), kinase-2 (LIVLDD), and kinase-3a (DWFGxGSR). These conserved NBS motifs are featured in *N*, *RPS2*, L^6, and several other resistance genes (refs. 5–9). The DNA sequences corresponding to the P-loop and a portion of the kinase-3a in each PCR clone were contributed by primers, and may not be the exact genomic sequences. These sequences were thus excluded from the calculation of sequence similarities, although they should in general agree with the actual genomic sequences because of the stringent PCR conditions in combination with the high degeneracy of oligonucleotide primers. The presence of an internal kinase-2 motif, which is independent of the primer sequences, in the PCR clones,

Table 2. Degenerate oligonucleotide primers based on the amino acid sequences of two highly conserved motifs of the NBS in tobacco *N* and *Arabidopsis RPS2* genes

Amino acid sequence		Oligonucleotide primer	
N	*RPS2*	Name	Sequences* (5′ to 3′)
^{216}GMGGVGKT	^{182}GPGGVGKT	NBS-F1	GGAATGGGNGGNGTNGGNAARAC
^{324}SRIIITTR	^{284}CKVMFTTR	NBS-R1	YCTAGTTGTRAYDATDAYYYTRC

*Codes for mixed bases: R = A/G, Y = C/T, D = A/G/T, H = A/C/T, and N = A/G/C/T.

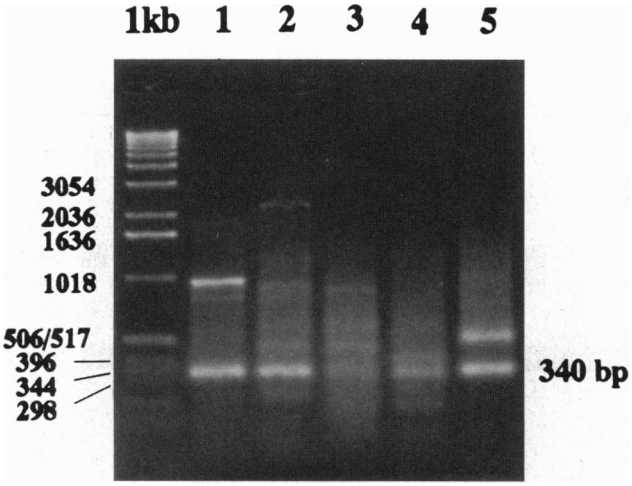

FIG. 1. PCR products amplified with the degenerate primers NBS-F1 and NBS-R1 designed from amino acid sequences of the NBS region of tobacco *N* and *Arabidopsis RPS2* genes. The template DNA used in lanes 1–5 are soybean (*Glycine max*), rice (*Oryza sativa*), barley (*Hordeum vulgare*), tobacco (*Nicotiana tabacum*), and potato (*Solanum tuberosum*), respectively. The "1 kb ladder" (Life Technologies) was used as size markers.

appears to confirm that these clones correspond to NBS-containing genes.

The amino acid sequences deduced from the 11 classes of the soybean NBS clones have 19.0%, 25.5%, and 29.5% average similarity to the corresponding NBS regions of *RPS2*, *N*, and L^6, respectively. For comparison, the amino acid sequence similarity is 14.9% between *RPS2* and *N*, 24.5% between *N* and L^6, and 14.9% between L^6 and *RPS2* in this region. In addition to the 3 NBS motifs, 9 (classes *a–i*) of the 11 soybean PCR clones contain other amino acid residues, e.g., FDxxCFL (positions 28–34) and LQxxLLSELL (50–59), which are conserved in the corresponding regions of the *N* and L^6 genes, but not in that of *RPS2* (Fig. 2). Based on a phylogenetic analysis (not shown) using the NBS amino acid sequences, the 11 soybean families fell into two major clusters: 9 classes (*a–i*) as 1 cluster, and the remaining 2 classes (*j* and *k*) as another. Classes *a-i* are highly homologous to the *N* and L^6 genes, with an average similarity value of 28.1% and 32.1%, respectively. They are, however, less homologous (18.7% average similarity) to the NBS region of the *RPS2* gene. Hereafter, we will refer to these classes as *N*-homologous sequences. Classes *j* and *k*, are reasonably homologous to *RPS2* (20.2%), but have slightly lower homology to *N* (13.9%) and L^6 (17.6), and are therefore hereafter referred to as *RPS2*-homologous sequences.

Table 3. Eleven classes of the superfamily of NBS-containing, tobacco *N*- and *Arabidopsis RPS2*-homologous DNA sequences

Class	NBS clones*	Association with resistance genes†	Linkage group‡
a	**17**, 8, 22, 29, 44, 50, 51, 60, 65, 70, 301		J
b	**5**, 57	*Rsv1, Rpv, Rps3*	F
c	**299**, 39, 49		G
d	**317**, 4, 7, 58, 64, 74	*Rps2, Rj2, rmd*	J
e	**307**, 6, 11, 32, 45	*Rps1*	N
f	**19**, 27, 47, 52, 69		E
g	**48**, 12		E, C
h	**43**, 1, 21, 25, 26, 28, 62, 63, 78		
i	**66**	*Rps2, Rj2, rmd*	J
j	**61**	*Rsv1, Rpv, Rps3*	E, F
k	**13**, 23		

Five classes are positionally associated with known soybean disease resistance genes as indicated by RFLP analysis using NILs and mapping populations.

*The DNA sequences of the NBS clones in boldface are shown in Fig. 2.

†The positional association of these NBS-containing multigene families with the corresponding disease-resistance genes was indicated by RFLP analysis using soybean NILs followed by linkage mapping.

‡The linkage group in the U.S. Department of Agriculture/Iowa State University RFLP map (18).

Using nbs5 of class *b* as a probe, a soybean genomic DNA clone (designated as BL-23) was isolated and partially sequenced. The partial sequence of this genomic clone not only contains NBS and LRR motifs, but also exhibits a high level of homology to other areas of the tobacco *N* and *Arabidopsis RPS2* genes. In particular, all of the six motifs that are conserved between *L6* and *N* (as reported in ref. 6) are present in BL-23 (data not shown). These PCR clones thus represent 11 multigene families that may be considered as a superfamily of *N*- or *RPS2*-homologous genes.

Identification of Candidate Disease-Resistance Genes from the NBS-Containing DNA Sequences by Genetic Mapping. Association of the NBS-containing DNA sequences with soybean disease-resistance genes was tested using NILs followed by segregation analysis with RFLP markers. Inheritance and chromosomal locations of genes conferring resistance to several soybean diseases have been reported. For example, the *Rps2* and *rmd* genes conferring resistance to *Phytophthora* root rot and powdery mildew (19), respectively, are tightly [2.3 centimorgans (cM)] linked to each other and to an ineffective nodulation gene, *Rj2*. In this study, the class *d* multi-gene family, as detected by nbs317 (Table 3), is polymorphic between the resistant and susceptible lines of three pairs of *Rps2–Rj2* NILs: L76-2060 vs. Clark, L82-1449 vs. Harosoy, and L76-1988 vs. Williams (Table 1, Fig. 3*A*). As is evident from the RFLP patterns, all three resistant NILs carry alleles of the class *d* family, not from their respective recurrent parents, but from the *Rps2* and *Rj2* donor line (CNS). Similarly, the class *d* family in two *rmd* NILs (L88-8226 and L90-7978) is not from the recurrent parent (Williams), but from their *rmd* donor lines (PI 86972-1 and Jefferson, respectively). Thus, NIL analysis suggested a close positional association between this subfamily and the chromosomal block containing these three soybean genes. This observation was confirmed by RFLP mapping in an F_2 population, which located nbs317 to the previously reported (20) *Rps2* chromosomal region in the "J" linkage group. In addition, RFLP analysis (not shown) with the same NILs, followed by linkage mapping, showed that the class *i* family, detected by nbs66 (Table 3), is also in the same vicinity as *Rps2*, *Rj2*, and *rmd*. The clones nbs66 and nbs317 mapped 3.0 cM away from RFLP locus A233 on linkage group "J" in 92 progeny from the cross of D26 × Lee 68. The existence of two NBS-containing multigene families (classes *d* and *i*) in the same chromosomal region as the three soybean genes should provide a pool of candidate sequences for the cloning of these resistance genes.

The *Rsv1*, *Rpv*, and *Rps3* genes for resistance to soybean mosaic virus, peanut mottle virus, and *Phytophthora* root rot, respectively, are closely linked to each other and have been previously mapped to linkage group "F" of the soybean RFLP map (16, 20). Class *b* (using clone nbs5) detected polymorphism between Williams and seven NILs that carry one of these three resistance genes. RFLP results for four of these NILs ,including L78-379 (contains *Rsv1*), L83-529 (*Rsv1-b*), L85-2308 (*Rpv*), and L83-570 (*Rps3*) are shown in Fig. 3*B*. These results show that the resistant NILs have the same pattern as the donor lines. The class *b* subfamily mapped to linkage group "F" and only three recombinants were observed between nbs5 and *Rsv1* in 243 progeny from the PI 96983 × Lee 68 cross. In addition to class *b*, class *j* subfamily (nbs61)

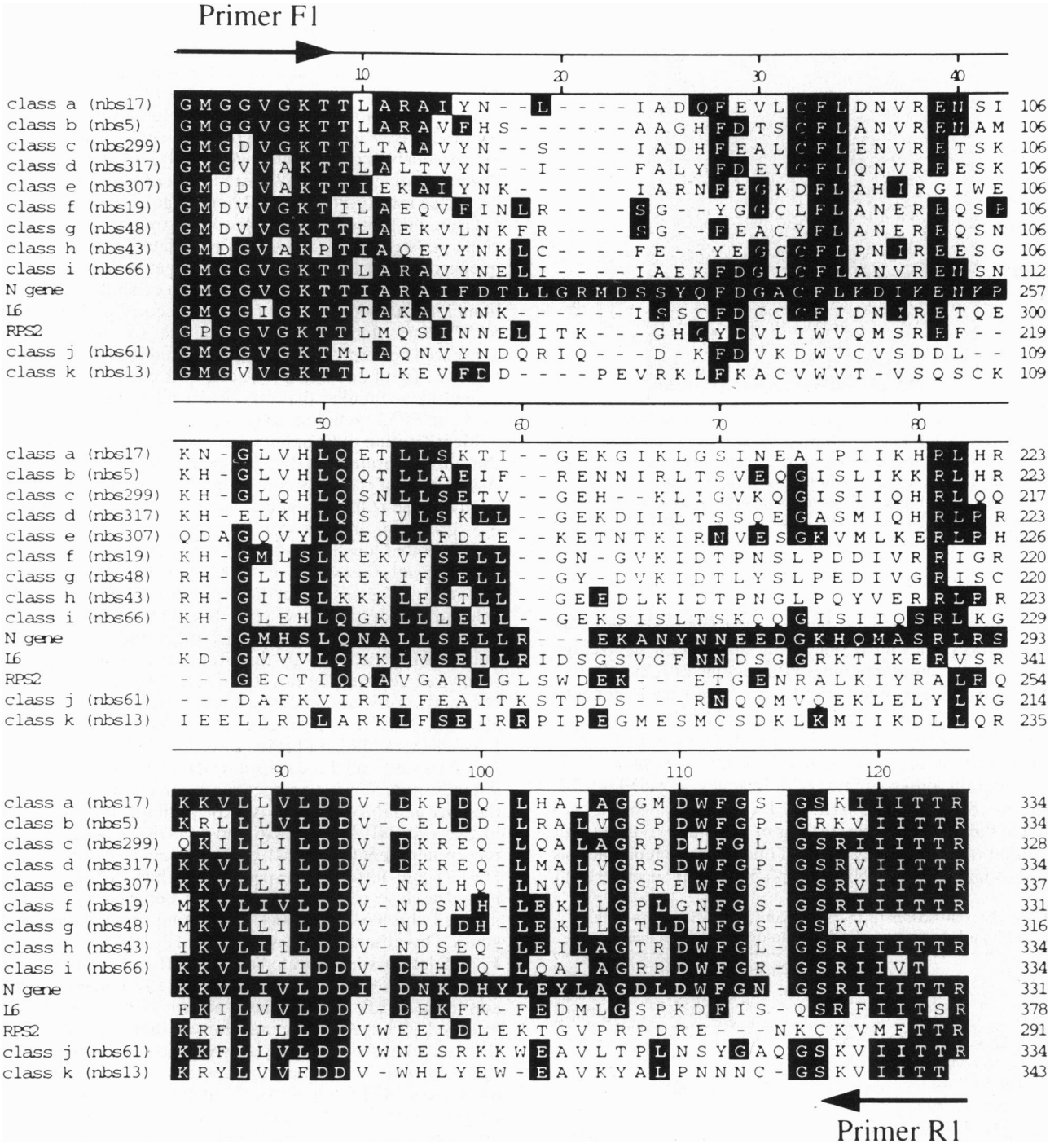

FIG. 2. Alignment of the deduced amino acid sequences of 11 classes (*a–k*) of soybean NBS clones with that of the disease-resistance genes of tobacco *N*, flax L^6, and *Arabidopsis RPS2* using the CLUSTAL method with the PAM250 residue weight table in the LASERGENE computer program (DNAStar). The amino acid sequences shown are residues 216–331 of the *N* gene, 265–378 of L^6, and 182–291 of *RPS2*. The letters in shaded areas match the residue of the *N* gene. It should be noted that the first and last eight amino acids of the PCR clones were contributed by the degenerate primer sequences used and therefore may not correspond to genomic sequences exactly.

also detected polymorphism between all seven NILs of *Rsv1*, *Rpv*, or *Rps3*. Linkage analysis using 150 individuals from the F_2 population of V71-370 × PI 407.162 showed that this class of *RPS2*-homologous subfamily is also in the chromosomal region of the three disease-resistance genes on linkage group "F" closely linked (0.30 cM) to nbs5. Thus, two subfamilies of the NBS-containing genes, one *N*-homologous (class *b*) and the other *RPS2*-homologous (class *j*), have been located in the vicinity of *Rsv1*, *Rpv*, and *Rps3*.

A multigene family detected by class *e* clones (e.g., nbs307) is positionally associated with yet another *Phytophthora*-resistance gene, *Rps1*. The RFLP pattern of this multigene family in six soybean NILs that carry *Rps1* from various sources are identical to their corresponding *Rps1* donor lines but different from their recurrent parents. These NILs include two of Clark (L75-3901 and L77-2015) and one of Williams (L77-1794) and three combinatorial NILs of Williams (L81-4420, L85-2378, and L87-0482, each carrying *Rps1*-k and a different unlinked resistance gene). Subsequent linkage mapping in the F_2 population of V71-370 × PI 407.162 indicated that nbs307 cosegregates with the RFLP locus A280 in the *Rps1* chromosomal region (20) on the "N" linkage group.

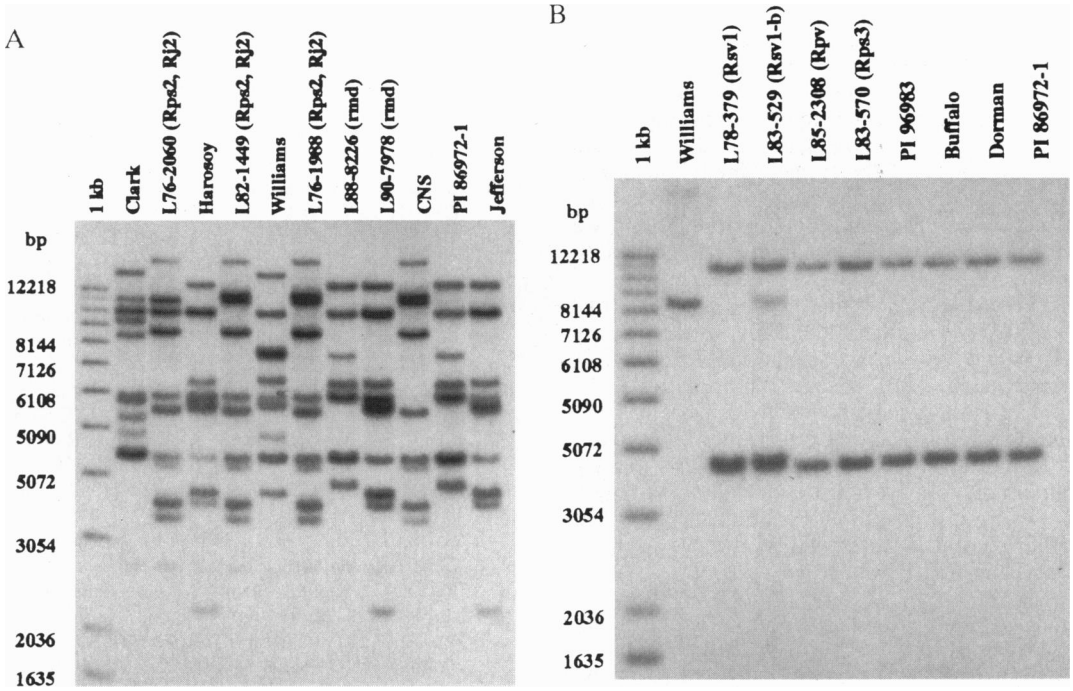

FIG. 3. (*A*) Positional association of the class *d* subfamily of NBS-containing genes with three closely linked soybean genes *Rps2*, *Rj2*, and *rmd*, as indicated by RFLP analysis using a set of soybean NILs. The *Rps2* and *rmd* genes confer resistance to *Phytophthora* root rot and powdery mildew, respectively, and *Rj2* is a non-nodulation gene (20). L76-2060, L82-1449, and L76-1988 carry *Rps2* and *Rj2* from the donor CNS, and are NILs of Clark, Harosoy, and Williams, respectively (see Table 1). L88-8226 and L90-7978 are Williams NILs that have *rmd* from PI 86972-1 and Jefferson, respectively. (*B*) Positional association of class *b* subfamily with clustered resistance genes *Rsv1*, *Rpv*, and *Rps3*. L78-379, L83-529, L85-2308, and L83-570 are NILs of Williams, each of which carries one of the resistance genes (see Table 1). PI 96983, Buffalo, Dorman, and PI 86972-1 are the donor parents of *Rsv1*, *Rsv1-b*, *Rpv*, and *Rps3*, respectively.

DISCUSSION

The conserved NBS and LRR structures among several disease-resistance genes naturally has led to the speculations of cloning additional resistance genes based on the homology to, for example, the NBS sequences (e.g., ref. 1). This seemingly simple approach, however, can be complicated by several factors. First, there might be a plethora of genes that contain NBS regions but that are not related to resistance genes. If only one or a small fraction of them are disease-resistance genes, the cloning of the NBS-containing DNA sequences will be of little value toward the cloning of resistance genes. Second, multiple *N*- and *RPS2*-homologous resistance genes may be located throughout the genome in a given plant species. If so, the sequence homology among these genetically independent and functionally distinct disease-resistance genes will present a difficulty in isolating individual clones, which correspond to a specific resistance gene by hybridization. And third, if multiple NBS/LRR-resistance genes exist as clustered multigene families on a chromosomal region, a practical method will be needed to distinguish different members of the multigene family and to match them to resistance genes or clusters.

In this study we have cloned the NBS region of multiple *N*- and *RPS2*-homologous sequences from soybean by means of PCR with degenerate oligonucleotide primers. We have found that the NBS-containing genes, as expected, are abundant in soybean. However, they can be divided into at least 11 multigene families, each of which consists of only a small number of genes. All 11 families contain the same 3 NBS motifs conserved among *N*, *RPS2*, and related disease-resistance genes (5, 6). Moreover, they are so homologous to the NBS region of the tobacco *N*, flax L^6, and *Arabidopsis RPS2* genes that they should not be described as just "P-loop proteins" or NBS-containing genes. Nine of them are highly homologous to the *N* and L^6 genes, and two are more similar to *RPS2* in *Arabidopsis*. These 11 *N*- or *RPS2*-homologous multigene families constitute a superfamily of NBS-containing genes, at least some of which may be involved in disease resistance in soybean.

Each subfamily exhibits unique, non-overlapping RFLP patterns on soybean Southern blots when probed by its corresponding PCR-derived clone. Each of them can be investigated for their positional association with the known resistance genes by NIL screening, followed by linkage mapping. RFLP analysis of the *N*- and *RPS2*-homologous subfamilies suggests a connection between these NBS-containing sequences and soybean disease-resistance genes. As summarized in Table 3, we have determined chromosomal locations of 9 of the 11 classes of the NBS-containing clones. Thus far, the map locations of at least five subfamilies (classes *b*, *d*, *e*, *i*, and *j*) coincide with known soybean disease-resistance gene clusters including *Rps1*, *Rps2*, *Rps3*, *rmd*, *Rsv1*, and *Rpv* on three different linkage groups. The possibility is remote that these positional associations would happen by chance, rather they are indicative of the involvement of at least some of the NBS-containing sequences in disease resistance.

The NBS-containing sequences appear to be clustered in several soybean chromosomal regions, as are the disease-resistance genes (1, 15). Two different subfamilies (classes *b* and *j*) have been mapped to a chromosomal region near *Rsv1*, *Rpv*, and *Rps3* and two other classes (*d* and *i*) have been mapped to the vicinity of the clustered *Rps2*, *Rj2*, and *rmd* genes. Additional resistance genes, not yet identified by mapping, may also be located on these two NBS-containing chromosomal regions. High-resolution mapping will be a valuable tool for determining which subfamily these known clustered resistance genes belong to and, subsequently, for distinguishing individual members of the candidate subfamily that confer resistance to a specific disease.

This study has provided valuable insights on the feasibility of a much more efficient sequence-based approach for the cloning of disease-resistance genes by capitalizing on the

success in map-based cloning (1, 9, 10). Nonetheless, there is work yet to be done toward the actual sequence-based cloning of resistance genes. For instance, although the five resistance gene-related subfamilies are in the chromosomal region of the corresponding resistance genes or gene clusters, we have not identified individual NBS-containing genes that co-segregate with disease-resistance genes. This is largely due to the lack of phenotypic data for disease reaction and the inconsistency among marker data from different mapping populations; we are currently collaborating with other research groups to address this issue.

The cloning of the disease-resistance genes from the five NBS subfamilies that have been identified as containing candidate genes would involve the isolation of full-length cDNA or genomic clones and complementation by DNA transformation. The class-specificity in DNA hybridizations using PCR clones isolated in this study as probes allows their use in the screening of a cDNA or genomic DNA library without the problem of cross hybridization. The remaining six subfamilies (classes *a*, *c*, *f*, *g*, *h*, and *k*) should be useful in screening for other resistance genes, which have not yet been located on the soybean RFLP linkage map.

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