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Two Classes of Highly Similar Coiled Coil-Nucleotide Binding-Leucine Rich Repeat Genes Isolated from the *Rps1*-k Locus Encode *Phytophthora* Resistance in Soybean

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A series of single genes protect soybean from the root and stem disease caused by the oomycete pathogen *Phytophthora sojae*. In the last two decades, *Rps1*-k has been the most stable and widely used *Phytophthora* resistance gene for the major soybean-producing regions of the United States. Four highly similar genes encoding coiled coil-nucleotide binding-leucine rich repeat (CC-NB-LRR)-type proteins were isolated from the *Rps1*-k locus. These genes were grouped into two classes based on their sequence identity. Class I contains three genes with identical open reading frames (ORF) and 5′ end regions. Two of these genes were also identical at the 3′ untranslated regions; the third gene showed a recombination breakpoint in the 3′ untranslated region resulting in the combination of 3′ end sequences of members from both classes. Reverse transcription-polymerase chain reaction analyses suggested that members of both classes of genes are transcribed at low levels. Representative members from each gene class were expressed in transgenic soybean plants. Analyses of independent R_0, R_1, R_2, and R_3 progeny populations suggested that both gene classes confer *Phytophthora* resistance in soybean. A possible evolutionary mechanism for the Class I gene family is proposed.

Constantly under attack by various pathogens, plants have evolved a network of preformed and inducible responses against pathogen invasion (Hammond-Kosack and Jones 1996). Active defense responses are induced following the recognition of pathogenic isolates by host resistance (*R*) genes. Proteins encoded by *R* genes are thought to recognize pathogen proteins encoded by avirulence (A*vr*) genes. The recognition between matching pairs of host and pathogen genes was first described by Flor (1955) in the flax and *Melampsora lini* interaction. Over 40 *R* genes have been cloned and characterized, and based on their predicted protein structures, cloned *R* genes can be broadly classified into several large groups (Hulbert et al. 2001; Martin et al. 2003). The majority of R proteins contain nucleotide binding (NB) and leucine-rich repeat (LRR) domains. The NB-LRR group can be further divided into three classes: i) Class I genes carry an N-terminal coiled-coil or leucine zipper domain, ii) Class II genes contain a toll interleukin 1 receptor domain showing homology to a domain found in *Drosophila* toll and mammalian interleukin-1 receptors, and iii) Class III includes NB-LRR genes with unusual functional motifs such as *RRS1-R* and *LR21* (Deslandes et al. 2002; Huang et al. 2003; Pan et al. 2000; Whitham et al. 1994).

Cloning and characterization of a large number of *R* genes have shown no association between *R* gene structures and types of encoded resistance. For example, CC-NB-LRR-type *R* genes from various plant species confer resistance against viruses, bacteria, fungi, oomycetes, nematodes, and aphids (Ballvora et al. 2002; Bent et al. 1994; Ori et al. 1997; Vos et al. 1998; Whitham et al. 1994). The cloning of *R* genes and their corresponding *Avr* genes led to the demonstration of direct interactions between R and Avr proteins for a limited number of plant-pathogen interactions (Lahaye 2004). Recent data, however, showed that R proteins can guard complexes of proteins consisting of one or more host factors, so-called virulence targets, and pathogen avirulence factors in order to prevent disease development. In the absence of the guard, the plant is susceptible. Presumably R proteins monitor the common host factors for initiation of active defense responses (Marathe and Dinesh-Kumar 2003). Multiple mechanisms may determine the outcome of plant-pathogen interactions. For example, Shao and associates (2003) demonstrated that *RPS5*-mediated resistance is specifically activated upon cleavage of the host protein PBS1 by the corresponding avirulent protein avrPphB.

Soybean is a major oil seed crop. In North America, soybean often suffers from root and stem rot disease caused by the oomycete pathogen, *Phytophthora sojae*; the annual crop losses from this disease are estimated to be about $273 million (Wrather et al. 2001). Resistance encoded by *Rps* (resistance to *P. sojae*) genes has been providing significant protection against this disease for the last four decades. Among 15 *Rps* genes, *Rps1*-k has been conferring resistance against a large number of the *P. sojae* races and, therefore, has been widely used in soybean cultivars grown in the midwestern United States (Sandhu et al. 2004; Schmitthenner et al. 1994). Despite the economic importance of disease resistance in soybean, the progress toward cloning *R* genes has been very slow. This is primarily because of the complexity of the soybean genome for positional cloning experiments and difficulties associated with transformation procedures for gene identification.

Single dominant avirulence genes corresponding to 11 of the *Rps* genes have been mapped. Several *P. sojae Avr* genes have

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been targeted for positional cloning, and recently, *Avr1b* has been cloned (Shan et al. 2004; Tyler 2002). Two genes, *Avr1b-1* and *Avr1b-2*, are required for expression of the Avr1b-encoded avirulence function. Both genes are physically linked and were cloned from the *Avr1b* locus. *Avr1b-2* regulates the accumulation of the *Avr1b-1* transcripts. *Avr1b-1* encodes a secreted protein that elicits a resistance response in soybean cultivars carrying *Rps1*-b (Shan et al. 2004).

Candidate genes for *Rsv1*, *Rps2*, and *Rps4* have recently been reported (Graham et al. 2002; Hayes et al. 2004; Sandhu et al. 2004). Only *Rpg1-b*, conferring bacterial resistance, has recently been isolated (Ashfield et al. 2004). No other *R* genes from any legume species have been cloned. We have isolated a large family of coiled coil-nucleotide binding-leucine rich repeat (CC-NB-LRR)-type genes from the *Rps1*-k region (Bhattacharyya et al. 2005). Here, we report that four members of the gene family are located at the *Rps1*-k locus. These four genes are grouped into two classes, one of which contains three identical genes. A member from each class has been shown to confer resistance in transgenic soybean plants. A possible mechanism for the evolution of highly similar functional *Rps* genes is discussed.

RESULTS

Identification and characterization of highly similar candidate genes from the *Rps1*-k region.

We have isolated a contiguous, approximately 145-kb DNA fragment from the *Rps1*-k locus (Bhattacharyya et al. 2005). Three overlapping bacterial artificial chromosome (BAC) clones, BAC18, BAC43, and BAC99, span this DNA fragment. All three BACs were sequenced to near completion. Except for a predicted pseudo serine/threonine kinase and four CC-NB-LRR-type disease resistance genes, no other disease resistance gene–like sequences were identified from these BAC clones. Therefore, we designated the four CC-NB-LRR-type genes as the candidate *Rps1*-k genes *Rps1*-k-1 to *Rps1*-k-4 and grouped them into two classes (accession numbers AY963292 and AY963293). Class I consists of *Rps1*-k-1, *Rps1*-k-3, and *Rps1*-k-4, whereas Class II contains *Rps1*-k-2. *Rps1*-k-1 and *Rps1*-k-4 are identical genes but were isolated from two nonoverlapping BACs, BAC18 and BAC99. Therefore, they are considered as two independent genes. At the 5′ untranslated region and the open reading frame (ORF), *Rps1*-k-1, *Rps1*-k-3, and *Rps1*-k-4 are identical. However, the 3′ untranslated region of *Rps1*-k-3 evolved from a recombination event between 3′ end sequences of Class I and II genes (Fig. 1). The recombination breakpoint in *Rps1*-k-3 was confirmed through molecular analyses of binary clones, BAC clones, and soybean genomic DNA (data not shown). The nucleic acid and deduced amino acid sequence identities between ORFs of Class I and II genes were 93 and 89.9%, respectively. Six structural domains were identified among the deduced candidate Rps1-k proteins (Fig. 2). The N-terminal region (domain A) did not show similarity to any known motifs or domains. Domain B contained the CC motif. The putative NB-ARC (nucleotide-binding adaptor conserved in Apaf-1, apoptosis protease activating factor-1, R gene products, and CED-4) domain was identified in domain C (van der Biezen and Jones 1998). A short spacer sequence (domain D) was observed between the NB-ARC domain and the LRR region (domain E). In *Rps1*-k-2 (Class II), the C-terminal LRR region contains 27 imperfect LRR, one of which is absent in Class I Rps1-k proteins (Fig. 2). The C-terminus (domain F) did not show identity to any known motifs or domains.

Candidate *Rps1*-k genes are transcribed at low levels.

In order to determine the gene structure of the candidate genes and establish that they are transcribed genes, we screened an unamplified cDNA library carrying approximately 4.6×10^6 PFU. Seven cDNAs representing four classes of genes were isolated (Bhattacharyya et al. 2005). Sequence alignments between cDNAs and candidate gene sequences revealed two introns located in the 3′ untranslated region (Fig. 1). However, none of these cDNAs showed 100% identity to any of the candidate *Rps1*-k genes (data not shown).

A two-step reverse transcription-polymerase chain reaction (RT-PCR) approach was utilized to identify transcripts of the candidate *Rps1*-k gene family. Total RNAs isolated from leaves of cultivar Williams 82 were reverse transcribed and then PCR-amplified using candidate *Rps1*-k-1– and *Rps1*-k-2–specific primers (Fig. 3A). The first PCR was performed using *Rps1*-k-1– and *Rps1*-k-2–specific forward primers and a com-

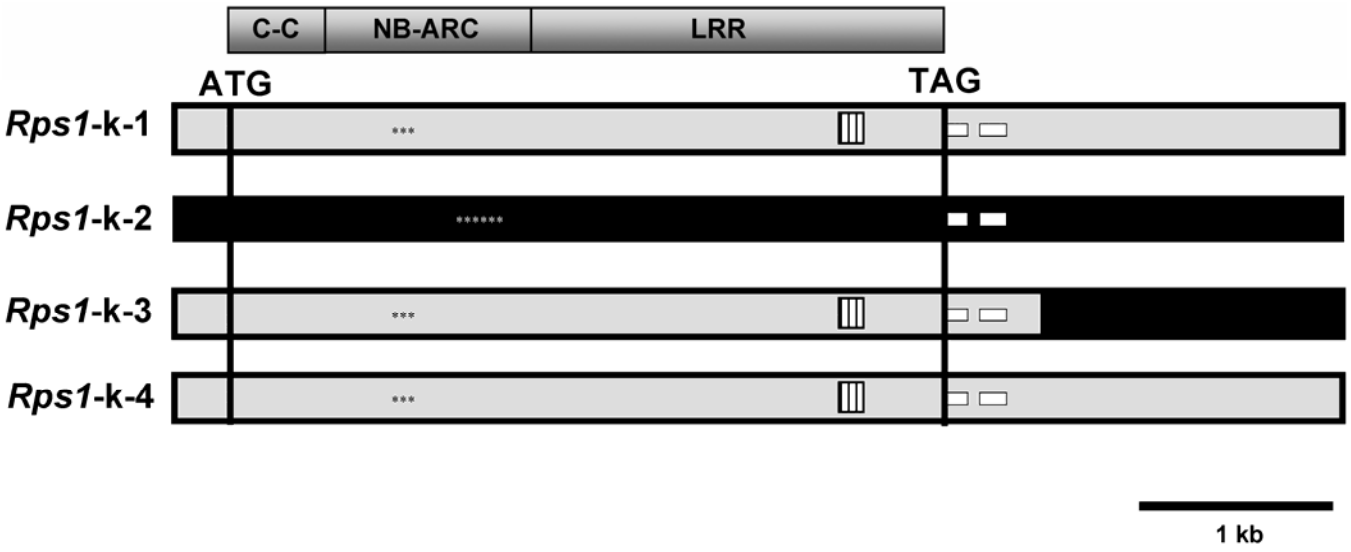

Fig. 1. The *Rps1*-k gene family is comprised of two classes of highly similar genes. Class I contains *Rps1*-k-1, *Rps1*-k-3, and *Rps1*-k-4. *Rps1*-k-1 and *Rps1*-k-4, showing 100% identity (gray color), were isolated from two nonoverlapping bacterial artificial chromosome clones. *Rps1*-k-3 contains a recombination breakpoint at the 3′ untranslated region (black color for exchanged sequences from *Rps1*-k-2). C-C = coiled-coil domain; NB-ARC = nucleotide-binding adaptor conserved in Apaf-1, apoptosis protease activating factor-1, R gene products, and CED-4 (van der Biezen and Jones 1998) domain; LRR = leucine rich repeat domain; * indicates single deletion; the square with vertical lines represents a 63-nt deletion; the white rectangles depict introns.

mon reverse primer. The targeted regions included two introns, and therefore, PCR products from contaminating genomic DNA were 344 bp larger than the RT-PCR products (Fig. 3B). The second PCR was conducted using the diluted gel-purified *Rps1*-k-1– or *Rps1*-k-2–specific RT-PCR products as templates. *Rps1*-k-1– or *Rps1*-k-2–specific primer pairs were used in the second PCR step (Fig. 3C). PCR products of the second PCR step were directly sequenced and compared with the *Rps1*-k-1 and *Rps1*-k-2 gene sequences. Complete identities were observed between RT-PCR products and the corresponding *Rps1*-k gene sequences (data not shown). We amplified 573 and 648 bp of the LRR domains of *Rps1*-k-1 and *Rps1*-k-2, respectively. These RT-PCR products contained five to six copies of the consensus xxLxLxx (x can be any amino acid residues) repeat sequence of the LRR region. The amplified regions between the two classes of genes were distinguished by the presence of a 63-nt deletion in the Class I *Rps1*-k genes. The solvent-exposed residues within the consensus xxLxLxx repeat sequence of the LRR domain have been shown to be highly divergent and under substantial diversifying selection (Meyers et al. 1998; Parniske et al. 1997). Therefore, it is very unlikely that RT-PCR products originated from transcripts of paralogous *Rps1*-k gene sequences rather than from that of the *Rps1*-k candidate genes, and we conclude that both classes of candidate *Rps1*-k genes are transcribed at very low levels.

Candidate *Rps1*-k genes conferred stable race-specific *Phytophthora* resistance in transgenic soybean plants.

To establish the functional identity of candidate *Rps1*-k genes, three binary plasmids, p43-JP1, p99-6A, and p43-10, carrying candidate *Rps1*-k-1, *Rps1*-k-2, and *Rps1*-k-3 genes, respectively, were introduced into the soybean cultivar Williams 79 using *Agrobacterium*-mediated transformation (Paz et al. 2004). One transformant for *Rps1*-k-1, six independent transformants for *Rps1*-k-2, and three for *Rps1*-k-3 were generated. Trifoliates of independent transformants were detached from the greenhouse-grown, basta-resistant transgenic soybean plants. Most of the R_0 transformants showed *Phytophthora* resistance against *P. sojae* race 4 (Fig. 4A). Phenotypic analysis of the R_1 generation of independent transformants demonstrated that all three genes confer resistance in soybean leaves against the *P. sojae* race 4 and isolate 997A-2-3 (Fig. 4 and Table 1). Progenies of the single transformant T-1-1 containing *Rps1*-k-1, two independent transformants, T-2-1 and T-2-6, carrying *Rps1*-k-2, and three, T-3-1, T-3-2, and T-3-3, carrying *Rps1*-k-3, showed a 3:1 segregation ratio for resistance and susceptibility

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A    MAAALVGGAFLSAFLDVFDRLASPEFVDLIR

B    GKKLSKKLLQKLETTLRVVGAVLDDAEKKQITNTNVKHWLNDLKHAVYEADDLLDHVFTKAATQNKVRDLFSRFSDRKIV
     SKLEDIVVTLESHLKLKESLDLKESAVENLSW

C    KAPSTSLEDGSHIYGREKDKEAIIKLLSEDNSDGREVSVVPIVGMGGVGKTTLAQLV
                                                    P loop
     YNDENLKQIFDFDFKAWVCVSQEFDVLKVTKTIIEAVTGKACKLNDLNLLHLELMDK
     LKDKKFLIVLDDVWTEDYVDWRLLKKPFNRGIIRRSKILLTTRSEKTASVVQTVHTY
           Kinase-2                              Kinase-3a
     HLNQLSNEDCWSVFANHACLSTESNENTATLEKIGKEIVKKCNGLPLAAESLGGMLR
     RKHDIGDWNNILNSDIWELSESECKVIPALRLSYHYLPPHLKRCFVYCSLYPQDYEF
     EKNELILLWMAEDLLKKPRKGRTLEEVGHEYFDDLVSRSFFQRS-RTSSWPHRKCF
     VMHDLMHDLATS

D    LGGDFYRSEELGKETKINTKTRHLSFAKENSSVLDNFDVIGRAKFLRTFLSIINFEAAPFNNEEAQCIIMSKLM

E         xxLxLxx
     YL   RVLSFCD        FQSLDSLPDSIGKLI
     HL   RYLDLSF        SRIETLPKSLCNLY
     NL   QTLKLCS        CRKLTKLPSDMRNLV
     NL   RHLGIAY        TPIKEMPRGMGKLN
     HL   QHLDFFV        VGKHEENGIKELGGLS
     NL   RGQLEIRK       LENVSQSDEALEARMMDKK
     HI   NSLQLEW        SGCNNNSTNFQLEIDVLCKLQPHF
     NI   ESLEIKG        YEGTRFPDWMGNSSYC
     NM   ISLKLRD        CHNCSMLPSLGQLP
     SL   KDLGIAR        LNRLKTIDAGFYKNEECRSGTSFP
     SL   ESLSIDD        MPCWEVWSSFDSEAFP
     VL   NSLEIRD        CPKLEGSLPNHLP
     AL   TKLVIRN        CELLVSSLPTAP
     AI   QSLEICK        SNKVALHAFPL
     LV   ETIEVEG        SPMVESVIEAITNIQPT
     CL   RSLTLRD        CSSAVSFPGGRLPE
     SL   KSLSIKD        LKKLEFPTQHKHE
     LL   ETLSIES        SCDSLTSLPLVTFP
     NL   RYLSIEK        CENMEYLLVSGAESFK
     SL   CYLLIYK        CPNFVSFWREGLPAP
     NL   ITFSVWG        SDKLKSLPDEMSTLLP
     KL   EDLTISN        CPEIESFPKRGMPP
     NL   RRVEIVN        CEKLLSGLAWPSMG
     ML   THLNVGG        PCDGIKSFPKEGLLPP
     SL   TSLSLYD        LSNLEMLDCTGLLHLT
     SL   QQLQIFG        CPKLENMAGESLPF
     SL   IKLTMVE        CPLLEKRCRMKHPQ

F    IWPKVSHIPGIKVGNRWI
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Fig. 2. Structure of the predicted Rps1-k proteins. The predicted, complete amino acid sequence of *Rps1*-k-2 is used to describe the structure (six domains A to F). The bold residues are the sites that vary between the Class I and Class II Rps1-k proteins. Domain A is the N-terminus with no homologies to known motifs or domains. Domain B is the coiled-coil domain predicted using the COILS program (Lupas, 1997). Domain C is the NB-ARC (nucleotide-binding adaptor conserved in Apaf-1, apoptosis protease activating factor-1, R gene products, and CED-4) domain. Conserved P loop, kinase-2, and kinase-3a sequences, GLPL, and MHD motifs are underlined. Domain D is the spacer that separates NB-ARC and LRR domains. Domain E is the leucine-rich repeat (LRR) domain. The consensus sequence in the LRR region is indicated by xxLxLxx just above the alignment of LRR sequences (L can be replaced by V, F, or M). The leucine-zipper–like motif identified in the LRR domain is underlined. One 21–amino acid LRR repeat absent in the Class I Rps1-k proteins is shown in italics and underlined. Domain F represents the C-terminus, which does not contain any known motifs or domains. The structural domains of predicted proteins were identified primarily using Pfam and by comparison with previously described *R* genes.

in either R_1 or R_2 generation (Table 1). Progenies of the basta-resistant T-2-4 transformant were all susceptible to the pathogen, presumably due to absence of transgene expression and, therefore, were considered as the negative control.

Wounded hypocotyls of R_2 or R_3 populations containing candidate *Rps1*-k genes were inoculated with both avirulent and virulent *P. sojae* isolates, in order to determine if the resistance conferred in transgenic plants was race-specific. Race 4, used in the leaf inoculation experiment, was found to be more aggressive on wounded Williams 82 hypocotyls of light-grown seedlings, and the cultivar containing *Rps1*-k consistently failed to produce a resistant phenotype. Race 18 and isolate 997A-2-3 were avirulent, while race 25 was virulent to Williams 82. R_2 and R_3 progenies containing either *Rps1*-k-1 or *Rps1*-k-2 were resistant to race 18 and isolate 997A-2-3 but were susceptible to race 25 (Table 2). The activity of both *Rps1*-k-1 and *Rps1*-k-2 genes was also tested in the recipient cultivar Williams that does not contain any known *Rps* genes (Table 2, the 7aD- and 30-85- transformants). These results suggested that both Class I and II genes encode race-specific *Phytophthora* resistance.

Cosegregation of an *Rps1-k*-2 transgene with the complemented race-specific resistance.

R_2 families were investigated to determine the association of integrated transgenes with the disease-resistant phenotype. R_2 plants of two independent R_0 plants, T-2-1 and T-2-6, carrying *Rps1*-k-2, and R_2 progenies of T-3-1 carrying *Rps1*-k-3 were investigated for the integration of transgenes. Progenies of 10 R_1 families developed from individual R_0 plants were also studied. R_2 progenies segregated for resistance and susceptibility.

R_2 families developed from these transformants were investigated for association between integrated transgenes and the complemented resistant phenotype. Inoculation of wounded hypocotyls of 10 light-grown $R_{1:2}$ families originating from the T-2-6 R_0 transformant carrying *Rps1*-k-2 showed a 1:2:1 segregation ratio for i) homozygous resistant phenotype, ii) segregating resistance and susceptible phenotypes, and iii) homozygous susceptible phenotype. Southern analysis showed that multiple copies of the transgene were integrated into the soybean genome. Progenies of randomly selected R_2 plants showed that the complemented resistant phenotype was associated with the integration of a single *Rps1*-k-2 transgene copy.

Rps1-k–specific resistance has been shown to be expressed in etiolated hypocotyls (Ward et al. 1979). Therefore, we investigated dark-grown seedlings for expression of *Rps1*-k-2 transgene-mediated resistance. Symptoms were recorded 24 h following inoculation of intact hypocotyls with *P. sojae* zoospores. DNA blot analysis of selected resistant and susceptible seedlings again showed complete association between the expression of resistant phenotype and integration of a specific *Rps1*-k-2 transgene copy (Fig. 5, arrow). Although many copies of *Rps1*-k-2 transgenes were segregating, only a single *Rps1*-k-2 transgene copy was shown to associate with the expression of *Phytophthora* resistance in both light- and dark-grown seedlings. Presumably, other integrated transgene copies failed to confer any *Phytophthora* resistance. Among R_2 progenies of T-2-5, no association between transgene copies and complemented resistant phenotype was observed.

Association of *Rps1*-k-3 transgenes with the disease resistant phenotype among R_2 progenies descended from T-3-1 was not evident. Investigation of additional transformants gener-

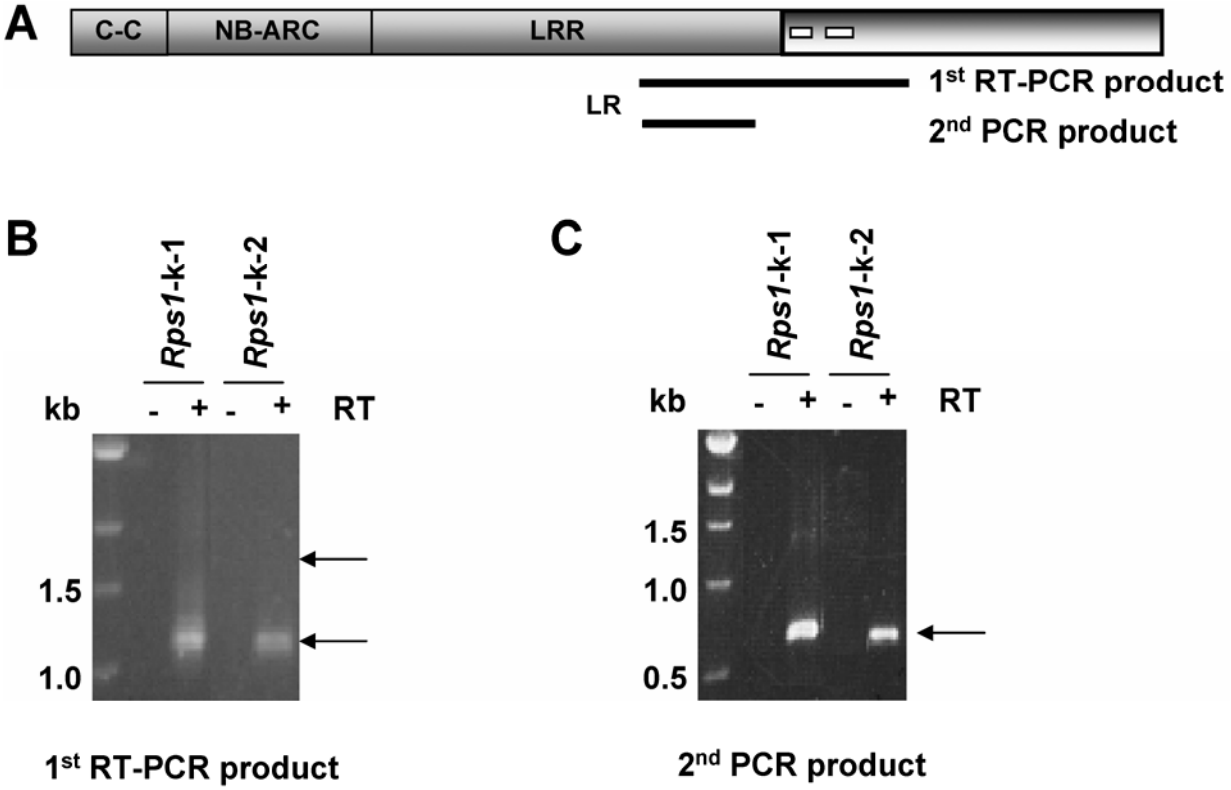

Fig. 3. Detection of candidate *Rps1*-k-specific transcripts. **A,** Schematic representation of the region considered for reverse transcription-polymerase chain reaction (RT-PCR) analyses.. Two white rectangles at the 3'-untranslated region are introns. The longer line indicates the region amplified through PCR following reverse transcription of total leaf RNAs. The shorter line shows the region considered for the second PCR. **B,** First RT-PCR products. RT = reverse transcriptase, + = with reverse transcriptase, and – = without reverse transcriptase. The lower arrow shows the product obtained from the first RT-PCR. The upper arrow shows the PCR product from contaminating genomic DNA. **C,** Second PCR products. Sequence comparison revealed 100% identity between the cDNA and gene sequences (data not shown).

ated by transforming the cultivar Williams with *Rps1*-k-1 also did not result in any clear-cut cosegregation of *Rps1*-k-1 transgenes with the complemented resistant phenotype. Williams does not carry any known Rps genes. Transgenic Williams lines carrying *Rps1*-k-1 transgenes showed resistance against race 1, race 18, and isolate 997A-2-3. Transcript levels of the *Rps1*-k gene family are extremely low. Seven cDNA clones representing four gene members were isolated from screening 4.6 million PFU of an unamplified cDNA library (Bhattacharyya et al. 2005). However, none of these cDNAs showed complete sequence identity with any members of Class I or II genes (data not shown). Transcripts of these genes were detected only in a highly sensitive two-step RT-PCR experiment (Fig. 3). Recently, we have observed that transient overexpression of *Rps1*-k-2 led to cell death (N. N. Narayanan and M. K. Bhattacharyya, *unpublished data*). Nearly undetectable transcript levels together with the cell death phenotype caused by overexpression of *Rps1*-k-2 suggested that, during transformation, expression of these genes at a higher level would be detrimental to the regeneration process. Although candidate genes with their own promoters were used in complementation analyses, the site of integration of these genes can greatly influence the levels of their expression (Butaye et al. 2004). Our failure to obtain a clear-cut cosegregation between transgene copies and *Phytophthora* resistance among transgenic lines could be attributed to low, unstable expression levels of more than one transgene copy among the transformants. Highly active transgenes were most likely silenced. This may also explain why we failed to observe any *Phytophthora* resistance among progenies of four *Rps1*-k-2 transformants (Table 1).

Diversifying selection and frequency of mutation in *Rps1*-k genes.

The solvent-exposed residues within the consensus repeat sequence xxLxLxx of LRR have been shown to be highly divergent and under substantial diversifying selection (Meyers et al. 1998; Parniske et al. 1997). Mondragon-Palomino and associates (2002) found strong positive selection in the LRR region by studying 163 NB-LRR genes of *Arabidopsis thaliana*. To determine the selection pressure exerted on the *Rps1*-k gene family, ratios of nonsynonymous and synonymous substitution rates were calculated for different regions of the two classes of genes. The nonsynonymous substitution rate observed for the xxLxLxx repeat sequence of the LRR domain was statistically much higher than the nonsynonymous substitution rate ($P < 0.0001$ at 5% level; Kumar et al. 2004; Table 3). This suggested that diversifying selection has been imposed on these solvent-exposed residues.

Phylogenetic analysis of the *Rps1*-k gene family and other CC-NB-LRR R genes.

Blastx and tblastn were carried out to study the relationship between *Rps1*-k-2 and other plant disease-resistance genes and

Table 1. Segregation of resistant and susceptible R_1 progenies following inoculation with *Phytophthora sojae* race 4[a]

R_0 transformant[b]	R	S	$X^2_{(3:1)}$	P ($X^2_{(3:1)}$)
T-1-1[c]	10	2	0.444	0.505
T-2-1[c]	11	6	0.961	0.327
T-2-2	2	1	39.185	<0.0001
T-2-3	2	15	36.255	<0.0001
T-2-4	0	16	48.000	<0.0001
T-2-5	5	10	13.899	0.0002
T-2-6[d]	–	–	–	–
T-3-1[c]	13	5	0.074	0.785
T-3-2[c]	11	7	1.852	0.174
T-3-3[c]	13	5	0.074	0.785

[a] Resistant and susceptible phenotypes were quantified by measuring lesion spread (mm/day) in individual segregants.
[b] Designations for transgenic plants: T- = transgenic plant; the first number indicates the *Rps1*-k gene number, e.g., T-1 for *Rps1*-k-1, and the second number is the transformant number (R_0).
[c] Showed a 3:1 resistant/susceptible segregation ratio.
[d] Data unavailable for the R_1 generation. A 3:1 resistant/susceptible segregation ratio was observed in the R_2 generation.

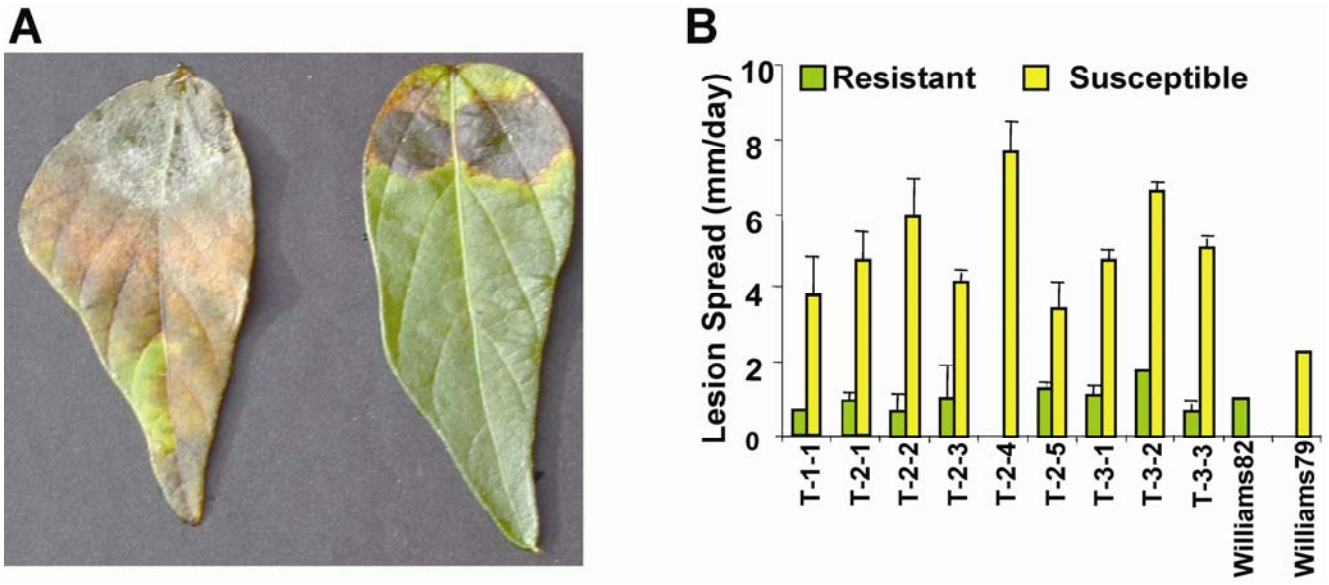

Fig. 4. Soybean transformants carrying candidate *Rps1*-k genes confer *Phytophthora* resistance. **A,** A leaf (on the right) of a transformant (R_0) showed hypersensitive cell death and typical resistance response, whereas the leaf on the left from cultivar Williams 79, used as a recipient for the transformation, showed the typical susceptible response. **B,** Symptom development among R_1 progeny populations. Lesion sizes were recorded 2 and 3 days following inoculation with *P. sojae* race 4, and lesion spread (mm/day) was determined by subtracting the spread on day 3 from that on day 2 in millimeters. Standard errors of lesion spread among resistant (green bar) or susceptible (yellow bar) progenies are shown with lines at the top of each bar. Four disease lesions were measured for each plant. χ^2 analyses of the data presented in B indicated a 3:1 segregation ratio for resistant to susceptible phenotypes for some of the R_1 populations. Designations for transgenic plants: T- = Transgenic plant, the first number indicates the *Rps1*-k gene number, e.g., T-1 for *Rps1*-k-1 and the second number is the transformant number (R_0).

resistance-gene analogs (RGA). Complete amino acid sequences of the RGA or *R* gene sequences (E-value < e^{-110}) were either retrieved from GenBank or manually annotated by aligning deduced protein sequences using the ExPASy translate tool. Most of the cloned NB-LRR genes with a genetically validated resistance specificity were also collected from GenBank. A multiple sequence alignment of all retrieved protein sequences was performed, and a neighbor-joining tree was generated from the alignment. From this preliminary tree, CC-NB-LRR-type R proteins and representative RGA that are closely related to Rps1-k were selected to generate the final tree (Fig. 6). Rps1-k-2 showed high amino acid identities with the sequences from three legume species, including *Glycine max*, *Medicago truncatula*, and *Lotus corniculatus*. Deduced protein sequences of two *L. corniculatus* var. *japonicus* RGA showed the highest identity to the Rps1-k-2 protein sequence (59%) and formed a very strongly supported group within the *Rps1*-k gene family. This suggests that evolution of lotus RGA and *Rps1*-k sequences may predate the separation of *Lotus* and *Glycine* genera. Although the possibility of convergent evolution of *Rps1*-k and lotus RGA sequences cannot be completely ruled out at this stage, evolution of these sequences from a single progenitor gene is the most likely alternative. If this is true, *Rps1*-k is a member of an ancient *R* gene family. Among cloned *R* genes, *Rps1*-k is closely related to *RPG1-b*, *I2C*, *Xa1*, *Rp1-D*, and *RB*.

No close relationship among *R* genes conferring resistance against a specific type of pathogen was observed. For example, *Rps1*-k is highly diverged from *R1*, a potato *R* gene that confers resistance against another *Phytophthora* pathogen, *P. infestans*. Potato *Rx* and *Gpa2* are paralogs, but they confer virus

Table 2. Responses of soybean cultivars or transformants to *Phytophthora sojae* race or isolate

	***P. sojae* race/isolate responses**[a]				
Cultivar	**Race 1**	**Race 4**	**Isolate 997A-2-3**	**Race 18**	**Race 25**
Williams (*rps1*)	S	S	S	S	S
Williams 79 (*Rps1*-c)	R	S	S	S	S
Williams 82 (*Rps1*-k)	R	R	R	R	S
Transformant T-1-1(*Rps1*-k-1)	…[b]	R	R	R	S
Transformant T-2-1(*Rps1*-k-2)[c]	…	R	R	R	S
Transformant T-2-6 (*Rps1*-k-2)	…	R	R	R	S
Transformant T-3-1(*Rps1*-k-3)	…	R	R	R	S
Transformant T-3-2 (*Rps1*-k-3)	…	R	R	R	S
Transformant T-3-3 (*Rps1*-k-3)	…	R	R	R	S
Transformant 7aD-3 (*Rps1*-k-1)	R	…	R	R	S
Transformant 7aD-6 (*Rps1*-k-1)	R	…	R	R	S
Transformant 7aD-9 (*Rps1*-k-1)	R	…	R	R	S
Transformant 30-85-1 (*Rps1*-k-2)	R	…	R	RS	S
Transformant 30-85-5 (*Rps1*-k-2)	R	…	R	RS	S
Transformant 30-85-6 (*Rps1*-k-2)	R	…	R	RS	S

[a] R = resistant; S = susceptible; RS = resistant response with some spread of the lesions. … indicates data not available.
[b] Data is not available because the recipient cultivar, Williams 79, is resistant to race 1.
[c] Responses of transformants are based on progeny testing.

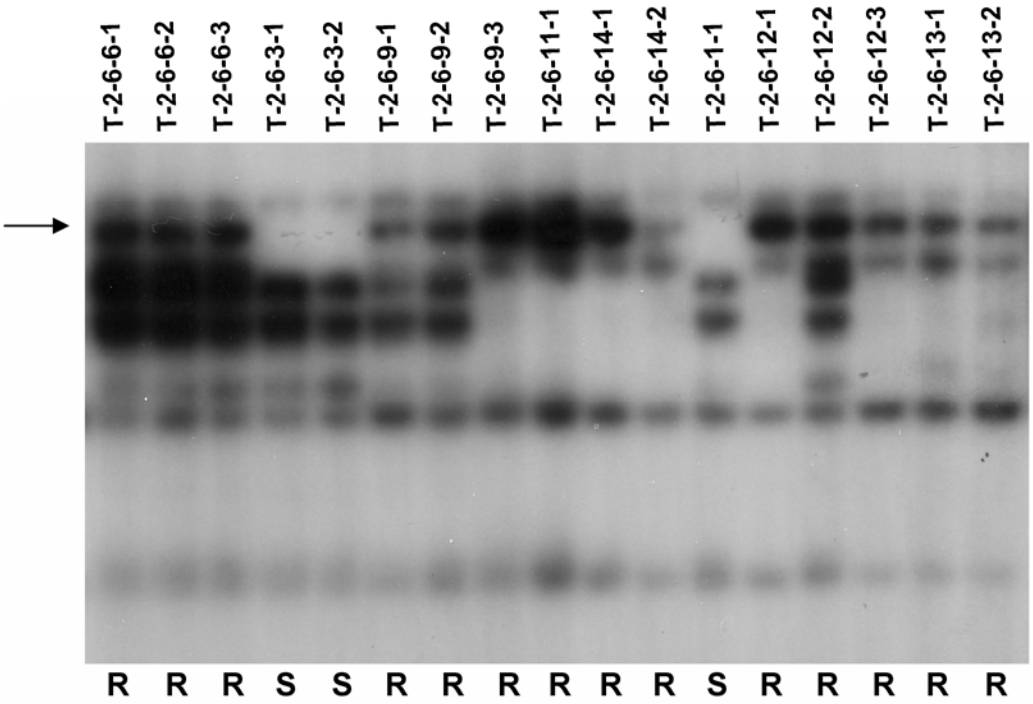

Fig. 5. Cosegregation of a transgene *Rps1*-k-2 copy with the complemented resistant phenotype. Intact, etiolated hypocotyls of R_3 progenies of the T-2-6 transformant containing the *Rps1*-k-2 transgene were inoculated with zoospore suspensions of the *Phytophthora sojae* isolate 997A-2-3. Randomly selected resistant and susceptible R_3 progenies were analyzed for segregation of transgene copies by carrying out DNA blot analysis. The *bar* gene was used as the probe in this analysis. The arrow shows the transgene copy that cosegregated with the complemented resistant phenotype.

and nematode resistance, respectively (van der Vossen et al. 2000). R proteins with high sequence similarity may not require the same signaling component. For example, although MLA1 and MLA6 proteins share 91.2% identity, MLA1 is *Rar1*-independent whereas MLA6 is *Rar1*-dependent (Halterman et al. 2001; Zhou et al. 2001). The phylogenetic tree indicated that most clades are family- or species-specific, which is consistent with the results of phylogentic analyses conducted for NB sequences of different plant families and species (Cannon et al. 2002; Meyers et al. 1999).

DISCUSSION

Two classes of CC-NB-LRR genes conferring *Phytophthora* resistance mapped to the *Rps1*-k locus.

In previous work, we isolated an approximately 145-kb contiguous DNA fragment from the *Rps1*-k locus (Bhattacharyya et al. 2005). Sequencing of this gene-poor fragment allowed us to locate four CC-NB-LRR genes as candidates for *Rps1*-k. Based on sequence similarities, we grouped these genes into two classes. Of these, Class II contains a single gene, *Rps1*-k-2, that showed stable expression of resistance in R_1 and R_2 generations of a transformants. Segregation analysis of the *Rps1*-k-2 transgene confirmed that the gene is a functional *Phytophthora* resistance gene (Figs. 4 and 5; Tables 1 and 2).

The other three CC-NB-LRR Class I genes are identical in their nucleotide sequences. Cloning and characterization of these CC-NB-LRR genes were carried out through the subcloning of three overlapping BAC clones in a binary plasmid vector followed by sequencing. Two of the identical Class I genes, *Rps1*-k-1 and *Rps1*-k-4, were cloned from two nonoverlapping BACs that shared parts of the *Rps1*-k locus. The presence of a recombination breakpoint in the 3′ untranslated regions distinguishes the third Class I gene, *Rps1*-k-3, from *Rps1*-k-1 and *Rps1*-k-4. The functions of *Rps1*-k-1 and *Rps1*-k-3 were studied in transgenic soybean plants. Multiple transgene copies were present in all transformants. However, among segregating R_2 progenies developed from transforma-

Table 3. Rates of nonsynonymous (Ka) and synonymous (Ks) substitutions in the evolution of the *Rps1*-k gene family

Region/domain	Ka/Ks[a]
ORF	0.625
Coiled-coil domain	0.29
Nucleotide binding site domain	0.33
xxLxLxx[b]	2.33
C-terminal region[c]	1

[a] The ratio of Ka and Ks substitution in variable sites was calculated by comparing the open reading frame (ORF) sequences of Class I (identical ORF of *Rps1*-k-1, *Rps1*-k-3, and *Rps1*-k-4) and Class II (*Rps1*-k-2) genes. The SNAP program was used in calculating the substitution values.

[b] Conserved hydrophobic sites (L, V, F, and M) were not included in the calculation of the Ka/Ks ratio of the xxLxLxx region.

[c] Includes all of leucine-rich repeat and F domains except xxLxLxx motifs.

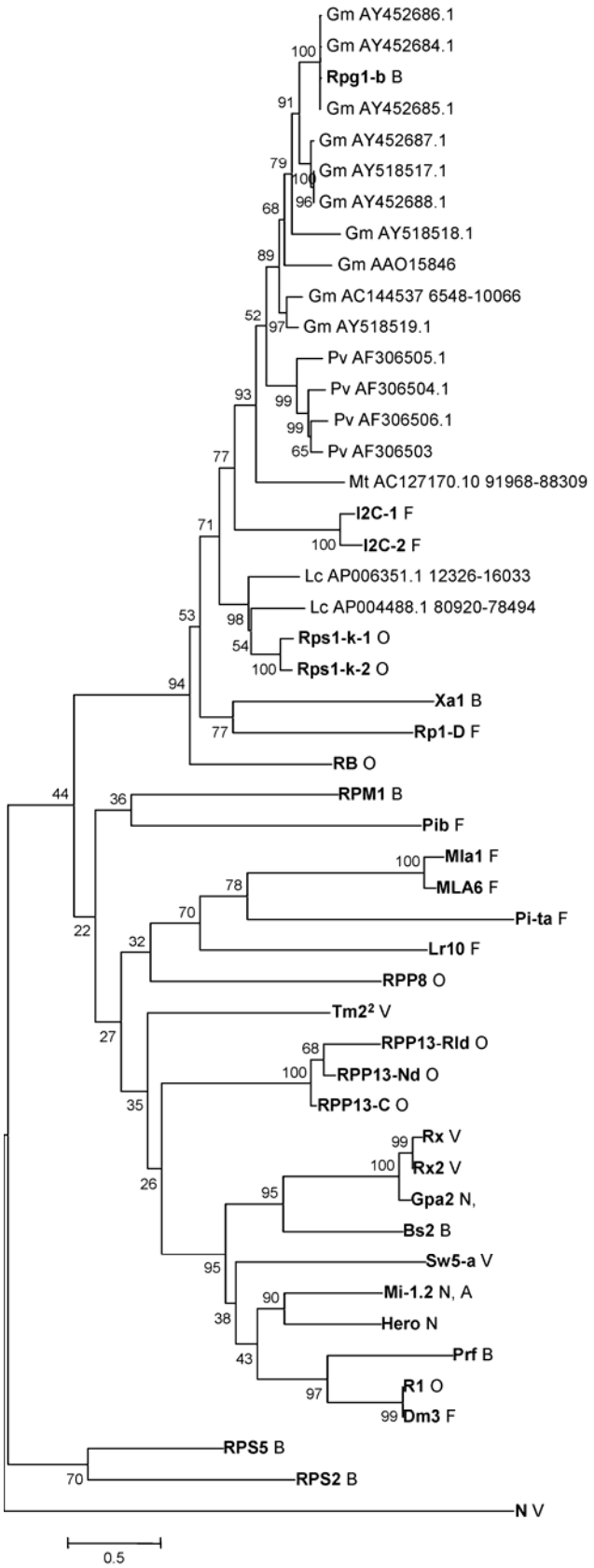

Fig. 6. Phylogenetic analysis of *Rps1*-k and related sequences. A neighbor-joining tree was constructed with the Molecular Evolutionary Genetics Analysis program (version 2.1) (Kumar et al. 2004). Full-length amino acid sequences of the *R* genes and resistance gene analogs (RGAs) were aligned using ClustalW for phylogenetic analysis. The bootstrap values (percentages of 1,000 trees generated) for the nodes are indicated. The *N* gene from tobacco was used as an outgroup sequence. Each characterized R protein is in bold font. The taxon name designation for functional R proteins is the name of each R protein followed by the pathogen against which it confers resistance. B = bacterium, F = fungus, N = nematode, O = oomycete, V = virus, RPP13-Rld = RPP13 from *Arabidopsis thaliana* cv. Rld, RPP13-Nd = RPP13 from *Arabidopsis thaliana* cv. Neiderzens, and RPP13-C = RPP13 from *Arabidopsis thaliana* cv. Columbia. Each RGA was identified with a species prefix (Gm = *Glycine max*, Lc = *Lotus corniculatus*, Mt = *Medicago truncatula*, Os = *Oryza sativa*, and Pv = *Phaseolus vulgaris*) followed by its GenBank accession number and the position from the bacterial artificial chromosome, if needed (as of October 11, 2004).

tion of either the cultivar Williams (*rps1*) or Williams 79 (*Rps1*-c) (Table 2), no association of *Rps1*-k-1 or *Rps1*-k-3 transgenes with the complemented *Phytophthora*-resistant phenotype was observed (data not presented). Transcript levels of the *Rps1*-k gene family were very low and detectible only in highly sensitive RT-PCR experiments (Fig. 3). It is possible that an increased level of Rps1-k-1 or Rps1-k-3 protein is deleterious to plant cells, as was observed for Rps1-k-2 in transient overexpression experiments (N. N. Narayanan and M. K. Bhattacharyya, *unpublished data*). Therefore, most likely a selection pressure for poorly transcribed or nontranscribed transgene copies was exerted during transformation and regeneration to avoid any detrimental effects originating from high expression levels of *Rps1*-k-1 or *Rps1*-k-3 transgenes. A low level of expression from more than one transgene could account for the complemented resistant phenotype observed among transformants carrying *Rps1*-k-3. From analyses of the ethylene mutant, *etr1*, it was concluded that there were most likely two *Rps* genes in the *Rps1*-k locus, one of which requires the ethylene-signal pathway for expression of *Phytophthora* resistance (Hoffman et al. 1999). We therefore conclude that the Class I genes are most likely functional (Table 1).

Evolution of the *Rps1*-k gene family.

Progenitors of two classes of *Rps1*-k genes differ by 58 synonymous substitutions in the ORFs. Therefore, we calculated that the two progenitor genes probably started to diverge about 10 million years ago (Cronn et al. 2002). Although we have identified only four CC-NB-LRR genes at the *Rps1*-k locus, there are many additional members of the gene family in the *Rps1* region (Bhattacharyya et al. 2005). Therefore, it is possible that paralogous sequences of the Class II gene are located in the *Rps1*-k–adjacent region.

Complete sequence identity among Class I *Rps1*-k genes is unusual. To date, no two identical *R* genes have been identified from the same haplotype. In flax, *L3* and *L10* carry identical ORFs while *L4* differed from these two by a single nucleotide (Ellis et al. 1999). However, *L3*, *L4*, and *L10* were characterized from three independent haplotypes. The only example of an *R* locus containing highly similar genes is the tomato *Cf2* locus, which carries two nearly identical resistance genes. These two genes differ only by three nucleotide residues (Dixon et al. 1996). Class I *Rps1*-k genes are the only examples of identical *R* genes isolated from a single locus.

Concerted evolution has been shown to have a major role in the evolution of tandemly arranged, identical repeat sequence families, such as snRNAs in humans and rDNA in yeast (Gangloff et al. 1996). It is unlikely that the high identity observed among members of the *Rps1*-k gene family resulted from concerted evolution. Homogenization, a requirement for concerted selection, acts against the diversification and generation of novel race-specificities. Parniske and his coworkers (1997) proposed that sequence homogenization could be prevented by the suppression of unequal recombination between two polymorphic, intergenic sequences. The relationship between physical and genetic distances at the *Rps1*-k region is about 1:1 Mb/cM, and the region is not recombination-prone for sequence homogenization (Bhattacharyya et al. 2005). Gene conversion, a major sequence homogenization mechanism for concerted selection, was probably not the cause of 100% identity between Class I *Rps1*-k genes of about 9 to 10 kb of DNA including 5′ and 3′ end sequences. Gene conversion tracts are mostly limited to ORFs and are relatively small (Drouin 2002).

Complete identity among Class I *Rps1*-k genes suggests that the genes were duplicated very recently through unequal crossing over, which is considered to be an important mechanism for the evolution of disease-resistance genes. In maize, unequal crossing over is the main mechanism of meiotic instability at the *Rp1* region (Sudupak et al. 1993). In the *Arabidopsis* ecotype Columbia, the nonfunctional, chimeric *rpp8* gene most likely evolved from unequal crossing over between the functional oomycete resistance gene *RPP8* and its homolog, *RPH8A* (McDowell et al. 1998). It has been proposed that the *HRT* gene encoding viral resistance evolved by unequal crossing over between progenitor genes related to *RPP8* and *RPH8A* (Cooley et al. 2000). Unequal crossing over has also been considered to play an important role in the generation of alleles at the *Rsv1* locus in soybean (Hayes et al. 2004). Unequal crossing over most likely occurs from the pairing of diverse haplotypes following cross-fertilization. This leads to tandem accumulation of similar but usually not identical paralogous genes, because diverse haplotypes are expected to accumulate mutations in paralogous genes.

Two unequal crossing over events were detected at the *Rps1*-k region, one at the physically linked TC1 and 18R loci (Bhattacharyya et al. 2005) and the other one at the 3′ end of *Rps1*-k-3 (Fig. 1). Soybean is predominantly a self-pollinated species, and it is very unlikely that three identical genes evolved from unequal crossing over events between diverse haplotypes of two genotypes. Considering the highly self-pollinated nature of soybean, the probability of open pollination between two diverse genotypes for unequal crossing over events in the recent past is very small. It is very unlikely that unequal crossing over events between *Rps1*-k and *rps1*-k haplotypes can lead to the evolution of identical, functional Class I *Rps1*-k genes during introgression of the gene into Williams 82 from Kingwa, because the recurrent parent Williams does not contain any known functional *Rps1* genes for contributing toward the gene duplication phenomenon. Considering the predominantly self-pollinated nature of soybean and 100% identity among Class I genes in a small genomic region, we propose that, during meiosis, slippage in pairing or mispairing between tandem paralogous rather than orthologous sequences of two sister chromatids of a homozygous plant resulted in the evolution of three identical Class I *Rps1*-k genes. Tightly linked, paralogous genes can mispair during meiosis, resulting in gene duplication, deletions, and evolution of chimeric genes. Jelesko and associates (2004) recently provided experimental evidence for recombination between misaligned paralogous sequences of sister chromatids during meiosis in the self-pollinated species, *Arabidopsis*. The presence of such a local gene duplication mechanism from misaligned sister chromatids in a self-pollinated plant species such as soybean can result in substrates for the generation of new genetic variations for some traits that require rapid evolution.

MATERIALS AND METHODS

Subcloning and sequencing of BAC clones.

Three overlapping BAC clones, BAC18, BAC43, and BAC99, carrying the *Rps1*-k locus, were sequenced by applying a shotgun cloning approach. To minimize *Escherichia coli* DNA contamination, BAC DNA samples were prepared using the Qiagen large construct kit (Qiagen, Valencia, CA, U.S.A.). Two shotgun libraries for each BAC clone were constructed. One of these libraries was constructed using the Topo shotgun subcloning kit (Invitrogen, Carlsbad, CA, U.S.A.). Individual BAC DNA samples were nebulized under pressure at 5 psi for 10 s. Fragments (5 to 10 kb) were blunt-ended, dephosphorylated, and ligated to the vector pCR4BluntTOPO. The second library was constructed by using partially digested *Sau*3AI DNA fragments. Following partial restriction, the partially digested DNA fragments were fractionated on an agarose gel, and frag-

ments of approximately 20 kb were purified and ligated into the dephosphorylated *Bam*HI site of the binary vector, pTF101.1 (Paz et al. 2004). Ligated DNA samples were electroporated into *E. coli* DH10Bα using the Cell porator *E coli* pulser (Invitrogen, Carlsbad, CA, U.S.A.). The resultant recombinant plasmids, generated from cloning into pCR4BluntTOPO and pTF101.1, were named TOPO and pTF101.1 clones, respectively. Colonies from each library were picked randomly and stored in 96-well microtiter plates. Plasmid DNA was prepared using the Montage plasmid miniprep$_{96}$ kit (Millipore, Bedford, MA, U.S.A.). Both ends of each clone were sequenced in an ABI PRISM 3700 analyzer at the DNA Sequencing Facility, Iowa State University.

The sequence data were assembled utilizing Phred/Phrap software on a PC with a Linux operating system. The resulting contigs of each BAC were manually ordered into a scaffold by using the read-pairs. Primer walking was applied to fill out most of the remaining gaps. The assembled sequences were searched against GenBank using the BlastX algorithm. A series of primers for every 200 to 300 bp of each DNA strand were designed based on the consensus sequence of the candidate *Rps1*-k genes. The primers were then used in sequencing the selected pTF101.1 clones as described in the next section.

Sequence analyses of CC-NB-LRR clones.

Sequences of each insert end in pTF101.1 clones were investigated for possible homology to candidate *Rps1*-k gene sequences. pTF101.1 clones showing no homologies to the candidate *Rps1*-k gene sequences were selected for Southern blotting experiments and were hybridized to NBS and LRR domain-specific probes developed from one of the *Rps1*-k genes. The pTF101.1 clones hybridizing to both probes were classified into seven putative groups by conducting DNA fingerprint analysis. A total of 13 clones selected from these seven groups were sequenced, using primers designed based on the consensus CC-NB-LRR sequences generated from the comparison of BAC contig sequences described in the previous section. Each nucleotide was sequenced at least three times. The sequence reads of each clone were assembled using the Vector NTI Suite 6 program (v.6 for PC, InforMax Inc.; Invitrogen). Four CC-NB-LLR genes were identified from sequencing of pTF101.1 clones. The genes were predicted using the Genscan program and BlastX algorithm search.

Generation of transgenic soybean plants.

Three binary plasmids in the pTF101.1vector, p43-JP1 (*Rps1*-k-1), p99-6A (*Rps1*-k-2), and p43-10 (*Rps1*-k-3), were electroporated into *Agrobacterium tumefaciens* EHA101. The agrobacteria containing the binary plasmids were used to transform cotyledonary explants of the cultivars Williams (*rps1*) or Williams 79 (*Rps1*-c) at the plant transformation facility at Iowa State University (Table 2; Paz et al. 2004).

A total of 16 seeds of each R_0 transformant were germinated, and 3-day-old seedlings were transferred to soil in small paper pots. After growth for 5 days in the greenhouse, followed by hardening in sunlight for 2 days, R_1 plants containing transgenes *Rps1*-k-1, *Rps1*-k-2, and *Rps1*-k-3 were transplanted at the Bruner farm, Iowa State University, in 2002. R_2 seeds of individual plants were harvested, stored, and threshed after about four months. In 2003, R_3 seeds were generated by growing R_2 plants at the Bruner farm.

Growing of soybean and *Phytophthora sojae*.

Transgenic and nontransgenic soybean seedlings were grown under dark or light conditions in growth chambers at the Agronomy Department, Iowa State University (Bhattacharyya and Ward 1986; Ward et al. 1979). *P. sojae* race 1, race 4, race 18, race 25, and isolate 997A-2-3 were grown in the dark at 22°C, and zoospores were prepared from 6-day-old cultures (Table 2; Ward et al. 1979).

Evaluation of transgenic soybean plants for resistance against *Phytophthora sojae*.

Trifoliate leaves of R_0 transformants were inoculated with *P. sojae* race 4 as follows. Trifoliate leaves were detached and placed in petri dishes (90-mm diameter) containing Whatman filter papers moistened with 10 ml of water. Petioles were kept under a film of water, and each half of the leaf blade was inoculated with a single droplet of zoospore suspension (Bhattacharyya and Ward 1986). Subsequently, unifoliate leaves of R_1 progenies of independent transformants containing either *Rps1*-k-1, *Rps1*-k-2, or *Rps1*-k-3 transgene were tested for *Phytophthora* resistance. Symptoms were evaluated 48 and 72 h following inoculation. The average lesion spread in millimeters per day was then determined for all lesions of an individual class of disease phenotype. A resistant phenotype was characterized by dark brown lesions that spread very slowly, whereas a susceptible phenotype was characterized by a very light brown lesion that spread rapidly.

Wounded hypocotyls instead of leaves of R_2 or R_3 populations were inoculated, because the wounded-hypocotyl inoculation method requires less space than the leaf inoculation procedure and a large number of plants can be infected in a small space under uniform environmental conditions (Haas and Buzzell 1976). For the wounded-hypocotyl inoculation method, soybean seedlings were grown in coarse vermiculite for 7 to 8 days, until the cotyledons expanded completely. *P. sojae* race 997A-2-3 was grown in V8 agar diluted by one-fourth, until the mycelia covered the entire petri plate (90-mm diameter). The culture was cut into strips and was placed in a 10-ml BD syringe (Becton, Dickinson and Co., Franklin, NJ, U.S.A.). The culture was then macerated by passing through a precision glide 18G × $1^1/_2$ needle (Becton, Dickinson and Co.). With the help of the needle tip, a slit of about 1 cm long was made in the hypocotyl just below the cotyledonary nodes. About 0.2 to 0.4 ml of the macerated culture slurry was inserted into the slit with the help of a syringe. The plants were incubated under a 16-h light and 8-h dark period for disease development. Disease phenotypes were recorded 48, 60, 72, and 84 h following inoculation. Dark-grown seedlings were inoculated with *P. sojae* zoospore suspensions, as described by Ward and associates (1979), and were evaluated 24 h following inoculation.

DNA gel blot analysis.

Soybean genomic DNA was prepared from individual transgenic plants according to White and Kaper (1989). DNA from each genotype (10 μg) was digested overnight with *Hin*dIII and was fractionated on a 0.8% agarose gel. The selectable marker gene *bar* was used as a probe (Paz et al. 2004). The gene was PCR-amplified from the plasmid pTF101.1 by using two primers (upstream forward primer: 5′-CAGCTGCCAGAA ACCCACGT-3′; and downstream reverse primer: 5′-CTGCAC CATCGTCAACCACT-3′). Prehybridization and hybridization of DNA filters were carried out according to Kasuga and associates (1997).

RT-PCR.

Total RNA was isolated from soybean leaf tissues by using the RNeasy plant mini kit (Qiagen). For RT-PCR experiments, cDNAs were synthesized from total RNAs using M-MLV reverse transcriptase (Promega, Madison, WI, U.S.A.). Primers for the first round of PCR experiments were: ClassI:F, 5′-ACC TGGATCCCTGAAGAGTCTGTATATCTC-3′; ClassII:F, 5′-C

CCAAGCTTGCAGAGTCATTTAAGAGTCTGTGTTATTTG TTA-3′; Common:R, 5′-CCCAAGCTTAATTCACTTGGTAC ACCTTTTCATCCTTCAC-3′. Primers for the second round of PCR experiments were: ClassI:F, 5′-ACCTGGATCCCTGAA GAGTCTGTATATCTC-3′; ClassI:R 5′-AATTGGATCCGAG AGTCAGGAAGCCTTTCTCCCA-3′; ClassII:F, 5′-CCCAAG CTTGCAGAGTCATTTAAGAGTCTGTGTTATTTGTTA-3′; ClassII:R 5′-GCGGGATCCTTTTTCCAGCAAAGGACATTC CACC-3′.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The Expert Protein Analysis System (ExPASy) proteomics server's translate tool: us.expasy.org/tools/dna.html
Los Alamos National Laboratory's SNAP (Synonymous/Non-synonymous Analysis Program): hiv-web.lanl.gov/content/hiv-db/SNAP/WEBSNAP/SNAP.html
National Center for Biotechnology Information Blast webpage: www.ncbi.nlm.nih.gov/BLAST/
The New GENSCAN Web Server at MIT webpage: genes.mit.edu/GENSCAN.html
The Wellcome Trust Sanger Institute's Pfam database (protein family alignments and HMMs): www.sanger.ac.uk/Software/Pfam/search.shtml