Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 11746–11750, October 1996
Genetics

Resistance gene analogs are conserved and clustered in soybean

(disease/mapping/multi-gene families/evolution)

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Communicated by Steven D. Tanksley, Cornell University, Ithaca, NY, August 19, 1996 (received for review May 3, 1996)

ABSTRACT **Sequences of cloned resistance genes from a wide range of plant taxa reveal significant similarities in sequence homology and structural motifs. This is observed among genes conferring resistance to viral, bacterial, and fungal pathogens. In this study, oligonucleotide primers designed for conserved sequences from coding regions of disease resistance genes *N* (tobacco), *RPS2* (*Arabidopsis*) and *L6* (flax) were used to amplify related sequences from soybean [*Glycine max* (L.) Merr.]. Sequencing of amplification products indicated that at least nine classes of resistance gene analogs (RGAs) were detected. Genetic mapping of members of these classes located them to eight different linkage groups. Several RGA loci mapped near known resistance genes. A bacterial artificial chromosome library of soybean DNA was screened using primers and probes specific for eight RGA classes and clones were identified containing sequences unique to seven classes. Individual bacterial artificial chromosomes contained 2–10 members of single RGA classes. Clustering and sequence similarity of members of RGA classes suggests a common process in their evolution. Our data indicate that it may be possible to use sequence homologies from conserved motifs of cloned resistance genes to identify candidate resistance loci from widely diverse plant taxa.**

The sequences of cloned plant disease resistance genes show that the majority, whether conferring resistance to viral, bacterial, or fungal pathogens, contain similar sequences and structural motifs. The *Arabidopsis* genes, *RPS2* (1, 2) and *RPM1* (3), conferring resistance to the bacterial blight pathogen *Pseudomonas syringae*, the tobacco gene *N* (4, 5), conferring resistance to tobacco mosaic virus, the rice gene *Xa21* (6), conferring resistance to *Xanthomonas oryzae*, the flax gene *L6* (7), conferring resistance to a rust fungus, and the tomato gene *Cf-9* (8), conferring resistance to the fungal pathogen *Clasdoporium fulvum*, all contain leucine-rich repeats that encode protein motifs often associated with protein–protein interactions or ligand binding (9). Motifs for a conserved nucleotide binding site are also found in the *RPS2*, *RPM1*, *N*, and *L6* coding region. It has been proposed that the similarities among resistance genes may make it possible to take advantage of sequence homologies to identify other resistance genes (10). Southern hybridization with a clone from the *Pto* locus of tomato to genomic DNA of six dicotyledonous and five monocotyledonous species detected homologs in all species (11) and suggested that gene families of these homologs may exist, similar to that observed in tomato. Similarly, conserved motifs from receptor proteins have been used to identify multigene families of odorant receptors in rat (12) and conserved motifs from regulatory proteins have been used to identify other homeobox proteins in *Xenopus* (13).

Although not all resistance genes have been demonstrated to reside in clusters, tight linkage associations of many resistance genes have been well established. Genetic linkage of resistance genes has been reported in maize for the *Rp1* cluster (14–16), in barley for the *Mla* cluster (17–20), in lettuce for a wide range of *Dm* loci (21, 22), in oat for the *Pc* cluster (23), and in flax for the *L* gene cluster (24–26). Clustering of resistance genes suggests that a common genetic mechanism has been involved in their evolution (16).

In this paper we demonstrate that the nucleotide conservation observed within disease resistance genes cloned from widely diverse taxa can be used to advantage to isolate sequences with strikingly similar motifs from a species from which no disease resistance genes have yet been cloned. We demonstrate that resistance gene analogs (RGAs) exhibit microclustering in the genome, that clusters of RGAs contain only members of the same family, and that mapping of RGA sequences can place genetic markers in close proximity to known resistance genes.

MATERIALS AND METHODS

Nucleic Acid Manipulations. Soybean genomic DNA was prepared as described in Dellaporta *et al.* (27). RNA was prepared according to Sambrook *et al.* (28). Electrophoresis, blotting, and hybridization was done using standard techniques (28). Restriction enzyme digestions were conducted using conditions recommended by the manufacturers.

PCR Amplification, Cloning, Sequence Analysis. Regions of amino acid identity in the *N* gene from tobacco, the *RPS2* gene from *Arabidopsis*, and the *L6* gene from flax were used to design degenerate primers. Primer LM638 was designed from the conserved P-loop sequence. Primer LM637 was designed from a second region of amino acid identity which in the *RPS2* protein is proposed to reside in a transmembrane region. Primer sequences were as follows: LM638, 5′-GGIGGIGTIGGIAAIACIAC-3′, and LM637, 5′-A(A/G)IGCTA(A/G)IGGIA(A/G)ICC-3′. PCR was performed in a total volume of 100 μl, with a 3-min initial denaturation step at 94°C followed by 35 cycles as follows: 94°C for 1 min, 45°C for 30 sec, and 72°C for 30 sec. PCR products were cloned into the pGEM-T vector (Promega). Clones were sequenced using the Applied Biosystems model 377 PRISM automated sequencer, or manually using the Sequenase DNA sequencing kit (United States Biochemical). Multiple sequences were obtained for each RGA class.

DNA sequence analysis was carried out with the DNASIS (Hitachi) and GCG (University of Wisconsin Genetics Computer Group, Madison) sequence analysis packages. Alignment of sequences was done using the PILEUP function of the Genetics Computer Group. Phylogenetic analysis of amino

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Abbreviations: RGA, resistance gene analog; BAC, bacterial artificial chromosome.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accessions nos. U55803–U55812).

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acid sequences was performed using MEGA version 1.0 (29). The tobacco *N* sequence was used as an outgroup.

Genetic Mapping of RGAs. RGA sequences were mapped in a *G. max* × *G. soja* population containing 56 individuals (30) using MAPMAKER (31). Additionally, one soybean bacterial artificial chromosome (BAC) was mapped in a *G. max* × *G. max* population containing 196 individuals and in which the disease resistance genes *Rps2* and *Rmd*, and the *Rhizobium* responsive gene *Rj2*, were mapped (32). Data sets from linkage group J from both populations were combined and an integrated map was constructed using the computer program JOINMAP (33). The Kosambi mapping function was selected and a minimum logarithim of odds (lod) score of 3 required for a two-point linkage to be included in analyses. The order of "anchored loci" defined by JOINMAP output agreed with the published order. Therefore, specification of fixed sequences was not necessary.

BAC Library Construction and Screening. The soybean BAC library was constructed as described (34) from megabase-sized DNA isolated from the soybean cultivar Williams 82. The 4–5 genome equivalent library contains ≈40,000 individually picked clones with an average insert size of 150 kb and it is stored in 384-well microtiter dishes. Three-dimensional BAC pools for PCR screening were set up using entire plates and individual rows or columns from groups of 20 plates. Plasmid DNA was purified from each pool by standard alkaline lysis midiprep technique (28). Class-specific primers were designed for each RGA class (Table 1). PCR amplification was done as described above using an annealing temperature of 55°C. Microtiter dishes were replicated onto nylon membranes (Zeta-Probe GT; Bio-Rad) and selected membranes were used to confirm results of PCR-based screening.

RESULTS

Cloning and Sequence Analysis. PCR amplification of soybean genomic DNA using degenerate RGA primers resulted in a product that appeared to be a large, single band on a 1% agarose gel. However, digestion of this product with several restriction enzymes recognizing 4-bp sites resulted in many fragments, whose sum was much greater than the molecular weight of the original PCR product. The presence of a heterogeneous PCR product suggested the involvement of a multigene family. These PCR products were cloned and ≈450 clones were analyzed. The clones were grouped into nine classes which did not cross-hybridize under stringent hybridization conditions [0.1× standard saline citrate (SSC)/0.1% SDS/60°C wash]. Clones representing each class were hybridized to Southern blots of soybean genomic DNA digested with various restriction enzymes to identify polymorphisms useful for genetic mapping of RGAs and to estimate copy number. Four classes were used as probes on Northern blots containing total RNA from different soybean organs (leaves, stems, and roots). Lower levels of RGA message were detected in stems and roots compared with that observed from leaves (Fig. 1). Differential expression or accumulation was not observed in these tissues as a result of wounding or *Phytophthora* inoculation (data not shown). One to five clones from each class were sequenced, and the deduced amino acid sequences of representative clones from each class are shown in Fig. 2. Among sequenced clones only class 4 sequences showed heterogeneity (two groups). Alignment of the amino acid sequences established that the cloned RGAs contain two additional conserved nucleotide binding protein domains also present in *N*, *RPS2*, and *L6*. A search of GenBank using the BLAST algorithm revealed that the RGA sequences were most similar to the *L6*, *N*, and *RPS2* gene products. Remote similarity, limited to the conserved motifs was found to other P-loop containing proteins (myosin heavy chain homolog, *Arabidopsis*; ATPase, *Plasmodium*).

Pairwise comparisons between different classes and between each class and the homologous *N* and/or *L6* sequences revealed that amino acid identities ranged from 30 to 66%; similarities ranged from 50 to 75%. Clone RGA9 is likely to be a pseudogene because it contains multiple stop codons and frame-shift mutations. However it did show strong similarity with RGA4 (77%). Genetic mapping placed RGA9 and RGA4 at one position on linkage group H. Class 4 contained two subclasses, a and b, which showed 88% amino acid identity. These subclasses differed at the nucleotide level but were not distinguishable by Southern blot analysis. Classes 3 and 7 were 66% identical at the amino acid level; classes 5 and 8 were 51% identical.

Table 1. Sequences of class-specific RGA primer pairs

RGA class	Sequences, 5′ → 3′
RGA1	AGTTTATAATT(C/T)(C/G)ATTGCT-ACTACGATTCAAGACGTCCT
RGA2	AGTTTATAATT(C/T)(C/G)ATTGCT-CACACGGTTTAAAATTCTCA
RGA3	AGTTTATAATT(C/T)(C/G)ATTGCT-CTCTCGATTCAAAATATCAT
RGA4	TGTTACTGCTTTGTTTGGTA-TACATCATGTGTTACCTCT
RGA5	TGCTAGAAAAGTCTATGAAG-TCAATCATTTCTTTGCACAA
RGA6	AGCCAAAGCCATCTACAGT-AACTACATTTCTTGCAAGT
RGA7	AGTTTATAATT(C/T)(C/G)ATTGCT-CCGAAGCATAAGTTGCTG
RGA8	AGCGAGAGTTGTATTTAAG-AGCCACTTTTGACAACTGC

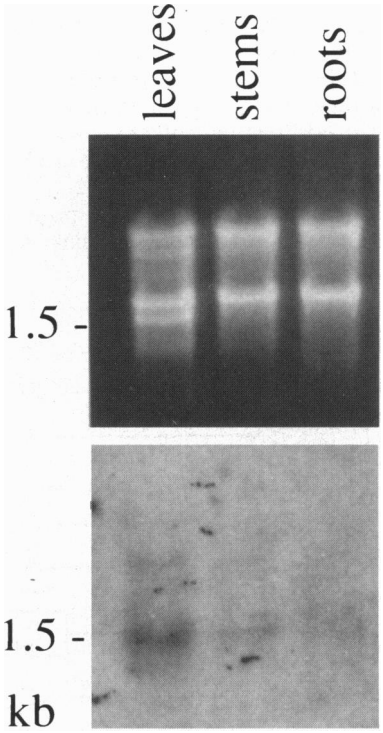

FIG. 1. Northern blot analysis of total soybean RNA from leaves, stems, and roots. (*Upper*) Ethidium bromide-stained RNA gel. (*Bottom*) Results of hybridization of a Northern blot of this gel with an RGA3 probe. Hybridizations using RGA1, RGA2, and RGA4 probes yielded similar results.

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                 .20                 .40                 .60                 .80                 .100
RGA4a  GGVGKTTLVTALFGKISP.....QYDARCFIDDLNKKCGN..FGAISAQKQLLSLALHQG.....NMEIHNLSHGTMLIRTRL
RGA4b  GGVGKTTLVTALFGKISP.....QYDARCFIDDLNKYCGD..FGATSAQKQLLCQALNQG.....NMEIHNLSHGTMLVRTRL
RGA3   GGVGKTTLAVAVYNSIAG.....HFEASCFLENVRETSN.K.KGLQHLQSILLSKTVGE.....KKIKLTNWREGIPIIKHKL
RGA7   GGVGKTTLAAAVYNSIAD.....HFEALCFLENVRETSK.K.HGIQHLQSNLLSETVGE.....H..KLIGVKQGISIIQHRL
RGA1   GGVGKTTLALAVYNLIAL.....HFDESCFLQNVREESN.K.HGLKHLQSIILSKLLGE.....KDINLTSWQEGASMIQHRL
RGA2   GGVGKTTIAREVYNLIAD.....QFEWLCFLDNVRENSI.K.HGLVHLQKTLLSKTIGE.....SSIKLGSVHEGIPIIKHRF
RGA5   GGVGKTTIARKVYEAIKG.....DFDVSCFLENIREVS..KTNGLVHIQK.ELSNLGVSCFLEKCKTNGLVPIVEEVFRDQLRIVDFDNLHDGKMIIANSL
RGA8   GGVGKTTLARVVFKKIRN.....KFDISCFLENVREISQ.NCDGMLSLQGKLLSHM.....KMKDLKIQNLDEGKSIIGGIL
RGA6   GGVGKTTSAKAIYSQIHR.....RFMDKSFIESIRSVCETDSKGHVHLQEQLLSDVLNT.....KVKVHSIGMGTTIIEKRL
N      GGVGKTTIARAIFDTLLGRMDSSYQFDGACFLKDIKE....NKRGMHSLQNALLSELLRE.....KANYNNEEDGKHQMASRL
L6     GGIGKTTTAKAVYNKISSC.....FDCCCFIDNIRET..QEKDGVVVLQKKLVSEILRI.....DSGSVGFNNDSGGRKTIKERV

                 .120                .140                .160                .180                .200
RGA4a  CHLKTLIVLDNVDQVEQLENLAL.HREYLGEGSRTIIISTNMHILRNYGVD..GVYNVQLLNKDKALQLLCKKAFKSDD.IVKGYEEVTHDVLKYVNGLPLA
RGA4b  RRLKTLIVLDNVDQVEQLEN.CL.HPEYLGEGSRIIIISKNMHILKNYGVY..KVYNVQLLKKDKALQLLCKKAFKSDD.IEKGYEEVTYDVLKYVNGLPLA
RGA3   KQKKVLLILDDVDEHKHLQAI.IGSPDWFGCGSRVIITTRNEHLLALHNVK..ITYKVRELNEKHALQLLTQKAFELEKEVDSSYNDILNRALIYASGLPLA
RGA7   QQQKILLILDDVDKREQLQAL.AGRPDLFGLGSRVIITTRDKQLLACHGVE..RTYEVNELNEEHALELLSWKAFKLEK.VDPFYKDVLNRAATYASGLPLA
RGA1   QRKKVLLILDDVDKRQQLKAI.VGRPDWFGPGSRVIITTRDKHILKYHEVE..RTYEVKVLNQSAALQLLKWNAFKREKN.DPSYEDVLNRVVTYASGLPLA
RGA2   LLKKVLLVVDDVDDPDQLQAI.VGGTDWFGSASSVIITTRDKHLLTCHGVT..STYEVDGLNKEEALKLLSGTAFKIDK.VDPCYMRILNRVVTYASGLPLA
RGA5   SNKKVLLVLDDVSELSQLENLA.GKQEWFGPGSRVIITTRDKHLLKTHGVH..LTCKARALAQNEALQLICLKAFKRDQPKKG.YLNLCKEMIECARGLPLA
RGA8   FNNNVLLVLDDVNDIRQLENFSVNDQKWLGPGSRIIIITRDMEVLRSHGTV..ESYKIDLLNSGESLQLFSQKAFKRDQPLEH.ILQLSKVAVQQAGGLPLA
RGA6   SGKRVLIVLDDVNEIGQLENL.CGNCEWFGQGSVIIITTRDVGLLNLFKVD..YVYKMEEMDENESLELFCLHAFGEPNPRED.FNELARNVVAYCGGLPLA
N      RSKKVLIVLDDIDNKDHYLEYLAGDLDWFGNGSRIIITTRDKHLIEKNDI....IYEVTALPDHESIQLFKQHAFGKEVPNEN.FEKLSLEVVNYAKGLPLA
L6     SRFKILVVLDDVDEKFKFEDMLGSPKDFISQ.SRFIITSRSMRVLGTLNENQCKLYEVGSMSKPRSLELFSKHAFKKNTP.PSYYETLANDVVDTTAGLPLT
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FIG. 2. Alignment of the deduced amino acid sequences of RGAs from soybean. Arrows indicate location of PCR primers used to amplify RGA sequences. Underlined regions correspond to additional conserved domains. Dotted regions indicate gaps in sequence introduced to maximize alignment. The tobacco *N* and the flax *L6* amino acid sequences are included for comparison.

Amino acid sequences were aligned and neighbor-joining analysis resulted in the production of a phylogenetic tree with the nine RGA sequences divided into several subclades (Fig. 3).

Mapping of RGA Loci. Genetic mapping of members of the different classes placed them on 8 of the 26 linkage groups comprising the soybean genetic map (Fig. 4). The RGA loci mapped both singly and in clusters and were located on several of the linkage groups to which known disease resistance genes have been mapped. A class 6 RGA mapped to linkage group N1 near *Rps1* (35). A large cluster of RGAs representing five of the different classes mapped to linkage group J and encompassed a resistance gene cluster including *Rmd*, *Rps2*, and *Rj2* (32) and near a quantitative trait locus for soybean cyst nematode resistance (N. Young, personal communication). We found no map positions near other known resistance genes: *Rhg4*, linkage group A2 (36); *Rps3* (35), *Rsv* (37), linkage group F; and *Rps4* (35), linkage group G.

To determine more precisely the map positions of RGAs relative to mapped resistance genes on linkage group J the composite map shown in Fig. 5 was constructed using the computer program JOINMAP (33) and markers common to both populations. These data indicate that at least two RGAs map within this multigene cluster.

BAC Library Screening. Our initial screen of the BAC library using the original degenerate primers was unsuccessful because the primers also amplified products from DH10B, the *Escherichia coli* host used to grow and maintain the library (data not shown). To overcome this obstacle we designed class-specific primers for eight of the RGA classes (Table 1) and identified 50 BACs representing seven classes. Copy number of RGA sequences within each BAC was estimated by digesting the BACs with restriction enzymes that did not have recognition sites within the RGA probe sequences and by hybridizing with each class-specific probe. Fig. 6 demonstrates results for six BACs belonging to classes 1, 2, and 3. Class 1 BACs each contained 3–10 copies of the RGA1 sequence. This agreed with the 8–10 copies expected based on genomic Southern hybridization patterns (data not shown). Genomic Southern blot analyses predicted two to four copies of class 2 RGAs and thus far the class 2 BACs isolated appear to have two to three copies of the class 2 RGA sequence. We identified eight BACs containing class 3 RGAs. Each of these BACs appeared to have two to five copies of RGAs. Restriction digests indicated that some of the BACs have common hy-

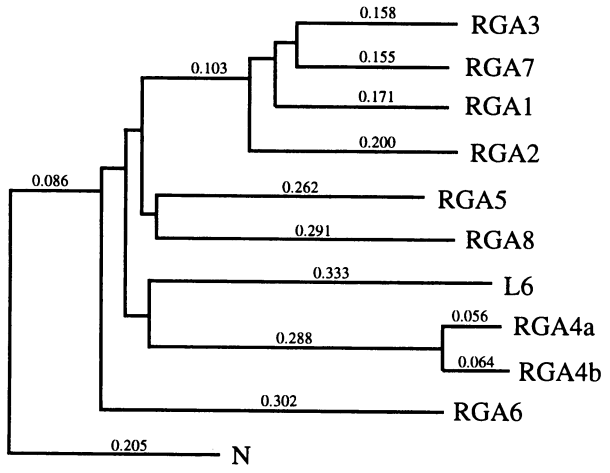

FIG. 3. Phylogenetic tree based on alignment of amino acid sequences of tobacco *N*, flax *L6*, and nine soybean RGA classes. Numbers above lines indicate branch length (proportion of amino acid differences distinguishing classes).

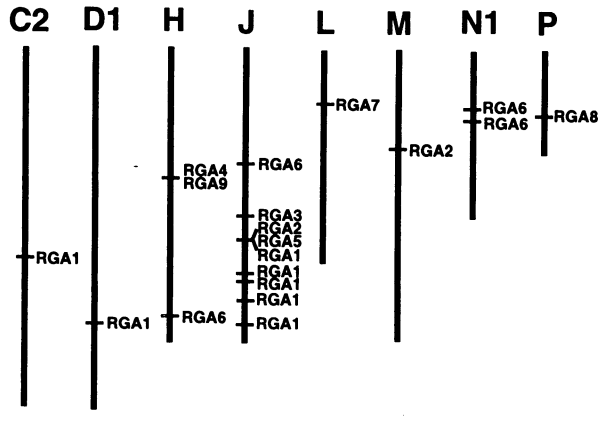

FIG. 4. Distribution of RGA markers on the soybean genetic map.

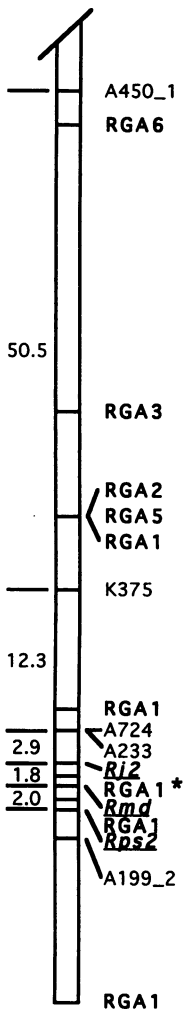

FIG. 5. A portion of linkage group J showing the integration of RGA positions and the map positions of *Rps2*, *Rmd*, and *Rj2*. The map was generated through the use of JOINMAP (33). Anchored markers used to create this composite map were K375, A724, and A233. Numbers to the left of the map indicate estimated genetic distances between selected loci in centimorgans. The position of the RGA1 identified with an asterisk (*) has been confirmed by independant mapping (see *Materials and Methods*).

bridizing fragments and appear to overlap, forming a contig (see, for example Fig. 6*C*, lanes 4–6), although some BACs may represent duplicated chromosomal segments. This cluster appears to contain eight RGA copies. Based on the size of individual BACs the cluster spans ≈200–300 kb, indicating that the distance between individual RGA copies averages 20 kb. The class 3 RGA mapped to only one position on the soybean map (Fig. 4, linkage group J; and Fig. 5). Based on genomic Southern blot analyses, we expected five to eight copies of class 3 RGAs; therefore, these BACs may contain the entire RGA class 3 cluster.

DISCUSSION

Nearly 50 restriction fragment length polymorphism were mapped using 13 restriction enzymes and 9 RGA class sequences as probes. Many polymorphisms were likely due to sequence variations among class members. However, given the microclustering of RGA class members, and the small population size of 56 individuals in which these were mapped, it was not possible to distinguish very tightly linked loci from a single

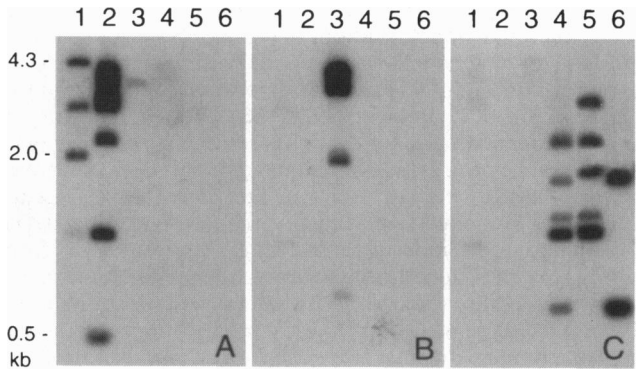

FIG. 6. Southern hybridization of identical membranes containing six *Hae*III digested BACs probed with RGA1 (*A*), RGA2 (*B*), and RGA3 (*C*). Numbered lanes indicate individual BACs. Molecular weights are shown on the left. Note that each BAC contains only one RGA family.

locus. Therefore, these polymorphisms resolved to 16 positions on 8 different linkage groups.

Although very few resistance genes have been mapped in soybean, we mapped RGA sequences close to several known genes. One reported gene cluster in soybean, consisting of the *Rps2* locus conferring resistance to the fungal pathogen *Phytophthora sojae* Kaufmann & Gerdemann, the *Rmd* locus conferring resistance to the powdery mildew pathogen *Microsphaera diffusa* Cooke & Peck, and the *Rj2* locus controlling a nodulation response to *Bradyrhizobia japonicum* (Kirchner) Jordan, maps within a 3.8-centimorgan region of linkage group J (32). A QTL associated with resistance to soybean cyst nematode resistance is also placed near this region (N. Young, personal communication). Surprisingly, a large group of RGAs representing five of the nine classes mapped in the region of this cluster. The consensus map generated by JOINMAP (33) placed two RGAs within this 3.8-centimorgan cluster. This was confirmed by independant mapping of an RGA1 BAC within the population segregating for all three genes. In this population we mapped the BAC within the 1.8-centimorgan region between *Rj2* and *Rmd* (Fig. 5). This finding demonstrates that mapping of RGA sequences can be beneficial in landing markers tightly linked to known resistance genes and possibly in identifying candidate resistance loci.

The fact that members of tight clusters of resistance genes can confer resistance to different pathogens is not surprising considering that members of the same gene family often maintain only partial redundancy; they retain a shared set of preserved functions but acquire unique specificities (12, 38). For example, this would allow tightly linked members of the same family to retain structural motifs necessary to function in similar pathways (e.g., disease resistance), while each could respond to unique signals.

Analysis of microclusters of RGAs may have important implications in identifying functional genes for any number of signal-responsive traits. In tomato the *Pto* gene belongs to a complex locus consisting of a tightly linked cluster of five to seven genes. *Pto* confers resistance to *P. syringae* (11), while the tightly linked homolog, *Fen*, confers sensitivity to an organophosphate insecticide (39, 40). Similar examples of related genes that have acquired unique roles can be found among gene families involved in the regulation of floral identity, in the reception of specific light spectra, and in cell differentiation (see ref. 38). The clustering of signal-responsive genes suggests that common genetic processes—e.g., unequal crossing-over and gene conversion—have acted upon them during their evolution; although no conserved mechanisms by which these results are obtained has been established (16).

It is unlikely that each member of an evolving gene family will remain functional. Without positive or negative selection acting to retain function of gene copies within a gene family the accumulation of deleterious mutation would quickly result in the silencing of redundant genes (38). However, within an environment in which a population is continually challenged by a broad range of stressful conditions (e.g., pathogens), it could be possible for a rich repetoire of functionally similar genes responsive to unique signals to develop (see refs. 12 and 41).

Our findings demonstrate that conserved sequences from resistance genes cloned from a diverse range of plant taxa can be used to identify evolutionarily related genes from soybean. These related sequences are distributed throughout the genome, exist in microclusters of gene classes, and are associated with known resistance genes. The identification of candidate resistance genes by restriction fragment length polymorphism mapping using RGA sequences may, however, have limitations. Indeed, it is likely that the silenced pseudogenes, by virtue of accumulation of mutation, will be the source of polymorphisms between genotypes. If this is true, we can predict that it will be these sequences that are mapped. Therefore, genetic mapping of RGA sequences may be more important in landing markers close to resistance genes for subsequent map-based cloning, than for direct identification of resistance genes.

We are grateful to Dr. C. Nickell (Agricultural Research Service, U.S. Department of Agriculture) for making available germplasm used in this study. We are also grateful for the financial support from the Iowa Soybean Promotion Board. This paper is a joint contribution of Midwest Area, Agricultural Research Service, U.S. Department of Agriculture, Field Crops Research Unit and Journal Paper No. J-16991 of the Iowa Agricultural and Home Economics Experiment Station, Ames; Project 3236.

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