Resistance Gene Candidates Identified by PCR with Degenerate Oligonucleotide Primers Map to Clusters of Resistance Genes in Lettuce

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The recent cloning of genes for resistance against diverse pathogens from a variety of plants has revealed that many share conserved sequence motifs. This provides the possibility of isolating numerous additional resistance genes by polymerase chain reaction (PCR) with degenerate oligonucleotide primers. We amplified resistance gene candidates (RGCs) from lettuce with multiple combinations of primers with low degeneracy designed from motifs in the nucleotide binding sites (NBSs) of *RPS2* of *Arabidopsis thaliana* and *N* of tobacco. Genomic DNA, cDNA, and bacterial artificial chromosome (BAC) clones were successfully used as templates. Four families of sequences were identified that had the same similarity to each other as to resistance genes from other species. The relationship of the amplified products to resistance genes was evaluated by several sequence and genetic criteria. The amplified products contained open reading frames with additional sequences characteristic of NBSs. Hybridization of RGCs to genomic DNA and to BAC clones revealed large numbers of related sequences. Genetic analysis demonstrated the existence of clustered multi-gene families for each of the four RGC sequences. This parallels classical genetic data on clustering of disease resistance genes. Two of the four families mapped to known clusters of resistance genes; these two families were therefore studied in greater detail. Additional evidence that these RGCs could be resistance genes was gained by the identification of leucine-rich repeat (LRR) regions in sequences adjoining the NBS similar to those in *RPM1* and *RPS2* of *A. thaliana*. Fluorescent in situ hybridization confirmed the clustered genomic distribution of these sequences. The use of PCR with degenerate oligonucleotide primers is therefore an efficient method to identify numerous RGCs in plants.

Resistance to many diseases, particularly those caused by biotrophic fungal pathogens, is determined by individual members of families of dominant genes, each member conferring resistance to specific strains of the pathogen. When parallel genetic studies have been made of host and pathogen, specificity has often been shown to be conferred by a “gene-for-gene” interaction; for every gene for resistance there is a corresponding gene for avirulence in the pathogen (Flor 1956). The gene-for-gene interaction has now been demonstrated or inferred in over 20 diseases and probably functions in many more (Crute 1986; Flor 1971). Resistance genes have been shown by classical genetics to be clustered in the genomes of several species. They may be groups of genetically separable loci or apparently multiallelic series (Islam and Shepherd 1991; Jorgensen 1992; Pryor 1987). In lettuce, 27 of the 29 known resistance specificities to seven diseases are clustered in five linkage groups (Bonnier et al. 1994; Farrara et al. 1987; Kesseli et al. 1993; Maisonneuve et al. 1994; Robbins et al. 1994; Witsenboer et al. 1995). Different genes within the same cluster may determine resistance to taxonomically diverse pathogens. On the basis of this clustered distribution and by inference from other cell-cell recognition systems, resistance genes have been hypothesized to be functionally and evolutionarily related (Michelmore et al. 1987; Pryor 1987).

Considerable progress has been made recently in cloning disease resistance genes from several plant species (Bent 1996; Jones and Jones 1997). These genes were isolated by transposon tagging or map-based cloning after many years of effort. Apart from *Hm1* (Johal and Briggs 1992), all seem to encode components of signal transduction pathways. Many contain similar sequence motifs, even though they determine resistance to very different pathogens, including a virus, fungi, and bacteria. All, except *Pto*, encode leucine-rich repeats (LRRs). *RPS2*, *RPM1*, *I2*, *N*, *RPP5*, and *L6*, but not *Cf9*, *Cf2*, or *Xa21*, also share sequences characteristic of the ATP/GTP binding sites (nucleotide binding site, NBS) of P-loop proteins (reviewed in Hammond-Kosack and Jones 1997). The presence of conserved domains in resistance genes presents the opportunity to clone numerous additional resistance genes from diverse species by polymerase chain reaction (PCR) with degenerate oligonucleotide primers to the conserved motifs.

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Degenerate primers have been used to isolate a variety of P-loop-containing proteins from mammals. These include *ARF* (ADP-ribosylation factor; Clark et al. 1993), *RAB* (*ras*-related GTP-binding protein; Yu et al. 1993), and *SAR* (secretion associated-*ras* related; Shen et al. 1993) members of the *ras* superfamily of genes encoding small or monomeric GTP-binding proteins. Although there are many P-loop-containing proteins in mammals, the motifs within the NBSs tend to be sufficiently conserved within each family but different enough between families to allow specific amplification of individual families by PCR. Degenerate oligonucleotide primers have been used to isolate NBS-containing sequences from soybean, potato, rice, and *Arabidopsis thaliana* (Kanazin et al. 1996; Leister et al. 1996, 1998; Yu et al. 1996; Aarts et al. 1998). Several of these mapped to known resistance genes and one was shown to have an LRR region 3' to the NBS sequence. However, it is possible that diverse NBS-containing proteins are common in plants. It was necessary to determine whether the sequences so far obtained for resistance genes were sufficiently specific for resistance genes or whether there would be amplification of numerous sequences that were not apparently resistance genes (Michelmore 1996).

In this paper, we describe the use of PCR to amplify resistance gene candidates (RGCs) from lettuce. Multiple primers with low degeneracy, particularly at the 3' end, were designed based on the sequences of three known resistance genes—*N*, *L6*, and *RPS2*—from tobacco, flax, and *A. thaliana*, respectively. These were tested on a variety of templates. The presence of multiple sequence motifs characteristic of resistance genes, genetic cosegregation with known resistance genes,

and the presence of clustered multigene families indicated that at least half of the families identified were amplified from resistance genes.

RESULTS

Amplification with degenerate primers.

Forty-eight combinations of sense and antisense primers (Table 1) were tested on a panel of nine templates consisting of two genomic DNA samples, two cDNA preparations, and three bacterial artificial chromosome (BAC) clones from the *Dm3* region; plasmids containing *L6* and *RPS2* were included as positive controls. As expected, amplification from *L6* and *RPS2* resulted in fragments of 549 and 540 bp, respectively. Nine combinations of primers resulted in fragments of approximately this size with multiple templates and their reaction products were cloned.

Sequence analysis of amplified products.

A total of 65 clones were sequenced. Four of the nine primer combinations collectively yielded 32 sequences with continuous open reading frames that were similar to the NBSs of known resistance genes (Table 2; Fig. 1). Seven of the 11 clones amplified from genomic DNA or cDNA templates with the primer pair PLOOPGA/GLPL6 were 564 bp long; they were identical to each other and named RGC1 (GenBank accession no. AF017751). All 16 clones amplified from genomic DNA or cDNA with the primers PLOOPAA/GLP6 were the same as RGC1. The PLOOPGA/GLPL4 pair of primers yielded two sequences that were similar but not identical to

Table 1. Degenerate oligonucleotide primers used to amplify resistance gene candidates from lettuce

Target motif	Designation	Sequence ^a	Degeneracy
P-loop sense direction	PLOOPAA	(CUA) ₄ GAATTCGGNGTNGGNA <u>AA</u> CA <u>CA</u> C	64
	PLOOPAG	(CUA) ₄ GAATTCGGNGTNGGNA <u>AA</u> ACGAC	64
	PLOOPGA	(CUA) ₄ GAATTCGGNGTNGGNAAGACA <u>CA</u> C	64
	PLOOPGG	(CUA) ₄ GAATTCGGNGTNGGNAAGACGAC	64
	PLOOPAC	(CUA) ₄ GAATTCGGNGTNGGNA <u>AA</u> ACCAC	64
	PLOOPAT	(CUA) ₄ GAATTCGGNGTNGGNA <u>AA</u> ACTAC	64
	PLOOPGC	(CUA) ₄ GAATTCGGNGTNGGNAAGACCAC	64
	PLOOPGT	(CUA) ₄ GAATTCGGNGTNGGNAAGACTAC	64
GLPL anti-sense direction	GLPL1	(CUA) ₄ GTCGACAGNGC <u>NA</u> GNGGNAGGCC	256
	GLPL2	(CUA) ₄ GTCGACAGNGC <u>NA</u> GNGGNAGACC	256
	GLPL3	(CUA) ₄ GTCGACAGNGC <u>NA</u> GNGGNAGTCC	256
	GLPL4	(CUA) ₄ GTCGACAGNGC <u>NA</u> GNGGNAGCCC	256
	GLPL5	(CUA) ₄ GTCGACA <u>ANG</u> CC <u>AA</u> NGGCA <u>AA</u> ACC	16
	GLPL6	(CUA) ₄ GTCGACA <u>ANG</u> CC <u>AA</u> NGGCAATCC	16

^a Bases that vary between primers are underlined. Degenerate positions at which all four bases were used are indicated by N.

Table 2. Characteristics of resistance gene candidates (RGCs) isolated from lettuce

RGC family	Template	Primers	Number ^a	Size (bp) ^b	Copy number ^c	<i>Dm</i> linkage
RGC1	genomic DNA	PLOOPGA+GLPL6	6/6	528	10	<i>Dm4</i> , <i>Dm13</i>
	cDNA	PLOOPGA+GLPL6	1/5			
	genomic DNA	PLOOPGA+GLPL6	15/15			
	cDNA	PLOOPGA+GLPL6	1/1			
	genomic DNA	PLOOPGA+GLPL4	2/7			
RGC2	BAC H8	PLOOPGG+GLPL3	3/3	516	20	<i>Dm1</i> , <i>Dm3</i>
RGC3	genomic DNA	PLOOPGA+GLPL4	3/7	471	2	Unlinked
RGC4	genomic DNA	PLOOPGA+GLPL4	1/7	513	15	Unlinked

^a Number of RGC sequences out of total number of clones sequenced.

^b Size of fragment amplified from the nucleotide binding site without the primer tails.

^c Approximate copy number estimated from genomic Southern blot analysis and numbers of clones in the bacterial artificial chromosome (BAC) library.

RGC1 (55% deduced amino acid identity). PLOOPGA/GLPL4 also yielded two other distinct sequences with similarity to the NBS of resistance genes; these were less than 30% identical to RGC1 and to each other and were therefore designated RGC3 (GenBank accession no. AF017753) and RGC4 (GenBank accession no. AF017754). All three clones sequenced from BAC clone H8 were 552 bp long and identical to each other, but less than 32% identical to RGC1, RGC3, and RGC4; they were designated RGC2 (GenBank accession no. AF017752). The 28 clones sequenced from five other primer combinations had no similarity to any NBS motifs and therefore were not studied further. Therefore, half of the clones sequenced contained NBS motifs representing four RGC families.

Comparison of the deduced amino acid sequences of the RGC sequences to products of known resistance genes revealed that the RGC sequences are as similar to each other as they are to resistance genes from other species. This is the same level of similarity shown between the known resistance genes (Table 3). The greatest similarity (44%) was between RGC4 and *N* from tobacco. Sequence comparisons were done with and without the regions homologous to the primers for RGC1 and RGC2 because their genomic sequences were determined by inverse PCR (IPCR) (see below). The regions homologous to the primers could not be included in sequence comparisons with RGC3 and RGC4 because they did not map to known clusters of disease resistance genes (see below) and therefore their flanking sequences were not determined.

Genetic analysis of RGCs.

In genomic Southern analyses, all four RGCs revealed multiple fragments of varying intensity. The number of bands was highly dependent on the stringency of hybridization (data not shown). Each RGC sequence was initially analyzed by bulked

segregant analysis (BSA) with pools of resistant and susceptible individuals for each of the four clusters of resistance genes.

BSA demonstrated that RGC1 was linked to both the *Dm4,7* and *Dm13* clusters (Fig. 2). Segregation analysis showed that the polymorphic bands detected by RGC1 cosegregated and were closely linked to *Dm13*; they were more loosely linked to *Dm4,7*. Only one recombinant was detected between RGC1 and *Dm13*. After we had characterized several RGC1 family members (see below), a divergent sequence (58% identical to RGC1a) was selected for further segregation analysis; this probe revealed a different set of cosegregating polymorphic bands. These cosegregated with *Dm13*.

Table 3. Comparison of the resistance gene candidates (RGCs) from lettuce to resistance genes cloned from other species^a

Comparisons with primers	Comparisons without primer regions						
	RGC1	RGC2	RGC3	RGC4	L6	N	RPS2
RGC1		25	25	27	26	23	28
RGC2	28		31	19	22	20	29
RGC3				25	22	26	29
RGC4					36	44	20
L6	29	22				36	19
N	31	21			40		20
RPS2	35	33			26	26	

^a Values shown are for percent identity of the deduced amino acid sequences. Sequence comparisons for the upper right half were made without the regions homologous to the primers, as the genomic sequences for these regions were not available for RGC3 and 4 (see text). Sequence comparisons for the lower left half included the genomic sequences homologous to the primers. RGC1, RGC2, RGC3, and RGC4: sequences amplified from lettuce with degenerate primers. L6, resistance to *Melampsora lini* in flax (Lawrence et al. 1995). N, resistance to tobacco mosaic virus in tobacco (Whitham et al. 1994). RPS2, resistance to *Pseudomonas syringae* in *Arabidopsis thaliana* (Bent et al. 1994; Mindrinos et al. 1994).

Fig. 1. Sequence similarity between nucleotide binding sites (NBSs) of resistance gene candidates (RGCs) from lettuce, and other resistance genes. Conceptual translations of the NBS regions RGC1a, RGC2a, RGC3a, RGC4a, L6 from flax, *N* from tobacco, and *RPS2* from *Arabidopsis thaliana* were aligned by the Clustal method with PAM250 residue weight table. Consensus residues are highlighted in black.

BSA with RGC2 demonstrated that this family of sequences mapped to the *Dm1*, *Dm3* cluster of resistance genes. At least some loci were expected to map to this cluster because RGC2a was derived from BAC H8, which was known to be from the *Dm3* region. All six polymorphic bands that distinguished the parents of our mapping population cosegregated with *Dm3*. Most of the genetic analysis was made with a related family member, RGC2b (72% identical to RGC2a), because it was derived from BAC H15 (see below) and was a candidate for the *Dm3* gene (Okubara et al. 1997; Meyers 1998). To provide finer genetic resolution, RGC2 was also mapped with a panel of *Dm3* deletion mutants (Anderson et al. 1996). Eleven of 18 fragments were missing in the largest deletion mutant, demonstrating that several RGC2 family members are physically located very close to *Dm3* (Fig. 3). No fragment was missing in all deletion mutants. However, this is not unexpected; there is extensive duplication around the *Dm3* region and despite developing BAC contigs in the region we have yet to obtain any hybridization marker that is missing in all the deletion mutants (Anderson et al. 1996; Meyers 1998).

RGC3 and RGC4 both detected multiple fragments. Both of the fragments detected by RGC3 were polymorphic in the mapping population. Five out of 17 fragments detected by RGC4 were polymorphic. With each probe, all polymorphic bands cosegregated. RGC3 and RGC4 segregated independently of each other and did not map to any of the four mapped clusters of resistance genes. RGC3 mapped between markers GLh17 and CL1007; RGC4 mapped between CL1117 and CL1034 (Kesseli et al. 1994).

Fig. 2. Bulked segregant analysis of resistance gene candidate (RGC) RGC1a against the four known clusters of resistance genes in lettuce. RGC1a was hybridized to pooled DNA samples of genomic DNA digested with *EcoRV* from resistant and susceptible individuals for the *Dm1/3*, the *Dm4/7*, the *Dm5/8*, and the *Dm13* clusters. The parental genotypes, Calmar (C: *Dm7*, *Dm8*, *Dm13*) and Kordaat (K: *Dm1*, *Dm3*, *Dm4*), were included for comparison. The parental allele present in each pooled DNA sample is indicated (C or K). The intensely hybridizing band relative to other fragments probably represents a conserved *EcoRV* fragment within multiple family members. Sizes are indicated in kb.

Isolation of additional family members by hybridization and genomic copy numbers.

To isolate additional sequences of RGC1 and RGC2, we screened our genomic BAC library. Twenty-three clones were identified that hybridized to RGC1 and 41 clones that hybridized to RGC2. As this library is thought to represent approximately two genome equivalents (Frijters et al. 1997), there are probably at least 10 and 20 members in the RGC1 and RGC2 families, respectively. These numbers are similar to the numbers of fragments observed in genomic Southernblots at moderate stringency. All the clones that hybridized to

Fig. 3. Southern analysis of the resistance gene candidate (RGC) RGC2b family with deletion mutants of *Dm3*. RGC2b was hybridized to genomic DNA digested with *HindIII* from a panel of deletion mutants (Anderson et al. 1996). Mutant *Dm3r1608* contains the largest deletion; mutants with the smallest deletions (1208 to 1885) are on the right. Deletions were generated in cv. Diana, which is included for comparison. Sizes are indicated in kb.

RGC2 also hybridized to marker AC15; this marker detects a small multigene family and had already been shown by deletion mutant analysis to be clustered around *Dm3* (Anderson et al. 1996). This provided further evidence for clustering of RGC2 sequences.

With primers conserved within each family, part of the NBS was amplified from each unique BAC clone and sequenced. This revealed that members within the RGC1 and RGC2 families ranged from 54 to 98% identical at the deduced amino acid level. The most divergent members only weakly cross-hybridized to each other. Currently, we consider RGC sequences to be part of the same family of sequences if they are at least 50% identical at the deduced amino acid level and map to the same region of the chromosome. Cross-hybridization was not a useful criterion as it is dependent on the stringency of the hybridization conditions used.

Sequence analysis of flanking regions.

To obtain further evidence that we had amplified resistance genes, we amplified the regions flanking the NBSs of RGC1b and RGC2a by IPCR of BAC clones. These products were then directly sequenced without cloning to minimize the introduction of PCR artifacts. Sequence analysis with BLASTX of 546 bp of the region 5' to the NBS of RGC2a failed to detect any homology to known resistance genes. Comparison of the 500 bp 5' to RGC1b detected significant similarity to the 5' region of candidates for the *I2* resistance gene in tomato (22 out of 55 amino acids identical). The sequence of the 1.5-kb region C-terminal to the NBSs of both RGC1b and RGC2a contained LRRs. These sequences detected similarity to several known resistance genes as well as resistance gene homologs. RGC1 was most similar to *RPM1* from *A. thaliana* and *I2C* from tomato. RGC2 was most similar to *RPS2* from *A. thaliana*. This region does not contain LRRs as regular as those present in some resistance genes, such as the *Cf* genes from tomato. Nevertheless, the repeat structure seems to be comparable with that of the flax resistance gene, *L6* (Jones and Jones 1997). Therefore, the presence of LRR regions is further evidence that the sequences we amplified with degenerate oligonucleotide primers are probably resistance genes.

Analysis of genomic distribution by FISH.

The location and clustering of RGC sequences was also analyzed with fluorescent in situ hybridization (FISH) of BAC clones containing either RGC1 or RGC2 to mitotic chromosomes. Each of the three RGC1-containing BACs revealed one strong Cy3 signal close to the centromere on the short arm of a pair of fairly large chromosomes (Fig. 4A,C). The three RGC2-containing BACs revealed one clear region of hybridization at the telomere of the short arm of a small pair of chromosomes (Fig. 4B,D). The low level of hybridization to the rest of the genome is attributable to incomplete blocking of retrotransposons and other repetitive elements that are present in these BACs (Meyers 1998). In the absence of C_0t1 DNA, strong hybridization occurs to all the chromosomes.

DISCUSSION

At least some members of the RGC1 and RGC2 families are probably resistance genes based on three criteria: the pres-

ence of multiple sequence motifs characteristic of resistance genes, genetic cosegregation with known resistance genes, and distribution in the genome as clustered, multigene families. The presence of LRR regions in a similar C-terminal position relative to the NBS as in cloned resistance genes provides stronger evidence than sequence similarity to NBS regions alone. The clustering of RGC1 and RGC2 sequences at the same position as the known clusters of resistance genes makes them strong candidates for encoding resistance genes. The hybridization patterns and genetic distribution of the RGC sequences are similar to those of cloned resistance genes in other species. There are resistance genes in lettuce that segregate independently of the four known clusters, but they have not been mapped relative to molecular markers. Therefore, the lack of observed cosegregation of RGC3 and RGC4 with the mapped resistance genes does not preclude them from encoding resistance genes.

Definitive proof that these sequences are resistance genes requires transgenic complementation. Such experiments are underway for *Dm3*. However, it is a non-trivial and time-consuming task to determine which individual member of a complex multigene family encodes an individual specificity. A rate-limiting step is the genetic resolution needed to distinguish individual family members either by recombination or by mutation analysis. Also, isolates recognizing individual specificities may not be available. Dissection of such complex loci will be aided by PCR-based approaches with primers specific to individual RGC sequences.

The four families of RGC sequences that we identified from lettuce are as different from each other as they are from resistance genes from other species. Members within each family could be identified by cross-hybridization. However, there was insufficient sequence similarity for cross-hybridization between families that mapped to different locations in the genome. This parallels our experience with resistance genes from different species used as probes. We have detected no hybridization (even at low hybridization stringency) with clones of the resistance genes *RPS2*, *N*, *L6*, or *Prf* to genomic Southern of lettuce or to BAC clones from the major cluster of resistance genes, even to clones that were subsequently shown to contain RGC sequences (M. van Damme, unpublished). Therefore, heterologous hybridization seems unlikely to be a successful strategy for cloning resistance genes, except from closely related species, in which case hybridization may detect orthologous genes.

We assigned sequences into distinct families based on sequence and genetic criteria. The 50% threshold for sequence similarity is low and deliberately conservative. The genetic data indicated that all sequences with similarities greater than 50% were clustered in the lettuce genome. Previous investigations of soybean and potato (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996) identified greater numbers of sequences but these were assigned to families on the basis of cross-hybridization. Distinct sequences mapped to the same region of the genome and therefore the number of distinct families may have been overestimated.

PCR with degenerate oligonucleotide primers has great potential for amplifying numerous resistance genes from diverse species. The degenerate primers that we designed from other resistance genes were specific enough to preferentially amplify resistance genes rather than NBS-containing proteins in

general. We utilized a conservative approach and tested numerous primers with little degeneracy, particularly at the 3' end. The now published studies on soybean and potato used primers with greater degeneracy or incorporated inosines (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996); this may allow the identification of more sequences with fewer primers. However, the use of multiple combinations of primers with low degeneracy should assay as comprehensive a range of sequences as more-degenerate primers and increases the chances of isolating different RGC sequences. As more resistance genes are cloned and additional RGC sequences are isolated, it will become feasible to design primers that will be increasingly selective for resistance genes.

The presence of many cross-hybridizing bands in genomic Southern raises the question as to how many are functional genes and whether all the genes detected are resistance genes.

Resistance genes may be only a subset of the genes detected; closely related genes may have roles in other recognition pathways. A definitive answer will require extensive functional analysis. However, plants need the ability to defend themselves against diverse challenges; therefore, there is no reason to suppose that the ability of plants to detect non-self is any less elaborate than that of mammals. Molecular data are increasingly consistent with plants having hundreds of resistance genes arrayed in complex clusters that are continuously evolving new recognition specificities.

The cloning of RGC sequences opens up the possibility of both conventional and transgenic approaches to increasing disease resistance of plants (Michelmore 1995). The RGC sequences can be used immediately as genetic markers for the analysis of germplasm, increasing the speed of introgression of resistance genes, or as a substitute for pathogen screens

Fig. 4. Fluorescent in situ hybridization (FISH) of resistance gene candidate (RGC)-containing bacterial artificial chromosome (BAC) clones to lettuce chromosomes. **A**, Interstitial localization of Cy3 signals (arrowed) in a late prophase cell probed with BAC H124 containing RGC1a. **B**, Hybridization (arrowed) with BAC H15 to the telomeric regions of a pair of chromosomes in a metaphase cell. **C**, Hybridization (arrowed) of BAC E136 containing RGC1c, to the short arm close to the centromere of a pair of chromosomes in an early metaphase cell. **D**, A pair of Cy3 signals detected with BAC H15 containing RGC2b located terminally on the short arm of a pair of chromosomes in a mid-prophase cell. All probes were blocked with a 50× excess of unlabeled C₆t1 DNA except in (**D**), in which a 150× excess was used.

when such are difficult or involve exotic organisms. The utility of RGC sequences as molecular markers for breeding purposes will be increased by the development of oligonucleotide primers that allow whole families or specific members to be assayed by PCR. Knowledge of which sequence of a family encodes a particular specificity is not required for this application. RGC sequences also allow a candidate cloning approach to isolate numerous known resistance genes although, as discussed above, it is not straightforward to identify genes with defined specificities. Efficient isolation of numerous genes will permit several transgenic approaches for disease resistance. Cloned resistance genes can be pyramided into single transgenic constructs or transferred into sexually incompatible species.

MATERIALS AND METHODS

DNA templates.

Lettuce genomic DNA was extracted from cv. Diana and a mutant line derived from cv. Diana (Okubara et al. 1997) by a modified CTAB protocol as described previously (Bernatzky and Tanksley 1986). To generate single-stranded cDNA templates, RNA was isolated from cv. Diana and the mutant following the procedure of Jones et al. (1995); first-strand cDNA was synthesized by Superscript reverse transcriptase from 1 µg of total RNA as specified by the manufacturer (Gibco BRL Life Technologies, Gaithersburg, MD). BAC clone templates from the *Dm3* region were isolated from a BAC library of over 53,000 clones with marker AC15 (Frijters et al. 1997). AC15 detects a small, multigene family that has been shown, with deletion mutants, to be closely linked to *Dm3* (Anderson et al. 1996). Bacterial plasmids containing clones of *L6* from flax (courtesy of J. Ellis, CSIRO, Canberra) and *RPS2* from *A. thaliana* (courtesy of B. Staskawicz, University of California, Berkeley) were used as positive controls.

PCR with degenerate oligonucleotide primers.

Oligonucleotide primers were designed based on conserved motifs in the NBSs of *L6*, *RPS2*, and *N* (Table 1). These primers were designed to have low degeneracy, particularly at the 3' end. Eight primers were made corresponding to the GVGKTT motif in the sense direction; each had 64-fold degeneracy. Six primers were made to the GLPLAL motif in the anti-sense direction; with either 16- or 256-fold degeneracy. Oligonucleotides included (CUA)₄ at the 5' end of the sense primers and (CAU)₄ at the 5' end of the antisense primers to allow rapid cloning of the PCR products into pAMP1 (Gibco BRL Life Technologies).

PCR amplification was performed in 50-µl reaction volume with 1 µM for each of a pair of sense and antisense primers. The templates were denatured by heating to 94°C for 2 min. This was followed by 35 cycles of 30 s at 94°C, 1 min at 50°C, 2 min at 72°C, with a single final extension of 5 min at 72°C.

Genetic analysis.

BSA (Michelmore et al. 1991) was performed to obtain a preliminary indication of the linkage relationships of the amplified products and known clusters of resistance genes. DNA from approximately 10 individuals was pooled for each susceptible and resistant bulk. Amplified products were then mapped by restriction fragment length polymorphism (RFLP)

analysis with 66 individuals from our intraspecific mapping population (Kesseli et al. 1994). Resistance genes from four clusters as well as over 600 markers have now been mapped on this population. Linkage was analyzed with MAPMAKER (Lander et al. 1987). Due to low recombination in the *Dm3* region, sequences were mapped relative to *Dm3* with a panel of deletion mutants that provided greater genetic resolution than the mapping population (Anderson et al. 1996). All blots were washed twice at 63°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)/0.1% sodium dodecyl sulfate (SDS) for 20 min, followed by one wash at 63°C in 1× SSC/0.1% SDS for 10 or 30 min and then a final wash of 0.5× SSC/0.1% SDS for 20 min.

Screening of BAC libraries.

Over 53,000 BAC clones containing lettuce genomic DNA with an average insert size of 111 kb (Frijters et al. 1997) were screened with two of the amplified products. High-density filters, each containing 1,536 clones, were hybridized to ³²P-labeled probes. Filters were washed at 65°C with 40 mM Na₂PO₄/0.1% SDS for 5 min followed by 20 min in the same solution.

Sequence analysis and nomenclature.

Double-stranded plasmid DNA clones and PCR products were sequenced with an ABI377 automated sequencer and fluorescently labeled di-deoxy terminators (ABI PRISM Ready Reaction kits; PE Applied Biosystems, Foster City, CA). Sequences were assembled with Sequencher (GeneCodes, Ann Arbor, MI), DNASTar (Lasergene, Madison, WI), and Genetics Computer Group (GCG, Madison, WI) software. Database searches were performed with BLASTX (Altschul et al. 1990) and FASTA (GCG) algorithms.

Sequences flanking the NBS region for RGC2 and for some of RGC1 were obtained by IPCR on BAC DNA templates and the products sequenced directly. IPCR worked less well for RGC1. Therefore, RGC1 was sequenced by the long-range sequencer protocol (Hagiwara and Harris 1996).

Sequences that were less than 40% identical at the deduced amino acid level were considered to belong to different families and given distinct RGC numbers (RGC#). Sequences that were at least 50% identical were considered to be potentially members of the same family; they were given the same RGC# designation and distinguished by a letter suffix. No sequences were between 40 and 50% identical.

FISH.

Chromosome spreads were made from protoplasts of root tips as described by Jewell and Islam-Faridi (1994). Three BAC clones containing members of RGC1 and three different BACs containing members of RGC2 were used as probes. The BAC DNA was labeled with digoxigenin-11-dUTP by nick-translation (Boehringer Mannheim, Indianapolis, IN). The first part of the FISH procedure was essentially as described by Rayburn and Gill (1985) except that the slides were treated with RNase-A (75 µg/ml) in 2× SSC for 45 to 60 min at 37°C. C₀t1 DNA (Zwick et al. 1996) of cv. Diana was in several-fold excess of the probe DNA to block the repetitive sequences in the BAC clones hybridizing to the chromosomes. The probe was incubated with denatured C₀t1 DNA for 25 min at 37°C prior to applying the mixture to the chromosomes spread on

slides. The washes after hybridization and Cy3 detection followed the protocols of Islam-Faridi and Mujeeb-Kazi (1995) and Gomez et al. (1997).

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