The Major Resistance Gene Cluster in Lettuce Is Highly Duplicated and Spans Several Megabases

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At least 10 Dm genes conferring resistance to the oomycete downy mildew fungus Bremia lactucae map to the major resistance cluster in lettuce. We investigated the structure of this cluster in the lettuce cultivar Diana, which contains Dm3. A deletion breakpoint map of the chromosomal region flanking Dm3 was saturated with a variety of molecular markers. Several of these markers are components of a family of resistance gene candidates (RGC2) that encode a nucleotide binding site and a leucine-rich repeat region. These motifs are characteristic of plant disease resistance genes. Bacterial artificial chromosome clones were identified by using duplicated restriction fragment length polymorphism markers from the region, including the nucleotide binding site-encoding region of RGC2. Twenty-two distinct members of the RGC2 family were characterized from the bacterial artificial chromosomes; at least two additional family members exist. The RGC2 family is highly divergent; the nucleotide identity was as low as 53% between the most distantly related copies. These RGC2 genes span at least 3.5 Mb. Eighteen members were mapped on the deletion breakpoint map. A comparison between the phylogenetic and physical relationships of these sequences demonstrated that closely related copies are physically separated from one another and indicated that complex rearrangements have shaped this region. Analysis of low-copy genomic sequences detected no genes, including RGC2, in the Dm3 region, other than sequences related to retrotransposons and transposable elements. The related but divergent family of RGC2 genes may act as a resource for the generation of new resistance phenotypes through infrequent recombination or unequal crossing over.

INTRODUCTION

Disease resistance genes frequently occur in tightly linked clusters (Pryor, 1987; Crute and Pink, 1996; Michelmore and Meyers, 1998). Clusters of plant resistance genes were first established by use of classic genetic techniques; detailed molecular analyses are now beginning to unravel the complexity of these loci and the underlying mechanisms determining their structure (Parmiske et al., 1997; Song et al., 1997). It is becoming increasingly apparent that such clusters may be both common and complex genomic regions in plants.

Clusters of resistance genes have been identified in diverse plant species. More than 30 different resistance specificities to the single fungal pathogen responsible for flax rust disease, Melampsora lini, have been mapped to five linkage groups (Flor, 1971; Islam and Shepherd, 1991). These loci exemplify two possible genetic arrangements that may exist for clusters of resistance genes: the flax L locus contains at least 13 allelic rust resistance specificities, and the more complex M locus exists as a tandem array of at least seven genes (Islam and Shepherd, 1991). In maize, multiple Rp genes, both linked and allelic, have been observed to mediate resistance to the rust fungus Puccinia sorghi; 16 genetically separable loci were mapped to a single cluster known as the Rp1 complex (Saxena and Hooker, 1968; Hulbert, 1997). Complex disease resistance clusters also have been identified in lettuce (Farrara et al., 1987; Witsenboer et al., 1995), Arabidopsis (Kunkel, 1996; Holub, 1997), rice (Song et al., 1995), barley (Jorgensen, 1994), tomato (Jones et al., 1993), and other plant species (reviewed in Michelmore and Meyers, 1998). Several of the specificities within these genetically well-defined resistance loci have been targeted for molecular cloning and analysis.

The molecular characterization of resistance gene clusters has been advanced by the recent cloning of plant disease resistance genes from diverse species. Most of these genes contain regions encoding leucine-rich repeats (LRRs), with

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or without either a nucleotide binding site (NBS) or a kinase domain (reviewed in Baker et al., 1997; Hammond-Kosack and Jones, 1997). These structurally similar gene products exhibit dramatic differences in specificity and can confer resistance to fungal, viral, nematode, or bacterial pathogens. Many, but not all, resistance genes are members of multigene families. DNA gel blot hybridization and genetic analysis indicate that the Pto and Cf genes of tomato, the Xa21 gene of rice, and the N gene of tobacco all exist as tandem arrays of sequences (Martin et al., 1993; Whitham et al., 1994; Song et al., 1995; Dixon et al., 1996; Pamiske et al., 1997). Sequences similar to resistance genes are being isolated via degenerate oligonucleotides or identified from cDNA libraries and are often linked to known resistance loci (Kanazin et al., 1996; Leister et al., 1996; Yu et al., 1996; Botella et al., 1997; Lagudah et al., 1997; Aarts et al., 1998). These resistance gene candidates also exist as multigene families, although their function has yet to be demonstrated. The multigenic structure of resistance gene loci from widely varied plant species suggests an important role for gene duplication and sequence divergence in the evolution of these gene families.

To understand the evolution of clusters of disease resistance genes, we have focused on the largest cluster of resistance genes in lettuce. Host-pathogen studies have identified at least 15 dominant resistance genes (Dm genes) in lettuce (Kesseli et al., 1994; Witsenboer et al., 1995) that match dominant avirulence genes in lettuce downy mildew (Bremia lactucae; Iltott et al., 1989). Most of these resistance genes map to three major clusters (Kesseli et al., 1994; Witsenboer et al., 1995). The largest cluster contains at least 10 Dm resistance genes as well as a gene for a resistance to root aphid (Farrara et al., 1987; Bonnier et al., 1994; T. Nakahara and R.W. Michelmore, unpublished data). In lettuce cultivar Diana, this cluster includes the downy mildew resistance genes Dm3 and Dm1, which are separated by 9 centimorgans (Kesseli et al., 1994). Several low-copy randomly amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) markers near Dm3 are components of localized duplications; long-range restriction mapping indicated that the region containing most of these duplications spanned 1.5 Mb (Anderson et al., 1996). A linear order of deletion breakpoints was determined based on the presence or absence of these duplicated markers in nine fast neutron-induced dm3 mutants (Okubara et al., 1994; Anderson et al., 1996). Sequences similar to the NBS of resistance genes were amplified from lettuce by use of degenerate oligonucleotides. One of these sequences mapped to the Dm3 locus and detected a localized family of sequences. These resistance gene candidates (RGCs) from the Dm3 locus (RGC2) encode an LRR region in addition to the NBS (Shen et al., 1998). Nine full-length copies of this gene, ranging in size from 7 to >13 kb, have been sequenced and analyzed in detail (Meyers et al., 1998).

Twenty-two members of the RGC2 gene family and intergenic sequences from the Dm3 region were characterized in this study. The region was dissected using deletion mutants, densely spaced molecular markers, and a collection of lettuce bacterial artificial chromosome (BAC) clones containing the RGC2 gene family. Eighteen RGC2 sequences could be positioned using the deletion breakpoint map. Sequence comparisons and phylogenetic analyses within the RGC2 family demonstrated that these sequences exhibit a high level of sequence diversity. A size estimate for the region suggests that the duplicated sequences comprise an extensive gene family spread over >3.5 Mb. Resistance gene loci may represent one of the largest and most diverse types of gene families in plants.

RESULTS

Localization of Additional Molecular Markers on the Deletion Breakpoint Map

The original deletion breakpoint map was constructed using a panel of nine fast neutron-induced dm3 mutants to locate 12 markers, including markers such as AC15800, that were used in this study (Anderson et al., 1996). None of these markers was missing in all of the deletion mutants, indicating that we had not saturated the region, the markers were duplicated, or the closest breakpoints overlapped within the Dm3 gene and no region was missing in all the mutants. Subsequently, two markers, microsatellite MSAT15-34 and the hybridization marker IPCR800, were identified from the sequence flanking a dm3 T-DNA insertion mutant that indicated a region was missing in all the mutants (Okubara et al., 1997). Both of these markers are duplicated within the Dm3 region; one copy of each marker was missing in all mutants.

To identify additional markers, we saturated Dm-containing regions with RAPD and amplified polymorphism (AFLP) markers. Initially, 336 RAPD primers and 80 AFLP primer pairs were screened against a panel of 12 genotypes. Six templates represented pooled DNA samples for bulked segregant analysis (Michelmore et al., 1991) of each of the major Dm clusters plus DNA samples of six fast neutron-induced Dm mutants. Most of the markers identified with this panel were polymorphic between the bulked samples but were not missing in the deletion mutants; therefore, they were only loosely linked to Dm genes (Figure 1). Consequently, 500 more RAPD primers and 648 AFLP primer pairs were screened against a panel of DNA samples from four fast neutron-induced Dm mutants. The largest deletion mutant, dm3r1608 (Okubara et al., 1997), was used to identify markers missing in the Dm3 region. Markers missing in dm3r1608 were then mapped on the complete panel of nine Dm3 deletion mutants. A total of ~58,000 RAPD and AFLP loci were screened: 9000 RAPD loci, 9000 AFLP loci by using the panel of 12 DNAs, and 40,000 loci by using the panel
of four mutants. This level of saturation should have provided an average spacing of one marker every ~50 kb through the genome. Seven markers from different primer pairs were identified as missing in dm3r1608 and mapped on the complete panel. One AFLP band, B13CG01, was missing in all mutants, and the remaining six markers mapped to the left of Dm3 (Figure 2).

Genomic DNA gel blots using the NBS-encoding region from RGC2B identifies numerous copies of this sequence that were missing in the dm3 deletion mutants (Shen et al., 1998). Most bands were localized in the Dm3 region in a manner consistent with our deletion map (Figure 2). One band, NBS2B band L, gave a pattern apparently inconsistent with the map. However, as previously noted by Anderson et al. (1996), multiple identical copies of a marker would obstruct correct mapping. When markers are duplicated on one side of Dm3, the proximal copy (relative to Dm3) is masked by the presence of the distal copy. When markers are duplicated on both sides of Dm3, a more complicated mapping pattern is observed. NBS2B band L is most parsimoniously explained by the presence of two copies flanking Dm3 (Figure 2). A similar situation exists for AC15800 band C, which was incorrectly mapped previously (Anderson et al., 1996); this hybridization pattern can most easily be explained by the presence of at least two copies positioned as shown in Figure 2.

Figure 1. Genetic Map of the Major Cluster of Resistance Genes in Lettuce.

The genetic map at the left was derived from the analysis of an intraspecific cross using cultivars Calmar × Kordaat (Kesseli et al., 1994). Distances are in centimorgans. RAPD and AFLP loci were identified using bulked segregant analysis and the deletion mutants of cultivar Diana. Polymorphic fragment sizes (in base pairs) are identified using bulked segregant analysis and the deletion mutants are shown in subscript. Cosegregating RAPD and AFLP loci that were missing in multiple deletion mutants are shown in the second and third columns. The precise genetic location of AFLP markers that differentiated resistant and susceptible bulked segregants for both Dm1 and Dm3 but were present in all deletion mutants was not determined (fourth column). Numbers within parentheses next to AFLP markers indicate multiple polymorphic bands identified using this primer pair. The resistance genes at far right have been shown by classic genetics to be linked to this cluster (Farrara et al., 1987; Maisonneuve et al., 1994; R.W. Michelmore, unpublished results). Filled boxes indicate that cosegregating markers are found adjacent to Dm1 and Dm3; open boxes indicate that flanking AFLP markers are nearby but not close enough to be missing in deletions surrounding these genes.

Isolation and Analysis of BAC Clones in the Dm3 Region

Forty-eight BAC clones were identified by use of the Dm3-specific RFLP markers AC15800 or the NBS-encoding region of RGC2B (NBS2B) as hybridization probes. Secondary screens detected both markers in all selected BAC clones; in retrospect, this was not surprising, because both markers were later shown to be from within the RGC2 gene (Figure 4A). Hybridization, sequence data, and the microsatellite MSATE6 indicated that RGC2 genes were present as single copies in 47 of the 48 BACs. The largest BAC, BAC H1 (210 kb), contained two copies of MSATE6, AC15800, and the NBS-encoding region of RGC2 (data not shown); sequence data confirmed the presence of two copies of these markers. The average length of the BACs containing the RGC2 genes was 120 kb. Therefore, the average spacing between RGC2 genes is at least this distance.

The 48 BAC clones were initially assembled into 21 groups according to AC15800 and NBS hybridization patterns. Groups of BACs were named according to the RGC2 sequence contained on these BACs. A unique AC15800 and NBS2B banding pattern defined each group. Sequence analysis and the MSATE6 bands confirmed 20 of the BAC groups, although sequence data divided one group identified by hybridization analysis in two. Two regions of the RGC2 gene were sequenced from each BAC: a 1.4-kb region that contains the NBS and an 800-bp region comprising the AC15800 marker (Figure 4B and Table 1). It was subsequently found that the AC15800 marker spans an intron–exon boundary;
the AC15800 marker only amplified from 17 BAC groups because of variable sequences in the intron. Only one of the two RGC2 copies could be amplified and sequenced from BAC H1 (RGC2Q but not RGC2R). Therefore, the 48 clones contained a total of 23 different copies of the RGC2 gene (Table 2).

The 48 BAC clones represented nearly all of the copies of RGC2 present in the genome. An average of ~2.2 BAC clones were identified for each RGC2 copy; this was consistent with the two to three times genomic coverage calculated for the libraries (Frijters et al., 1997; Z. Zhang and R.W. Michelmore, unpublished data). Hybridizations with AC15800 and the NBS-encoding region of RGC2B identify at least 10 and 18 bands, respectively (Anderson et al., 1996; Shen et al., 1998). Several lines of evidence indicated that one RGC2 copy (RGC2X) had not been cloned: four groups of BACs containing IPCR800 and the associated microsatellite MSAT15-34 were identified rather than the five expected; one copy of each of the markers NBS2B, AC15800, SCINT2, and MSATE6 was identified in genomic DNA but was not present on a BAC clone. All of these missing copies mapped adjacent to Dm3 in the deletion mutants (Figure 2). The colocalization of the markers absent from the BAC clones indicates that there are few, if any, additional RGC2 members that were not cloned. Therefore, there are probably 24 copies of the RGC2 gene in cultivar Diana.

Positioning of BAC Groups in the Dm3 Region by Using the Deletion Mutant Map

Two approaches were used to position the BAC groups. Molecular markers from the Dm3 region were assayed on the BAC clones, and markers derived from RGC2 sequences on the BACs were located on the deletion breakpoint map. The use of multiple markers to localize the BAC groups reduced the likelihood of misplacement due to duplications. Two multipolymerase chain reaction (PCR)-
based markers, MSATE6 and SCINT2, were developed from sequence comparisons between RGC2 genes. Of the 48 clones, only two BACs (containing RGC2T and RGC2V) could not be positioned unequivocally because of insufficient marker information; these BACs did not contain markers that map immediately adjacent to Dm3 and have not been completely characterized. Sixteen of the 22 BAC groups, including BAC H1, which contains two RGC2 genes, were positioned between the breakpoints in the largest deletion mutant (Figure 2). In addition, four groups of BACs contained RGC2 markers that were outside the region missing in our largest deletion mutant; of these, two have been mapped to the left or right of Dm3 by use of recombinants selected from a large F2 population segregating for Dm3 (Figure 2; D.B. Chin, unpublished data).

**Figure 3.** Hybridization of the Region 3′ to RGC2B to the Panel of Deletion Mutants.

Autoradiography showed variable loss of markers in deletion mutants that correspond with specific members of the RGC2B gene family. Genomic DNA from wild-type Diana and the nine deletion mutants was cut with HindIII and probed with 651END, which is an 800-bp fragment 4 kb 3′ to the poly(A) site of RGC2B. The arrowheads to the right indicate mapped loci (Figure 2), designated by letters; the RGC2 family member(s) that corresponds to each band is noted within parentheses with a 2. The three high molecular weight bands were not identified among the BAC clones. Positions of DNA standards are indicated at left in kilobases.

![Figure 3](image)

**Figure 4.** Genomic Structure of RGC2 Homologs Showing Positions of Relevant Markers.

(A) Diagram of a typical RGC2 gene. Markers AM14 and AC15800 (Anderson et al., 1996), IPCR800 and MSAT15-34 (Okubara et al., 1997), and MSATE6, SCINT2, and NBS2B were used to map the deletion breakpoints and BAC groups (Figure 2).

(B) The 1.4-kb region in the 5′ end and AC15800 in the middle of the coding region were sequenced for phylogenetic analysis and to confirm the identity of the sequences contained on the BACs.

(C) Relative positions of the primers used for sequencing and for amplifying markers (Table 1). Half arrows indicate positions and directions of primers.

(D) The gene segments (10) used for phylogenetic analysis to examine the nine RGC2 gene sequences for evidence of recombination and gene conversion.

**Estimation of the Size of the Dm3 Region**

We estimated the degree of overlap between BACs within a group by using HindIII digests and AFLP fingerprints. AFLP fingerprinting using primers with one discriminatory base produced an average of 10 fragments per primer pair per BAC (Meyers, 1998). AFLP fingerprints frequently showed identical bands present in diverse BACs that mapped to nonadjacent locations consistent with a high level of duplication throughout the region. AFLP fingerprints confirmed the BAC groupings generated with other markers; however, they did not allow the unambiguous identification of overlaps between groups because it was impossible to determine the difference between duplications and overlaps.

HindIII digests of BAC clones provided a more comprehensive analysis of each group of BACs. The size of each group of BACs was determined by summing the unique and common fragments within a group (Table 2). Groups determined to be potential neighbors on the basis of their position on
the deletion breakpoint map were examined for overlapping fragments. However, there was not sufficient overlap or resolution to identify unambiguous small overlaps between groups. Hybridization with either whole BACs or end clones did not provide additional resolution or further evidence of sequence duplication throughout the region. Although small overlaps cannot be excluded, large overlaps between BAC groups were not present.

The size of the region containing the duplicated RGC2 sequences was estimated by summing the sizes of the BAC groups. Assuming little or no overlap between 22 BAC groups, the region that we have cloned must be at least 3 Mb (Table 2). Twelve of these BAC groups were >150 kb; however, only one BAC contained two copies of RGC2. This was the largest RGC2-containing BAC (H1; 210 kb), suggesting a minimum spacing of 150 kb between RGC2 copies. The BAC group containing RGC2I and RGC2B (BACs H10 and H15; see below) determined that these copies are between 100 and 255 kb apart. Both lines of evidence indicate that the average distance between RGC2 copies is at least 145 kb. Therefore, the 24 members of the RGC2 gene family span a minimum of 3.5 Mb. This is significantly greater than the previous estimate of 1.5 Mb based on summation of long-range restriction fragments detected by AC15800 (Anderson et al., 1996). The difference may result from the lack of detection of all RGC2 copies in the earlier analysis; approximately half of the fragments detected by AC15800 in our subsequent analysis of BAC clones were not present in conventional genomic DNA gel blots. The estimate of 3.5 Mb is conservative; the region containing the RGC2 multigene family may be considerably larger because of gaps between the BAC groups.

Identification of Dm3 Candidate Sequences

One of the goals of this project was the identification of candidate sequences for the Dm3 resistance gene. Several lines of evidence indicate that RGC2B is Dm3. Markers specific to RGC2B were missing in all deletion mutants. Several of these markers were present in the single BAC (H15) containing RGC2B. The marker SCRGC2B (Table 1) is amplified specifically from the 5'9 sequence of RGC2B and is missing in all deletion mutants. The insertion of a T-DNA element into RGC2B correlated with a loss of Dm3 function (Okubara et al., 1997). Although SCRGC2B, the T-DNA insertion site, and other RGC2B-specific markers were present on BAC H15, sequence analysis indicated that this BAC did not contain the complete gene (Figure 4A; Meyers et al., 1998). Therefore, a genomic λ library was screened, and a 20-kb clone was isolated that contained the complete RGC2B gene as well as ~4 kb of both upstream and downstream sequences. Transgenic complementation is currently under way with this clone.

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### Table 1. Oligonucleotide Primers Used for PCR

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*Positions of primers within RGC2 genes are shown in Figure 4C.
The region missing in all deletion mutants was characterized in detail to determine whether there were additional RGC2 sequences that could be candidates for Dm3. Ends of the BACs in the region immediately adjacent to Dm3 were isolated by using inverse PCR. Overlap was detected between BAC H10 (carrying RGC2I) and BAC H15 (carrying RGC2B) by amplification and DNA gel blot hybridization with these end clones. One end clone from BAC H10 was a single copy in the region, present in BAC H15, and present in deletion mutant dm3r1885 (Figure 2). One BAC H15 end clone was duplicated on several BACs; the corresponding fragment from BAC H10 was sequenced to distinguish between identity and duplication of closely related sequences. The BAC H10 copy was identical to that of BAC H15, whereas copies from other BAC clones were not. Therefore, BAC H10 and BAC H15 represent contiguous sequence between RGC2I and RGC2B. All RGC2I-specific markers were present in deletion mutant dm3r1885 (Figure 2). Therefore, RGC2I is not Dm3. As discussed above, one RGC2 gene that was not present in our BAC library, RGC2X, is adjacent to RGC2B. Therefore, the gap between RGC2B and the right-hand breakpoints in mutants dm3r129 and dm3r1208 was not present on a BAC clone and could not be analyzed. However, markers specific to RGC2X are present in both dm3r129 and dm3r1208 (Figure 2); therefore, RGC2X is unlikely to be Dm3.

Determination of Gene Density in the Dm3 Region

Random and low-copy DNA fragments from the Dm3 region were sequenced to search for other genes duplicated locally in the Dm3 region. A total of 15.7 kb of sequences resulted from the sequencing of end clones from BACs H1, H5, H10, H15, H149, E7, E29, and E33. In addition, low-copy fragments were identified by reverse genomic DNA gel blot hybridizations to libraries made from partial digests of three BACs (H15 [RGC2B], E32 [RGC2H], and H2 [RGC2A]) by using total genomic DNA as a probe. These BACs represented a total of 425 kb of genomic DNA, including ~90 kb from BAC H15 upstream of the Dm3 candidate, RGC2B; BACs H2 and E32 contained diverse RGC2 sequences that mapped to the left of Dm3 (Figure 2). DNA sequences totaling ~50 kb were obtained from 93 sequencing reads of clones containing low-copy DNA from the three BAC subclone libraries. The frequency of reads with similarity to the RGC2 gene on each BAC served as a check for saturation of sampling for low-copy sequences. Twenty-nine low-copy subclones were identical to the sequence in or 5' of the full-length RGC2 genes (sequence described in Meyers et al., 1998). In total, these sequences represented 47% of the complete RGC2 genes and the 5' flanking region. Therefore, any additional duplicated and sizeable genes should have been detectable with this level of sampling.

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<td>E7, E36, E54, H208</td>
<td>90, 60, 150, 50</td>
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<td>AF072283</td>
<td>E23</td>
<td>150</td>
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<td>RGC2Q</td>
<td>AF072284</td>
<td>H1, H4</td>
<td>210, 175</td>
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<tr>
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<td>(Not sequenced)</td>
<td>(H1^p)</td>
<td>(210^p)</td>
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<td>H201</td>
<td>100</td>
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<td>AF072287</td>
<td>H207</td>
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<td>AF072288</td>
<td>E155</td>
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<tr>
<td>RGC2X</td>
<td>NA^c</td>
<td>Not present in BAC library</td>
<td>See text</td>
<td>NA</td>
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^a ND, not determined.
^b Second copy present on BAC H1.
^c NA, not available.
The only significant homologies found were to mobile elements and plant cyclin genes. Retrotransposable elements were identified by four subclones that had significant similarity to the copia-like class of recently isolated from intergenic regions of the maize genome (best BLASTX score = 122; P = 2 × 10^{-30}; SanMiguel et al., 1996). One clone identified transposable element sequences in the databases, with BLASTX scores of 79 to the TNP2 element of snapdragon (Antirrhinum; GenBank accession number X57297) and 69 to the maize transposon En-1 (GenBank accession number S29329). Retrotransposon-like sequences (best BLASTX score = 144; P = 8 × 10^{-46} to GenBank accession number 226407) also were identified in three end clones: one end of each of the BACs H1, H5, and H15. BLAST searches using the remaining end clone sequences found no significant similarity to sequences in the databases. Hybridization using the retroelements probed to the BACs in the RGC2 groups indicated that retroelement copy number varied from low to high copy in the Dm3 region (data not shown). Sequences with similarity to plant cyclins, proteins involved in cell cycle regulation, were identified from subclones of two BAC sequences (best BLASTX score = 68.5; P = 2 × 10^{-21} to GenBank accession number X82036). These clones contained four regions spanning 750 bp that are conserved in cyclins; however, the conserved sequences were interspersed with stop codons, and one clone contained a poly(A) motif, suggesting that this sequence could be an ancient processed pseudogene.

Rapid amplification of cDNA ends (Frohman et al., 1988) analysis of lettuce cDNA by using primers from the cyclin sequences detected no transcripts (data not shown). Hybridization to the 48 RGC2-containing BACs indicated that the cyclin fragment was present in only three copies in the Dm3 region (data not shown). Therefore, the cyclin-related sequences in the Dm3 region are unlikely to be functional. No open reading frames >300 bp were found in the majority of low-copy sequences, and there was no significant similarity to sequences in the databases. Consequently, there was no evidence for additional functional genes on these BACs; however, a single-copy or small gene could have been missed in this analysis.

**Sequence Comparisons of the RGC2 Family**

Segments from the majority of the RGC2 copies were sequenced to determine the evolutionary relationship within the multigene family and to investigate the genetic mechanisms shaping the cluster. Initially, we sequenced two segments: an 800-bp 3' region corresponding to the AC15800 marker and a more 5' 1.4-kb region encoding the NBS and the first six LRRs (Figure 4C). Eighteen unique AC15800 sequences were obtained first and used to group the BACs based on sequence identity (see above). AC15800 did not amplify from the remaining four groups of BACs. The AC15800 marker was later shown to span the intron 3/exon 4 boundary of the RGC2 gene, including ~400 bp of the LRR-encoding region in exon 4 (Figure 4C). Twenty-two sequences composed entirely of open reading frame were obtained for the segment containing the NBS homology. This segment was used for pairwise comparisons and phylogenetic analysis of the RGC2 family.

Pairwise comparisons of the 1.4-kb sequences demonstrated a high level of diversity within the RGC2 gene family. The nucleotide sequence identity ranged from 53 to 97% between the 22 copies (Table 3). The phylogenetic relationships of the RGC2 family members were determined by use of several tree-building methods, all of which gave nearly identical results; neighbor-joining (Figure 5A) and maximum parsimony (Figure 5B) trees are shown. Bootstrap values indicated that the RGC2 family was composed of several well-supported subfamilies, single divergent members, and some less well supported subfamilies (Figure 5B). The subfamily containing RGC2B (the Dm3 candidate), RGC2S, RGC2C, and RGC2D (as well as the missing copy RGC2X) was also supported by other marker data, notably the presence of IPRC800 and MSAT15-34.

There was no correlation between the phylogenetic relationships and physical position of the RGC2 sequences (Figure 5B). Closely related sequences mapped to different, nonadjacent locations on the deletion breakpoint map (Figure 2). The complexity of the relationship between phylogeny and physical position indicated that many rearrangements have occurred during the evolution of this region. Therefore, it is impossible to reconstruct the history of duplications and deletions in the Dm3 region from the analysis of a single haplotype.

Trees generated from different regions of the RGC2 gene were then compared for evidence for chimeric genes that could have resulted from gene conversion or unequal crossing over between family members. Initially, phylogenetic trees were generated using the AC15800 segment from 18 RGC2 copies (data not shown); however, the bootstrap values were low, and therefore, the trees were not sufficiently robust to detect unequal crossing over. Toward the end of the study, we obtained the full-length sequences for nine RGC2 genes (Meyers et al., 1998). The full-length sequences were aligned and divided into ~600-bp segments, taking into account functional domains and the position of introns (Figure 4D). Phylogenetic trees were generated for each segment and compared. Trees generated for segments 5' to the large intron (segments 1 to 6, Figure 4D) were robust trees, with bootstrap values for the majority of nodes >70% and often >90% (Figure 6).

The phylogenetic relationship for most genes was invariant, indicating that there has been no unequal crossing over or significant lengths of gene conversion between these genes. However, the phylogenetic relationships of RGC2S and RGC2B relative to RGC2C and RGC2D or RGC2J changed dramatically between the first and fourth segments (Figures 6A and 6B). Inspection of the sequences for these genes indicated that a crossover had occurred between
The complex multigene family at the Dm3 region is the largest resistance gene locus thus far characterized at the molecular level. Based on the size of our BAC clones, we have estimated that the ~24 members of the family span at least 3.5 Mb, with an average spacing of ~145 kb. Clusters of resistance genes from other species contain fewer members and are spaced much closer together (7 to 70 kb apart). The M locus for rust resistance in flax has ~15 members localized in <1 Mb (Anderson et al., 1997). The rice Xa21 cluster has most of the eight resistance gene homologs in a larger cluster, five members in both the Cf-9 and Cf-4 clusters are found within 1 kb (Parmiske et al., 1997). The I2 locus of tomato is composed of seven homologs spanning ~90 kb (Simons et al., 1998). In soybean, RGC sequences clustered near the Rps2 and Rmd resistance loci are spaced approximately every 20 kb (Kanazin et al., 1996). Nine RPP5-related sequences in Arabidopsis are spread over 70 kb (Bevan et al., 1998). The increased number and spacing of RGCs in the Dm3 region could be a reflection of the larger genome size of lettuce, 2.3 × 10^8 bp (Arumuganathan and Earle, 1991; Michealson et al., 1991; D. Galbraith and R.W. Michelmore, unpublished data) compared with these other species. It remains to be determined whether the organization observed for the Dm3 region is typical for species with moderately sized genomes and whether species with larger genomes have correspondingly larger clusters.

Differences in genome size and intergenic spacing among plants have been attributed to an accumulation of various
types of transposable elements in plant species with larger genomes (Wessler et al., 1995; SanMiguel et al., 1996). Mobile genetic elements, particularly retrotransposons, may comprise a significant portion of most plant genomes. In rice, transposon-like elements both flank and interrupt some members of the Xa21 gene family (Song et al., 1997). The RGC2 family in lettuce is interspersed with sequences similar to a variety of mobile elements, although there was no evidence for these sequences within RGC2 genes (this study; Meyers et al., 1998). Transposons could have played a role in the evolution of the Dm3 region through expansion or translocation of RGC sequences. Mobile elements can cause either localized duplications and deletions of genes (Walker et al., 1995) or more extensive chromosomal rearrangements (Fedoroff, 1989). Variation in RGC2 copy number within the Dm3 region occurs in diverse lettuce germplasm, detected by hybridization with AC15 800 (Anderson et al., 1996). Furthermore, sequences related to triose phosphate isomerase are linked to at least three separate Dm clusters (Paran and Michelmore, 1993), suggesting that these clusters resulted from duplications of an ancient cluster. Determination of the role of transposons in the evolution of resistance gene clusters requires the detailed characterization of multiple haplotypes.

There was no evidence for functional genes in the Dm3 region other than RGC2 homologs and transposon-related sequences. Limited localized duplications may have resulted in the cyclin-related fragments that were observed in several BACs. The Cf-4/9 homologs in tomato are interspersed with fragments of Lox genes, which may have played a role in the duplication of that region (Parniske et al., 1997). The Pto cluster of protein kinase homologs also contains a single NBS-LRR gene, Prf, that is necessary for the function of two members of the Pto cluster (Salmeron et al., 1996). Although we found no evidence for duplicated large genes in the region, a single small gene, such as a protein kinase, could have been missed.

Additional RGC2 Copies Could Encode Other Resistance Genes

The function of the ~24 RGC2 sequences in cultivar Diana has yet to be determined. Mutation analysis indicates that

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(A) Neighbor-joining tree from distance matrices constructed according to Kimura’s two-parameter method by using the DNA sequence of the 1.4-kb region that includes the NBS from each RGC2 gene. The branch lengths are proportional to genetic distance.

(B) Maximum parsimony tree using the same DNA sequences as given in (A). Bootstrap values are indicated as calculated for nodes supported with >70% of 100 replicates. Branch lengths are not scaled. The vertical line to the right represents the chromosome with the mapped positions of the RGC2 copies in the Dm3 region. A bracket next to the RGC2 copies indicates uncertainty in gene order.

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Figure 5. Phylogenetic and Physical Relationships of RGC2 Copies.
only a single gene encodes Dm3 specificity (Okubara et al., 1997). Multiple members of this family are expressed (Meyers et al., 1998) and may represent functional resistance genes. It is difficult to demonstrate the function of these genes in the absence of known pathogens detected by these genes. This is an increasing problem with the many resistance gene homologs of unknown function identified by these genes in the absence of known pathogens detected (Meyers et al., 1998). Random PCR-based amplified regions (SCARs). The duplicated when cloned and used as RFLPs or converted to sequence robust markers. RAPD markers were particularly informative when occurring between segments 1 and 4.

(A) Phylogenetic relationship of RGC2 genes based on the nucleotide sequence of segment 1.
(B) Phylogenetic relationship of RGC2 genes based on the nucleotide sequence of segment 4.

The analysis of the complex RGC2 region required both genetic and physical analyses. Critical components included the deletion mutant map, many molecular markers in the Dm3 region, genomic clones containing the RGC2 multigene family, and sequence analysis of numerous RGC2 copies. Screens using the largest deletion mutant, dm3r1608, enabled us to rapidly identify additional markers in the region. The multiple deletion mutants allowed mapping of the markers and clones within a highly duplicated region in which recombination is repressed (Anderson et al., 1996) and simultaneously allowed us to refine the position of the deletion breakpoints.

Multiple markers were necessary to confirm map positions of the clones. Different types of markers varied in their usefulness for mapping the BAC clones on the deletion mutants because of differences in reliability and duplications in the Dm3 region. Low-copy hybridization (ISH) using BAC H15, which contains RGC2B, are localized to a telomeric position on one chromosome (Shen et al., 1998). At least 10 distinct resistance specificities from a variety of other lettuce genotypes map to the Dm3 locus; however, these are distinct loci, and varying amounts of recombination have been detected between Dm3 and these other genes (Farrara et al., 1987; Kesseli et al., 1994). Even though recombinants were detected between these Dm genes, RGC2 family members could encode some of these genes because the frequency of recombination in the region depends on the degree of similarity between the parents. However, it is unlikely that all are encoded by a RGC2 family member; for example, Dm1 (also present in cultivar Diana) maps 10 centimorgans away from Dm3 (Kesseli et al., 1994; Figure 1). Dissimilar families of RGCs may encode the genetically distinct resistance genes linked to Dm3. Multiple divergent NBS-containing genes are closely linked in soybean (Kanazin et al., 1996). The evolutionary or functional significance of closely linked families of distinct NBS-LRR genes is presently unknown.

Analysis of Resistance Gene Clusters Requires the Integration of Genetic and Physical Approaches

...
nature of the region was actually an advantage; single cloned RAPD fragments frequently provided informative markers for multiple BACs. AFLP markers were not as useful as RAPDs because they were difficult to convert to SCAR or RFLP markers. Sequencing of multiple RGC2 genes allowed markers to be designed that detected individual BACs, specific subgroups, or all BACs.

Saturation of the region with markers as well as the development of RGC2-specific markers reduced the need for a contiguous set of BAC clones across the entire Dm3 region. The physical structure of the region could be ascertained by the positional and phylogenetic analysis of the BAC groups. Marker analysis indicated that most RGC2 copies from the Dm3 region were contained within the BAC clones. Sequence data and a variety of informative markers allowed us to construct robust groups of BACs and to distinguish between identical and duplicated RGC2 sequences. However, fingerprinting the BACs with AFLPs and HindIII fragments was not sufficiently informative to identify unambiguously small overlaps between groups of BACs. To identify genuine overlaps requires the development of informative PCR-based markers for the ends of each group of BACs, as was done for BACs H10 and H15.

A large array of resistance genes provides a variety of opportunities for plants to counter the challenge of variable pathogens. Pathogen populations are under selection to evade detection by a resistance gene and gain access to the host plant. There are two ways that arrays of resistance genes could be advantageous. Clusters could either provide building blocks for rapidly evolving genes with new recognition capabilities or act as reservoirs of unique specificities. These alternatives are not mutually exclusive. A resistance locus containing a large family of related genes could allow both independent evolution as well as periodic shuffling of domains through recombination or gene conversion. Not all genes may be active; function may be restored by infrequent recombination between active and inactive copies. In the human major histocompatibility complex (MHC), the class I genes are present in ~20 copies, only three of which appear to be functional (Trowsdale, 1993). The additional copies appear to be pseudogenes; the sequence variation encoded in these pseudogenes may be recycled through recombination or gene conversion with functional genes. Many RGC2 copies are expressed, although some are pseudogenes (Meyers et al., 1998). Therefore, it is possible that some RGC2 members serve as a reservoir of diversity.

The Large, Duplicated Nature of Resistance Gene Clusters Has Several Evolutionary Consequences

The position and rate of recombination events influence the structure and function of the RGC2 genes. Reciprocal recombination between alleles (or orthologs, in the case of genes introgressed from other species) alters sequences within individual family members, potentially changing the specificity of the gene. This type of recombination is probably important for generating variation in allelic series such as the L locus in flax. The repeated nature of the LRR region in resistance genes provides the possibility of unequal crossing over within alleles. This has been observed in mutants at the M locus in flax (Anderson et al., 1997) and RPP5 in Arabidopsis (Parker et al., 1997); in addition, the size of the LRR region varies between alleles of L (Ellis et al., 1997). Moreover, RGC2 genes also vary in the number of LRR repeats (Meyers et al., 1998). Unequal crossing over within coding regions of paralogs generates chimeric genes; evidence of such events has been found at the Xa21 locus of rice (Song et al., 1997). Furthermore, meiotic instability, alterations in resistance specificity, and the appearance of disease lesion mimics are associated with a high frequency of unequal crossovers at the Rp1 locus in maize (Sudupak et al., 1993; Richter et al., 1995; Hu et al., 1996). Unequal crossover events in regions flanking genes alter the number of genes but do not generate new specificities. Recombination events in the flanking regions have been detected at the Cf-4/s cluster in tomato (Parniske et al., 1997). Hybridization of AC15800 indicated that the copy number of RGC2 genes varies greatly between genotypes (Anderson et al., 1996); however, this does not reveal whether unequal crossing over has occurred within or outside of RGC2 sequences. Phylogenetic analysis of RGC2 genes did indicate that unequal crossing over has infrequently generated chimeric genes during the evolution of this cluster.

A variety of genetic events may be important in the evolution of new resistance specificities in plants. The RGC2 family provides evidence for both recombination and diversifying selection (this study; Meyers et al., 1998). These evolutionary mechanisms are critical components for producing and maintaining sequence diversity in other multigene families involved in nonhost recognition. Infrequent recombination within the mammalian MHC and Ig gene clusters has resulted in gene duplication and occasional loss of function or deletion of duplicated sequences (Hughes and Yeager, 1997; Nei et al., 1997). The lack of congruency between physical position in the RGC2 locus and sequence similarity indicates that there has been a complex series of recombination events resulting in duplications and deletions as well as chimeric genes. Frequent recombination would decrease variation through concerted evolution, resulting in the homogenization of sequences due to gene conversion within a multigene family (Smith, 1973; Dover, 1982). There is no evidence for such a homogenization of sequences in the RGC2 cluster; sequence variability in this multigene family is high. Therefore, rates of unequal crossing over and gene conversion must be below levels required for concerted evolution. Diversifying selection is a major force increasing variation in MHC and Ig genes (Hughes and Nei, 1988; Ota and Nei, 1994; Nei et al., 1997). Diversifying selection also seems to be important in increasing the sequence diversity of the RGC2 family members and other resistance genes (Parniske et al., 1997; Meyers et al., 1998; Wang et al., 1998). The
combined effects of infrequent recombination and diversifying selection on individual genes may permit plants to evolve resistance to rapidly changing pathogens.

METHODS

Identification of RAPD and AFLP Markers

Two sets of lettuce (Lactuca sativa) genomic DNAs were screened to identify markers linked to Dm genes. The initial set of DNAs was composed of resistant and susceptible bulked DNAs for the Dm1/3, Dm4/7, and Dm5/8 clusters as well as six deletion mutants representing Dm1, Dm3, Dm5/8, and Dm7 (mutants were dm1b, dm1d, dm3r1208/dm3r1885 [these two DNAs were pooled to create a minimum deletion around Dm3], dm3r1608, dm5s8a, and dm7a; mutants described in Okubara et al., 1994). These DNAs were screened with 336 randomly amplified polymorphic DNA (RAPD) primers (Operon Technologies Inc., Alameda, CA; Williams et al., 1991) and 80 amplified polymorphism (AFLP) primer pairs with EcoRI and MseI adapters and three selective bases per primer (Keygene, Wageningen, The Netherlands; Vos et al., 1995). To increase the speed of marker screening, we reduced the set of DNAs to a panel of four deletion mutants (dm1d, dm3r1608, dm5s8a, and dm7a). These four DNAs were screened with 500 RAPD primers and 648 combinations of AFLP primer pairs (EcoRI [4 selective bases] and MseI [3 selective bases]).

Isolation of BAC Clones

Genomic bacterial artificial chromosome (BAC) libraries of lettuce had previously been constructed from cultivar Diana by using partial HindIII and EcoRI digests (Frijters et al., 1997; Z. Zhang and R.W. Michelmore, unpublished data). These libraries represent two to three genome equivalents in 76,000 clones with an average insert size of 115 kb. Clones were gridded onto Hybond N+ membranes (Amersham Corp., Arlington Heights, IL) with 1536 BACs per filter. Duplicate filters of both libraries were screened by hybridizing with empty BAC vector and removed. Approximately three BAC equivalents for each digest of each BAC were selected. Clones containing BAC vector sequences were identified by hybridizing with fluorescently labeled BAC DNA digested with HindIII.

Fragment length polymorphism (RFLP) markers AC15800 (Anderson et al., 1996), AM14 (Anderson et al., 1996), K13 (Anderson et al., 1996), RGC2 (Shen et al., 1998), and IPCR800 (Okubara et al., 1997). AFLP fingerprints (Vos et al., 1995) were obtained for individual BAC clones by digestion of BAC DNA and analysis of the resulting fragment sizes. Five micrograms of miniprepped plasmid DNA was digested with HindIII, separated on 1% agarose, and transferred to a Hybond N+ membrane (Amersham Corp.) for DNA gel blot analysis. Fragments observed on the agarose gel were compared between BACs to identify duplicated bands as potentially overlapping fragments. In several cases, overlap was checked by DNA gel blot hybridization using radioactively labeled BAC DNA digested with HindIII.

Low-Copy-Number Subclones

Six libraries were constructed by partially digesting BACs H15, H2, and E32 with either T5ps091 or Sau3Al (New England Biolabs, Beverly, MA). Enzyme digests were done according to standard techniques (Sambrook et al., 1989), with limiting enzyme concentrations used to obtain partial digests. Fragments > 3 kb were gel purified and cloned into either EcoRI- or BamHI-digested pUC119 (for T5ps091 or Sau3Al libraries, respectively). The majority of resulting transformants contained inserts of 1.5 to 2 kb. Transformants representing approximately three BAC equivalents for each digest of each BAC were screened by reverse genomic DNA gel blot hybridizations. Lettuce genomic DNA was labeled by the random hexamer method (Amersham Corp.) and used to probe the clones arrayed on membranes. Hybridization intensity was determined using a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Colonies with hybridization intensities comparable to control single-copy clones were selected. Clones containing BAC vector sequences were identified by hybridizing with empty BAC vector and removed. Approximately 15 low-copy clones from each library (30 from each BAC) with inserts > 0.5 kb were sequenced and analyzed by BLAST searches (Altschul et al., 1997).

PCR-Based BAC End Clone Rescue

Inverse PCR primers were designed with pairs of primers adjacent to either side of the BAC cloning site (Table 1). For BACs with inserts at the HindIII site (designated BAC H), inverse PCR templates were constructed from miniprepped BAC DNA and separately digested in 20-μL reactions with each of the four-base cutters MseI, HaeIII, and T5ps091. Digestd DNA was then diluted in 100 μL of T4 ligase buffer with 40 units of T4 ligase (New England Biolabs) and ligated overnight. Two microliters of this template was amplified using either the primer pairs ENDCln1/ENDCln2 or ENDCln3/ENDCln4 (Table 1).
to amplify each end of the BAC. In a similar manner, BACs with lettuce genomic inserts at the EcoRI cloning site (designated BAC E) were analyzed by use of inverse PCR templates constructed as above, using MseI, HaeIII, and Sau3AI digests and primer pairs ENDC11/ENDC14 or T7 (Promega, Madison, WI)/ENDC1N5 (Table 1); to amplify the left or right ends, respectively. PCR products were sequenced and analyzed by BLAST searches (Altschul et al., 1997).

**PCR-Based Sequencing and Analysis**

All PCRs were performed in 30 cycles with a 58 to 60°C annealing temperature. Half of the PCR product was checked on an agarose gel, and the other half was treated with exonuclease I and shrimp alkaline phosphatase (Amersham Corp.), according to the supplier’s instructions. When possible, PCR products were then sequenced directly (see below). PCRs resulting in multiple bands or high backgrounds were cloned using the pGEMT vector system (Promega), according to the manufacturer’s instructions; multiple clones were sequenced to eliminate PCR artifacts.

Sequencing reactions were performed with a dye terminator cycle sequencing kit (Applied Biosystems Inc.) and either the original PCR primers or standard Sp6, T7, M13 (-21), or M13 reverse primers (Promega). Reactions were then resolved on an ABI 377 automated sequencer (Applied Biosystems Inc.). Sequence data were evaluated using Sequencher (Gene Codes, Ann Arbor, MI) for sequence editing and contig assembly. The DNAStar (Lasergene, Madison, WI) and GenBank accession numbers for sequences used in the phylogenetic analysis are given in Table 2.

**ACKNOWLEDGMENTS**

We gratefully acknowledge the technical assistance of Pejman Rohani and Michelle Lai. B.C.M. was supported in part by a National Science Foundation graduate research fellowship. This project was supported by the U.S. Department of Agriculture National Research Initiative Competitive Grant Program (Grant No. 95-37300-1571).

Received June 12, 1998; accepted September 14, 1998.

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The Major Resistance Gene Cluster in Lettuce Is Highly Duplicated and Spans Several Megabases
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*Plant Cell* 1998;10;1817-1832
DOI 10.1105/tpc.10.11.1817

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