Genetic and Molecular Characterization of the Maize rp3 Rust Resistance Locus

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ABSTRACT

In maize, the Rp3 gene confers resistance to common rust caused by Puccinia sorghi. Flanking marker analysis of rust-susceptible rp3 variants suggested that most of them arose via unequal crossing over, indicating that rp3 is a complex locus like rp1. The PIC13 probe identifies a nucleotide binding site-leucine-rich repeat (NBS-LRR) gene family that maps to the complex. Rp3 variants show losses of PIC13 family members relative to the resistant parents when probed with PIC13, indicating that the Rp3 gene is a member of this family. Gel blots and sequence analysis suggest that at least 9 family members are at the locus in most Rp3-carrying lines and that at least 5 of these are transcribed in the Rp3-A haplotype. The coding regions of 14 family members, isolated from three different Rp3-carrying haplotypes, had DNA sequence identities from 93 to 99%. Partial sequencing of clones of a BAC contig spanning the rp3 locus in the maize inbred line B73 identified five different PIC13 paralogues in a region of ~140 kb.

PLANT genomes carry large arrays of genes for the detection of pathogen attack and the induction of appropriate defense responses (Meyers et al. 1999; Pan et al. 2000). Resistance (R) genes recognize the products or function of specific pathogen-encoded avirulence genes (Scofield et al. 1996; Tang et al. 1996; Jia et al. 2000). These R genes are often members of families of tightly linked genes (Hulbert et al. 2001). Some of these gene clusters appear to have been generated by ancient duplication events, since the members show limited homology in their coding regions. Members of other families show high levels of homology indicating a more recent origin. Meiotic mispairing and recombination occurs between the members of some resistance gene families leading to the reassortment of functional domains and presumably generating variation important in the evolution of new resistance gene specificities (Ellis et al. 2000; Sun et al. 2001).

The rp1 complex is the best-characterized resistance gene family from maize. The genes in the rp1 complex belong to the most common class of resistance genes: those that code for nucleotide binding site-leucine-rich repeat (NBS-LRR) proteins (Collins et al. 1999). Unequal recombination events are frequent in rp1 homozygotes and heterozygotes. These generate new combinations of family members (haplotypes; Collins et al. 1999) and generate novel genes by intragenic recombination (Sun et al. 2001). The rp1 haplotypes of different maize lines vary considerably in the number of rp1 genes they carry. Most carry between 5 and 20 rp1 genes, but haplotypes with only a single rp1 gene have been observed (Sun et al. 2001; T. Pryor, unpublished observations). Sequence analysis of the rp1 genes has indicated that both mutation and intragenic recombination between paralogues contribute to the evolution of the resistance gene family (Collins et al. 1999; Sun et al. 2001).

Here we report the characterization of a second rust resistance locus from maize, rp3. Like rp1, rp3 controls race-specific resistance to Puccinia sorghi Schwein., the fungus causing maize common rust. While rp1 maps near the terminus of maize chromosome 10 (Rhoades 1935; Jiang et al. 1996), the rp3 locus resides near the centromere on chromosome 3 (Saxena and Hooker 1974; Sanz-Alferez et al. 1995). As with rp1, rp3 alleles or closely linked genes conferring resistance were identified in several different maize accessions in surveys conducted by Hooker and co-workers in the 1960s (Hooker and Russell 1962; Hagan and Hooker 1965; Wilkinson and Hooker 1968). We previously isolated a resistance gene analogue designated PIC13 from maize and found it to be tightly linked to the rp3 locus (Collins et al. 1998). In the current analysis, further evidence is presented that PIC13 is homologous to a gene family that includes the gene(s) coding for the Rp3 specificity. Molecular characterization of this gene family and its behavior in meiosis indicates it is a complex locus with many similarities to the rp1 complex.

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AF489541–AF489554.

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**MATERIALS AND METHODS**

**Nucleic acid isolation, purification, and gel blot analysis:** Genomic DNA was isolated from young leaf tissue and gel blot analysis was performed essentially as previously described (Hulbert and Bennetzen 1991). In all experiments using RNA, total RNA was isolated from ground, frozen tissue using TRIZOL reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s protocol. Wizard PCR purification columns (Promega, Madison, WI) were used to purify all PCR products before cloning or sequencing. Protocols for making and screening gel blots were taken from Sambrook et al. (1989). All probes were [α-32P]dCTP labeled by random priming (Feinberg and Vogelstein 1983).

**Genetic materials:** Rp3 near-isogenic lines (NILs) in the B14, H95, and R168 genetic backgrounds were used as the source for Rp3 resistance in genetic experiments. Rp3-A and Rp3-B lines that were homozygous for the rp3 locus but heterozygous for flanking restriction fragment length polymorphism (RFLP) markers were constructed to test the stability of Rp3 homozygotes. Hooker and co-workers had repeatedly crossed the six Rp3-carrying haplotypes (Rp3-A–Rp3-F) into the R168 and B14 genetic backgrounds. Examination of these lines with rp3-linked RFLP markers indicated that the introgressed region in the pairs of Rp3-A and Rp3-B lines were sufficiently small to carry recurrent parent alleles at loci within 5 cM of the rp3 locus. Crossing the lines with the same Rp3 haplotype in the two different backgrounds created the Rp3 homozygous test lines with heterozygous flanking markers. Thus, the Rp3-A line was made by crossing an Rp3-A-R168 NIL to an Rp3-A-B14 NIL. The F1 was test crossed to a susceptible inbred line and progeny because it was found to share 100% identity with test lines with heterozygous flanking markers. Thus, the Rp3-A was placed in the B14, H95, and R168 genetic backgrounds were used as the source for Rp3 resistance in genetic experiments.

**Genetic library:** Two maize genomic libraries were constructed using DNA from seedling leaves of the Rp3-A haplotype, and a third was constructed from the variant Rp3-A/D4. After partial digestion with Sau3AI, DNA fragments were size fractionated by 25 hr of ultracentrifugation through a 10–40% sucrose step gradient. Only those fragments ≥9.0 kb in size were dialyzed, precipitated, and ligated into λ-vectors. BamHI-digested ZAP Express and BamHI-digested λ-DashII arms (Stratagene, La Jolla, CA) were used to construct the two Rp3-A libraries, and Rp3-AD4 genomic fragments were ligated into BamHI-digested λ-DashII arms.

The use of two probes, one from the NBS and one from the LRR domain of a cloned and sequenced PIC13 family member, allowed identification of λ-clones carrying full-length, intact genes from the Rp3-AD4 library. Plaques showing positive hybridization to both NBS and LRR regions were purified away from nonhybridizing plaques by dilutions. High-titer phage stocks were stored in 7.0% dimethyl sulfoxide (DMSO) at −80°. A pair of PCR primers (F1, AACGAAAGGCAAGGGCCACTTCTAGGAAAC; GSF1, GSF2, AATCAGGGGCAACGTGTTTCGCTGTTAAGG; R3, GSF3, GSF4, GSF5, GSF6, GSF7, TGCCTGCTACGTGTTAAGG; R3, GSF3, LRR1, CGATGTCAGCTGTCCAGTGTAATG). Note that the LRR1 primer is located within the coding region (~600 bp 5′ of the predicted translation termination codon; therefore this pair is not predicted to amplify a complete coding region sequence.)

The following forward primers were designed to be specific for the 5′ flanking region of the Rp3-AD4 gene: GSF3, GSF4, TAGAAACAAGGACAAAATAACAGC; GSF5, CGCTCCGA AAAGGCATCAAGC; GSF6, ATTAGGTAAAGGATGAGTGCTC TC; GSF7, TGACTGAAGGCCACAGAC; and GSF8, GCCCAA ACTAACCAACCATTGAAG. Reverse gene-specific primers were designed from the 3′ flanking region of the Rp3-AD4 gene: GSR1, GSR2, ACACGAGCTGATGAGTGCTCAGTGTAATG. Basic amplification conditions were: 95° for 2 min, 10 cycles of 95° for 30 sec, 54° for 30 sec, 72° for 5 min 30 sec (or 1 min of extension/1.0 kb length of predicted size of product), followed by 20 cycles of 95° for 30 sec, 54° for 30 sec, 72° for 5 min 30 sec + 10 sec per cycle, and a final extension of 10 min at 72°. For any particular amplification experiment, the thermocycler was reprogrammed for an annealing temperature that was within ±5° of each primer’s Tm value. PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen,

**Rust inoculation and screening:** Growth in a 3:1 soil:peat moss mix in 38 × 61 × 8-cm flats, greenhouse-reared 8-day-old maize seedlings were inoculated with fresh *P. sorghi* isolate (Sigma, St. Louis). Infection was initiated by overnight incubation (~16 hr) inside a mist tent in the greenhouse. Plants were screened at 7–8 days postinoculation. Rust resistance was scored on a scale of 0 to 4, with a 0 score assigned to completely resistant plants showing no sporulation. A rating of 1 indicated a high level of resistance with only one or a few pustules per leaf. Plants with a 2 rating had larger numbers of pustules per leaf, but maintained clear necrotic hypersensitive reactions, with most of the fungal pustule. Ratings of 3 were given to plants with larger numbers of pustules per leaf but mounting only a weak, visible resistance response such as chlorotic zones around some of the pustules. Plants that were completely susceptible and displayed no noticeable necrosis were given a 4 rating.

**Flanking marker analysis:** Rp3 linkage to maps distal to the gene was selected. The clone was chosen for further subcloning and sequencing efforts because it was found to share 100% identity with the DNA sequence collected from the unique Rp3-AD4 HpaI fragment. The clone was Sau3AI partially digested and ligated into pUC19, and a subclone containing the full coding region of the gene was selected.

**Long-range PCR and DNA sequencing of family members:** To amplify full-length coding regions from PIC13 family members, Herculease-enhanced DNA polymerase (Stratagene) was used in long-range PCR amplification experiments using genomic DNA as the template. The following PCR primer pairs were used: (1) F1, GSR1, CGACTCTTCGACCCACTTTAGGAAAC; GSF1, GSF2, AATCAGGGGCAACGTGTTTCGCTGTTAAGG; R3, GSF3, GSF4, GSF5, GSF6, GSF7, TGACTGAAGGCCACAGAC; and GSF8, GCCCAA ACTAACCAACCATTGAAG. Reverse gene-specific primers were designed from the 3′ flanking region of the Rp3-AD4 gene: GSR1, GSR2, ACACGAGCTGATGAGTGCTCAGTGTAATG. Basic amplification conditions were: 95° for 2 min, 10 cycles of 95° for 30 sec, 54° for 30 sec, 72° for 5 min 30 sec (or 1 min of extension/1.0 kb length of predicted size of product), followed by 20 cycles of 95° for 30 sec, 54° for 30 sec, 72° for 5 min 30 sec + 10 sec per cycle, and a final extension of 10 min at 72°. For any particular amplification experiment, the thermocycler was reprogrammed for an annealing temperature that was within ±5° of each primer’s Tm value. PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen,
Carlsbad, CA) essentially by the manufacturer’s suggested protocol. In all following experiments in which PCR products or cDNAs were cloned, PCR2.1-TOPо was the vector used unless it is stated otherwise. All PCR primers were synthesized at Integrated DNA Technologies (Coralville, IA).

All DNA sequencing was done at the DNA Sequencing and Genotyping Facility, Department of Plant Pathology, Kansas State University. Alignments were made with the aid of ClustalW 1.8 at the Baylor College of Medicine search launcher site (http://searchlauncher.bcm.tmc.edu:9331/), and/or SeqWeb, version 2 (Wisconsin Sequence Analysis Package, Genetics Computer Group). The web server (http://www.ncbi.nlm.nih.gov/blast/index.html) was used to predict possible coiled-coil protein structure in these genes (LUPAS 1997). Database searches were conducted using the BLASTX algorithm (http://www.ncbi.nlm.nih.gov/blast/index.html).

5’ and 3’ rapid amplification of DNA ends: Analysis of 5’ and 3’ transcript ends derived from an Rp3-A-carrying maize line was performed by rapid amplification of cDNA ends (RACE). These protocols used total RNA isolated from expanded Rp3-A seedling leaves. The 5’ RACE system, version 2.0 (Life Technologies), was used according to manufacturer’s recommendations. Following the tailing reaction step, a nested PCR approach was used, with two rounds of PCR, each using a different reverse PCR primer under stringent annealing parameters (60°C). Both reverse primers were designed from conserved NBS regions of PIC13 family members. For nested PCR, we first used the 5’ Abridged Anchor Primer supplied combinations in PCR reactions, the resulting products were fractionated in 0.7% agarose gels. BAC clones were initiated from conserved sequence in the LRR region of PIC13 and fractionated in 0.7% agarose gels. BAC clones were initiated from conserved sequence in the LRR region of PIC13 differentiated using this original collection of rust iso-

These plasmids were digested to completion with HindIII and fractionated in 0.7% agarose gels. BAC clones were initially grouped by determining which clones shared the most identified HindIII fragments. The clones were then progressively and continually reordered on subsequent agarose gels so that similar clones were adjacent for ease of comparison. Southern blots were probed with sequences from NBS and LRR regions of a PIC13 gene, entire HindIII-digested BAC clones, or specific HindIII fragments from particular BAC clones.

Using PCR primer pairs (F1, NBSR1 and LRRF1, LRRR1), we amplified the NBS and LRR regions, respectively, from selected BAC templates. Amplification products were sequenced and compared as an aid in constructing a gene order across the contig. Cycling parameters and PCR product handling was done as described above in Genomic library. PCR primers (B73NBSF, CCTGCTACTCATGCTAATTTC and B73NBSR, CAAATACGTGAGCTAAGAGC) were designed so as to flank two insertions/deletions in the NBS region of the genes carried on the BAC clones. Size polymorphism in the products allowed differentiation of the genes carrying these insertions/deletions. Two forward PCR primers (B73F1, CCCATCGTGAAATTGATACCAAGGC) and two reverse primers (B73R1, TGTAAGGTGCATACTG and B73R2, TAAAGGCTGTCGACTTAG) were designed from conserved areas within the LRR region of the genes. After these primers were used in various pair combinations in PCR reactions, the resulting products were sequenced to differentiate the five genes carried on the BACs.

RESULTS

The Rp3-mediated resistance specificity: Six Rp3 alleles have been designated Rp3-A–Rp3-F. These alleles were originally identified from six different maize accessions on the basis of their resistance reaction to eight P. sorghi biotypes (WILKINSON and HOOKER 1968). Hooker and co-workers subsequently crossed resistance genes from each of these six sources into the maize inbred line R168 to create near-isogenic lines. It is not clear if the six Rp3 NILs in the R168 background could be differentiated using this original collection of rust isolates, and these isolates are no longer available. With the exception of the Rp3-C NIL, none of the other five lines carrying presumptive Rp3 alleles could be distinguished in field or greenhouse rust infection assays (PATALK 1987; GROTH et al. 1992) or by infection with a further 16 rust biotypes collected between 1975 and 1994 (HULBERT et al. 1991; our unpublished data). The Rp3-C NIL has been shown to carry an Rp1 gene (either Rp1-A or Rp1-F), which probably accounts for the observed differentiation of this NIL from other Rp3 NILs (SANZ-ALFEREZ et al. 1995). With the exception of several rust biotypes that could recognize the Rp1-A or F alleles in the Rp3-C NIL, a range of rusts with varying virulence gives identical reaction phenotypes on all six Rp3 NILs (Figure 1), suggesting, on the basis of the criterion of rust infection type, that all six Rp3 NILs carry the same resistance gene specificity. Previous studies (COLLINS et al. 1998) have shown that a resistance gene analogue, PIC13, cosegregated with the Rp3 resistance
gene. In this study we confirm this observation and demonstrate that DNA from five of six Rp3 NILs has identical Southern patterns when digested with 18 different cytosine methylation-insensitive restriction endonucleases and probed with PIC13. The exception, Rp3-D, consistently had one extra hybridizing restriction fragment in most of the different enzyme digestions (Figure 2). Examination of restriction fragments from the other five Rp3 lines, in test cross F2 and backcross progeny, revealed that almost all the PIC13-hybridizing fragments from the Rp3 parental line cosegregated with rust resistance while the PIC13-homologous fragments from the susceptible parent segregated with susceptibility. With some enzymes, an occasional PIC13-homologous fragment was observed not to map to Rp3, but this rare observation was not investigated further. A similar analysis using 17 cytosine methylation-sensitive enzymes could distinguish all Rp3 NILs, except for Rp3-A and Rp3-C, which were identical with all enzymes. However, the different methylation patterns do not necessarily indicate a different DNA sequence.

Thus two lines of evidence, the Rp3 resistance specificity and PIC13 hybridization pattern at the rp3 locus, suggest that five of the six presumptive allelic variants are identical. The exception is the Rp3-D NIL that clearly carries at least one extra PIC13-homologous restriction fragment. Flanking chromosomal regions are polymorphic between each Rp3 NIL, a character that has been exploited in examining the nature of recombination events between Rp3 variants.

The rp3 locus is meiotically unstable: The genetic transmission of resistance was analyzed in large families to examine the meiotic stability and structure of the rp3 locus and to assess the feasibility of a transposon-tagging approach to clone the Rp3 gene (Table 1). Most testcross populations, made by crossing heterozygotes of different Rp3 lines to susceptible rp3/rp3 lines, produced rare susceptible variants when inoculated with P. sorghi rust biotype KS1. The susceptible variants were associated with crossovers in the rp3 region as determined by analysis of the closely flanking RFLP markers umc18 and umc10. The largest number of recombinants was obtained from a testcross of an Rp3-A/Rp3-D heterozygote, where five susceptible recombinants were identified from 8994 progeny. All five had the Rp3-A parent allele at umc18 and the Rp3-D parent allele at umc10, indicating that the recombination events all occurred to the umc18 side of the Rp3-A gene and to the umc10 side of the Rp3-D gene. This result could be expected if Rp3-A and Rp3-D were not alleles and mapped 0.06 cM apart, with Rp3-A mapping closer than Rp3-D to the distal umc10 locus.

Alternatively, if rp3 is a complex locus like rp1, then the recombination between Rp3-A and Rp3-D could be due to mispairing and unequal crossing over. To test this, it should be possible to identify crossover-derived susceptible variants from homozygotes. Hybrids homozygous for Rp3-A or Rp3-B but heterozygous for flanking RFLP markers were constructed (see Materials and Methods) and crossed to a susceptible (rp3/rp3) line. One susceptible plant was identified in 4236 progeny from the cross of the Rp3-A homozygote. This susceptible variant had a nonparental combination of flowering
TABLE 1
Susceptible variants derived from crosses with Rp3 lines

<table>
<thead>
<tr>
<th>Type of cross</th>
<th>Parents</th>
<th>Segregation*</th>
<th>Flanking marker analyses†</th>
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<tr>
<td></td>
<td></td>
<td>R: Int:S</td>
<td>Variant</td>
</tr>
<tr>
<td></td>
<td>Rp3-B-R168/ Rp3-B-B14* × rp3/rp3</td>
<td>22,775:0:0</td>
<td>Rp3-AB1</td>
</tr>
<tr>
<td>Rp3 heterozygotes × rp3/rp3</td>
<td>Rp3-A/Rp3-B × rp3/rp3</td>
<td>5,176:0:3</td>
<td>Rp3-AB2</td>
</tr>
<tr>
<td></td>
<td>Rp3-A/Rp3-B × rp3/rp3</td>
<td>5,176:0:3</td>
<td>Rp3-AB3</td>
</tr>
<tr>
<td></td>
<td>Rp3-B/A × rp3/rp3</td>
<td>2,697:0:2</td>
<td>Rp3-AC1</td>
</tr>
<tr>
<td></td>
<td>Rp3-A/Rp3-C × rp3/rp3</td>
<td>2,697:0:2</td>
<td>Rp3-AC2</td>
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<td></td>
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<td>Rp3-AD2</td>
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<td>Rp3-AD3</td>
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<td>Rp3-AD4</td>
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<td>Rp3-AD5</td>
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<td></td>
<td>Rp3-AD6</td>
</tr>
<tr>
<td>B. Mutator background</td>
<td>Rp3-B/Rp3-B-Mu × rp3/rp3</td>
<td>37,524:0:4</td>
<td>Rp3-AD7</td>
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<td></td>
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<td>Rp3-AD8</td>
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<td>Rp3-AD9</td>
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<td>Rp3-AD10</td>
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</tbody>
</table>

* Populations were screened with a P. sorghi isolate that was avirulent on the Rp3 parental lines. R, resistance; Int, intermediate; and S, susceptible rust reaction. Flanking marker analyses were conducted on susceptible variants where possible.

† The centromere proximal marker used was umc18 and the distal marker was umc10. The distal marker could not differentiate between Rp3-A, -C, and -E. ND, not done.

Marker alleles (Table 1), indicating it arose by an unequal crossover event. In a similar cross with an Rp3-B homozygote, no susceptible progeny were identified among 22,775 progeny. A second Rp3-B population was made by crossing an Rp3-B homozygote in a background carrying active Mutator (Mu) transposable elements to a rust-susceptible (rp3/rp3) line. Four susceptible individuals were identified from 37,528 progeny of this cross (Table 1B). No Mu elements were observed to cosegregate with the rp3 locus in the progeny of any of these four variants, indicating that they were probably not caused by transposon insertion. Flanking markers could not be assayed in this second Rp3-B population, but results from hybridization with a PIC13 probe (below) were consistent with an origin by recombination for the susceptible variants.

Resistance specificities and phenotypes of Rp3 recombinants: A total of 17 individuals were selected from the crosses of Rp3 homozygotes and heterozygotes (Table 1) due to their complete loss of resistance to rust biotype KS1. Seed was obtained from all 17 individuals, either by self-fertilization or by outcrossing to rp3/rp3 plants when self-fertilization was not possible. Inoculations of ~12 progeny from each variant with isolate KS1 found all progeny to be susceptible (reaction type 4), verifying that resistance to this isolate had been lost. To determine if any altered specificities had been created (Richter et al. 1995), the progeny were inoculated with rust isolates AF1, HI1, KS1, IN1, IN2, and IN3 (Hulbert et al. 1991). Progeny from the four variants derived in the Mutator transposable element background were examined only with isolates KS1 and IN1. No resistance was observed among the progeny from any of these variants to any of the rust biotypes.

The variant Rp3-AD4, isolated from the Rp3-A/Rp3-D testcross population, displayed a unique intermediate resistance reaction phenotype. It is the only Rp3 variant with a phenotype. When inoculated with rust isolates that are avirulent on Rp3 (isolates AF1, IN1, IN3, and KS1), the Rp3-AD4 line typically showed reaction type 2 or 3, with reduced numbers of uredinia surrounded by oblong necrotic rings (Figure 3). Rp3-AD4 had the same specificity as its parental alleles, except when challenged with biotype IN2. Rp3-AD4 appeared completely susceptible (reaction type 4) to IN2 while its parents, Rp3-A and Rp3-D, were intermediate.

Genetic analysis of recombinants indicates the PIC13 family includes the Rp3 gene: Crossing over in the rp3 area could be assayed only in crosses where the resistant parent was heterozygous at RFLP markers flanking the locus (Table 1). This included the 1 rust-susceptible variant recovered from a testcross of an Rp3-A homozygote, 3 variants recovered from a testcross of an Rp3-A/Rp3-B heterozygote, 6 from a testcross of an Rp3-A/Rp3-D heterozygote, and 1 from a testcross of an Rp3-A/Rp3-F heterozygote. Of these 11, 9 had recombinant flanking markers, indicating that they probably arose by crossover events in the Rp3 complex. The single susceptible variant from the Rp3-A homozygote, having a nonparental combina-
both flanking markers from its Rp3-B parent, while variant Rp3-AB3 displayed both flanking markers from its Rp3-A parent.

Homozygotes derived from all of the susceptible variants were examined with the PIC13 probe in five different restriction enzyme digests, BamHI, BglII, HpaII, NsiI, and SacI (Figure 4). Comparisons of the PIC13-hybridizing fragments of the progeny with those of the parents were consistent with the hypothesis that they were generated from recombination events within the PIC13 family. Nearly all of the susceptible progeny were missing one or more PIC13-hybridizing fragments that were present in both parents (Figure 4A). The rust-susceptible variant from the Rp3-A homozygote and the four variants from Rp3-B homozygotes were also missing parental restriction fragments. This would be expected if they were derived by unequal crossovers between family members flanking or including the Rp3 genes. In this regard, the four variants from the Rp3-B homozygotes in the Mutator background were similar to the other crossover-derived variants and are therefore likely to be crossover variants and not insertion mutants. The one exceptional variant, showing no missing parental fragments, was one of the two NCO variants (Rp3-AB3) from an Rp3-A × Rp3-B heterozygote. This appeared identical.

Figure 3.—One recombinant haplotype, Rp3-AD4, exhibits an intermediate rust resistance reaction compared to its rust-resistant parents Rp3-A and Rp3-D. The photograph was taken ~7 days after inoculation with the rust biotype IN1.

Figure 4.—Crossover-generated deletions and novel restriction fragments in 18 spontaneous rust-susceptible Rp3 variants. (A) A gel blot demonstrates absence of certain NsiI fragments from the majority of the variants (indicated by arrows). DNAs of homozygous variants were restricted with NsiI and hybridized with an NBS region probe. Lanes 1–6 are DNAs of the Rp3-A, -B, -C, -D, -E, and -F resistant parents, respectively. The remaining lanes are DNAs from susceptible variants derived from crosses with an Rp3-B homozygote from a Mutator background (lanes 7–10), an Rp3-A/Rp3-B heterozygote (lanes 11–13), an Rp3-A/Rp3-C heterozygote (lanes 14 and 15), an Rp3-A/Rp3-D heterozygote (lanes 16–21), an Rp3-A/Rp3-E heterozygote (lane 22), and an Rp3-A/Rp3-F heterozygote (lane 23). (B) Shown is a novel 9-kb SacI fragment that occurred in most of the variants when hybridized with the same NBS probe used in A (arrow). Lanes 1–4 contain rust-susceptible variants from crosses with Rp3-A/ Rp3-D heterozygotes, lanes 5–7 carry DNAs of susceptible variants from Rp3-A/Rp3-B heterozygotes, and lanes 8 and 9 have variants from Rp3-A/Rp3-C heterozygotes. One susceptible variant derived from an Rp3-A/Rp3-E heterozygote (lane 10) is followed by three variants derived from testcrosses with Rp3-B homozygotes from a Mutator background (lanes 11–13). The remaining lanes (14–18) carry DNA of resistant Rp3-A, -B, -C, -D, and -E parental haplotypes. Size markers, in kilobases, are shown on the left side of each gel blot.
to the Rp3-B parent in all enzyme digests, indicating it was probably derived from a mutation or possibly a conversion event that did not noticeably change the restriction fragments of the parental haplotype. The other NCO variant from this cross appeared more similar to the crossover-derived variants in that it was missing parental restriction fragments in most restriction enzyme digests. It is possible this variant was derived from a crossover event, but had an additional crossover between the locus and one of the flanking markers.

In addition to missing restriction fragments, all variants except one (Rp3-AB3, one of the two NCO variants) displayed a novel-sized PIC13-hybridizing fragment with at least one restriction endonuclease. The presence of novel PIC13-hybridizing restriction fragments indicates that crossovers generating the novel PIC13 haplotypes were occurring in or very near the PIC13 gene family members. Most of the variant progeny lines showed a novel 9.0-kb SacI fragment and were missing an \( \sim 12.0 \)-kb fragment present in the parents (Figure 4B). The only progeny lines that did not show this novel SacI fragment were the NCO-type variant Rp3-AB3 and three of the four variants from the Rp3-B homozygotes. All four variants from the Rp3-B homozygotes had novel bands in EcoRI, NsdI, and XbaI digests. The variant Rp3-AD4 also appeared to be a consequence of a recombination within the PIC13-homologous gene family; there was an exchange of flanking markers (Table 1A) and a novel-sized PIC13 HpaII restriction fragment of 3.5 kb that cosegregates with the Rp3-AD4 intermediate resistance phenotype (Figure 5). Smaller-sized (<1.5 kb) hybridizing HpaII fragments were observed in the Rp3-AD4 variant relative to the resistant Rp3-A and Rp3-D haplotypes.

Isolation and characterization of PIC13 family members: Using the PIC13 probe, nine genomic clones were isolated from an Rp3-A λ-library. Subclones from these nine positive clones were sequenced. None of them carried a complete open reading frame (ORF), but two of them overlapped to give a single 3.3-kb ORF, which was predicted to encode a complete NBS-LRR protein, suggesting that there was only one coding exon. Alignment of these sequences permitted the design of PCR primers from conserved regions near the predicted ends of the genes. Primers from conserved regions within the coding region were used in RACE experiments to determine the 5′ and 3′ ends of the mRNA and to identify any introns. Examination of these sequences and a nearly full-length cDNA clone (~500 bp short of the 3′ end of the coding region) that was isolated from an Rp3-A haplotype gene confirmed that the coding region is free of introns. One small intron of 238 bp was identified in the 5′-untranslated region (UTR) ending 39 bp upstream of the predicted translation start codon. A second intron of 414 bp was detected in the 3′-UTR at 22 bases downstream of the predicted translation stop. A similar intron arrangement was seen in the Rp1-D gene (Sun et al. 2001). The ORF was predicted to encode an NBS-LRR protein. COILS analysis (Lupas 1997) predicted a high probability (\( P \approx 0.9 \)) that the gene coded for an amino-terminal coiled-coil domain, thus placing it in the CC-NBS-LRR class of resistance genes. The NBS domain displayed amino acid motifs conserved among known resistance proteins as described by Collins et al. (1998). When compared with the cytoplasmic LRR consensus (LxxLxxLxxLxx(N/C/T)x(x)Ipxx; Jones and Jones 1997), the LRR motif of the predicted protein could be broken into \( \sim 20 \) imperfect leucine-rich repeat units. The first 14 repeats were from 20 to 27 amino acid residues in length. Following the fourteenth repeat was a stretch of 65 residues that could not be arranged
Figure 6.—Amino acid alignment of the coding region of PIC13 paralogues from three Rp3 haplotypes. Four genes from Rp3-A, five from Rp3-D, and four from Rp3-AD4 haplotypes are represented. Conserved amino acid motifs common to most NBS-LRR genes such as the P-loop (GSGKTT), kinase-2 (LAVLDDV), GLPL (GVPLAI), and MHD, are underscored in the consensus sequence. Dots represent amino acids identical to the Rp3-AD4 consensus sequence. Deletions or missing sequence at the 3' end are indicated by blank spaces. Corresponding DNA sequences are available as GenBank accession nos. AF489541–AF489554.
into repeats. The remaining 6 units were quite variable in length (23–43 residues) and fit the consensus very poorly.

Genomic PIC13 family member sequences were obtained from the Rp3-A and Rp3-D haplotypes in addition to the Rp3-AD4 haplotype, which was derived from recombination between the Rp3-A and Rp3-D haplotypes. Genes from Rp3-AD4 were isolated from a genomic library found that they fell into at least four different groups. From the Rp3-D haplotype, five (Rp3-AD41 to Rp3-AD45) displayed uninterrupted ORFs between 3585 and 3753 bp, which showed DNA sequence identities of 94–96%.

One of the five genes isolated from the Rp3-AD4 haplotype (Rp3-AD45) appeared to be a pseudogene on the basis of a disruption of its ORF by a 2594-bp retrotransposon containing a 1635-bp ORF. The whole transposon product showed 52% amino acid identity to a putative non-LTR retroelement reverse transcriptase from Arabidopsis thaliana (GenBank accession no. AP002521). The insertion is located 300 bp upstream of the NBS/LRR motif. With the retrotransposon DNA sequence removed, this Rp3-AD4 gene is 94–95% identical at the DNA level to the other four Rp3-AD4 family members throughout their entire length. The removal of the retrotransposon also restores a full-length ORF (3486 bp) with no stop codons, suggesting that the insertion was a relatively recent evolutionary event.

Several combinations of PCR primers were used to amplify genes from the Rp3-A and Rp3-D haplotypes. Whenever possible, primer pairs flanking the coding region were used so as to amplify the complete ORF. Thirty-five PCR clones were isolated from Rp3-A genomic DNA template and partially sequenced. Analysis of these and the nine partial clones sequenced from the Rp3-D haplotype, five different groups were identified from 22 PCR-amplified sequences. From within each haplotype, one gene of...
each group was fully sequenced (Rp3-A1 to -A4 and Rp3-D1 to -D5). The intact, single ORFs of these nine genes were compared with those of the four fully sequenced Rp3-AD4 genes to determine the degree of similarity among the family members and to determine if any of the genes isolated from the Rp3-AD4 haplotype might appear to be a recombinant of two different genes in the parental haplotypes (Figure 6).

Genes isolated from the Rp3-A haplotype were between 95 and >99% identical in DNA sequence, while the Rp3-D-derived genes showed identities ranging from 95 to 98%. In one case, the coding regions of two Rp3-A haplotype genes (Rp3-A1 and -A3) were found to differ only by one nonsynonymous nucleotide substitution over 3753 bp of their coding regions. In another case, only three nonsynonymous nucleotide substitutions in 2844 bp were all that separated two other Rp3-A genes (A2 and A3). In this light, it is likely that some of the partially sequenced clones, ignored after appearing identical to other clones already in hand, may have actually represented different genes.

A range of DNA sequence similarities was also observed when genes isolated from the Rp3-A and Rp3-D haplotypes were compared with one another and to the Rp3-AD4 haplotype. In an extreme case, one Rp3-A gene and one Rp3-D gene (A1 and D1) were predicted to encode the same protein. Their coding regions of 3753 bp differed by only a single, synonymous nucleotide substitution. In another case, a gene (AD41) from the Rp3-AD4 haplotype was found to be identical to the A1 gene from the Rp3-A haplotype, although this is likely the same gene since the line carrying the Rp3-A haplotype was one of the Rp3-AD4 parents. At the other extreme, a gene (A3) from the Rp3-A haplotype was only 90% identical in DNA sequence (85% identical in predicted amino acid sequence) to a gene (AD44) from the Rp3-AD4 haplotype. This is roughly equivalent to the sequence differences among some of the more distinct rp1 genes. For example, the two most different genes in the Rp1-D haplotype (rp1-dp2 and rp1-dp8) were also only 85% identical in predicted amino acid sequence. Sequence comparisons of the genes in the Rp3 haplotypes also provide evidence for intragenic recombination events between different family members as previously recorded for genes at rp1 (Sun et al. 2001) and the tomato Cf4/Cf9 locus (Parniske et al. 1997). For example, genes D2 and D4 and AD43 were nearly identical, with only four nonsynonymous nucleotide substitutions for the first 2.9 kb of the coding region, at which point they diverged. After this point, D2 was nearly identical to D3 and AD43, while D4 became nearly identical to D5 and AD42.

Expression analysis of the PIC13 gene family: Alignment of a 525-bp region from 28 3’ RACE cDNAs from the Rp3-A haplotype indicated they corresponded to five different genes. Surprisingly, the sequences from these five transcripts were similar, but not identical to any of the four genomic Rp3-A clones described above, thus providing evidence of at least nine genes in this haplotype. Sequence data from seven RT-PCR clones suggested that at least six genes were transcribed in the Rp3-AD4 haplotype.

Expression of genes from the PIC13 family was tested in various tissues by RNA blot analysis using a 3.6-kb probe derived from the total coding region of a PIC13 family member. No expression was observed in roots or mesocotyl tissues. The observed expression in leaves was not altered in P. sorghi-inoculated tissue as compared with the control (mock inoculated). A transcript of ~1.5 kb was absent from fully expanded leaves (Figure 7) but present in immature leaves (Figure 8). Developmentally regulated transcript levels were also observed at the rp1 locus (Collins et al. 1999). Differences in transcript size were also apparent when expanded leaf tissues from different maize lines were compared. Clear differences in both expression level and hybridization pattern were found to exist between the six Rp3 haplotypes in the H95 genetic background when the same 3.6-kb probe was used (Figure 7). A hybridizing transcript of ~7.5 kb was observed in the Rp3-B, -C, and -F haplotypes. In Rp3-A, -D, and -E, however, this fragment was absent or less noticeable. All haplotypes had a hybridizing transcript of ~4.5–5.0 kb in size, which was in agreement with the size expected from sequence data. The origins

![Figure 7.—PIC13-homologous transcripts from maize lines with Rp3-A through F haplotypes. From each lane, total RNA was isolated from expanded leaf tissue and gel blotted. The RNA blot was hybridized with a 3.6-kb probe derived from the coding region of a cloned PIC13 family member. The size markers shown on the left are derived from a 9.49- to 0.24-kb RNA ladder. The formaldehyde-treated, 1% agarose gel (bottom) is ethidium bromide stained to show the relative loading (~10 μg/lane) of total RNA.](image-url)
of the larger transcripts are not clear, but they may be from an uncharacterized family member or from alternative splicing of introns (Ayliffe et al. 1999; Collins et al. 1999; Dinesh-Kumar and Baker 2000; Halterman et al. 2001). Truncated gene products could account for the smaller transcripts observed. For example, an estimated 1.5-kb transcript was predicted from the isolated PIC13 family member that carried the retroelement insertion.

The observed polymorphic RNA transcripts were repeatable and cosegregated with the rp3 locus. Total RNA from 12 homozygous resistant and 12 susceptible F2 seedlings derived from the F1 Rp3-B/rp3 (identified by sequential inoculation with the rust biotypes IN1 and then IN2) were assayed on gels and showed that the polymorphic 5.0- and 7.5-kb species as well as the higher expression of the 1.5-kb transcript cosegregate perfectly with the rp3 locus. Transcripts of ~1.5 kb were present in both resistant and susceptible seedling RNA, but transcripts of this size were consistently more abundant in resistant plants (Figure 8).

Characterization of the novel HpaII fragment from the Rp3-AD4 haplotype: The Rp3-AD4 haplotype is associated with an altered rust resistance phenotype, recombination of flanking markers, and a novel-sized 3.5-kb HpaII fragment with homology to the 5′ half of PIC13 genes including the NBS region. Agarose gel-purified 3.5-kb HpaII DNA fragments were used as template with primers from conserved sequences of the NBS region to amplify an 880-bp product, which was then cloned, sequenced, and compared to the five PIC13 family members characterized from the Rp3-AD4 haplotype.

Of the five characterized PIC13 family members from the Rp3-AD4 haplotype, only the AD42 gene showed perfect DNA sequence identity with the novel 3.5-kb HpaII fragment. However, when AD42 was compared to the characterized genes from the parental Rp3-A and Rp3-D haplotypes, it did not appear as a recombinant of any characterized genes from these two parental haplotypes. The first 1001 amino acids encoded by the AD42 gene are identical with the D3 gene, while the remainder of the gene encodes for an amino acid sequence that is indistinguishable from that encoded by either the D4 or the D5 gene (Figure 6). Attempts to isolate a gene from the Rp3-A haplotype that could have been a presumptive progenitor of the Rp3-AD4 variant were unsuccessful. Thus far, it cannot be demonstrated that the AD42 gene arose from a recombination event between Rp3-A and Rp3-D genes.

Physical characterization of the PIC13 gene family in B73: The number of PIC13 paralogues and the distance between them were determined in the maize inbred B73 (rp3/rp3). DNA from 10 maize inbred lines was digested with various restriction endonucleases, gel blotted, and probed with the NBS region of a PIC13 gene family member (Figure 9). B73 typically had the smallest number of PIC13-homologous fragments, indicating it carries the fewest family members of the 10 lines tested. In a complete HindIII digest, B73 displayed at least four PIC13-homologous fragments (data not shown).

Fourteen PIC13-hybridizing BAC clones, ranging in size from 90 to 140 kb, were isolated from CUGI’s ZMMBBb library. The BACs were arranged into a single overlapping contig by identification of common HindIII restriction fragments and by partial sequence analysis. The distribution of PIC13-homologous genes within the BACs was determined by probing HindIII-digested BAC clones and using the PCR primer pair LRRF1 and LRRR1. Amplification products from each BAC clone template were either sequenced directly or cloned and then sequenced. The number of different sequences identified on each of the BACs by sequencing of PCR products ranged from one to four. A total of five different genes were amplified and designated prp3-B73a–e. It is likely these represent all the genes from the B73 haplotype. It appears that the gene family lies within a region of ~130–140 kb, with one BAC clone, 0215F09, carrying all five PIC13-homologous genes (Figure 10).

**Figure 8.**—Polymorphic PIC13-homologous transcripts map to the Rp3 locus. Total RNA was isolated from immature leaf tissue from homozygous resistant and homozygous susceptible plants selected from segregating Rp3-B F2 progeny and gel blotted. The RNA blot was hybridized to E4A, a 3.6-kb probe derived from the entire coding region of a cloned PIC13 family member. The size markers shown on the left are derived from a 9.49- to 0.24-kb RNA ladder. The ethidium bromide-stained agarose gel (bottom) shows the relative loading of ~10 μg of total RNA per lane.

**DISCUSSION**

Genetic analysis indicated that rp3 is a complex locus, and a family of NBS-LRR genes identified by the PIC13 probe maps to the locus. This probe was originally isolated by PCR amplification of resistance gene-like se-
the family members that control the phenotype. Occasional NCO variants may occur by mutation. Analysis of these deletion variants can allow identification of the family member controlling the resistance phenotype. At the \( rp3 \) locus, this is complicated by the large number of family members in \( Rp3 \)-carrying lines and the similarity between them. \( Rp3 \)-carrying lines have nine or more family members that are difficult to distinguish in gel blots and, in some cases, even by sequence analysis. The family member conferring \( Rp3 \)-mediated resistance has not yet been positively identified.

Studies at the \( rp1 \) complex of maize have indicated that unequal crossing over is a frequent event and that the crossovers are often intragenic (Sun et al. 2001). In contrast, at the lettuce \( Dm3 \) locus and the \( Pto \) locus of tomato, genes in orthologous positions in different lines appear to be more similar than paralogues, thereby suggesting that meiotic mispairing and recombination is uncommon (Michelmore and Meyers 1998; Chin et al. 2001). The present analysis of \( rp3 \) indicates it behaves more like the \( rp1 \) complex. When probed with PIC13, differences in gene copy number among maize lines indicate that unequal crossing over occurs at the locus. Furthermore, susceptible variants from \( Rp3 \) homozygotes and heterozygotes are usually associated with recombination events that delete family members. Analysis of the PIC13 gene family indicates that some paralogues in the same haplotype can be nearly identical in DNA sequence and that others appear to be recombinant versions of other pairs of genes. The patterns of polymorphism in the gene family therefore indicate that they are frequently reassorted into new combinations and that these recombination events, at least sometimes, occur within the coding regions.

Hooker and Saxena (1967) coined the term “reversal of dominance” when attempting to explain how \( Rp3 \) could confer dominant resistance to one rust biotype and recessive resistance to another. They postulated that the dominant \( Rp3 \) gene could be linked to a recessive gene, though they were not able to break this possible linkage. Furthermore, we failed to identify separate dominant and recessive resistance genes in recombinants for

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**Figure 9.** Maize lines carry multiple, polymorphic PIC13 paralogues. Genomic DNAs were restricted with NsiI, gel blotted, and hybridized with a probe from the NBS region of a PIC13 gene family member. DNA marker sizes, in kilobases, are shown on the left.

**Figure 10.** A BAC contig was assembled across the \( rp3 \) locus in the maize line B73. CUGI’s address designation for each clone is shown on the left. Size estimations for each clone (in parentheses) were obtained by summing the molecular weights of all fragments in a complete \( HindIII \) digest. Solid boxes represent where the NBS and LRR region probes hybridized, giving the approximate location of the five PIC13 family members, prp3-B73a–e.
the \( \text{rp3} \) locus. The \( \text{Rp3} \)-carrying lines confer a recessive resistance against biotype IN2 from our current rust biotype collection. Our examination of 1 noncrossover and 16 crossover-derived variants showed that, in addition to losing the dominant \( \text{Rp3} \) gene, all had lost resistance against IN2. A more likely alternative is that \( \text{Rp3} \) resistance to some biotypes may be due to the Avr factor in these biotypes being expressed at lower levels or interacting less strongly with the \( \text{Rp3} \) resistance gene product. Heterozygosity of Avr loci may lead to weaker resistance; Kolmer and Dyck (1994) found that wheat leaf rust isolates that were heterozygous for avirulence genes often showed intermediate levels of avirulence. Recessionally inherited resistance genes are often considered to be functionally different from dominant R genes (Büschesges et al. 1997; Deslandes et al. 2002), but the results with \( \text{Rp3} \) imply that at least some of these will be simple cases of weaker R gene or Avr gene expression and/or weaker interactions between Avr and R gene products.

Different members of the same gene family can encode different resistance specificities when they detect different pathogen factors (effectors) whose production is controlled by different \( \text{Avr} \) genes. Examples include the \( \text{Gf-2} /5 \) (Dixon et al. 1996, 1998) and \( \text{Gf-4} /9 \) (Jones et al. 1994; Parniske et al. 1997; Thomas et al. 1997; Takken et al. 2000) loci in tomato, the \( M \) (Anderson et al. 1997) and \( P \) (Dodds et al. 2001) loci of flax, and maize \( \text{rp1} \) (Saxena and Hooker 1968). Only a single specificity could be differentiated for \( \text{Rp3} \) when a series of rust biotypes were inoculated onto the six \( \text{Rp3} \) alleles. Unlike \( \text{rp1} \), no obvious novel specificities or lesion mimic phenotypes were identified in any of the variants generated in the present study. The \( \text{Rp3-AD4} \) variant differed from the parental \( \text{Rp3} \) genes in being fully susceptible to rust biotype IN2, but this is the same isolate that the parental genes show only partial resistance to. The observation that \( \text{Rp3-AD4} \) provided less resistance than the parental genes to all of the rust biotypes tested indicates that the recombinant gene probably just provides a reduced resistance with the same recognition specificity. The level of effective \( \text{Rp3} \) gene activity in \( \text{Rp3-AD4} \) homozygotes and \( \text{Rp3} \) heterozygotes appears to be below a threshold needed to provide noticeable levels of resistance to rust biotype IN2 in greenhouse seedling assays. A reduced-resistance variant, similar to \( \text{Rp3-AD4} \), has been identified at the \( \text{rp1} \) complex. The \( \text{Rp1-D}^{*5} \) gene is a recombinant gene with the LRR derived from \( \text{Rp1-D} \) (Sun et al. 2001). It displays reduced levels of resistance, but confers resistance to the same spectrum of rust isolates as the parental \( \text{Rp1-D} \) (Richter et al. 1995). The \( \text{Rp3-AD4} \) was also associated with a crossover somewhere within the haplotype and may have a similar origin. Differences in methylation patterns among some of the PIC13 family members were also apparent when the \( \text{Rp3-AD4} \) haplotype was compared to the parental haplotypes. These small hybridizing \( HpaI \) fragments that indicate the methylation state of the \( \text{Rp3-AD4} \) haplotype has changed relative to the two parents. It is therefore possible that the reduced resistance from the \( \text{Rp3-AD4} \) gene was due to a reduced expression associated with methylation changes. In Arabidopsis, an NBS-LRR resistance gene cluster containing \( \text{RPP5} \) was recently found to be subject to epigenetic variation associated with DNA methylation (Stokes et al. 2001). One gene in the cluster was altered, leading to its overexpression. This apparently triggered the constitutive expression of pathogenesis-related genes, resulting in dwarfing and elevated disease resistance.

The maize \( \text{rp3} \) and \( \text{rp1} \) loci appear genetically and molecularly similar. Their genes are structurally similar, with intronless coding regions and small introns in the untranslated regions. They are not closely related by sequence, however, as the \( \text{Rp3} \) genes are only \( \sim 25\% \) identical to the different \( \text{Rpl} \) genes in predicted amino acid sequences. Phylogenetic analysis of cereal NBS-LRR genes provides additional evidence they are not closely related, placing the two gene families in different clades (J. Bai and S. H. Hulbert, unpublished data). Both loci map to R-gene-rich areas and are composed of gene families with structurally variable haplotypes in different maize lines. Most genes at both loci appear to potentially code for NBS-LRR proteins with few obvious pseudogenes. Many, if not most, genes in haplotypes of both loci are transcribed, although most of these genes have no known phenotypes. Genes at both loci show patches of sequence affinities, where genes in the same haplotype are identical for large stretches, showing the importance of exchange in their evolution. To date, \( \text{rp3} \) is only the second rust resistance locus to be characterized from maize. As additional maize R gene loci are examined, a clearer picture will emerge as to the commonality of events such as mispairing and unequal crossing over and their resulting impact on the evolution of disease resistance.

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