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-^{32}P]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 F_1 was test crossed to a susceptible inbred line and the resulting populations were screened with a *P. sorghi* isolate that was avirulent on the *Rp3*-carrying lines. Similarly, an *Rp3-B* line was constructed by crossing an *Rp3-B*-R168 NIL to an *Rp3-B*-B14 NIL. Flanking marker analyses were conducted on susceptible variants using the centromere proximal marker *umc18* and the distal marker *umc10*. All susceptible variants from crosses with *Rp3* homozygotes and heterozygotes (Table 1) were self-fertilized until individuals homozygous for the variant haplotypes could be identified. The linked RFLP markers *umc10*, *umc18*, and *umc102* were used to identify homozygotes.

Rust inoculation and screening: Grown 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* urediospores. Spores were diluted to a concentration of ~10 mg/ml in Soltrol oil (Phillips Chemical Company, Phillips, TX) and the suspension was applied to the leaves with a chromatography sprayer (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 penetrations resulting in chlorotic or necrotic zones around the pustule. Ratings of 3 were given to plants with large numbers of rust 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: RFLP probes UMC10 (distal) and UMC18 (proximal), flanking the *rp3* locus, were used to determine which susceptible variants carried nonparental combinations of flanking markers. *Rp3* has been placed ~4 cM from *umc10* and ~2 cM from *umc18* (Sanz-Alferez *et al.* 1995). Marker *umc10* was reported to map proximal and *umc18* to map distal to *rp3*, but this was when *rp3* was thought to occupy a position on the long arm of chromosome 3. A current map of chromosome 3 (Davis *et al.* 1999) places both the *umc10* and *umc18* loci on the short arm near the centromere but with a distance between them of ~14 cM.

Genomic 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-AD4*. After partial digestion with *Sau*3A I, 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. *Bam*HI-digested ZAP Express and *Bam*HI-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 *Bam*HI-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, AACGAAGCAG TTAATCTATTCTTCTC; NBSR1, GTCACTCCTTCCTTTCA CAAT) designed to amplify ~854 bp of the 5′ region was used to amplify from each of the high-titer stocks. The PCR products were sequenced directly.

λ-clone 7a 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 Hpa*II fragment. The clone was *Sau*3A I 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, Herculase-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, CGACTTTCGACGCCACTTAGAT GGAAGC; (2) F1, R2, AATCACTTGCCGACTGGT; (3) F2, TGCGTATTCACTGGTCTTAGGG; R3, TGTTTCCATCAAG TCCAAGA; (4) GSF3, TAGCAAACAGAGAAAATAAACAG, R3; and (5) GSF3, LRRR1, CAGTGGATGCTCTCAGGTA AATG. (Note that the LRRR1 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 gene specific for the 5′ flanking region of the *Rp3-AD42* gene: GSF3, GSF4, TAGAAACAAGAATAACATAAAG;, GSF5, CGCTCCGA AAAGGCATCAACG; GSF6, ATTGAGGTAAAGATGAACAG TC; GSF7, TGACTGAAGCCACAAGC; and GSF8, GCCCAA ACTAAAACCATTCAGGA. Four reverse, gene-specific primers were designed from the 3′ flanking region of the *Rp3-AD42* gene: GSR1, GSR2, ACACGACATGTAATACGAGGCA GCA; GSR3, CTTAGATGGAAGCAGTGCAACAAAC; and GSR4, TTGTTTTCCAAAGTTGATGCAC.

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-TOPO 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.ch.embnet.org/software/coils/COILS_doc.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°). 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 in the 5′ RACE kit and our reverse primer NBSR1. A 1-μl aliquot was taken after 10 full cycles of PCR and used as the template for nested PCR using the 5′ Abridged Anchor Primer and a second reverse primer. This nested primer (NBSR2, GCCTTTATCACCAACTGTTTGCA) anneals 235 bp upstream of NBSR1. In this second round of PCR, the following cycling parameters were used: 40 cycles of 94° for 2 min, 60° for 30 sec, 72° for 1 min 30 sec, and then a final extension step at 72° for 10 min. The resulting cDNAs were cloned and the QIAprep spin miniprep kit (QIAGEN, Valencia, CA) was used to purify the plasmids prior to sequencing.

For 3′ RACE experiments, first-strand cDNA was synthesized from 10 μg of total RNA with a ProSTAR RT-PCR kit (Stratagene), using a modified 27 mer, oligo(dT)-*Bam*HI primer (GGATCCTTTTTTTTTTTTTTTTTTTTT). A forward PCR primer (LRRF1, AACCACCATCAAAAATTGAGAAGCT), designed from conserved sequence in the LRR region of PIC13 family members, was coupled with the reverse oligo(dT)-*Bam*HI primer to amplify target cDNAs. The forward primer site was predicted to lie ~2.0 kb upstream from translation termination. Advantage-HF polymerase mix (CLONTECH, Palo Alto, CA) was used for PCR amplification. Cycling parameters were: 20 cycles of 91° for 1 min 30 sec, 54° for 1 min 30 sec, 72° for 2 min 30 sec, and then a final extension step at 72° for 10 min. The PCR product migrating at the predicted size of 2.0 kb was separated from nonspecific amplification products by electrophoresis through a 1× TAE-1.0 mm guanine buffer agarose gel. After gel excision, this fragment was purified using a GENECLEAN III kit (BIO 101, Vista, CA) and the freshly purified PCR product was cloned. Plasmids were purified using a modified alkaline-lysis/polyethylene glycol 8000 precipitation protocol (Tartof and Hobbs 1987). Twenty-eight 3′ RACE clones were end sequenced with M13 forward and M13 reverse sequencing primers.

Bacterial artificial chromosome clones: The Clemson University Genomics Institute (CUGI) bacterial artificial chromosome (BAC) library was screened with a putative *rp3* NBS region probe by Gernot Presting and co-workers. BAC DNA was isolated from the 14 hybridizing clones using a standard alkaline-lysis protocol (Genome Systems, St. Louis).

Purified plasmids were digested to completion with *Hin*dIII and fractionated in 0.7% agarose gels. BAC clones were initially grouped by determining which clones shared the most identical *Hin*dIII 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 *Hin*dIII-digested BAC clones, or specific *Hin*dIII 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, CCTCTCACTCATGCTAATTTCC and B73NBSR, CAATACAGTTGATACCAAGGC) 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, CCCATCTGACTGAATTAGTAC and B73F2, CCCATCTGAC TAAACTAGTAT) and two reverse primers (B73R1, TGTAAG GTCTGTGCACATGT and B73R2, TAAGGCCTGTGCACT TGA) 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 (Pataky 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

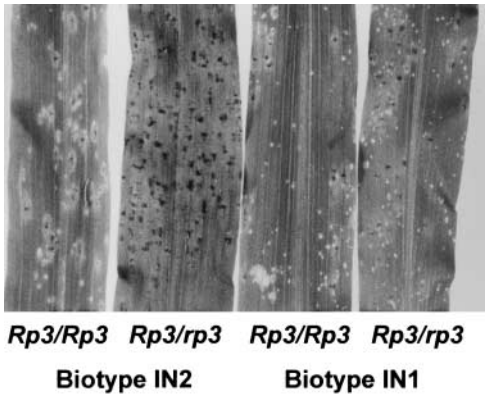

FIGURE 1.—The reaction phenotype of the six *Rp3* NILs cannot be differentiated when inoculated with most biotypes in the current *P. sorghi* collection. *Rp3* displays complete or nearly complete dominance with most biotypes (*e.g.*, IN1) but displays incomplete dominance of resistance when inoculated with the rust biotype IN2. The photograph was taken 7 days after inoculation.

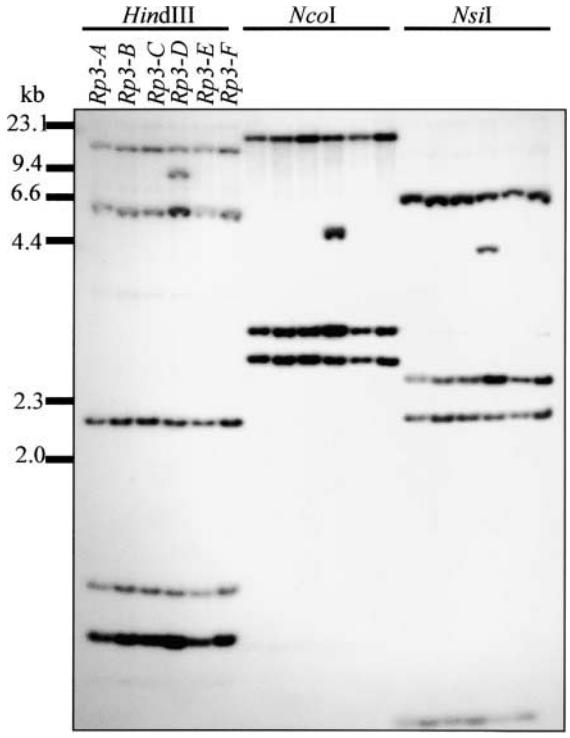

FIGURE 2.—Maize haplotypes *Rp3-A–Rp3-F*. Seedling DNAs were digested to completion with *Hin*dIII, *Nco*I, and *Nsi*I restriction endonucleases, separated by agarose gel electrophoresis, gel blotted, and hybridized with the NBS region of a PIC13 family member. Size markers, in kilobases, are shown on the left.

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 F_2 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 flanking

TABLE 1

Susceptible variants derived from crosses with *Rp3* lines

Type of cross	Parents	Segregation[a] R:Int:S	Flanking marker analyses[b]		
			Variant	Proximal	Distal
A. *Rp3* homozygotes × *rp3/rp3*	*Rp3-A*-R168/*Rp3-A*-B14[a] × *rp3/rp3*	4,236:0:1	*Rp3-AA*	*Rp3-A*-R168	*Rp3-A*-B14
	Rp3-B-R168/*Rp3-B*-B14[a] × *rp3/rp3*	22,775:0:0			
Rp3 heterozygotes × *rp3/rp3*	*Rp3-A/Rp3-B* × *rp3/rp3*	5,176:0:3	*Rp3-AB1*	*Rp3-A*-R168	*Rp3-B*-R168
			Rp3-AB2	*Rp3-B*-R168	*Rp3-B*-R168
			Rp3-AB3	*Rp3-A*-R168	*Rp3-A*-R168
	Rp3-A/Rp3-C × *rp3/rp3*	2,697:0:2	*Rp3-AC1*	*Rp3-A*-R168	ND[b]
			Rp3-AC2	*Rp3-A*-R168	ND[b]
	Rp3-A/Rp3-D × *rp3/rp3*	8,988:1:5	*Rp3-AD1*	*Rp3-D*-R168	*Rp3-A*-R168
			Rp3-AD2	*Rp3-D*-R168	*Rp3-A*-R168
			Rp3-AD3	*Rp3-D*-R168	*Rp3-A*-R168
			Rp3-AD4	*Rp3-D*-R168	*Rp3-A*-R168
			Rp3-AD5	*Rp3-D*-R168	*Rp3-A*-R168
			Rp3-AD6	*Rp3-D*-R168	*Rp3-A*-R168
	Rp3-A/Rp3-E × *rp3/rp3*	6,108:0:1	*Rp3-AE*	*Rp3-A*-R168	ND[b]
	Rp3-A/Rp3-F × *rp3/rp3*	3,102:0:1	*Rp3-AF*	*Rp3-A*-R168	*Rp3-F*-R168
	Rp3-C/Rp3-B × *rp3/rp3*	2,770:0:0			
	Rp3-C/Rp3-D × *rp3/rp3*	3,768:0:0			
B. Mutator background	*Rp3-B/Rp3-B-Mu* × *rp3/rp3*	37,524:0:4			

[a] 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.

[b] 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-

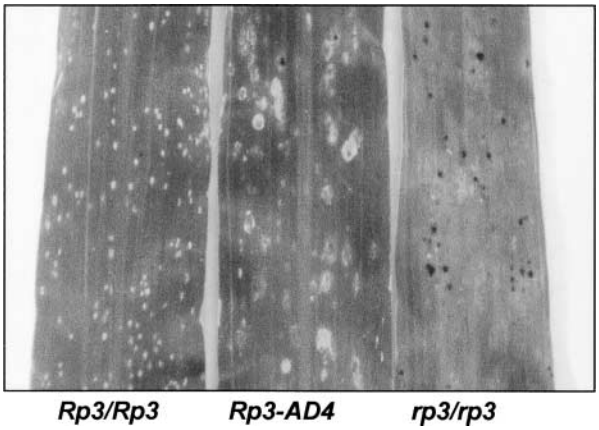

FIGURE 3.—One recombinant haplotype, *Rp3-AD4*, exhibits an intermediate rust resistance reaction compared to its rust-resistant parents *Rp3-A* and *Rp3-D*. The photograph was taken ~7 days after inoculation with the rust biotype IN1.

tion of flanking markers, indicates that mispairing and recombination can occur at *rp3*. Only two apparent non-crossover (NCO) variants were recovered from these crossing experiments. Both were identified from a test-cross of an *Rp3-A/Rp3-B* heterozygote to a susceptible (*rp3/rp3*) line. The variant designated *Rp3-AB2* retained 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, *Bam*HI, *Bgl*II, *Hpa*II, *Nsi*I, and *Sac*I (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

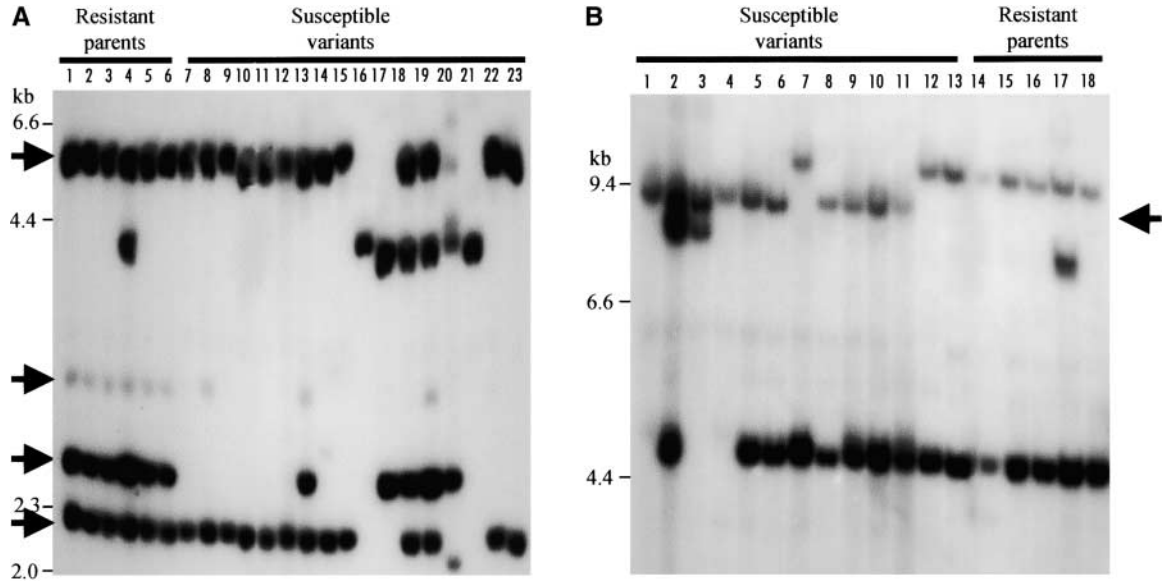

FIGURE 4.—Crossover-generated deletions and novel restriction fragments in 18 spontaneous rust-susceptible *Rp3* variants. (A) A gel blot demonstrates absence of certain *Nsi*I fragments from the majority of the variants (indicated by arrows). DNAs of homozygous variants were restricted with *Nsi*I and 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 *Sac*I 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 *Sac*I fragment and were missing an ~12.0-kb fragment present in the parents (Figure 4B). The only progeny lines that did not show this novel *Sac*I 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 *Eco*RI, *Nsi*I, and *Xba*I 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 *Hpa*II restriction fragment of 3.5 kb that cosegregates with the *Rp3-AD4* intermediate resistance phenotype (Figure 5). Smaller-sized (<1.5 kb) hybridizing *Hpa*II 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

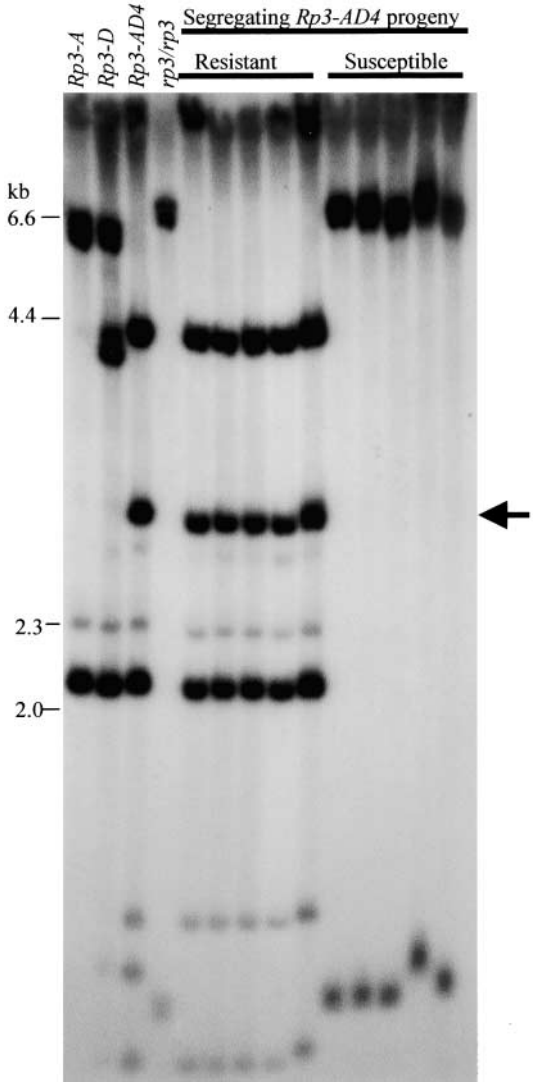

Figure 5.—Gel blot analysis of the reduced-resistance variant *Rp3-AD4* and the two parental *Rp3* haplotypes, *Rp3-A* and *Rp3-D*. DNAs were restricted with *Hpa*II and probed with the NBS region of a PIC13 gene family member. The recombinant haplotype carries restriction fragments from both parents as well as novel restriction fragments generated either by recombination or by alterations in methylation patterns. The arrow shows the strongly hybridizing, novel 3.5-kb fragment that maps to *rp3* and cosegregates with resistance.

NBS-LRR protein. COILS analysis (Lupas 1997) predicted a high probability ($P \geq 0.9$) 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 (LxxLxxLxxLxLxx(N/C/T)x(x)Ipxx; Jones and Jones 1997), the LRR motif of the predicted protein could be broken into ~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

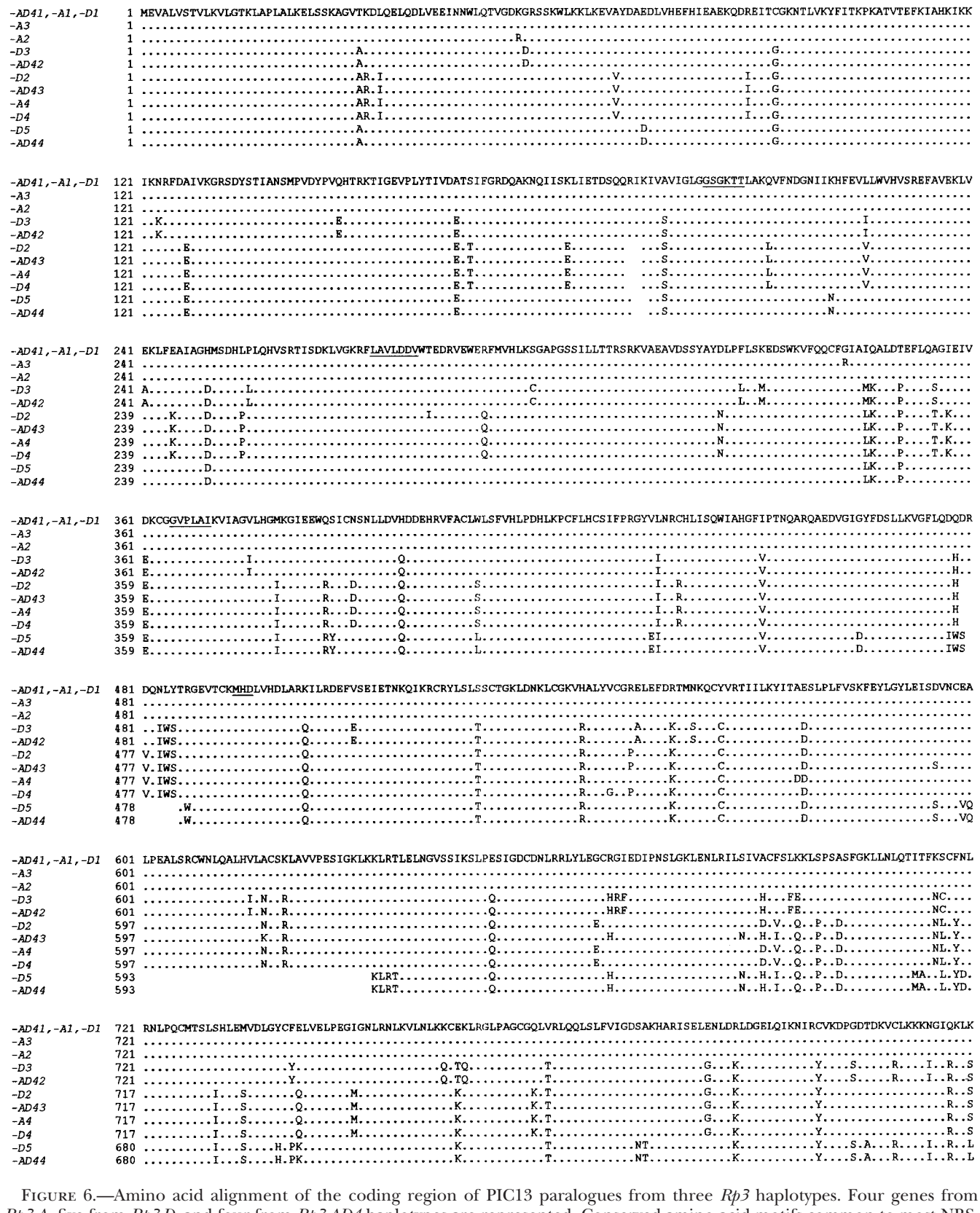

FIGURE 6.—Amino acid alignment of the coding region of PIC13 paralogues from three *Rp3* haplotypes. Four genes from *Rp3-A*, five from *Rp3-D*, and four from *Rp3-AD4* haplotypes are represented. Conserved amino acid motifs common to most NBS-LRR genes such as the P-loop (GSGKTT), kinase-2 (LAVLDDV), GLPL (GVPLAI), and MHD, are underscored in the consensus sequence. Dots represent amino acids identical to the *Rp3-AD41* 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.

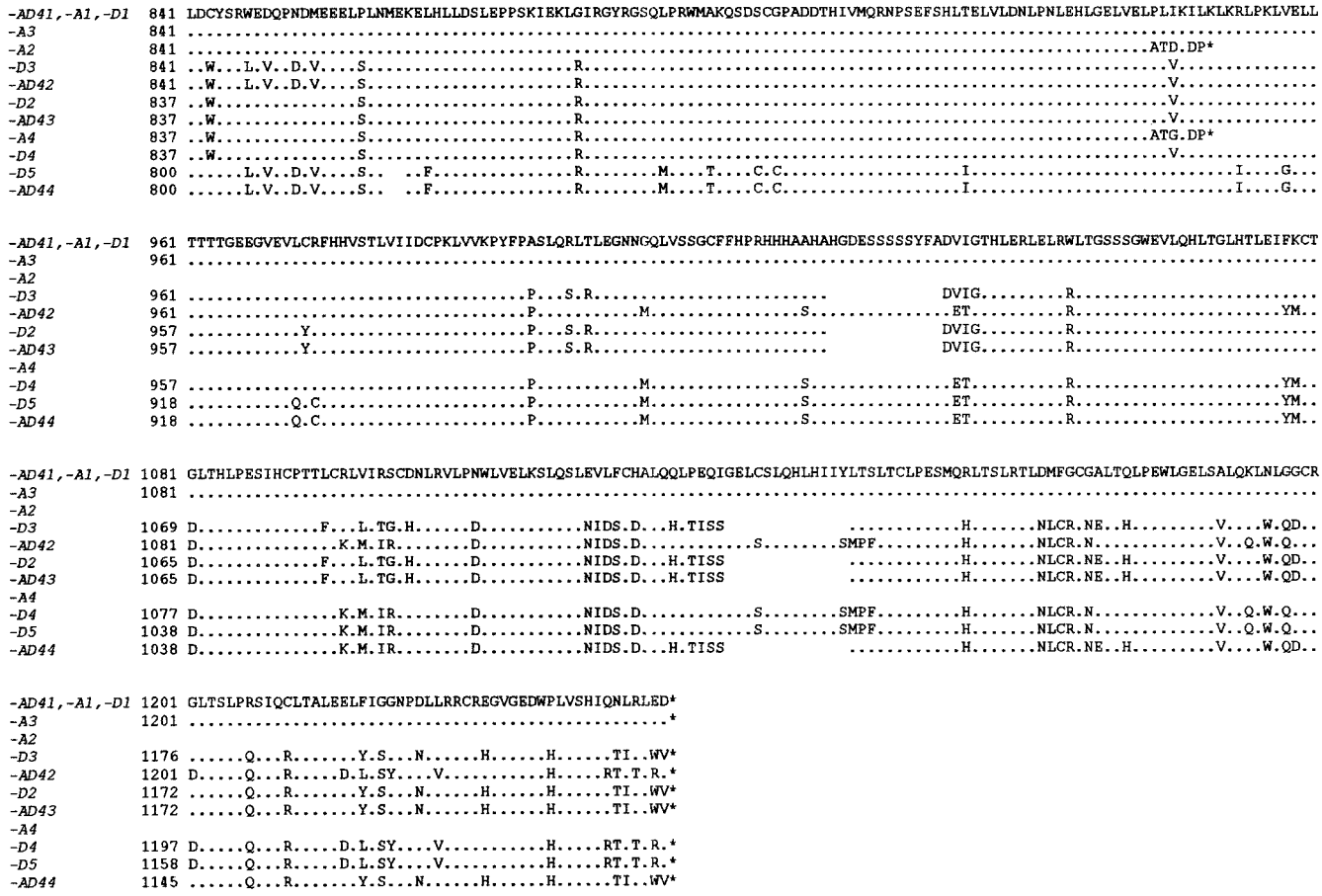

FIGURE 6.—*Continued.*

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 λ-library while genes from the two parental haplotypes were PCR amplified from genomic DNA templates. To account for PCR-induced errors in DNA sequence, an apparent change in any single base had to be present at the same position in two or more independent sequences for it not to be considered an artifact. Two gene sequences were considered to be similar or different from one another only if these criteria of "informative base pair differences" were met. These standards were implemented whenever PCR products were sequenced.

Twenty-one λ-clones carrying putative full-length genes from the *Rp3-AD4* haplotype were identified. These were grouped into five distinct classes that are based on the partial DNA sequence analyses of their NBS domains. A representative gene from each of the different groups was fully sequenced. Four of the *Rp3-AD4*-derived genes (*Rp3-AD41* to *-AD44*) 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 junction (MHD 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-A* genomic library found that they fell into at least four different groups. 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 93 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.

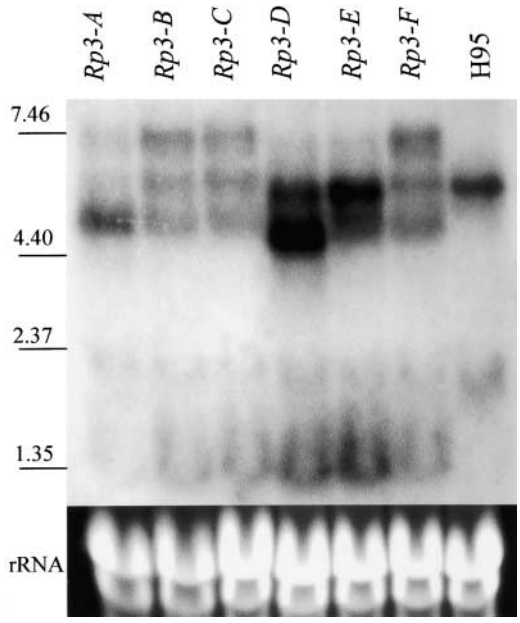

Figure 7.—PIC13-homologous transcripts from maize lines with *Rp3-A* through *F* haplotypes. From each line, total RNA was isolated from expanded leaf tissue and gel blotted. The RNA blot was hybridized with a 3.6-kb probe derived from the coding region of a cloned PIC13 family member. The size markers 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.

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

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 F_2 seedlings derived from the F_1 *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).

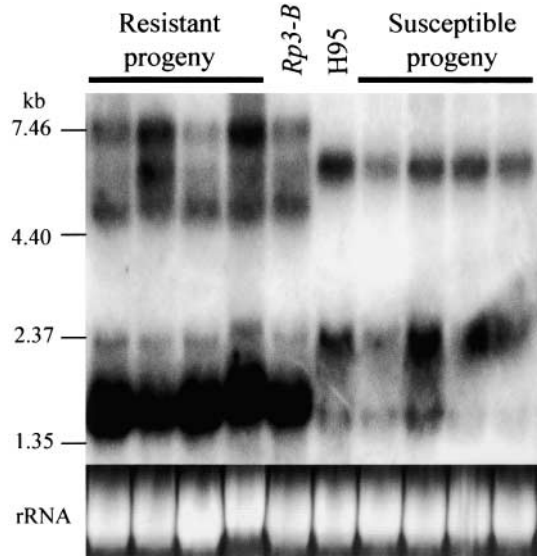

Figure 8.—Polymorphic PIC13-homologous transcripts map to the *Rp3* locus. Total RNA was isolated from immature leaf tissue from homozygous resistant and homozygous susceptible plants selected from segregating *Rp3-B* F_2 progeny and gel blotted. The RNA blot was hybridized to E4A, a 3.6-kb probe derived 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.

Characterization of the novel *Hpa*II 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 *Hpa*II fragment with homology to the 5′ half of PIC13 genes including the NBS region. Agarose gel-purified 3.5-kb *Hpa*II 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 *Hpa*II 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 *Hin*dIII 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 *Hin*dIII restriction fragments and by partial sequence analysis. The distribution of PIC13-homologous genes within the BACs was determined by probing *Hin*dIII-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).

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-

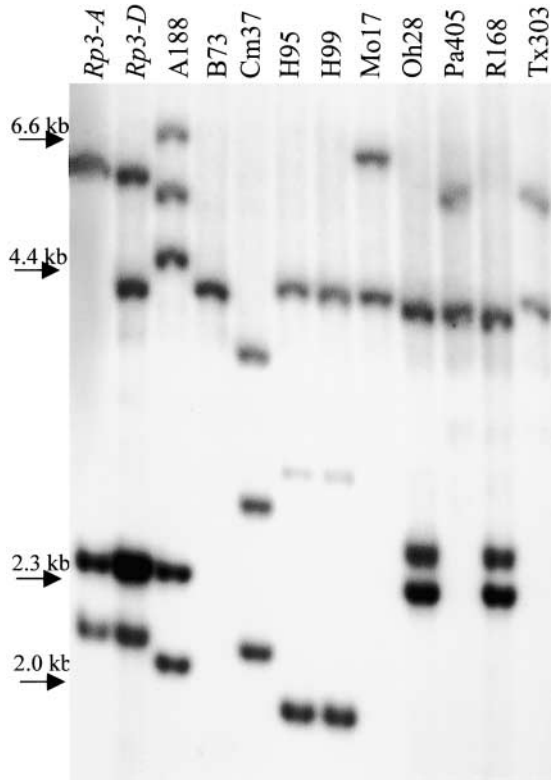

FIGURE 9.—Maize lines carry multiple, polymorphic PIC13 paralogues. Genomic DNAs were restricted with *Nsi*I, gel blotted, and hybridized with a probe from the NBS region of a PIC13 gene family member. DNA marker sizes, in kilobases, are shown on the left.

quences, using primers designed from conserved domains from this class of gene (COLLINS *et al.* 1998). Sixteen of 17 spontaneous susceptible variants from *Rp3* homozygotes and heterozygotes showed losses of PIC13 family members in gel blot analyses with the PIC13 probe. One additional variant with an altered resistance phenotype also showed a loss of one or more PIC13 family members. These results were essentially the same as when similar *Rp1* variants were examined with an *rp1* probe: most susceptible *Rp1* variants arose by crossover events that resulted in complete or partial deletion of 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

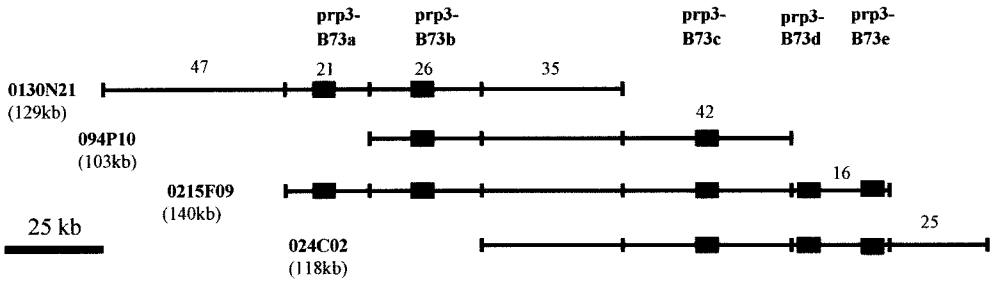

FIGURE 10.—A BAC contig was assembled across the *rp3* locus in the maize line B73. CUGI's address designation for each clone is shown on the left. Size estimations for each clone (in parentheses) were obtained by summing the molecular weights of all fragments in a complete *Hin*dIII digest. Solid boxes represent where the NBS and LRR region probes hybridized, giving the approximate location of the five PIC13 family members, prp3-B73a–e.

the *rp3* locus. The *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 *Rp3* gene, all had lost resistance against IN2. A more likely alternative is that *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 *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. Recessively inherited resistance genes are often considered to be functionally different from dominant R genes (Büschges *et al.* 1997; Deslandes *et al.* 2002), but the results with *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 *Avr* genes. Examples include the *Cf-2/5* (Dixon *et al.* 1996, 1998) and *Cf-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 *rp1* (Saxena and Hooker 1968). Only a single specificity could be differentiated for *Rp3* when a series of rust biotypes were inoculated onto the six *Rp3* alleles. Unlike *rp1*, no obvious novel specificities or lesion mimic phenotypes were identified in any of the variants generated in the present study. The *Rp3-AD4* variant differed from the parental *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 *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 *Rp3* gene activity in *Rp3-AD4* homozygotes and *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 *Rp3-AD4*, has been identified at the *rp1* complex. The *Rp1-D**5* gene is a recombinant gene with the LRR derived from *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 *Rp1-D* (Richter *et al.* 1995). The *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 *Rp3-AD4* haplotype was compared to the parental haplotypes. These small hybridizing *Hpa*II fragments that indicate the methylation state of the *Rp3-AD4* haplotype has changed relative to the two parents. It is therefore possible that the reduced resistance from the *Rp3-AD4* gene was due to a reduced expression associated with methylation changes. In Arabidopsis, an NBS-LRR resistance gene cluster containing *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 *rp3* and *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 *Rp3* genes are only ~25% identical to the different *Rp1* genes in predicted amino acid sequence. 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, *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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