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Molecular Characterization of the Maize *Rp1-D* Rust Resistance Haplotype and Its Mutants

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The *Rp1-D* gene for resistance to maize common rust (*Puccinia sorghi*) is a member of a complex locus (haplotype) composed of *Rp1-D* and approximately eight other gene homologs. The identity of *Rp1-D* was demonstrated by using two independent gene-tagging approaches with the transposons *Mutator* and *Dissociation. PIC20*, a disease resistance (*R*) gene analog probe previously mapped to the *rp1* locus, detected insertion of *Dissociation* in an *Rp1-D* mutation and excision in three revertants. Independent libraries probed with the *PIC20* or *Mutator* probes resulted in isolation of the same gene sequence. *Rp1-D* belongs to the nucleotide binding site, leucine-rich repeat class of *R* genes. However, unlike the rust resistance genes *M* and *L6* from flax, the maize *Rp1-D* gene does not encode an N-terminal domain with similarity to the signal transduction domains of the Drosophila Toll protein and mammalian interleukin-1 receptor. Although the abundance of transcripts of genes from the *rp1* complex changed with leaf age, there was no evidence of any change due to inoculation with avirulent or virulent rust biotypes. A set of 27 *Rp1-D* mutants displayed at least nine different deletions of *Rp1-D* gene family members that were consistent with unequal crossing-over events. One mutation (*Rp1-D*-24*) resulted in deletion of all but one gene family member. Other unique deletions were observed in the disease lesion mimic *Rp1-D*-21* and the partially susceptible mutant *Rp1-D*-5.* Different *rp1* specificities have distinct DNA fingerprints (haplotypes). Analysis of recombinants between *rp1* specificities indicated that recombination had occurred within the *rp1* gene complex. Similar analyses indicated that the rust *R* genes at the *rp5* locus, 2 centimorgans distal to *rp1*, are not closely related to *Rp1-D.*

INTRODUCTION

The *rp1* locus for resistance to maize common rust maps to the distal end of the short arm of maize chromosome 10 (Rhoades, 1935). Fourteen different resistances have been given the *rp1* designation on the basis of map position (Saxena and Hooker, 1968), and a number of these have been genetically recombined, suggesting that they are encoded by members of a gene cluster (Saxena and Hooker, 1968; Hulbert and Bennetzen, 1991). Different *rp1* genes spontaneously mutate to susceptibility at frequencies between 0.016 and 0.5% (Pryor, 1987; Bennetzen et al., 1988). It has been proposed that the instability of *rp1* genes is due to gene conversion or unequal crossing-over events between mispaired sequence repeats at the *rp1* locus during meiosis (Sudupak et al., 1993; Hu and Hulbert, 1994). Mutants of *rp1* also include disease lesion mimics (Hu et al., 1996) and mutants with novel resistance specificities (Richter et al., 1995). The molecular analysis of these mutants promises to shed light on the processes underlying resistance and the way in which natural plant populations generate variability at resistance loci.

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During the last 6 years, a number of gene-for-gene–type plant disease resistance (*R*) genes have been isolated (reviewed in Baker et al., 1997). The majority of these genes encode a putative nucleotide binding site (NBS) and a leucine-rich repeat (LRR) region. NBS-LRR *R* genes have been isolated from a range of monocot and dicot plant species and confer resistance to bacteria, viruses, fungi, nematodes, and insects (Baker et al., 1997; Milligan et al., 1998; Rossi et al., 1998; Vos et al., 1998). Conserved amino acid motifs in and around the putative NBS of NBS-LRR resistance proteins have facilitated the polymerase chain reaction (PCR) amplification of *R* gene–like sequences from plant genomes (Kanazin et al., 1996; Leister et al., 1996, 1998; Yu et al., 1996; Aarts et al., 1998; Shen et al., 1998; Speulman et al., 1998). Using this approach, we identified a sequence that hybridized with a small gene family at the *rp1* locus (Collins et al., 1998). In this study, we report the identification of the member of this gene family that confers the *Rp1-D* rust resistance specificity by using two independent transposon-tagging procedures. We also report that *rp1* transcription is unaffected by rust inoculation, describe deletion mutants of *Rp1-D* derived from probable unequal crossing-over events, and show that the *rp5* rust resistance gene located 2 centimorgans distal to *rp1* is not closely related to *Rp1-D.*

RESULTS

Tagging with *Mutator*

Mutator (*Mu*) tagging experiments were designed to generate mutants of the *Rp1-A*, *Rp1-B*, or *Rp1-D R* genes. Plants heterozygous for *rp1* genes (*Rp1-A*/*Rp1-D* and *Rp1-B*/*Rp1-D*) and containing multiple active *Mu* elements were pollinated by *Rp1-J* homozygotes to produce two families (1 and 2, respectively), and the progeny were screened with the rust biotype IN2, which is virulent on *Rp1-J*–containing plants but not on plants containing *Rp1-A*, *Rp1-B*, or *Rp1-D.* In family 1, 30 susceptible plants were identified from ~100,000 plants; in family 2, 27 susceptible plants were identified from ~45,000 plants.

To distinguish potential *Mu* insertions from recombination events between the *R* genes present in the heterozygous parents, we scored each susceptible individual for the restriction fragment length polymorphism (RFLP) markers bnl3.04 and ksu3, which closely flank the *rp1* locus (Hulbert and Bennetzen, 1991). Twenty-seven susceptible plants derived from family 1 were recombinant for flanking markers, whereas the remaining three (NCO1, NCO2, and NCO3) contained flanking marker alleles from the *Rp1-D* chromosome, indicating that they were derived from the *Rp1-D* gene by non-cross-over events (data not shown). Of the susceptible individuals identified in family 2, one was missing both flanking markers from *Rp1-B* and *Rp1-D* (suggesting that it was the result of a large deletion), 23 were recombinant, one had the flanking marker alleles of the *Rp1-B* parent, and the remaining two both contained the flanking marker alleles of the *Rp1-D* parent (NCO4 and NCO5; data not shown). The NCO1, NCO2, NCO3, and NCO4 individuals were crossed to maize lines homozygous for detectable *rp1* alleles (*Rp1-J* or *Rp1-C*) but lacking *Mu* elements, and the hybrids were backcrossed one or two times to these lines to reduce the number of *Mu* elements. Backcross families segregating for the mutant alleles were then subjected to RFLP analysis with *Mu* probes, and in one family of 90 progeny segregating for NCO 4, a HindIII restriction fragment of ~5.0 kb cosegregated with the mutant allele (data not shown).

Tagging with *Activator* and *Dissociation*

Experiments designed to obtain *Activator* (*Ac*) or *Dissociation* (*Ds*) insertion mutants of *Rp1-D* were performed concurrently with *Mu* tagging experiments. These tagging experiments, partially described by Pryor (1993), are summarized in Figure 1. The first mutant screen was performed by pollinating lines homozygous for *Rp1-D* and heterozygous for *Ac* with an *Rp1-M* line and inoculating the progeny with rust race R1, which is avirulent on *Rp1-D* but virulent on *Rp1-M.* Of ~171,000 progeny screened, 23 mutants with altered resistance phenotype were identified (Figure 1). These included 21 fully susceptible mutants, one partially susceptible mutant (*Rp1-D*-5*), and one susceptible mutant with a disease lesion mimic phenotype (*Rp1-D*-21*; Hu et al., 1996).

Only a small proportion of these mutants were expected to have been caused by *Ac*/*Ds* activity, because the frequency with which the mutations were obtained (0.014%) was similar to the mutation frequency of 0.016% observed for *Rp1-D* in a genetic background not known to contain any active transposons (Pryor, 1987). To help distinguish mutants caused by transposon insertion from those caused by other mutation events, such as deletions due to unequal crossing over, we tested the mutants for their ability to revert in the presence of *Ac* (Pryor, 1993). The disease lesion mimic *Rp1-D*-21* and 17 fully susceptible mutants were tested, and all were found to be stable. Although no resistant revertants were obtained from *Rp1-D*-5*, this partially susceptible line gave rise to fully susceptible progeny at a frequency of 0.23% in the presence of *Ac* (Figure 1). No such mutants of *Rp1-D*-5* were identified in the absence of *Ac* in a similar-sized sample (Figure 1), indicating that the instability of this line was *Ac* dependent.

Homozygous mutant lines were obtained by self-pollinating the F_1 mutant plants and using resistance assays to select F_2 or F_3 progeny lacking the *Rp1-M* gene derived from the pollen parent (Pryor, 1993). With rust race R1, the F_2 or F_3 progeny showed the same resistance phenotypes in the homozygotes as were observed in the original F_1 seedlings, except for *Rp1-D*-13*, which had been selected as a fully susceptible F_1 seedling but was fully resistant and indistinguishable from the *Rp1-D* parent in the homozygote. We hypothesized that *Rp1-D*-13* had contained a transposon in the *Rp1-D* gene, which excised to give a functional resistance allele before the homozygote was recovered, and that such a transposon may have moved to a location close to *Rp1-D*, given that transposons tend to transpose to linked sites (Greenblatt, 1984; Dooner and Belachew, 1989).

Anticipating that a closely linked transposon in *Rp1-D*-13* may give rise to a relatively high frequency of transposon insertions in *Rp1-D*, further tagging experiments were performed with the resistant *Rp1-D*-13* line. *Rp1-D*-13* gave rise to susceptible mutants at frequencies of 0.14 and 0.04% in the presence and absence of *Ac*, respectively (Figure 1), indicating *Ac*-dependent instability. Two of the mutants obtained from *Rp1-D*-13* in the presence of *Ac* (*Rp1-D*-13-2* and *Rp1-D*-13-3*) were tested for their ability to revert. Whereas *Rp1-D*-13-2* gave rise to resistant revertants at a frequency of 0.21%, no revertants were obtained for *Rp1-D*-13-3* (Figure 1). Thus, along with the partially susceptible *Rp1-D*-5* mutant, *Rp1-D*-13-2* was identified as a potential *Ds* insertion mutant.

DNA gel blot analysis with *Ac* and *Ds* probes identified a number of newly transposed elements in the *Rp1-D*-13-2* and *Rp1-D*-5* mutants. However, when segregating families

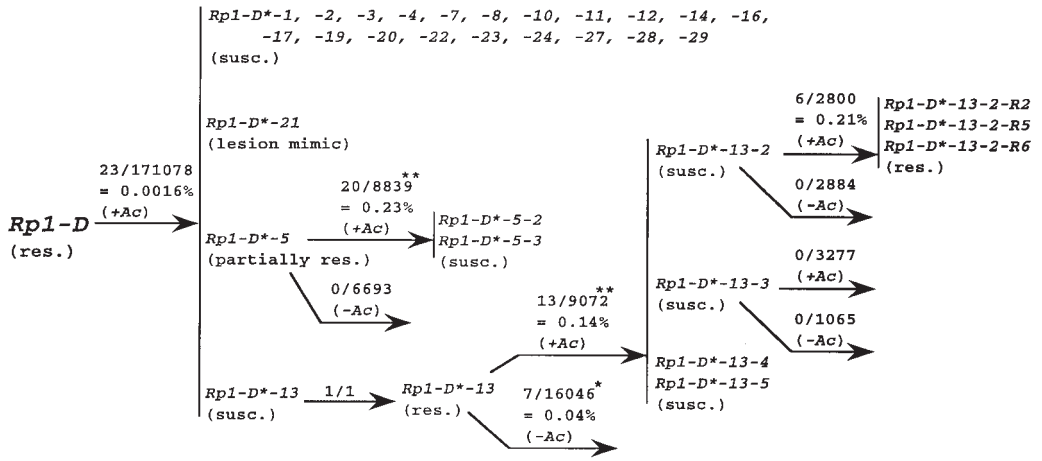

Figure 1. Summary of *Ac*/*Ds* Tagging Experiments.

Shown is a diagram summarizing experiments, performed previously (Pryor, 1993) and in this study, the goal of which was to tag *Rp1-D* with *Ac* or *Ds.* Mutants were identified in genetic backgrounds with (+Ac) or without (−Ac) *Ac*, either directly from wild-type *Rp1-D* homozygotes or in second- or third-round mutation experiments. Susceptible (susc.), partially susceptible, and disease lesion mimic mutants were identified, together with resistant (res.) revertants. The numbers of the mutants observed and the size of the testcross populations screened are indicated, together with the corresponding percentage of mutation frequencies. The *Rp1-D*-13* line was identified as a susceptible seedling in the initial screen, but when a line homozygous for the mutant chromosome was obtained, it was resistant—hence, the 1/1 reversion frequency. Mutation frequencies significantly greater than that previously observed for *Rp1-D* in the absence of any known transposons (0.016%; Pryor, 1987) were observed in *Rp1-D*-5* and *Rp1-D*-13*, at either the $P < 0.01$ level (**) or the $P < 0.05$ level (*).

were examined, none of these was found to be linked to *rp1* (data not shown). This search for transposable elements in the mutant genes was hampered by the extremely complex hybridization pattern shown by the probes, which reflected the high copy number of *Ac*- and *Ds*-related sequences in the maize genome.

In a previous exercise, we used PCR to isolate 11 non-cross-hybridizing classes of sequences from maize that encode products with similarity to the NBS regions of NBS-LRR resistance proteins (Collins et al., 1998). One of these *R* gene–like sequences (PIC20) hybridized with a small gene family of approximately nine members at the *rp1* locus. An examination of the potential *Ds* insertion mutants of *Rp1-D* and parental lines with the *PIC20* probe detected no differences between wild-type *Rp1-D* and the resistant *Rp1-D*-13* line (Figure 2). However, in the susceptible *Rp1-D*-13-2* mutant, one hybridizing 6.9-kb NcoI restriction fragment was replaced by another fragment ~400 bp longer, and in three resistant revertants of *Rp1-D*-13-2*, the hybridization pattern was once again indistinguishable from that of wild-type *Rp1-D* (Figure 2). Thus, the *PIC20* probe revealed the presence or absence of an insertion at the *rp1* locus in *Rp1-D*-13-2* and its revertants that was perfectly correlated with loss or gain of *Rp1-D* resistance, respectively. *PIC20* revealed deletions in three other mutants of *Rp1-D*-13*, in the partially susceptible line *Rp1-D*-5* and in two fully susceptible derivatives of *Rp1-D*-5* (Figure 2).

Cloning the *Rp1-D* Gene

A λ genomic DNA library of the NCO4 *Rp1-D* mutant containing the *Mu* insertion was made, and a clone containing the *rp1*-linked 5.0-kb HindIII fragment was isolated from it using a *Mu* probe. Sequencing showed that the clone contained a 1736-bp *Mu* element almost identical to *Mu1.7* (Taylor and Walbot, 1987), which, like other *Mu* insertions, was flanked by 9 bp of directly duplicated target sequence, in this case, 5′-ATCCTGGGT-3′. A genomic DNA library of the *Rp1-D*-13-2* mutant was also made and screened with *PIC20*, and one positive 13-kb clone was found by sequencing to contain a member of the *Ds1* family of transposable elements (Gerlach et al., 1987) within the region covered by the *PIC20* probe. The sequence of the 399-bp *Ds* element was 95 to 99% identical to those of other *Ds1*-type transposons represented in the databases, and like other *Ds* insertions, it was flanked on either side by 8 bp of directly duplicated target sequence, in this case, 5′-GCTGGAAG-3′. Sequencing of the entire sequence flanking the *Mu* element in the 5.0-kb HindIII clone and 5.6 kb of the sequence flanking the *Ds* element showed that the two transposable elements were inserted into the same NBS-LRR gene (GenBank accession number AF107293), with the *Mu* insertion site located 1219 bp downstream relative to the *Ds* insertion site. Thus, the two independent *Rp1-D* cloning approaches identified the same gene.

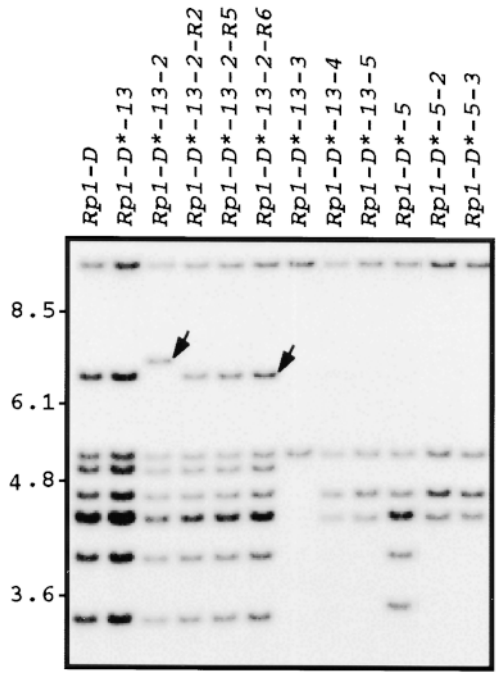

Figure 2. DNA Gel Blot Analysis of Potentially *Ac*/*Ds*-Tagged *Rp1-D* Mutants.

DNA of mutants and the parent *Rp1-D* line were cut with NcoI and probed with the *PIC20* probe, which is derived from the NBS-encoding region of *Rp1-D.* Arrows indicate restriction fragments altered in size by an insertion/excision correlated with loss/gain of *Rp1-D* resistance in the susceptible *Rp1-D***-13-2* mutant and its resistant revertants *Rp1-D***-13-2-R2*, *-R5*, and *-R6.* Mutants *Rp1-D***-5*, *Rp1-D***-5-2* and *-5-3*, and *Rp1-D***-13-3*, *-13-4*, and *-13-5* all showed deletions of *Rp1-D* gene family members. DNA length markers (in kilobases) are shown at left.

cDNA Analysis

RNA isolated from leaves of *Rp1-D* plants was used to construct a cDNA library, and clones hybridizing with the *PIC20* probe were identified from a screen of ~250,000 plaques. Ten of the positive clones were identical in sequence to the *Rp1-D* gene, and together with a clone of the 5′ end of the transcript obtained by PCR (see Methods), these provided complete coverage of the transcribed region and enabled the ends of the mRNA and the introns to be defined. Although no introns were identified in the coding region, one intron of 142 bp was identified in the 5′ untranslated region, and another of 79 bp was identified in the 3′ untranslated region 28 bp after the translation stop codon. The first intron was absent in the clone of the 5′ end of the transcript obtained by PCR but was present in the only *Rp1-D* cDNA clone from the library that spanned this region, indicating that the first intron is not always spliced from the *Rp1-D* transcript.

Two other cDNA clones of NBS-LRR genes were obtained in the cDNA library screen and were 94% identical to one another and to *Rp1-D.* The remaining positive clones were derived from a gene designated *rp1-Cin4* (GenBank accession number AF107294), composed of a 5′ domain related to *Rp1-D* and a 3′ region of 475 bp with similarity to the *Cin4* family of retrotransposon-like elements (i.e., 64% identity to the sequence with GenBank accession number Y00086; Schwarz-Sommer et al., 1987). Translation of *rp1-Cin4* results in a truncated protein containing a putative NBS but no LRRs (data not shown). Truncated proteins containing an NBS but no LRRs are predicted products of genes from other NBS-LRR resistance loci (Whitham et al., 1994; Dinesh-Kumar et al., 1995; Lawrence et al., 1995; Parker et al., 1997; Ayliffe et al., 1999). However, it has not been determined whether these presumptive truncated proteins play a role in resistance.

The RP1-D Protein

Rp1-D encodes a 1292–amino acid protein containing the P loop and kinase-2 nucleotide binding motifs (Traut, 1994) and an LRR domain (Figure 3) and therefore belongs to the NBS-LRR class of plant *R* genes (Baker et al., 1997). Amino acid sequence similarity to other NBS-LRR resistance proteins was also observed throughout the protein, especially in regions around the putative NBS previously shown to be well conserved in this class of resistance protein (Grant et al., 1995; Lawrence et al., 1995; Ori et al., 1997). Unlike the flax rust resistance proteins L6 and M (Lawrence et al., 1995; Anderson et al., 1997), the RP1-D protein lacks an N-terminal TIR domain (Baker et al., 1997) with similarity to the Drosophila Toll protein and the mammalian interleukin-1 receptor. The RP1-D protein also lacks a leucine zipper motif, such as the ones present at the N termini of the RPM1, RPS2, and MI-1 resistance proteins (Bent et al., 1994; Mindrinos et al., 1994; Grant et al., 1995; Milligan et al., 1998). The leucine-rich domain of the RP1-D protein contains ~24 repeats that resemble the consensus for cytoplasmic LRRs proposed by Jones and Jones (1997) (Figure 3). The repeats in RP1-D are irregular and are interrupted by a region that cannot be easily arranged into repeats (Figure 3).

cDNA analysis identified a class of *Rp1-D* transcripts that retained intron 1. In this class of transcripts, an in-frame ATG start codon within intron 1 could initiate translation of a protein with an N-terminal 16–amino acid extension relative to the protein translated from the fully spliced transcript. A number of plant genes are known to retain introns in a fraction of their transcripts, and this phenomenon is thought to reflect a relatively inefficient intron splicing mechanism in plants (Nash and Walbot, 1992; Simpson and Filipowicz, 1996). Therefore, the longer alternative version of the RP1-D protein may simply be the consequence of an inefficient splicing mechanism and may play no specialized role in resistance.

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mclqrkkkksfllnfMADLALALAGLRWAASPIVNELLTKAS
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GQCPLAAKVLGSRLCRKKDIAEWKAALKIGDLSDPFTSLLW
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DILHDFAESLSREDCFRLEDDNVTEIPCTVRHLSIHVHSMQ
KHKQIICKLHHLRTIICIDPLMDGPSDIFDGM

LRNQRKLRVLSLSFYN-SKNLPES
IGELKHLRYLNLIRTL-VSELPRS
LCTLYHLQLLWL--NHMVENLPDK
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LRQLKDLNELGGSLKVKNLENVIGKDEAVESK
LYLKSRLKELALEWSSENGMDAMDILEG
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RSYFENLESFQLSNCSLLEGLPPD
TELLRNCSRLRINFVPNLKELSN
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RVLFEDYVSLIRLMTLMMDDDISKHLQIIGSVLV
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LVLPSGLCELSLSSCS-ITDEALAIC
LGGLTSLRTLQLKYNMALTTLPSEKV
FEHLTKLDRLVVSGCLCLKSLGGLRAAPS
LSCFNCWDCPSLELARGAELMPLN
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LCFVEGLSSLHLKRLSLVDVANLTAKC
ISPFRVQESLTVSSSVLLNHMLM
AEGFTAPPNLTLLDCK-EPSVSFEE
PANLSSVKHLHFSCCE-TESLPRN
LKSVSSLESLSIERCPNIASLPDLPSS
LQRITILNC

PVLMKNCQEPDGESWPKISHVRWKSFPPKSIWLP
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Figure 3. The RP1-D Protein.

Underlined sequences in the N-terminal half of the protein correspond to the P loop and kinase-2 nucleotide binding motifs (Traut, 1994). The N-terminal extension resulting from retention of intron 1 is shown in lowercase letters. The C-terminal portion of the protein contains two sections with irregular LRRs (bracketed) separated by a region that cannot be easily arranged into repeats. The repeats resemble the consensus L--L--L--L-L--(N/C/T)-(-)L--IP-- proposed by Jones and Jones (1997) for cytoplasmic LRRs (where a hyphen is any residue). Residues in boldface indicate where the RP1-D repeats conform to this consensus or where the L and I residues are substituted for the residues F, I, M, or V.

Analysis of *rp1* Transcription

RNA gel blot analysis using the *PIC20* probe (NBS region) identified transcripts of two sizes that were present in approximately equal abundance in leaves of *Rp1-D* maize plants (Figure 4A). Only the larger transcript class also hybridized with a probe for the LRR-encoding region of *Rp1-D*, indicating that this transcript class is derived from NBS-LRR genes (Figure 4A). This transcript class represents a mixture, because the cDNA library screen showed that at least two other NBS-LRR members of the *Rp1-D* gene family were transcribed in addition to *Rp1-D.* The smaller transcript class was shown to be derived from the *rp1-Cin4* gene, because it hybridized with a probe made from the *Cin4*-related sequence in *rp1-Cin4* (Figure 4A).

Transcript levels of the *Rp1-D* gene family members were examined in leaves of *Rp1-D* homozygous seedlings inoculated with incompatible (R1) or compatible (R2) rust races. The levels of *rp1*-related transcripts were determined in seedlings at 0, 12, 36, and 60 hr after rust inoculation or

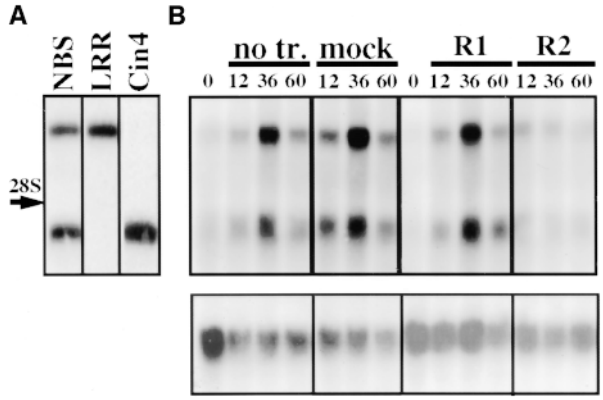

Figure 4. Analysis of *rp1* Transcription.

(A) Identification of NBS-LRR and *rp1-Cin4* transcripts. A single lane containing ~3.0 μg of poly(A)$^+$-enriched leaf RNA from homozygous *Rp1-D* plants was blotted and probed sequentially with probes for the NBS and LRR coding sequences of *Rp1-D* and the *Cin4*-related sequence present in *rp1-Cin4.* The NBS probe identified transcripts of two different sizes. The LRR and Cin4 probes indicated that the larger and smaller transcript classes were derived from NBS-LRR and *rp1-cin4* members of the *Rp1-D* gene family, respectively. The position of the 28S rRNA is indicated.

(B) Testing for effects of rust on *rp1* transcription. Ten-day-old homozygous *Rp1-D* seedlings were inoculated separately with the rust biotypes R1 (avirulent on *Rp1-D* plants) and R2 (virulent on *Rp1-D* plants), mock inoculated (sprayed with water minus rust spores), or left untreated (no tr.). The first leaf and the end half of the second leaf, corresponding to tissue exposed to rust spores in the inoculated samples, were taken for RNA extraction at the time of inoculation (0 hr) and at 12, 36, and 60 hr after inoculation. Some seedlings that were left unharvested were scored 8 days after inoculation to monitor the effectiveness of rust infection. Blots containing 20 μg of total RNA per lane were probed with the NBS *PIC20* probe (top gel). The same blots were also probed with the rRNA probe *rTA71* (bottom gel). The abundance of NBS-LRR or *rp1-Cin4* transcripts was not noticeably greater in the treated samples than in the untreated samples, indicating that transcription of *Rp1-D* gene family members is not induced by rust infection or by the inoculation procedure. In general, the abundance of both transcript types in treated and untreated seedlings increased between the 0- and 36-hr time points and then decreased between the 36- and 60-hr time points.

mock inoculation and in untreated controls. The results of one experiment are presented in Figure 4B. Transcript abundance increased at the 36-hr time point in the incompatible (R1) interaction and then decreased. However, the same pattern of transcript accumulation occurred in both the untreated and mock-treated control seedlings. In the experiment shown in Figure 4B, transcript accumulation was not observed in the equivalent compatible (R2) interaction. In other experiments (data not shown), transcript levels did accumulate following innoculation with the R2 race. The reason for this discrepancy is unknown. We conclude that whereas *rp1* transcript levels may be influenced by the stage of leaf development, there is no clear correlation between increases in levels of *rp1* transcripts and innoculation with incompatible or compatible rust races.

Rp1-D Deletion Mutants

Most of the mutations identified in the *Ac*/*Ds* tagging studies did not revert in the presence of *Ac*, suggesting that they were caused by events other than transposon insertion. To determine the nature of these mutations, we performed DNA gel blot analysis with the *PIC20* probe. All of the 27 mutants contained fewer hybridizing restriction fragments than did wild-type *Rp1-D* plants (Figure 5) but still contained the flanking RFLP markers bnl3.04 and ksu3 (data not shown), indicating that they had resulted from interstitial deletions. The *Rp1-D* coding region contains a 2.6-kb BglI fragment, a 2.0-kb HincII fragment, and a 1.6-kb HindIII-NsiI fragment detectable using the *PIC20* probe. Each mutant was missing at least one of these fragments (data not shown), indicating that the *Rp1-D* coding region had been deleted or rearranged.

On the basis of the hybridization patterns obtained with the restriction enzymes AccI, BglI, BglII, DraI, NcoI, and NsiI, the mutants were of at least nine different types. *Rp1-D*-5*, *-17*, *-19*, *-21*, and *-24* were each unique, whereas the other mutant classes contained two (*Rp1-D*-4* and *-13-3*), four (*Rp1-D*-7*, *-12*, *-28*, and *-5-2*), five (*Rp1-D*-10*, *-11*, *-14*, *-16*, and *-29*), and 11 (*Rp1-1*, *-2*, *-3*, *-8*, *-20*, *-22*, *-23*, *-27*, *-13-4*, *-13-5*, and *-5-3*) representatives. Representatives within each of these groups could not be distinguished by using the six restriction enzymes. Despite the instability shown by *Rp1-D*-13* and *Rp1-D*-5*, susceptible mutants derived from these lines (with the exception of *Rp1-D-13-2* tagged with *Ds*) were indistinguishable from mutants obtained directly from *Rp1-D* plants. An examination of families segregating for mutations from each class with the *PIC20* probe demonstrated that *PIC20* only identifies sequences at the *rp1* locus (data not shown). Hence, differences in the hybridization patterns between the mutants reflected the type of deletion that occurred at *rp1. Rp1-D*-24* carried the largest deletion, because only one hybridizing band was detected in this mutant with each restriction enzyme. The smallest deletion was carried by *Rp1-D*-5*, which, depending on the restriction enzyme, showed a loss of zero to two hybridizating bands.

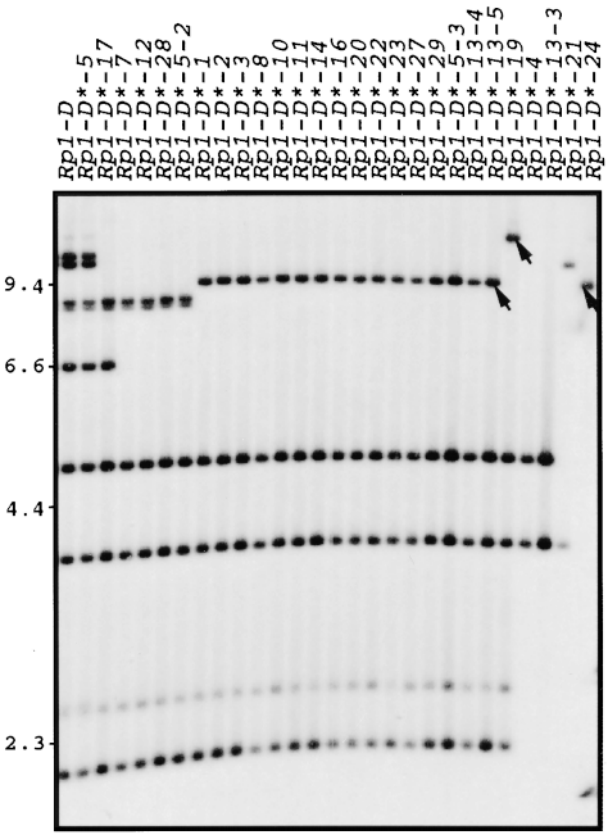

Figure 5. DNA Gel Blot Analysis of 27 *Rp1-D* Mutants.

DNA of wild-type *Rp1-D* plants and mutants was cut with the restriction enzyme AccI and probed with the *PIC20* probe for the NBS-encoding region of *Rp1-D.* The mutants showed a range of deletions of *Rp1-D* gene family members (*Rp1-D*-5* is indistinguishable from wild-type *Rp1-D* plants on this blot, but other restriction enzymes showed that this mutant contains a deletion). Mutants are arranged in order of decreasing deletion size. Novel-sized restriction fragments in the mutants are indicated by arrows. DNA length markers (in kilobases) are shown at left.

Fragments that differed in size from those in wild-type *Rp1-D* plants were identified in all of the deletion mutants, except for *Rp1-D*-4*, *-7*, *-12*, *-17*, *-21*, *-28*, *-5-2*, and *-13-3* (e.g., the AccI restriction fragments indicated by arrows in Figure 5). Detection of the novel restriction fragments with the *PIC20* probe indicated that the majority of the deletions involved recombination or other rearrangements within or close to the coding regions of *Rp1-D* gene family members.

rp1 Locus Haplotypes

The *rp1* genes described by Saxena and Hooker (1968) are available in a series of near-isogenic lines in the R168 back-

ground. DNA gel blot analysis with *Rp1-D* probes was used to examine DNAs of 13 of these R168 lines following digestion with a range of restriction enzymes (e.g., Figure 6). Some of the lines gave hybridization patterns that could not be clearly distinguished. Specifically, the *Rp1-C*, *Rp1-L*, and *Rp1-N* lines could not be distinguished from one another, nor could the *Rp1-E*, *Rp1-I*, and *Rp1-K* lines or the *Rp1-A* and *Rp1-F* lines (Figure 6). Those lines that showed indistinguishable hybridization patterns with *Rp1-D* probes could also not be distinguished by using our current set of 11 rust races (Hulbert et al., 1991). The fact that each of these lines contains unique flanking RFLP marker genotypes (Hong et al., 1993; S. Hulbert, unpublished data) makes it unlikely that the lines were mislabeled during handling. All of the remaining R168 lines showed unique hybridization patterns with *Rp1-D* probes. Of these lines, only *Rp1-J* and *Rp1-H* cannot be distinguished from one another by using our current collection of rust races (Hulbert et al., 1991).

The reason we could not distinguish the resistance specificities of some of the R168 lines is unknown. It may reflect limitations of the current set of rusts, but the identical hybridization patterns given by these lines with *Rp1-D* probes raise other possibilities. For example, the original set of rusts (no longer available) used by Hooker's group to characterize *rp1* genes may have been capable of differentiating the apparently similar haplotypes. Alternatively, some of the original rust resistance phenotypes may have been the result of identical *rp1* haplotypes, which were modified by unlinked genes that were eliminated during introgression into the R168 background.

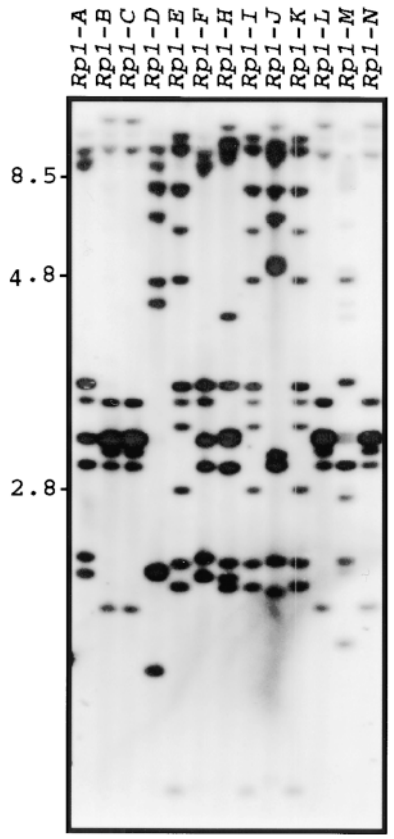

Figure 6. DNA Gel Blot Analysis Profiles of *rp1* Locus Haplotypes.

DNA of near-isogenic lines containing different *rp1* genes in the R168 background was cut with the restriction enzyme EcoRV and analyzed with a probe for the NBS-encoding domain of *Rp1-D.* Overall, the hybridization patterns reflected our ability to distinguish these lines by using our current collection of rust biotypes (see text for details). Although *Rp1-B* does not appear different from *Rp1-C*, *Rp1-L*, and *Rp1-N* here, it was clearly different from the other lines when other restriction enzymes were used. DNA length markers (in kilobases) are shown at left.

Recombination Analysis of Rust Resistance Specificities in the *rp1* Region

Different *rp1* rust resistance specificities can be genetically recombined, and chromosomes recombinant for two different *rp1* specificities (either double resistant or double susceptible) have been selected from testcross progeny by using appropriately chosen rust biotypes (Saxena and Hooker, 1968; Hulbert and Bennetzen, 1991; Hulbert et al., 1993). *Rp1-D*/*Rp1-J* recombinants subjected to DNA gel blot analysis with an *Rp1-D* probe for the NBS region display combinations of fragments from both parents, indicating that the recombination events had occurred within the cluster of *Rp1-D* homologous genes from each parent (Figure 7). Other recombinants for different pairs of *rp1* genes also showed combinations of hybridization bands from both parents when examined with *Rp1-D* probes (data not shown). Together, these data support the hypothesis that the other uncloned *rp1* specificities are encoded in gene families related to *Rp1-D.*

We have performed a similar analysis of lines recombinant for *Rp1-D* and the *Rp5* rust *R* gene, which is located 1 to 3 centimorgans distal to the *rp1* locus (Wilkinson and Hooker, 1968; Hulbert and Bennetzen, 1991). With the restriction enzyme EcoRV, the hybridization patterns of the double-resistant recombinants were identical to that of the *Rp1-D* parent, whereas the hybridization pattern of the double-susceptible recombinant was identical to that of the *Rp5* parent (Figure 7).

Although some of the parental EcoRV fragments shown in Figure 7 are monomorphic, analysis of these and other *Rp5*/*rp1 R* gene recombinants with a range of restriction enzymes has failed to identify an *Rp1-D*–related restriction fragment associated with *Rp5* resistance (data not shown). Analysis of randomly chosen progeny from segregating populations has also failed to identify any *Rp1-D*–related

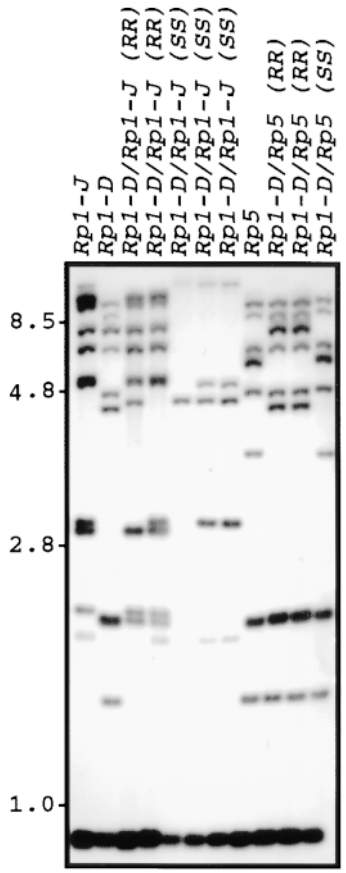

Figure 7. DNA Gel Blot Analysis of *Rp1-D*/*Rp1-J* and *Rp1-D*/*Rp5* Recombinants.

Lines homozygous for chromosomes and recombinant for pairs of rust resistance genes (*Rp1-D*/*Rp1-J* and *Rp1-D*/*Rp5*) were subjected to DNA gel blot analysis alongside the *Rp1-D*, *Rp1-J*, and *Rp5* parent lines. The restriction enzyme EcoRV and the *PIC20* probe for the NBS region were used. Of the *Rp1-D*/*Rp1-J* recombinants, two are double resistant (*RR*; containing both *Rp1-D* and *Rp1-J*) and three are double susceptible (*SS*; containing neither *Rp1-D* nor *Rp1-J*). Of the *Rp1-D*/*Rp5* recombinants, two are double resistant (*RR*; containing both *Rp1-D* and *Rp5*) and one is double susceptible (*SS*; containing neither *Rp1-D* nor *Rp5*). Each *Rp1-D*/*Rp1-J* recombinant displays a mixture of hybridization bands from both parents, suggesting that the *Rp1-J* gene belongs to the *Rp1-D* homologous gene cluster present in the *Rp1-J* parent. However, only bands from the *Rp1-D* parent are evident in each of the two double-resistant *Rp1-D*/*Rp5* recombinants, and only bands from the *Rp5* parent are evident in the double-susceptible *Rp1-D*/*Rp5* recombinant, suggesting that the nearby *Rp5 R* gene is not an *Rp1-D* homolog. DNA length markers (in kilobases) are shown at left.

sequences located more than a fraction of a centimorgan proximal or distal to the *rp1* genes (S. Hulbert, unpublished data). These data strongly suggest that the *Rp5* rust resistance gene is not closely related to the *rp1* genes.

DISCUSSION

Testing *R* Gene Identity

Regions of sequence conservation between the putative nucleotide binding domains of NBS-LRR resistance proteins have facilitated the PCR isolation of *R* gene–like sequences from a number of dicot and monocot species. Whereas some of these sequences have been shown to cosegregate with known *R* genes (Leister et al., 1996, 1998; Collins et al., 1998), verification of *R* gene identity requires either complementation in transgenic plants or a genetic approach such as point mutation or transposon tagging. In this study, independent insertions of *Ds* and *Mu* transposable elements into the same NBS-LRR gene were observed in *Rp1-D* mutants. In the case of the *Ds* insertion, excision of the *Ds* element was coincident with the *Ac*-dependent reversion to *Rp1-D* resistance. In lieu of a transgenic complementation test, these genetic data allowed unequivocal identification of the gene encoding the *Rp1-D* resistance specificity from among members of an NBS-LRR gene family that initially was identified by PCR.

Mutations of *Rp1-D*

Previous genetic studies of the *rp1* locus revealed the following features. Genes for different *rp1* resistance specificities can be combined in *cis*, suggesting that they belong to a gene cluster (Saxena and Hooker, 1968; Hulbert and Bennetzen, 1991). Lines homozygous for *rp1* rust resistance specificities give rise to susceptible individuals at high frequencies, indicating that *rp1* genes are unstable (Pryor, 1987; Bennetzen et al., 1988). The instability of *rp1* genes involves chromosome pairing, because *rp1* genes are stable in hemizygotes that have one copy of the *rp1* region deleted (T. Pryor, unpublished data). Most losses of *rp1*-mediated resistance in homozygotes involve meiotic recombination, as evidenced by the exchange of RFLP markers flanking the locus (Sudupak et al., 1993; Hu and Hulbert, 1994). The conclusions drawn from these data were that *rp1* resistance haplotypes probably consist of clusters of homologous genes that can undergo imperfect pairing and recombination in homozygotes or “unequal crossing-over,” resulting in deletions of active gene family members.

Our molecular analysis supports these conclusions. On the basis of DNA gel blot analysis with a probe for the NBS-

encoding region, the *Rp1-D* haplotype consists of a cluster of approximately nine homologous genes, including *Rp1-D.* In addition, 27 of the mutants obtained from homozygotes showed deletions of *Rp1-D* gene family members consistent with unequal crossing-over events. Theoretically, deletions caused by mispairing between a tandem array of repeated sequences could vary in size, depending on the degree of mispairing. The *Rp1-D***-24* mutant, which contains only one member of the *Rp1-D* family, appears to be a consequence of the most extreme form of mispairing, in which *Rp1-D* gene family members from opposite ends of the cluster paired and recombined, thereby deleting the intervening genetic material. Because the phenotype of the *Rp1-D***-24* mutant is normal, except for its rust susceptibility, this deletion suggests that there are no essential genes within the *rp1* complex. The other mutants showed a range of smaller deletions of at least six different sizes, presumably resulting from different, less extreme mispairing events.

The tomato *Cf-9* and *Cf-4* haplotypes that mediate resistance to *Cladosporium fulvum* each contain four homologs in addition to the respective *R* genes *Cf-9* and *Cf-4* (Parniske et al., 1997). Five recombination events between the *Cf-9* and *Cf-4* genes in *Cf-4*/*9* heterozygotes were identified by Thomas et al. (1997), and all of these arose from the same chromosome pairing configuration. The recombination events were found to have occurred in intergenic regions at sites showing relatively extensive similarity (Parniske et al., 1997). Therefore, it was proposed that the bias observed toward certain pairing/recombination events was due to the presence of these islands of intergenic sequence conservation that facilitated these events. This concept was supported by the study of Dooner and Martinez-Ferez (1997), in which points of recombination between alleles of the maize *bronze* gene were found to be significantly biased against regions that contained insertion/deletion differences or a high density of sequence polymorphisms. Although the *Rp1-D* haplotype appears to have mispaired in many different ways, the inability to distinguish many of the independent mutants by DNA gel blot analysis provided evidence for a bias toward certain mispairing/recombination events.

Recombination at the *rp1* locus can also give rise to disease lesion mimics (Hu et al., 1996). *Rp1-D***-21* and other lesion mimic mutants of *rp1* show loss of parental resistance and express a leaf-spotting phenotype, which histochemically resembles the hypersensitive lesions involved in the normal resistance response, suggesting that these mutants of *rp1* result from modification of *rp1* genes. Consistent with this observation is the fact that *Rp1-D***-21* contains a partial deletion of the *Rp1-D* haplotype, which is unique among the *rp1* mutants. A unique deletion was also identified in the partially susceptible mutant *Rp1-D***-5.* The low level of resistance in *Rp1-D***-5* shows the same specificity as wild-type *Rp1-D* resistance across a range of rust biotypes (Richter et al., 1995), suggesting either that this mutant may still contain the part of the *Rp1-D* gene involved in pathogen recognition or that two other homologs have combined to form a gene with the same resistance specificity as *Rp1-D.* Studies currently are under way to characterize the *Rp1-D***-21* and *Rp1-D***-5* mutations in further detail.

R Gene Transcription

Rust inoculation resulted in no detectable increase in the levels of *Rp1-D*–related mRNAs in seedling leaves. However, these transcripts increased and then decreased in both inoculated and uninoculated plants during the course of the experiment, suggesting that the expression of *Rp1-D* gene family members changes with development. Transcripts of the *Xa1* NBS-LRR bacterial resistance gene in rice were also reported to accumulate after pathogen inoculation and mock inoculation (Yoshimura et al., 1998). However, because controls receiving no treatment were not described, the possibility that this effect was due to plant development rather than pathogen inoculation cannot be ruled out completely. Possible developmental influences on transcription should be tested in any such experiment, especially in light of the present findings. Studies with the Arabidopsis *PTO* bacterial resistance gene, which encodes a protein kinase, and the *L6* flax rust resistance NBS-LRR gene have shown that transcription of these genes is not increased by pathogen inoculation (Martin et al., 1993; Ayliffe et al., 1999).

Relationship between *Rp1-D* and Other Rust *R* Genes in the *rp1* Region

Maize lines near-isogenic for different *rp1* resistance specificities display distinct and complex DNA hybridization patterns when an *Rp1-D* probe is used, indicating that these lines contain multiple related and closely linked genes. The analysis of recombinants between several pairs of *rp1* specificities using the *Rp1-D* probe demonstrated that recombination occurred within the *rp1* gene complex. From this observation, it is inferred that other specificities that map to the *rp1* locus may be encoded by genes closely related to *Rp1-D.* Confirmation will require mutational analysis or complementation in transgenic plants. A similar analysis of recombinants between *rp1* and *rp5*, which maps 1 to 3 centimorgans distal to *rp1*, did not identify any *Rp1-D*–related sequences that cosegregated with *rp5.* From this result, we infer that *rp5* is not closely related to *rp1*, although it may still be a member of the NBS-LRR superfamily of *R* genes.

Close genetic linkage has been observed between non-cross-hybridizing classes of NBS-encoding *R* gene–like sequences in a number of plant species (Kanazin et al., 1996; Collins et al., 1998; Leister et al., 1998; Spielmeyer et al., 1999). From these observations and those made in this article, the picture is emerging that plant chromosomes contain clusters of NBS-LRR genes. Without insertional mutagenesis or highly efficient cloning/complementation technology,

the identification of specific *R* genes by positional information alone will be difficult in most crop plants.

METHODS

Maize Lines

Lines with the *Pvv* (*variegated pericarp*) allele of the *P* gene containing an active *Activator* (*Ac*) element (Barklay and Brink, 1954) obtained from J. Kermicle (University of Wisconsin, Madison) were used as the source of *Ac* in the *Ac*/*Dissociation* (*Ds*) tagging experiments. Because *Ac* transposition is highest in maize lines containing only one *Ac* copy (Brink and Nilan, 1952), tagging was performed in lines heterozygous for *Pvv* over *Pww*, an allele of *P* that lacks *Ac.* Disease resistance (*R*) gene stability in the absence of *Ac* was tested in homozygous *Pww* lines. Absence of *Ac* activity was determined by testcrosses to a tester line (supplied by J. Kermicle), which carried a chromosome-breaking *Ds* located proximally to the gene *R* (colored aleurone). Absence of variegation for colored aleurone due to chromosome breakage indicated the absence of *Ac* activity. Lines containing multiple copies of *Mutator* (*Mu*) for use in *Mu* tagging experiments were obtained from G. Johal (University of Missouri, Columbia). R168 near-isogenic lines containing different *rp1* genes and *Rp5* were obtained from A. Hooker (formerly of the University of Illinois, Urbana).

cDNA and Genomic DNA Libraries

Genomic and cDNA libraries were constructed as recommended by the manufacturers of the vectors. HindIII fragments of 4 to 6 kb from the noncrossover event 4 (NCO4) *Rp1-D* mutant were purified using the GeneClean II kit (Bio-101, Vista, CA) and cloned into the λ DASH vector (Stratagene, La Jolla, CA). The *Rp1-D*-13-2* genomic library was made using partially digested Sau3AI DNA fragments that were size fractionated by glycerol gradient centrifugation and cloned into the BamHI site of the EMBL3 λ vector (Promega). To make the *Rp1-D* cDNA library, we used poly(A)$^+$-enriched RNA prepared from total leaf RNA by using Dynabeads oligo(dT)$_{25}$ (Dynal, Oslo, Norway) to make cDNA with the HybriZAP cDNA synthesis kit (Stratagene), and the cDNA was cloned into the HybriZAP two-hybrid λ vector (Stratagene). Plasmid stocks of purified cDNA clones were obtained by excising pAD-GAL4 phagemids from the HybriZAP vector in vivo and using them to transfect *Escherichia coli*, as recommended by the supplier of the vector.

Cloning of Transcript 5′ Ends

The 5′ ends of *Rp1-D* gene family member transcripts were amplified by using the SMART polymerase chain reaction (PCR) cDNA synthesis and Advantage cDNA PCR kits (Clontech, Palo Alto, CA), essentially using the protocol supplied with the kits, except that a nested PCR approach using several downstream primers complementary to the *Rp1-D* gene were used instead of the downstream primer provided. The sequences of the nested primers for sites increasingly close to the 5′ end of *Rp1-D* were P5 (5′-TTTGCTGCCAAAGGACATTGT-3′), P21 (5′-GCCCTCAAGGACATTGTACTC-3′), and P16 (5′-GGCCGCTTGAATCACCAGCTCGAACTG-3′). First-strand cDNA was made from total leaf RNA of *Rp1-D* plants in reactions containing the P5 primer and the SMART oligonucleotide. PCR was then performed in a 20-μL reaction containing one-tenth of the first-strand cDNA as template, the SMART oligonucleotide, and the P21 primer by using 35 cycles of 95°C for 30 sec, 50°C for 30 sec, and 72°C for 1.5 min. Using the same amplification conditions, we used one-thousandth of this reaction as a template in a 60-μL PCR containing the SMART oligonucleotide and the P16 primer. In the last amplification, Taq polymerase was used instead of the polymerase from the kit to facilitate cloning of the products into the T-easy plasmid vector (Promega).

Sequencing

λ DNA of purified genomic clones was prepared as described by Sambrook et al. (1989). λ DNA further purified by precipitation with polyethylene glycol or plasmid DNA of λ subclones or cDNA clones purified by the boiling method (Sambrook et al., 1989) was used as a template for sequencing using the Big-Dye sequencing system (Applied Biosystems, Foster City, CA). Sequence data were analyzed using computer programs from the Genetics Computer Group (Madison, WI) software package (Devereaux et al., 1984).

DNA and RNA Gel Blot Analyses

Gel blot analysis of maize genomic DNA was performed as previously described (Collins et al., 1998). Total maize leaf RNA for RNA gel blot analysis was extracted using the method of Logemann et al. (1987) and enriched for poly(A)$^+$ RNA by using the PolyATract mRNA isolation system (Promega). RNA samples were quantified by absorbance spectroscopy at 260 nm, subjected to denaturing agarose gel electrophoresis in 1.0% agarose gels containing formaldehyde (Sambrook et al., 1989), and blotted to a Hybond N membrane (Amersham), as recommended by the manufacturer of the membrane. Hybridization and autoradiography for RNA gel blot analysis were the same as given for DNA gel blot analysis. Membranes were hybridized with α-^{32}P-dCTP–labeled DNA probes overnight at 65°C and given four washes of ~7 min in 0.5 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 60°C before autoradiography. Clone *rTA71* (Gerlach and Bedbrook, 1979) was used for the detection of rRNAs.

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