Recombination Events Generating a Novel *Rp1* Race Specificity

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Submitted 30 August 2004. Accepted 8 November 2004.

Genes at the maize *Rp1* rust resistance complex often mispair in meiosis, which allows genes to recombine unequally, creating recombinant haplotypes. Four recombinant haplotypes were identified from progeny of an *Rp1-D/Rp1-I* heterozygote that conferred a nonparental resistance specificity designated *Rp1-I. Sequence comparisons of paralogs in the recombinant and parental haplotypes demonstrated that all four recombinants were derived from intergenic (between gene) recombination events. The sequence of paralogs in the *HRp1-I* parental haplotype indicated this haplotype includes 41 or more *rp1* genes, at least 31 of which are transcribed. The results indicate that most of the novel resistance specificities that have arisen spontaneously at *Rp1* are the result of reassortment of existing *Rp1* genes.**

Additional keywords: evolution, *R* gene.

Plants have evolved many different mechanisms to protect themselves from pathogen infection. The classic gene-for-gene mechanism allows the plant to recognize the invading pathogen, which in turn, activates the plant defense response system, resulting in resistance (Flor 1955; Keen 1990; Staskawicz et al. 1995). However, plant pathogen populations commonly alter or lose *Avr* genes, rendering them unrecognizable by the corresponding plant resistance gene protein, resulting in susceptibility (McDonald and Linde 2002). Therefore, plant populations must be able to generate new resistance specificities to survive. The patterns of nucleotide substitution when comparing alleles or family members of many resistance genes provides evidence that selection plays a key role in generating phenotypic diversity (Ellis et al. 2000; Mondragon-Palomino et al. 2003). Sequence comparisons of family members at complex *R* gene loci often show evidence of multiple exchanges, suggesting recombination also contributes to the generation of variants with novel race specificities (Hulbert et al. 2001; Parniske et al. 1997). Experimental examples of the creation of novel race specificities are very few. The best-characterized examples come from studies of the *L* locus of flax. This is a structurally simple locus with multiple alleles conferring different resistance specificities. Functional analysis of intragenic recombinants at the *L* locus have identified recombinants with resistance specificities different from either parental allele. Recombi-

nants between the *L6* and *L2* alleles conferred the *L2* or *L7* specificity and *L2/L10* and *L9/suL10* recombinants conferred previously uncharacterized specificities (Ellis et al. 1999, 2000).

Another resistance locus in which phenotypic variants have been selected is the *Rp1* rust resistance locus of maize. *Rp1* is a complex locus consisting of a highly variable cluster of nucleotide binding site (NBS)–leucine-rich repeat genes (Collins et al. 1999). The best-characterized *Rp1* haplotype is *HRp1-D*, which contains nine paralogs, seven of which are transcribed, including a truncated gene. Only one paralog is phenotypically detectable and is located on the most distal end of the array. Different *Rp1* haplotypes can vary in gene number and haplotype size due to mispairing and recombination in meiosis (Smith et al. 2004; Sun et al. 2001). The most common class of variants identified at *Rp1* are susceptible to all rust biotypes tested, but recombinant haplotypes with novel phenotypes also have been identified. These include haplotypes with nonspecific reactions to rust and lesion mimic phenotypes (Hu et al. 1996; 1997), haplotypes conferring reduced levels of resistance but parental race specificities (Sun et al. 2001), and haplotypes with nonparental race specificities (Richter et al. 1995). Many of these variants have been associated with intragenic recombination events (Sun et al. 2001). For most of those characterized, the crossovers occurred in the actual coding region (open reading frame) and not just in the gene (coding region and regulatory regions) and could, therefore, create novel resistance proteins. However, because of the complex nature of the locus, some of the phenotypes may be due to the combination of genes in the recombinant haplotypes rather than recombinant gene. For example, haplotypes combining the resistance genes from both parental haplotypes have been created (Hu and Hulbert 1996).

Many of the variants identified at *Rp1* have been generated by crosses with *HRp1-I*, *HRp1-E*, or *HRp1-K*. The specificity for these haplotypes was originally identified in three different accessions: *HRp1-I* from PI 163558 from Guatemala, *HRp1-E* from Line B49 from Argentina, and *HRp1-K* from Queretaro V 231-5 from Mexico. These three haplotypes cannot be distinguished with any of the rust biotypes in our collection. Furthermore, they are indistinguishable by gel-blot analysis with *Rp1* probes, indicating they carry the same *rp1* genes and confer the same specificity. One class of variants with the same resistance specificity and phenotype, herein designated *Rp1-I**, has been isolated in a number of different crosses with this haplotype. In the current analysis, we examine several genetic events that gave rise to this novel specificity. Our objectives were to identify and characterize these recombinant haplotypes and to characterize the types of recombination events that gave rise to them by comparisons with the parental haplotypes.

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RESULTS

Isolation of variants from a cross between *HRp1-D-Cuzco* and *HRp1-I*.

Two variants with nonparental race specificities were previously isolated from a test cross of an *Rp1-I/Rp1-D* heterozygote (Richter et al. 1995). The *Rp1-D* parent carried the well-characterized haplotype from the Kenyan cultivar Kitale (Sun et al. 2001). Both recombinants showed the *Rp1-I** specificity, with essentially the same resistance reactions when tested with a range of *Puccinia sorghi* biotypes. Preliminary characterization by gel-blot analysis indicated they had many genes, probably similar in numbers to *HRp1-I* (discussed below). An attempt was made to generate similar recombinants with fewer *rp1* paralogs by using a cross between the *Rp1-I* parent and a line with *HRp1-D-Cuzco*, which carries the *Rp1-D* gene but fewer paralogs (Smith et al. 2004). A population of 26,000 test cross individuals from this heterozygote were inoculated simultaneously with rust biotypes IN1 and IN2. The *HRp1-I* parent consistently showed no uredinia after inoculation (0 reaction type) with these biotypes, while the *HRp1-D-Cuzco* parent had only occasional uredinia (0 to 1 reaction type) (Fig. 1). The progeny seedlings had resistance reactions similar to the parents, with the exception of 22 individuals that had higher reaction types supporting many uredinia (2 or higher). The 22 selected individuals were grown to maturity and self-fertilized, and the progeny were re-examined with the two rust biotypes. Four of the 22 progeny lines segregated for the reduced resistance and were selected for further analysis. The remaining 18 lines segregated for the *Rp1-D* resistance phenotype and were, therefore, considered to be misclassified in the previous generation. An additional generation of self-fertilization provided lines homozygous for the genes conferring the reduced resistance. The homozygous lines were screened separately with rust biotypes IN1, IN2, IN3, and HI1.

When compared with the parental lines, the four progeny lines exhibited altered resistance phenotypes. One of the lines, whose haplotype was designated *HRp1-D2I4*, was highly susceptible (4 reaction type) to all four rust biotypes (Table 1). Another progeny line (*HRp1-D2I3*) appeared to have essentially the same resistance specificity as the *Rp1-D* parent but showed a reduced resistance reaction. *HRp1-D2I3* conferred a disease rating of 2 with each of biotypes IN1, IN2, and IN3 but was completely susceptible with biotype HI1. Thus, the pattern

Fig. 1. Resistance reaction phenotypes of *Rp1* parental and recombinant haplotypes. Fully expanded fourth leaf regions from lines homozygous for haplotypes *HRp1-D-Cuzco*, *HRp1-I*, *HRp1*, and *HRp1-D2I*, 7 days after inoculation with rust biotype IN2. *HRp1-D2I* was derived from a *HRp1-D-Cuzco* × *HRp1-I* cross.

of resistance and susceptibility to the different biotypes is the same as the *Rp1-D* line but the resistance reactions allow many more uredinia to form. Two of the progeny lines (*HRp1-D2I*, *HRp1-D2I2*) had resistance specificities that were different from each of the two parental haplotypes (Table 1). These lines specified resistance reactions of 4, 2, 4, and 1 with biotypes IN1, IN2, IN3, and HI1, respectively. Conversely, parental line *HRp1-I* specified complete resistance (0 reaction) with biotypes IN1, IN2, and HI1 but was completely susceptible (4 reaction) with biotype IN3. Parental line *HRp1-D-Cuzco* specified a disease rating of 0 to 1 with biotypes IN1, IN2, and IN3 but was completely susceptible (4 reaction) to biotype HI1. The resistance specificities of *HRp1-D2I* and *HRp1-D2I2* were essentially identical to the *Rp1-I** specificity. Other recombinants with this specificity include the previously identified *HRp1-DI1* and *HRp1-DI28* recombinants from the cross between *Rp1-I* and the other *Rp1-D* haplotype (from cv. Kitale).

Gel-blot analysis of variants with novel race specificities.

Maize lines homozygous for recombinant haplotypes (*HRp1-D2I*, *HRp1-D2I2*, *HRp1-D2I3*, *HRp1-DI1*, and *HRp1-DI28*) were examined by gel-blot analysis with multiple restriction enzymes. The four variants derived from crosses between *Rp1-D* and *Rp1-I* lines with the *Rp1-I** specificity all carried fragments from both parental haplotypes (*HRp1-I* and either *HRp1-D* or *HRp1-D-Cuzco*) (Fig. 2), indicating they carried paralogs from both parents in their recombinant haplotypes. After analysis with 15 different restriction enzymes, *HRp1-D2I* and *HRp1-D2I2* appeared identical in their *Rp1*-hybridizing restriction fragments. This would be expected if the two haplotypes were generated from identical or very similar recombination events. Similarly, *HRp1-DI1* and *HRp1-DI28* appeared identical to each other in every restriction digest examined (Fig. 2). These two recombinants and the *HRp1-I* parental haplotype had a large number of *Rp1*-hybridizing restriction fragments, suggesting they carried many genes. Conversely, the two recombinant haplotypes from the cross with *HRp1-D-Cuzco* had fewer *Rp1*-hybridizing restriction fragments in every restriction digest, suggesting they had fewer *Rp1* genes. Novel-sized restriction fragments were not observed in the parents or in any of the four recombinant haplotypes, when the DNAs were probed with *Rp1* probes.

Characterization of genes from *HRp1-I* and four recombinant haplotypes.

The lack of novel restriction fragments in the four recombinants with the *Rp1-I** specificity indicated they might not be derived from intragenic recombination events. This is not conclu-

Table 1. Resistance reaction types of parental and recombinant *Rp1* haplotypes

Haplotypes	Rust biotypes			
	IN1	IN2	IN3	HI1
Parental				
<i>HRp1-D-Cuzco</i>	0 to 1	0 to 1	0 to 1	4
<i>HRp1-D</i>	0 to 1	0 to 1	0 to 1	4
<i>HRp1-I</i>	0	0	4	0
Recombinant ^a				
<i>HRp1-D2I</i>	4	2	4	1
<i>HRp1-D2I2</i>	4	2	4	1
<i>HRp1-D2I3</i>	2	2	2	4
<i>HRp1-D2I4</i>	4	4	4	4
<i>HRp1-DI1</i>	4	2	4	1
<i>HRp1-DI28</i>	4	2	4	1

^a *HRp1-DI1* and *HRp1-DI28* were derived from a cross with the *Rp1-D* haplotype from cultivar Kitale, while the other recombinant haplotypes came from a cross with *HRp1-D-Cuzco*.

sive, however, since intragenic recombinants often have mostly parentally-sized restriction fragments (Sun et al. 2001). If they did arise from intragenic crossover events, the events could be characterized by identifying the recombinant genes. In the absence of nonparental restriction fragments, recombinant genes can be identified by comparing their DNA sequences to those of all the parental *Rp1* genes. The genes in the two different *Rp1-D*-carrying parental haplotypes have been characterized (Smith et al. 2004; Sun et al. 2001), but little sequence was available from *HRp1-I* genes. A λ library of genomic clones from a maize line homozygous for *HRp1-I* was constructed and screened for hybridization to two probes corresponding to the 5' and 3' regions of the *Rp1-D* gene. Both probes hybridized to 49 clones, identifying them as clones that potentially carry the full *Rp1* coding region. The clones were partially sequenced by polymerase chain reaction (PCR)-amplifying templates from each of the λ clones, using primer pairs corresponding to the 5' (P6/1520R) and 3' (P22/4890R) ends of the *Rp1* coding region (Table 2). Additional sequence was generated from some of the clones, and three of them were completely sequenced (GenBank accession numbers: *rp1-ip7*, AF344308; *rp1-ip9*, AF344309; and *rp1-ip11*, AF344311). Comparisons of the sequences indicated the clones were derived from at least 32 different genes (Table 3). In addition, sequences of several genes were identified in the recombinant haplotypes that were determined not to have come from the *Rp1-D* parent and therefore represent unidentified genes from *HRp1-I* (discussed below). In total, 37 different genes were identified in genomic sequences that originated from this haplotype.

Sequences of *rp1* genes were obtained from the four recombinants with the *Rp1-I** specificity by analyzing clones isolated from genomic λ libraries homozygous for the recombinant

haplotypes or from PCR-amplified clones. Genomic λ clones carrying full *Rp1* coding regions were isolated from *HRp1-D21*, *HRp1-D11*, and *HRp1-DI28* recombinants by identifying clones that hybridized to two probes corresponding to the 5' and 3' regions of the *Rp1-D* gene. λ Clones from each haplotype were partially sequenced in the 5' (after amplification with primer pair P6 and 1520R) and 3' (primer pair P22 and 4890R) regions and aligned to identify redundant clones and to estimate how many genes were present in each haplotype. The ends of genes from the *HRp1-D21* and *HRp1-D212* haplotypes were also characterized by sequencing the ends of cloned fragments amplified with primer pair P6 and 4890R (Table 2). *Rp1-D-Cuzco* or *Rp1-D-Kitale* haplotype genes not identified in each recombinant were searched for by amplifying genomic DNA from that recombinant haplotype with PCR primers specific for the genes. Any amplification products were cloned and sequenced to examine their identity.

Fourteen λ clones representing five different genes were examined from *HRp1-D21* (Table 3). Sequence analysis of 16 different PCR-amplified clones identified an additional five genes in this haplotype. Thirty-one PCR-amplified clones representing 11 different genes were identified in *HRp1-D212*. Two of the sequences identified in these two haplotypes matched genes from the *HRp1-D-Cuzco* parent. Sequence comparisons of genes from *HRp1-D21* and *HRp1-D212* showed they carry mostly the same genes (Table 3). Ten different genes were identified in *HRp1-D21*, all of which were identified in *HRp1-D212*. One of the 11 different genes identified in *HRp1-D212* was not identified in *HRp1-D21*. Since the two haplotypes were indistinguishable by Southern analysis, the differences found in gene content was likely due to the fact that the genes in the two haplotypes were not sampled exhaustively. If the two haplotypes were indeed identical, they should both carry two genes from *HRp1-D-Cuzco* and eight or more genes from *HRp1-I*. A similar situation was found when comparing genes from *HRp1-D11* and *HRp1-DI28*. Twenty-five λ clones representing 18 different genes and 20 λ clones representing 17 different genes were identified in *HRp1-D11* and *HRp1-DI28*, respectively (Table 3). Five of the sequences identified in the two haplotypes matched genes from the *HRp1-D* parent. Three additional genes from *HRp1-D* were identified in both recombinant haplotypes with primer pairs specific to these genes, demonstrating that both carried eight of the nine *rp1* paralogs from *HRp1-D-Kitale*. Eight additional *HRp1-I* genes were identified in *HRp1-D11*, and seven additional *HRp1-I* genes were identified in *HRp1-DI28* by cloning and sequencing amplification products generated with conserved primers. A total of 29 different genes were identified in *HRp1-D11*, three of which were not identified in *HRp1-DI28*. The 26 different genes identified in *HRp1-DI28* were all identified in *HRp1-D11*. *HRp1-D11* and *HRp1-DI28* were also indistinguishable by Southern blot analysis, so the differences between the genes identified in the two haplotypes were likely due to incomplete sampling. Thus, the two haplotypes probably carry the same eight genes from *HRp1-D-Kitale* and at least 21 genes from the *HRp1-I* parent.

Sequences from the genes in the recombinant haplotypes were aligned with sequences from *HRp1-D*, *HRp1-D-Cuzco*, and *HRp1-I*. Five genes were identified in the recombinant haplotypes that were not identical to any parental haplotype genes, three genes from *HRp1-D11* and two from *HRp1-D213*. None of these five genes matched sequences from the *Rp1-D* parental haplotypes at their 5' or 3' ends as would be expected of recombinant genes. Therefore, these genes were assumed to be *HRp1-I* genes that were not identified previously, since this haplotype was not sampled exhaustively. This brings the total number of predicted genes identified from *HRp1-I* by analysis of genomic sequences to 37.

Fig. 2. Comparisons of parental and recombinant haplotypes by gel-blot analysis. Fragment sizes are shown on the left and right in kilobases. **A**, DNAs from lines carrying *HRp1-D21*, *HRp1-D212*, *HRp1-D213*, *HRp1-I*, and *HRp1-D-Cuzco* were digested with *Xba*I and were probed with a probe corresponding to the 5' half of the *Rp1-D* coding region. *HRp1-D21* and *HRp1-D212* exhibited identical banding patterns. **B**, *Acc*I-digested *HRp1-D11*, *HRp1-DI28*, *HRp1-I*, and *HRp1-D* DNAs probed as in **A**. Recombinant haplotypes appeared identical in their restriction fragments and exhibited a recombinant banding pattern.

Transcriptional analysis of the *HRp1-I* genes in the parental and recombinant haplotypes.

If a small proportion of the genes in the different *rp1* haplotypes are transcribed, this information might be useful for identifying the genes that control the resistance phenotypes. To identify genes transcribed in *HRp1-I*, cDNA fragments were amplified by reverse transcriptase (RT)-PCR using primer pair P22 and 4890R. The resulting PCR products were then cloned and sequenced. The amplified fragments flanked a small intron in the 3' untranslated region (UTR), allowing any amplification from contaminating genomic DNA to be distinguished. Each of 65 clones examined carried an approximately 470-bp fragment, which was sequenced using M13 forward and reverse primers. A total of 29 unique sequences were identified among the 65 clones, 25 of which corresponded to genomic sequences identified. The remaining four sequences did not correspond to any of the *HRp1-I* genomic clones. Transcripts were also identified for two *HRp1-I* genomic clones with primer pairs ip23-238F/ip23-585 and ip2,6-381F/ip2,6-501R, using the same methods. Collectively, the 37 genomic sequences and four unique cDNAs suggest that *HRp1-I* carries at least 41 *rp1* genes, of which at least 31 are transcribed (Table 3).

Comparisons of the *Rp1-I* parental and recombinant haplotypes identified nine *Rp1-I* genes that were present in all four recombinant haplotypes with the *Rp1-I** specificity (Table 3). To determine which *HRp1-I* genes were transcribed in the four recombinant haplotypes, RT-PCR amplifications were performed on total RNA isolated from the recombinant haplotypes with primer pairs specific to each of the nine *HRp1-I* genes. The cDNAs were cloned, sequenced, and compared with *HRp1-I* genomic sequences. Transcripts were identified for *rp1-ip2*, *rp1-ip4*, *rp1-ip8*, *rp1-ip13*, *rp1-ip21*, and *rp1-ip23* in the four recombinant haplotypes and the parental *HRp1-I*

haplotype. Transcripts were not detected for the remaining three genes in *HRp1-I* or in any of the four recombinant haplotypes.

Localization of recombination exchange points.

The lack of recombinant genes identified by sequencing genes from the recombinant haplotypes demonstrated that either the recombinant genes were present in these haplotypes but not yet identified or that no recombinant genes were present in these haplotypes. Flanking marker analysis of the four haplotypes with the *Rp1-I** specificity demonstrated that all were associated with crossover events. All had the *Rp1-I* parent marker at the distally mapping restriction fragment length polymorphism marker bn13.04 and the *Rp1-D* parent allele at the centromere proximal marker npi285. Most, if not all, of the *HRp1-D* genes are oriented with their 3' ends towards the telomere (Sun et al. 2001). The flanking marker configuration of the recombinant haplotypes therefore implies that genes derived by intragenic crossing over should have the 5' end of an *HRp1-D* (or *HRp1-D-Cuzco*) gene and the 3' end of an *HRp1-I* gene. Since the genes in both *Rp1-D*-carrying haplotypes are fully sequenced, the presence of recombinant genes was scrutinized by searching for sequences from these haplotypes and determining if they belong to parental or recombinant genes. Two of the sequences identified in *HRp1-D2I* and *HRp1-D2I2* recombinant haplotypes corresponded to paralogs *Cuzco-p2* and *Cuzco-p3* from the *HRp1-D-Cuzco* parent (Table 3). Previous analysis of this haplotype indicated it carried five *rp1* genes (Smith et al. 2004). The presence of the other three genes in the recombinant haplotypes were examined by PCR with gene-specific primers designed near the end of the genes, but none of the three genes were found in either haplotype. Gene-specific 5' forward primers from the three genes were

Table 2. Primers used to amplify *Rp1* sequences

Primer name	Position ^a	Primer sequence	Specificity ^b /haplotype ^c
D-0F	-88F	ATTTTGAGACCGGAAAGATATA	S/ <i>Rp1D</i>
dp7-198F	-198F	ATTTTGAGACCGGAAAGATGTT	S/ <i>Rp1-D</i>
dp8-669F	-669F	CTGAATCTGTCCCTGTCGTAGAG	S/ <i>Rp1-D</i>
P6	67F	AAGCTTCAGCTTACCTCAGT	NS
ip4-252F	252-F	AGCCACTGTCCTAGATCCAACGT	S/ <i>Rp1-I</i>
ip23-238F	238-F	AGCCTACTATGATGCCGAGGACT	S/ <i>Rp1-I</i>
ip1,5-310F	310-F	CATAGGGGCAAGCTAGAATCCTA	S/ <i>Rp1-I</i>
201F1	266F	TTGAGGGCAAGGCCAAGAGCA	S/ <i>Rp1-D-Cuzco</i>
201F2	266F	TTGAGGGCAAGGCCAAGAGCGA	S/ <i>Rp1-D-Cuzco</i>
ip23-585R	585R	TGTGGT TGTGGGAACACTGGTAC	S/ <i>Rp1-I</i>
ip4-590R	590-R	ACAAGACAACAACCTGCTCAGGCAA	S/ <i>Rp1-I</i>
ip1,5-620R	620-R	TAGATTTTCTTCTCGGCAAGA	S/ <i>Rp1-I</i>
1520R	641-R	GTTTGGCCATTGTTGGATTGG	NS
2290F	1406F	GGGTGGCAGAAGGATTG	NS
dp7-1500R	1510R	ATGACATAGTACCGACCGTACCA	S/ <i>Rp1-D</i>
P15	2200R	GAGAATTGTCATTGGAAAGGAT	NS
dp1,3,8-R	2305-R	CCCTCTAGAACATCCAAATGCAG	S/ <i>Rp1-D</i>
324R1	3404R	ATGTTATGTTGGGGCAATGTT	S/ <i>Rp1-D-Cuzco</i>
324R2	3404R	ATGTTATGTTGGGGCATCGTT	S/ <i>Rp1-D-Cuzco</i>
P22	3460-F	TGTCCAGGAATCGCTCACGG	NS
ip2,6-381F	3481-F	CACCAAAATCTTACTCTTCCAGC	S/ <i>Rp1-I</i>
392R2	3766R	TCTTCAAGACGGGGCAACCT	S/ <i>Rp1-D-Cuzco</i>
ip3,12-441F	3771-F	AATCTCACATCTGTCAAGCGCCTGC	S/ <i>Rp1-I</i>
458R	3840R	AAGCTCTTCCAGCGAACGTGTAG	S/ <i>Rp1-D-Cuzco</i>
ip2,6-501R	3882-R	ATCAATATTTAAACATTTTAT	S/ <i>Rp1-I</i>
522R	3897R	AATTAGTAGAACCTGTACCTTCATTT	S/ <i>Rp1-D-Cuzco</i>
ip3,12-631R	4002-R	GACTAATACCAAACCTTGCTT	S/ <i>Rp1-I</i>
636R	4007R	CAAGAGGAGAGAGCAGTCGCA	S/ <i>Rp1-D-Cuzco</i>
4890R	4010R	CTTGAAGCTCCAGAGTTTCAGG	NS

^a Position of the 5' end of the homologous sequence of the *Rp1-D* gene. Position 1 corresponds to the predicted start codon. Negative position numbers correspond to the promoter region. F and R correspond to the sequences of the coding and noncoding strands of the *Rp1* gene from which the primers were designed.

^b S identifies specific primers designed for amplification of specific *Rp1* genes. NS identifies nonspecific primers designed to amplify most or all *Rp1* genes.

^c *Rp1* haplotype from which gene-specific primers were designed.

then used for PCR amplification of the recombinant haplotypes with a nonspecific reverse primer designed from conserved sequences at the 3' ends of the genes. Recombinant genes would be expected to amplify if recombination occurred

Table 3. *rp1* paralogs from *HRp1-I*, *HRp1-D*, and *HRp1-D-Cuzco*^a

Paralogs	Haplotypes analyzed ^b				
	HRp1-I	HRp1-DI1	HRp1-DI28	HRp1-D2I	HRp1-D2I2
<i>HRp1-I</i>					
<i>ip1</i>	+	+	+	+	+
<i>ip2</i>	+	+	+	+	+
<i>ip3</i>	+	+	+	+	+
<i>ip4</i>	+	+	+	+	+
<i>ip5</i>	+	+	+	+	+
<i>ip6</i>	+	+	+	–	–
<i>ip7</i>	+	–	–	–	–
<i>ip8</i>	+	+	+	–	+
<i>ip9</i>	+	+	+	–	–
<i>ip10</i>	+	+	+	–	–
<i>ip11</i>	+	–	–	–	–
<i>ip12</i>	+	+	+	–	–
<i>ip13</i>	+	+	+	+	+
<i>ip14</i>	+	+	+	–	–
<i>ip15</i>	+	–	–	–	–
<i>ip16</i>	+	–	–	–	–
<i>ip17</i>	+	+	+	–	–
<i>ip18</i>	+	+	+	–	–
<i>ip19</i>	+	–	–	–	–
<i>ip20</i>	+	+	+	–	–
<i>ip21</i>	+	+	+	+	+
<i>ip22</i>	+	–	–	–	–
<i>ip23</i>	+	+	+	+	+
<i>ip24</i>	+	+	+	–	–
<i>ip25</i>	+	–	–	–	–
<i>ip26</i>	+	–	–	–	–
<i>ip27</i>	+	–	–	–	–
<i>ip28</i>	+	–	–	–	–
<i>ip29</i>	+	–	–	–	–
<i>ip30</i>	+	–	–	–	–
<i>ip31</i>	+	–	–	–	–
<i>ip32</i>	+	–	–	–	–
<i>ip33</i>	+	–	–	–	–
<i>ip34</i>	+	–	–	–	–
<i>ip35</i>	+	–	–	–	–
<i>ip36</i>	+	–	–	–	–
<i>di1p1</i>	–	+	–	–	–
<i>di1p2</i>	–	–	–	–	–
<i>di1p3</i>	–	+	–	–	–
<i>d2i3p1</i>	–	–	–	–	–
<i>d2i3p2</i>	–	–	–	–	–
<i>HRp1-D-Kitale</i>					
<i>dp1</i>	N/A	+	+	N/A	N/A
<i>dp2</i>	N/A	+	+	N/A	N/A
<i>dp3</i>	N/A	+	+	N/A	N/A
<i>dp4</i>	N/A	+	+	N/A	N/A
<i>dp5</i>	N/A	+	+	N/A	N/A
<i>dp6</i>	N/A	+	+	N/A	N/A
<i>dp7</i>	N/A	+	+	N/A	N/A
<i>dp8</i>	N/A	+	+	N/A	N/A
<i>dp9(Rp1-D)</i>	N/A	–	–	N/A	N/A
<i>HRp1-D-Cuzco</i>					
<i>Cuzco-p1</i>	N/A	N/A	N/A	–	–
<i>Cuzco-p2</i>	N/A	N/A	N/A	+	+
<i>Cuzco-p3</i>	N/A	N/A	N/A	+	+
<i>Rp1-D-</i>					
<i>Cuzco</i>	N/A	N/A	N/A	–	–
<i>Cuzco-p4</i>	N/A	N/A	N/A	–	–

^a Presence of *rp1* paralogs from *HRp1-I*, *HRp1-D*, and *HRp1-D-Cuzco* in four recombinants, *HRp1-DI1*, *HRp1-DI28*, *HRp1-D2I*, and *HRp1-D2I2*, that confer the novel race specificity.

^b + Indicates a gene matching the sequence of this paralog was observed in the recombinant haplotype. – Indicates the paralog was not observed. Paralog designations in bold show transcribed genes. *HRp1-I* genes that were not observed in the recombinant haplotypes could be present.

within (intragenic) one of the three paralogs, but no recombinant genes were amplified. The fact that no genes could be amplified that were partially derived from the *HRp1-D-Cuzco* parent haplotype establishes that no recombinant *rp1* genes are present in the recombinant haplotypes and that the crossover events that gave rise to them occurred between the paralogs. The order of the paralogs in *HRp1-D-Cuzco* has not been determined. However, it is apparent that the three parental paralogs not identified in the recombinants are distal to the recombination exchange point, resulting in their loss from the recombinant haplotypes. The presence of *Rp1-I* paralogs in the recombinant haplotypes (Table 3) and the flanking marker configuration of the recombinants imply that the proximal two genes of the recombinant haplotype came from the *HRp1-D-Cuzco* parent and the distal remainder came from the *HRp1-I* parent.

The order of the paralogs in the *HRp1-D-Kitale* haplotype (Sun et al. 2001) has been determined, so the recombination exchange points could be determined in *HRp1-DI1* and *HRp1-DI28* (Fig. 3). *HRp1-DI1* and *HRp1-DI28* both carried paralogs *hrp1-1* through *hrp1-8* from the *HRp1-D* parent (Table 3), leaving only the distally mapping *Rp1-D* gene unaccounted for. If the recombinant haplotypes were derived from recombination events occurring within *rp1* genes, they would have to involve the *Rp1-D* gene. In this case, these haplotypes would be expected to carry a recombinant gene with the 5' end of the *Rp1-D* gene. To search for such a gene, a primer designed from the promoter region of the *Rp1-D* gene was used with a 3' conserved primer to amplify from the recombinant haplotypes. No *Rp1-D* sequences could be amplified from the recombinant haplotypes. The absence of *Rp1-D* sequences suggests that *HRp1-DI1* and *HRp1-DI28* were derived from crossovers between paralogs *hrp1-8* and *hrp1-9* (Fig. 3). The presence of *Rp1-I* paralogs in the recombinants (Table 3) and flanking marker analysis demonstrate that paralogs proximal to the recombination exchange point came from the *HRp1-D* parent and the distal half came from the *HRp1-I* parent.

Interestingly, the two recombinant haplotypes (*HRp1-D2I* and *HRp1-D2I2*) that utilize *HRp1-D-Cuzco* as the *Rp1-D* parent have fewer genes than the recombinants (*HRp1-DI1* and *HRp1-DI28*) that utilize *HRp1-D-Kitale* (Table 3). This was partially due to the *HRp1-D-Cuzco* parental haplotype, which carries fewer genes than *HRp1-D-Kitale*. *HRp1-D2I* and *HRp1-D2I2* also carry fewer *Rp1-I* genes when compared with *HRp1-DI1* and *HRp1-DI28*. All of the *Rp1-I* genes identified in *HRp1-D2I* and *HRp1-D2I2* were found in *HRp1-DI1* and *HRp1-DI28* (Table 3). However the latter two recombinant haplotypes carried 12 *Rp1-I* genes that were not present in *HRp1-D2I* and *HRp1-D2I2*, demonstrating recombination occurred at different positions in the *HRp1-I* parental haplotype.

Characterization of other recombinants from *HRp1-I*.

The *Rp1-I** specificity is the most common variant from crosses with *HRp1-I*, along with recombinants that are completely susceptible (Richter et al. 1995). Two other recombinants were previously identified with a different specificity from a cross between this haplotype and a rust-susceptible (*rp1*) maize accession carrying the *rp1*-linked *Oy* leaf color marker (Richter et al. 1995). The two recombinants were exceptional in that they were partially resistant to isolate IN3, which is virulent on lines carrying *Rp1-I*. Gel-blot analysis of these recombinants found most restriction fragments similar to the *HRp1-I* parent, but many digests had two or more restriction fragments that did not match this parent or our current stocks of *Oy* maize (data not shown). Since the *Oy* line was not inbred, it seems likely it was homozygous at *Oy* but het-

erogeneous at the *Rp1* locus. Since we cannot verify that the *Oy* parent haplotype used in the cross giving rise to these individuals had no rust resistance to any of the isolates used in the analysis, the origin of this novel race specificity is not clear.

DISCUSSION

Genes at the *Rp1* complex often mispair in meiosis, which provides an opportunity for different genes on a chromosomal array to recombine unequally. The ability of unequal crossing over to create new haplotypes as well as recombinant genes allows for a variety of possible mechanisms for the generation of nonparental race specificities (Hulbert et al. 1997) (Fig. 4). Intragenic crossing over creates recombinant genes, some of which could have nonparental specificities as demonstrated for the flax *L* locus (Ellis et al. 1999, 2000) (Fig. 4A).

The present analysis examined four *Rp1* variants with novel race specificities. All four recombinants were associated with crossovers in the *Rp1* complex. Surprisingly, none of the recombinant haplotypes were derived from recombination events that occurred within the *rp1* gene-coding regions, indicating they did not result from the creation of a gene coding for a novel protein.

An alternative model for the creation of novel specificities is crossing over in the regions between the *Rp1* genes reassorting the genes into a new combination with a nonparental specificity. This is possible when parental haplotypes carry more than one phenotypically detectable resistance gene (Fig. 4B). Insertional inactivation of the *Rp1-D* gene indicated it was the only gene in the *HRp1-D-Kitale* haplotype conferring resistance to any rust isolate in our collection (Collins et al. 1999). Haplotypes with more than a single phenotypically detectable gene are possible, however, since several have been constructed for experimental or breeding purposes (Hu and Hulbert 1996; Richter et al. 1995; Saxena and Hooker 1968). The *Rp1-I** specificity is clearly not the result of the combination of the *Rp1-D* and *Rp1-I* genes and, in fact, does not carry either parental gene because it is susceptible to rust isolates that both genes confer resistance to. Furthermore, the *Rp1-I** specificity does not require an *Rp1-D*-carrying line as a parent. Four other *Rp1-I** variants have been recovered in previous work (Richter et al. 1995) from three different crosses. All three crosses included *HRp1-I* as a parent, but the other parents carried *Rp1-B*, *Rp1-F*, and even an *rp1* line with no detectable resistance. The pattern suggests that the *Rp1-I** specificity somehow came from *HRp1-I*. If *HRp1-I* carried two detectable *Rp1* genes and one of these controlled the *Rp1-I** phenotype, it is plausible that recombination could have separated these genes and one of the recombinant classes would have the *Rp1-I** specificity. However, several aspects of the *Rp1-I* haplotype are inconsistent with a model in which two genes control the phenotype: i) the *Rp1-I* resistance phenotype is essentially

Fig. 3. Model illustrating the intergenic recombination exchange point as determined by the presence and absence of *rp1* paralogs in *HRp1-DI1* and *HRp1-DI28* recombinants. Open boxes represent the paralogs in *HRp1-D*. The filled box denotes the *Rp1-D* gene. '+' indicates that a gene matching the sequence of this paralog was observed in the recombinant haplotype. '-' indicates the paralog was not observed. The arrow pointing downward designates the intergenic recombination exchange point. Intergenic regions are not drawn to scale.

identical after inoculation with any rust isolate to which it confers resistance, while the resistance phenotypes of lines with two genes typically vary with different rust isolates, depending on which gene is conferring resistance; ii) no combination of two known *Rp1* genes, including *Rp1-I**, could together account for the *Rp1-I* specificity; and iii) if the *Rp1-I** gene is one of two genes controlling the *Rp1-I* specificity, we would expect to find recombinants with a gene contributing the other component of resistance, but no such recombinant has been found. It is possible there is another gene in the *HRp1-I* haplotype but this gene has a phenotype that is difficult to detect. This might be the case if it somehow interacted with the *Rp1-I** gene to confer resistance phenotypes that are much stronger than either gene by itself. Alternatively, it is possible the other gene confers a resistance similar to the *Rp1-I* specificity. In this case, recombination events that separate these two genes would result in the isolation of variants with the *Rp1-I** specificity or a specificity that was not noticeably different from the combination of the two. Recombinant haplotypes that carry both genes or only the *Rp1-I* gene would confer the *HRp1-I* parental phenotype and would not have been selected from the variant population. The *Rp1-I* gene is highly resistant (0 reaction) to rust isolates carrying its corresponding avirulence gene (Table 1). The *Rp1-I** haplotypes have either a lower resistance reaction (1 to 2) or are completely susceptible to the same rust biotypes. Therefore, the *Rp1-I** specificity would never be observed in *HRp1-I* or recombinant haplotypes in which both genes are present due to a high level of resistance reaction conferred by the *Rp1-I* gene.

Fig. 4. Possible mechanisms for the generation of *Rp1* haplotypes with nonparental race specificities. Boxes represent paralogs in a hypothetical *Rp1* haplotype. Open boxes represent phenotypically undetectable genes. Filled and hashed boxes signify functional genes. Arrows indicate the recombination exchange points. **A**, Intragenic crossing over between two phenotypically detectable genes creates recombinant genes with novel resistance specificities. Novel genes can also be created from recombination between silent and detectable genes. **B**, Crossovers that occur between *rp1* genes (intergenic crossing over) can reassort them and combine or separate the detectable genes, generating haplotypes with nonparental resistance specificities. **C**, Crossovers that occur in the regulatory regions of *rp1* genes can activate a nontranscribed gene, resulting in a nonparental resistance specificity.

Gene activation is another mechanism that has the potential to generate nonparental race specificities (Fig. 4C). If *HRp1-I* carried a gene controlling the *Rp1-I* specificity and another silent gene capable of coding for the *Rp1-I** specificity, recombination events near the *Rp1-I** gene (possibly in the promoter region) could transcriptionally activate it while separating it from the *Rp1-I* gene. Not all *Rp1* genes within a haplotype are transcribed. No transcripts could be identified for two of the nine genes in *HRp1-D-Kitale* (Sun et al. 2001), and the others appeared transcribed at variable levels. In addition, the two nontranscribed genes had no apparent defects and were able to code for predicted proteins very similar to *Rp1-D*. It is therefore plausible that some *Rp1* genes are potentially functional but not activated. Nine genes from *HRp1-I* were found in all four recombinants and could, therefore, be candidates for a gene activated in the recombinant haplotypes. However, transcriptional analysis with gene-specific primers indicated the six genes transcribed in the recombinants were transcribed in the *HRp1-I* parent and the three genes for which no transcripts were identified were not transcribed in the parent either. In addition, to activate the gene controlling the *Rp1-I** specificity in the *HRp1-I* parent, recombination would be expected to occur near this gene. However the *HRp1-D-Kitale* (*HRp1-D11*, *HRp1-D128*) and *HRp1-D-Cuzco* (*HRp1-D21*, *HRp1-D211*) recombination exchange points occurred at very different positions in *HRp1-I*, as evidenced by the fact that they carry very different numbers of genes from this haplotype. These results are inconsistent with a gene activation model for generating novel haplotypes. Thus, the gene reassortment model is the best plausible explanation for the generation of the nonparental race specificity in the four recombinant haplotypes.

Most of the *Rp1* phenotypic variants that have been previously molecularly characterized came from a line homozygous for *HRp1-D* from the cultivar Kitale. Of 22 spontaneous variants, 20 were found to be associated with intragenic recombination (Sun et al. 2001). While most of these variants were susceptible to all rust biotypes tested, *HRp1-D*5* had the specificity of the *Rp1-D* parent with a reduced level of resistance and *HRp1-D*21* showed the same highly necrotic reaction to all biotypes. If the *HRp1-D*21* specificity is considered to be a nonspecific resistance, none of these recombinants would be considered a novel race specificity. Therefore, it was surprising that all four of the present recombinants with the novel *Rp1-I** specificity were derived from crossovers between the *Rp1* coding regions. It is likely that the structure of *Rp1-D* haplotype from Kitale and the selection imposed while identifying the recombinants biased the types of recombinants identified. The *Rp1-D* gene lies at the distal end of the *rp1* gene array in this haplotype, and selection was imposed for the loss or alteration of this gene. Loss of a gene at the distal end of a tandem array from a homozygote by unequal crossing over would require that the crossover be within the gene unless the region distal to it shared homology to the intergenic sequences within the array. It is therefore possible that this initial molecular analysis of *rp1* recombinants is highly biased and that much of the recombination in the *Rp1* complex does not occur within the *Rp1* genes. This idea is also supported by the observation that the intergenic regions can be 38 kb or more and may carry other unrelated genes or, at least, gene fragments (Ramakrishna et al. 2002). While the two *Rp1-I** variants from the *HRp1-I* × *HRp1-Cusco* cross were not associated with recombinant *Rp1* genes, the *HRp1-D213* recombinant from this cross was (Smith et al. 2004). Both this and the *HRp1-D*5* recombinant were derived from intragenic crossovers between the *Rp1-D* gene and other paralogs and resulted in a reduced resistance phenotype. Taken together, the results from molecular characterization of recombinant haplotypes indicate that the

types of phenotypes selected for can bias whether the recombination events occurred within or between the *rp1* genes. To determine whether most recombination events in the *rp1* complex occur within or between the *rp1* genes, it will be necessary to examine unselected variants.

If the *Rp1-I* resistance is controlled by a single *Rp1* gene, identification of this gene could be useful for breeding purposes because it confers high levels of rust resistance in the field (Brown et al. 2001) and is resistant to most known rust biotypes (Hulbert et al. 1991). The very large numbers of genes in this haplotype and the fact that most are transcribed present a difficulty in identifying one or more genes controlling the phenotype. *HRp1-I* carries 41 or more genes, at least 31 of which are transcribed. A total of 16 of the transcribed *HRp1-I* genes were not identified in the recombinant haplotypes and are therefore good candidates for an *Rp1-I* gene. Similarly, nine genes from *HRp1-I* were found in all four of the recombinants with the *Rp1-I** specificity and are therefore candidates for one or more genes controlling the *Rp1-I** resistance. Transcripts were found for six of these.

The recombinants examined in the present study were considered to be among the most likely candidates for newly created genes with novel race specificities. They were selected from recombinant haplotypes identified by screening many thousands of individuals. The lack of recombinant genes in these haplotypes demonstrates that the generation of new genes with novel resistance specificities is a rare occurrence at *Rp1*. Instead, the evidence provided here suggests that intergenic crossing over is a mechanism utilized at *Rp1* to reassort existing genes into new haplotypes with novel specificities and is important for the evolution of this complex locus.

MATERIALS AND METHODS

Generation and screening of families for the identification of genetic variants.

Populations were generated by crossing two different *Rp1-D* haplotypes (*HRp1-D-Kitale* and *HRp1-D-Cuzco*) to *HRp1-I*. The *Rp1-D/Rp1-I* heterozygotes were test-crossed to *H95*, a rust-susceptible maize inbred line. The *Rp1-D-Kitale/Rp1-I* × *rp1/rp1* test-cross population (3,500 progeny) was screened previously for variant phenotypes with one rust biotype (Richter et al. 1995). Individuals showing a nonparental resistance reaction were identified and self-fertilized to generated homozygous lines. These were then examined for resistance phenotype after inoculation with 11 rust biotypes. An *Rp1-D-Cuzco/Rp1-I* × *rp1/rp1* test-cross population of 26,000 progeny were inoculated with two rust biotypes and were screened for variant phenotypes. Seedlings (8- to 10-day-old) were spray inoculated simultaneously with maize rust spores from biotypes IN1 and IN2. Inoculated seedlings were incubated at 100% humidity for 12 to 15 h and were scored for resistance 8 and 10 days later. Biotypes IN1 and IN2 are both avirulent on lines carrying either *Rp1-D* or *Rp1-I*. A resistance reaction rating scale of 0 to 4 was utilized to score seedlings from variant lines. A rating of 1 indicated a high level of resistance with one or a few uredinia per leaf. Leaves with a large number of uredinia with necrotic or chlorotic spots and a clear hypersensitive response denote a 2 rating. Ratings of 3 denote a large number of uredinia with more sporulation than a 2 rating but less than a 4 rating of complete susceptibility. Individuals with nonparental resistance reactions were self-fertilized, inoculated, and scored a second time to verify scoring. Lines homozygous for each variant haplotype were established from subsequent self-fertilized generations. Homozygous lines were screened separately with four rust biotypes (IN1, IN2, IN3, and HI1).

Identification of recombinant haplotypes.

Southern blot analyses of lines homozygous for the variant phenotypes were performed with an *rp1* fragment probe to verify the origin of the recombinant haplotypes. DNAs from four variant lines (*HRp1-D21*, *HRp1-D212*, *HRp1-D11*, and *HRp1-D128*) were digested with more than 15 restriction enzymes and were probed with an NBS probe corresponding to the 5' half of the *Rp1-D* coding region. To determine the presence of recombinant genes within the variant haplotypes, sequences derived from purified genomic λ clones or PCR-amplified genomic clones were aligned with sequences from the parental haplotypes (*HRp1-I* and *HRp1-D-Cuzco* or *HRp1-D*). PCR-amplified genomic clones were isolated from *HRp1-D21* and *HRp1-D212* recombinant haplotypes to increase the probability of identifying all of the genes in each haplotype. Genomic clones were amplified with primers designed from conserved regions among *Rp1* genes, which amplified an approximately 4-kb region that includes nearly the complete *Rp1* coding region (Table 2). To identify variants derived from crossover events, the sequences of both ends of the genes were compared with the sequences of all the characterized parental genes. Gene-specific primers were designed from the 5' and 3' ends of each paralog from *HRp1-D-Cuzco* or *HRp1-D* (Table 2). This enabled the identification of *Rp1-D-Cuzco* or *Rp1-D* haplotype genes in the recombinant haplotypes. The PCR products were cloned, partially sequenced, and compared with the genes in *HRp1-D-Cuzco* or *HRp1-D*. In the event the gene-specific primer pairs failed to amplify the recombinant haplotypes, which suggested either the complete absence of a paralog (intergenic recombination) or the creation of a recombinant gene (intragenic recombination), 5' gene-specific primers designed from the promoter region were coupled with a reverse primer in the 3' UTR distal to the gene-specific 3' primers to amplify the recombinant haplotype and determine which genetic event occurred.

PCR amplification of all genomic DNA templates was performed using enhanced DNA polymerase as described by the manufacturer (Stratagene, La Jolla, CA, U.S.A.). PCR products were gel-purified and cloned into the TOPO TA cloning vector (Invitrogen, Carlsbad, CA, U.S.A.). Recombinant plasmids were prepared by using Qiagen (Valencia, CA, U.S.A.) miniprep purification system. To verify insert sizes, clones were digested with *EcoRI* (NEB, Beverly, MA, U.S.A.) and were analyzed by agarose gel electrophoresis. Clones with the correct insert size were end-sequenced with M13 forward and reverse primers. Primer pairs from conserved regions of the *Rp1* genes were used to further sequence selected clones (Table 2).

Construction of genomic DNA libraries.

Genomic libraries were constructed and screened following standard protocols. Genomic DNAs isolated from homozygous maize lines (*HRp1-I*, *HRp1-D21*, and *HRp1-D128*) were partially digested with *Sau3a* enzyme (NEB). Partially digested DNAs ranging from 9 to 23 kb in size were ligated into a λ DASH II replacement vector and were packaged using a λ DASH II/*Bam*HI vector kit, as described by the manufacturer (Stratagene). An *Rp1* NBS region probe was used to screen genomic libraries for positive clones. Bacteriophage growth, purification, and DNA extraction was performed using standard protocols (Maniatis et al. 1982).

Amplification and cloning of *Rp1* cDNA sequences.

Tissue collected from fully expanded second maize leaves were used for total RNA isolation performed with Trizol reagent, as described by the manufacturer (Gibco-BRL, Rockville, MD, U.S.A.). ProStar first-strand RT-PCR kit (Stratagene) was used to perform RT-PCR. First-strand DNA synthesis was

carried out with an oligo dT primer. Following first-strand synthesis, the P22/4890R or ip23-238F/ip23-585R primer pairs (Table 2) were used to amplify *Rp1* cDNA sequences. The P22/4890R primer pair was designed from regions that were conserved among the *Rp1-D* paralogs and flank a 3' intron to ensure detection of genomic contamination. These primers amplify an approximately 600-bp fragment in genomic clones and a 470-bp fragment in cDNA clones due to the removal of the 3' intron. The ip23-238F/ip23-585R gene-specific primer pair was designed from a 5' region specific to the *ip-23* gene and amplify an approximately 350-bp fragment in genomic clones. The gene-specific primer pair does not flank an intron. Therefore, control reactions were done in parallel without adding reverse transcriptase; amplification of the control PCR reactions would imply genomic contamination. The PCR products from each fragment were purified using Wizard PCR Preps DNA purification system from Promega (Madison, WI, U.S.A.). The PCR products were cloned as described above and completely sequenced with M13 forward and reverse primers.

DNA sequence analysis.

The GCG Seqweb version 2 program or the Baylor College of Medicine basic local alignment search tool was used to align the sequences of all clones. Sequences were aligned to identify clones derived from the same gene and determine the number of unique genes identified in each haplotype. Because of the large numbers of sequences being compared, occasional errors in sequence determination were expected, especially when sequencing PCR-amplified templates. A criterion for scrutinizing sequences for classification of genes was therefore established. Polymorphic bases were only considered informative for grouping sequences from different clones if the polymorphism was shared by at least one other clone. Polymorphic bases that were unique to a single clone were considered possible sequencing errors. Only genes with multiple informative differences from any other gene were classified as unique genes. Sequences from cDNA clones were aligned, scrutinized, and classified in the same manner prior to alignment with genomic clones.

Gene-specific primers (Table 2) were designed for each gene (paralog) in the two *Rp1-D*-carrying haplotypes (*HRp1-D-Cuzco* and *HRp1-D-Kitale*) by aligning the sequences of the paralogs in each haplotype and scrutinizing the sequence for regions unique to each paralog. All sequencing was performed at Kansas State University sequencing facility.

ACKNOWLEDGMENTS

We thank D. Wood and J. Poland for technical assistance and the National Science Foundation for financial support (grant MCB-0090883). This article is contribution number 05-108-J from the Kansas Agricultural Experimental Station, Kansas State University, Manhattan, Kansas.

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