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ORIGINAL PAPER

Recombinant *Rp1* genes confer necrotic or nonspecific resistance phenotypes

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Abstract Genes at the *Rp1* rust resistance locus of maize confer race-specific resistance to the common rust fungus *Puccinia sorghi*. Three variant genes with nonspecific effects (*HRp1 -Kr1N*, *-D*21* and *-MD*19*) were found to be generated by intragenic crossing over within the LRR region. The LRR region of most NBS-LRR encoding genes is quite variable and codes for one of the regions in resistance gene proteins that controls specificity. Sequence comparisons demonstrated that the *Rp1-Kr1N* recombinant gene was identical to the N-terminus of the *rp1-kp2* gene and C-terminus of another gene from its *HRp1-K* grandparent. The *Rp1-D*21* recombinant gene consists of the N-terminus of the *rp1-dp2* gene and C-terminus of the *Rp1-D* gene from the parental haplotype. Similarly, a recombinant gene from the *Rp1-MD*19* haplotype has the N-terminus of an *rp1* gene from the *HRp1-M* parent and C-terminus of the *rp1-D19* gene from the *HRp1-D* parent. The recombinant *Rp1 -Kr1N*, *-D*21* and *-MD*19* genes activated defense responses in the absence of their AVR proteins triggering HR (hypersensitive response) in the absence of the pathogen. The results indicate that the frequent intragenic recombination events that occur in the *Rp1* gene cluster not only recombine the genes into novel haplotypes, but also create genes with nonspecific effects. Some of these may contribute to nonspecific quantitative resistance but others have severe consequences for the fitness of the plant.

Keywords *Rp1* · Recombination · Nonspecific resistance · Spontaneous necrotic phenotype · Hypersensitive response · Ectopically activated disease resistance gene

Nucleotide sequence data reported for *Rp1-Kr1N* is available in the GenBank database under the accession number GU942722.

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Introduction

The majority of disease resistance genes that have been characterized have been found to code for NBS-LRR proteins that confer race-specific resistance (McHale et al. 2006; Bozkurt et al. 2007). However, shifts in pathogen populations have proven that race-specific resistance is typically not durable. Quantitative disease resistance is considered more durable than simply inherited resistance but is controlled by multiple genes making it more difficult to manipulate genetically and it is generally not clear if the individual genes have nonspecific effects (Hooker 1967; Young 1996; Rosewarne et al. 2008). A few resistance genes have been identified that appear to have race-nonspecific effects, but they are not NBS-LRR genes (Brueggeman et al. 2002; Krattinger et al. 2009; Fu et al. 2009). Race-nonspecific

resistance is considered to be the most durable type of resistance. It can sometimes be controlled by a small number of loci and sometimes confers resistance to multiple pathogens (Krattinger et al. 2009).

Typical R proteins function to perceive pathogens by a direct or indirect interaction with specific AVR effectors. Once this recognition occurs, the R protein activates defense responses, typically including a rapid HR response (Greenberg et al. 1994). Aberrant NBS-LRR proteins have been generated that activate defense responses independently of their AVR proteins and therefore trigger HR in the absence of the pathogen. Such autoactive genes have been created by mutations at specific sites (Bendahmane et al. 2002; Shirano et al. 2002; Howles et al. 2005) or engineering truncated proteins or hybrid proteins by swapping domains from homologs or alleles (Hwang et al. 2000; Hwang and Williamson 2003; Frost et al. 2004; Howles et al. 2005; Rairdan and Moffett 2006; Zhang and Gassman 2007). These observations have led to the perception that regions of NBS-LRR proteins have roles as negative regulators of the defense signaling processes controlled by other regions of the protein (Belkhadir et al. 2004b). If these interdomain interactions are interfered with by the presence of the AVR protein or specific alterations of the protein itself, constitutive signaling can result.

Overexpression of NBS-LRR genes (Oldroyd and Staskawicz 1998; Tao et al. 2000; Bendahmane et al. 2002; Belkhadir et al. 2004a; Zhang et al. 2004; Chern et al. 2005) can also cause spontaneous activation of defense responses indicating a low level of AVR-independent basal activity of these proteins. Expression of the flax NBS-LRR gene L6 in tobacco has also been shown to cause spontaneous defense responses (Frost et al. 2004), but this may also be due to increased expression of the protein.

The *Rp1* rust resistance locus of maize consists of a cluster of NBS-LRR genes that confer race-specific resistance to the common rust fungus *Puccinia sorghi* (Collins et al. 1999). These genes often mispair in meiosis and recombine unequally which re-assorts them into new combinations creating haplotypes with novel gene combinations and sometimes novel genes. Several different *Rp1* recombinant haplotypes have been selected by their nonparental phenotypes. These variants consist of haplotypes with nonparental race specificities (Richter et al. 1995), haplotypes with reduced levels of resistance with parental race specificities (Sun et al. 2001) and haplotypes with spontaneous necrotic phenotypes with nonspecific rust resistance reactions (Hu et al. 1996; Hu et al. 1997). Some of these phenotypes are due to the novel combination of *rp1* genes but many of the phenotypes are the result of the generation of new *Rp1* genes by intragenic recombination (Sun et al. 2001; Smith and Hulbert 2005). The term "spontaneous necrotic phenotype" indicates the variants described in this work exhibited necrotic spotting resistance phenotypes similar to the hypersensitive response (HR), however without inoculation. *Rp1* haplotypes with spontaneous necrotic phenotypes and nonspecific rust resistance reactions were the focus of this investigation. The phenotypes of three haplotypes have been described (Hu et al. 1996). The *HRp1-Kr1N* variant exhibited a diffuse necrosis without necrotic spotting which is not inducible by rust inoculation. Necrosis begins at the tip of the leaf once the leaf is fully expanded then progresses down towards the base of the leaf. In addition, *HRp1-Kr1N* maintains the parental, *Rp1-Kr1*, resistance specificity. The *HRp1-D*21* and *HRp1-MD*19* variants exhibited necrotic spotting phenotypes without inoculation (Sun et al. 2001), but rust inoculation rapidly induces the necrotic spots. *HRp1-D*21* confers a nonspecific resistance with all common rust biotypes tested (*P. sorghi*).

This analysis will shed light on the factors at the *Rp1* locus that control race-specific and nonspecific interactions and condition cell death activating plant defenses in the absence of the pathogen.

Materials and methods

Library construction and screening

A *HRp1-Kr1N* genomic DNA library was constructed and screened following standard procedures (Maniatis et al. 1982). This haplotype is typically lethal as a homozygote, but homozygous seedlings sometimes reached the 3–4 leaf stage when cultured under summer greenhouse conditions where high temperatures (>28 C) are maintained at day and night. Genomic DNA isolated from a *HRp1-Kr1N* homozygous maize line was partially digested with *Sau*3a (NEB, Beverly, MA) and size fractionated on a sucrose gradient. DNA fragments ranging from 9 to 23 kb in size were ligated into a *Lambda* DASH II replacement vector and packaged using a *Lambda* DASH II/*Bam*HI Vector Kit as described by the manufacturer (Stratagene, LaJolla, CA). An *rp1* 3′ probe (amplified with primer pair P22 and 4890R) was used to screen the genomic library for cross-hybridizing clones. Bacteriophage growth, purification and DNA extraction was performed using standard molecular protocols (Maniatis et al. 1982).

DNA sequence alignment of all clones was performed using the GCG Seqweb version 2 program or the Baylor College of Medicine (searchlauncher.bcm.tmc.edu), Basic Local Alignment Search Tool. The sequences from the 5′ and 3′ regions of genes or full-length gene sequences were aligned and analyzed, to identify a recombinant gene, determine the recombination exchange point and verify correct construction of transformation constructs. All sequencing was performed at the Kansas State University Sequencing Facility.

Transformation constructs

A genomic fragment carrying the recombinant gene from *HRp1-D*21* with approximately 2 kb of sequence upstream of the predicted coding region was subcloned from the original genomic *Lambda* clone (Sun et al. 2001) into the pUC19 cloning vector. The plasmid construct, designated pUCRp1-D*21 was used in stable plant transformation experiments. Two additional recombinant *rp1* genes were constructed in vitro by exchanging fragments of genomic clones of the *rp1-dp2* and *Rp1-D* genes (Fig. 1) subcloned into pUC19. The Rp1-dp2-D-1 construct carries the promoter and NBS region from the *rp1-dp2* gene and the LRR regions from the *Rp1-D* parent. A 2.4-kb fragment carrying the promoter and NBS region was amplified from the *rp1-dp2* with an *rp1-dp2* specific forward primer (dp2-F1, Table 1) and a reverse common primer (2620R, Table 1). The resulting PCR product was double digested with *Bam*HI and *Nsi*I restriction enzymes (NEB, Beverly, MA), and gel purified. The 2.5-kb LRR region of this construct was obtained from a plasmid carrying the *Rp1-D* gene (Ayliffe et al. 2004) with *Eco*RI and *Nsi*I. The promoter/NBS and LRR fragments were cloned into a *Bam*HI/*Eco*RI digested pUC19 cloning vector. Selected clones were fully sequenced.

The Rp1-dp2-D-2 construct contains the *rp1-dp2* promoter, NBS-encoding region and the coding region from the first half of the LRR, with an *Rp1-D* fragment coding for the second half of the LRR (Fig. 1). A plasmid carrying *rp1-dp2* was digested with *Nco*I and *Eco*RI to release the fragment encoding the distal half of the LRR. This region was replaced by an *Eco*RI-*Nco*I fragment from a plasmid carrying the *Rp1-D* gene. Selected clones were fully sequenced and aligned with the original sequences to verify the construct.

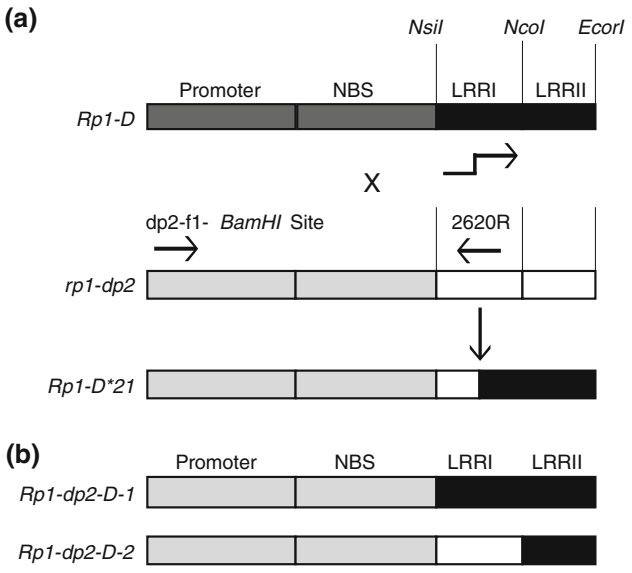

Fig. 1 Recombination events that gave rise to *Rp1-D*21* spontaneous necrotic variant and construction of chimeric genes *Rp1-dp2-D1* and *Rp1-dp2-D-2*. **a** Recombination event between *Rp1-D* and *rp1-dp2* paralogs gave rise to *Rp1-D*21*, which confers a spontaneous necrotic phenotype. b *Rp1-dp2-D-1* construct contains the *rp1-dp2* NBS region and LRRI-LRRII regions from the *Rp1-D* parental paralog. *Rp1-dp2-D-2* construct contains the *rp1-dp2* NBS-LRRI regions and the *Rp1-D* LRRII region. *Vertical arrows* indicate recombination exchange point. Horizontal arrows signify primer sites (dp2-f1 and 2620R)

A Lambda clone (*Kr1N-35-2-60)* carrying the putative *Rp1-Kr1N* gene was partially digested with *Sau*3A (NEB, Beverly, MA) and subcloned into *BamH*I digested pUC19 cloning vector. Clones were end-sequenced with M13 primers to identify clones with a complete coding region. A clone with a full coding region was completely sequenced using 13 *Rp1* primers (Table 1). This clone was then digested with *Eco*RI to remove the *Lambda* vector sequences, re-circularized and designated pKr1n-pUC19. The first 138 nucleotides of the clone is homologous to the predicted intron in the 5′ UTR of the *Rp1-D* gene followed by a 3,855 nucleotide ORF. To drive expression of the putative *Rp1-Kr1N* gene in plants, a ubiquitin promoter was added. An M13 forward primer was coupled with a reverse primer (pAHC17-R-GCATCAACATGTATACCT ATCCT) designed from the intron in the 5′ region of the maize *ubiquitin* gene to PCR amplify the *ubiquitin* promoter from the pAHC17 plasmid (Christensen et al. 1992). An approximately 1.4-kb fragment that included the *ubiquitin* promoter (~900 bp) and approximately 500 bp of the 5′ *ubiquitin* intron was amplified and cloned into an Invitrogen (Carlsbad, CA) TOPO TA cloning vector. A sequence-verified clone was then digested with *Eco*R1, and the 1.4-kb fragment ligated into the *EcoR1* in the pKr1n-pUC19 plasmid.

The maize *ubiquitin* promoter was used for the *Rp1-Kr1N* transient assay in place of the endogenous promoter as the maize *Rp1* endogenous promoter may not function well in other plant species. Previous work demonstrated that maize transgenic lines expressing the *Rp1-D* gene under the control of the *ubiquitin* promoter or its endogenous promoter gave high levels of *Rp1-D* transcripts (Ayliffe et al. 2004). However, wheat and barley transgenic lines expressing the *Rp1-D* gene under the control of its endogenous promoter gave low levels of *Rp1-D* transcripts that were mostly truncated.

Transformation constructs for both transient and stable plant transformation experiments were co-bombarded with constructs carrying selectable and or visible markers. The pAHC27 plasmid (Christensen et al. 1992) was used in transient assays to express the *uid*A reporter gene, which encodes *ß-glucuronidase* (GUS) and is driven by the maize *ubiquitin* promoter. The pBARGUS or pAHC20 plasmids were utilized to express the Bar and GUS reporter genes in stable plant transformations. pBARGUS contains a BAR

Table 1 Primers used to amplify *Rp1* sequences

Primer name	Position[a]	Primer sequence	Specificity[b]
hd2-F1	−1078 F	TTGAAAGGATCCTGCCCTTTATTACATCC	Specific
P6	67 F	AAGCTTCAGCTTACCTCAGT	Nonspecific
P7	338 F	CCTCCACTGCAACTACTGTC	Nonspecific
P8	638 F	AAGCTCAGCTAAGTACTCG	Nonspecific
K2	940 F	TCATAATGAGACAGAGTGGG	Nonspecific
2290F	1406 F	GGGTGGCAGAAGGATTTG	Nonspecific
Kr1N-1415F	1415 F	CTCTGGATCTTTCTTCCAATTGG	Specific
2620R	1740 R	GGATGGCCCAAGTGATATTTT	Nonspecific
P11	1860 F	TTGGTGAGCTGAAGCACCTC	Nonspecific
P12	2143 F	TTGAATGAGCTTGGTGGCAG	Nonspecific
P15	2200 R	GAGAATTGTCATTGGAAAGGAT	Nonspecific
Kr1N-2556R	2556 R	TTGCAACACCGAAATTGCAGGTG	Specific
P18	2592 F	ATCAATTGGTTGGTGCCCACTG	Nonspecific
3535F	2685 F	GATGTGGGAGGTGGATTCAGG	Nonspecific
DF	2824 F	ATTGGAAG TGTTCTGGTT CCGG	Specific
P19	3084 F	GATAGGTTGGTTGTAAGTG	Nonspecific
3990R	3116 R	GCTTACGTGCTGCTCCATCTC	Nonspecific
Kr1NF	3213 F	GTCCTTCTTTAGAGCTAGCACGG	Specific
Kr1NR	3337 R	TGCT CCCCATCCTTATCGATTGGC	Specific
P22	3460 F	TGTCCAGGAATCGCTCACGG	Nonspecific
4450R	3622 R	AGCCGTCAGTTTCATTTG	Nonspecific
4890R	4010 R	CTTGAAGCTCCAGAGTTCAGG	Nonspecific
DR4	4092 R	TAGCGGCGAAATACAAGCGGC	Specific
DR5	4121 R	GGCCACATGAATGATATAGC	Specific
DR3	4213 R	CCAATTGGATTGATTACAAGTGTG	Specific

Nonspecific primers were designed from conserved regions among the *HRp1-D* paralogs. Specific primers were designed from specific *Rp1* genes

[a] Position of 5′ end of the homologous sequence of the *Rp1-D* gene where position 1 corresponds to the predicted start codon. F and R correspond to the sequences of the coding and noncoding strands of the *Rp1* gene the primers were designed from

[b] Specific primers were designed for amplification of specific *Rp1* genes. Nonspecific primers were designed to amplify most or all *Rp1* genes

gene that encodes phosphinothricin acetyltransferase and the GUS (*ß-glucuronidase*) reporter gene under the control of the CaMV 35S promoter and *Adh1* promoter respectively (Fromm et al. 1990). pAHC20 contains a BAR reporter gene under the control of the *ubiquitin* promoter.

Generation of transgenic maize and wheat plants

A maize callus culture was established from immature embryos of HiII maize grown in the green house (Armstrong and Green 1985). The HiII maize population was highly susceptible to all the *P. sorghi* isolates used in this study. Wheat explants were prepared as pre-cultured (3–5 days) immature embryos from the spring wheat variety 'Bobwhite'. Transformation was performed with a particle inflow gun using tungsten as the micro carrier. Transgenic embryogenic maize callus was cultured under selection of 10 mg/l glufosinate for 4–5 months. Growing tissue was then selected, tested for GUS activity and regenerated to adult plants according to Songstad et al. (1996). Wheat callus was cultured for 2 weeks under selection of 5 mg/l glufosinate before shoot and root formation was initiated (modified from Altpeter et al. 1996). T_0 plants were transferred to soil and screened for transgene integration. Selected T_0 wheat plants were self-fertilized to generate T_1 progeny, while T_0 maize plants were crossed to HiII and H95 maize lines.

Transient assays and stable plant transformation

Maize and wheat lines stably expressing recombinant *rp1* genes were made using biolistic protocols as described previously (Ayliffe et al. 2004). The maize line HiII and the wheat line Bobwhite were utilized as the recipient lines. To verify that transgenes were being transcribed, RT-PCR was performed with total RNA isolated from transgenic maize callus clones or fully expanded second leaf segments of plants carrying the transgene. Total RNA was isolated

with Trizol Reagent (GIBCOBRL, Rockville, MD) as described by the manufacturer. RT-PCR was performed using a ProStar First-Strand RT-PCR Kit (Stratagene, LaJolla, CA). First strand synthesis was performed with an oligo dT primer. A Kr1N-1415F/Kr1N-2556R primer pair was used to specifically amplify Ubi-Kr1N cDNA sequences and a DF/DR3 primer pair was used to amplify Rp1-dp2-D-1 and Rp1-dp2-D-2 cDNAs (Table 1). These primers were designed from regions that were polymorphic among *Rp1* sequences and the sequences were specific to each transgene.

Transient transformation procedures were also carried out using biolistic transformation methods. Particle bombardment was performed with a particle inflow gun (60 psi of helium pressure) with an inside chamber vacuum of −28 psi and a target distance of 15 cm (Finer and McMullen 1991). Tungsten metal particles (20 µg of 99.9% 1 µm M10 equv., Atlantic Equipment Engineers, Bergenfield, NJ) were used as a DNA carrier. Three micrograms (1 µg/µl) of purified selectable marker plasmid and 3 µg of plasmid carrying the *Rp1* gene (1 µg/µl) were precipitated onto a 25 µl suspension of tungsten particles by adding 25 µl of 2.5 M $CaCl_2$ and 10 µl of 100 mM spermidine to the DNA sample. Following a 5-min incubation on ice, 50 µl of supernatant was removed. The particles coated with purified plasmid remained and 2 µl of the DNA/tungsten particle sample was used for each bombardment. Three treatments were used for transient assay transformations, one experimental and two controls. The experimental treatment consisted of the co-bombardment of pAHC27 and Ubi-Kr1N plasmids. One control treatment was the co-bombardment of pAHC27 and the Kr1N-pUC19 plasmids and a second control was the pAHC27 and Ubi-Rp1-D plasmids bombarded together. The pUbi-Rp1-D (Ayliffe et al. 2004) construct contains the *Rp1-D* gene coding region and 3′ UTR downstream of the maize *ubiquitin* promoter and was utilized as a control plasmid for transient assay experiments.

Leaf segments from fully expanded seedlings were placed on H_20/agar plates (8 g/500 mL) for transient transformation assays. Leaf sections were bombarded twice with DNA/tungsten particle samples. Plates containing bombarded leaf segments were placed in a growth chamber at 23°C overnight. Following incubation, a GUS histochemical assay was done to determine the viability of transformed cells (Jefferson 1987). Leaf segments were submerged in GUS assay stain and agitated gently overnight at 37°C. Leaf segments were destained for 4–6 h by gently shaking leaves in 100% EtOH for 1 h at 37°C followed by several incubations of 75% EtOH, changing the 75% EtOH solution every hour until the leaf segments were destained. GUS stained cells were then visualized under a dissecting microscope and counted to obtain numerical data for statistical analysis.

Statistical analyses

Statistical analysis of numerical data obtained from transient assay experiments was performed using SAS statistical program version 8.01 (SAS®, SAS Institute Inc., SAS Campus Cary NC, USA). SAS was used to test whether cells bombarded with the Ubi-Kr1N plasmid had fewer cells expressing the reporter gene than control bombardments in transient transformation experiments. The *P* (probability) value was set to 0.0001. All other parameters were set to default.

Results

Characterization of the *Rp1-D*21* recombinant gene

The *HRp1-D*21* variant conferring a spontaneous necrotic phenotype was originally identified in an *Rp1-D/Rp1-D* x *rp1/rp1* testcross population (Pryor 1987). Analysis of the variant haplotype indicated it was derived from an unequal crossing over event in which the most distal gene in the haplotype, *Rp1-D,* recombined with the gene that was second from the proximal end of the array, *rp1-dp2* (Sun et al. 2001). The resulting haplotype therefore carries the *rp1-dp1* gene and a recombinant gene, which consists of the 5′ portion of the *rp1-dp2* gene (NBS and first 280 amino acids of the LRRI) and the 3′ portion of the *Rp1-D* gene (remaining 480 amino acids coding for part of the LRRI-LRRII region).

To determine if the recombinant gene in *HRp1-D*21* conferred the nonspecific resistance phenotype, transgenic maize lines were made expressing the gene. PCR analysis confirmed genomic integration of the putative *Rp1-D*21* gene in six T0 plants, two of which produced T1 families by crossing to the HiII maize line. Both plants appeared normal before transplanting into soil but developed chlorotic spots soon within a week of transplanting to the greenhouse (Fig. 4d). T1 progeny of both lines segregated for the *Rp1-D*21* spontaneous necrotic phenotype described for plants carrying a native copy of the gene (Pryor 1993; Hu et al. 1996). The necrotic spotting phenotype segregated 24:42 and 39:45 (necrotic spots: normal). As with lines carrying the native copy of the gene, necrotic spots did not appear until leaf tissue was fully expanded and they typically appeared first near the tip of seedling leaves. *Rp1-D*21* transgenics responded rapidly to inoculation with rust isolates with a hypersensitive response even in leaves that were not fully expanded. This hypersensitive reaction was observed with *P. sorghi* as well as *P. triticina* confirming

the recombinant gene confers a nonspecific response to multiple rust species.

Identification and characterization of the *Rp1-Kr1N* recombinant gene

The *Rp1-Kr1N* variant, conferring a spontaneous necrotic phenotype, was identified in the progeny of a *HRp1-Kr1* homozygote (Hu et al. 1996). The *HRp1-Kr1* variant was previously recovered from an *Rp1-K*/*rp1* × *rp1*/*rp1* population (Richter et al. 1995) as a variant with a nonparental resistance specificity. Since the *HRp1-Kr1N* haplotype arose spontaneously from an *HRp1-Kr1* homozygote, DNAs of the two haplotypes were compared by gel blot analysis to determine if any novel restriction fragments had been generated. Southern blot analysis of homozygous DNAs isolated from *HRp1-KrN* identified a novel 2.1-kb fragment when digested with *Hin*dIII and probed with an LRR probe (*Rp1* 3′ LRR) corresponding to the 3′ region of the *Rp1-D* gene (Fig. 2). The novel fragment was not found in the parental haplotype and was therefore considered to be potentially associated with the *rp1* gene conferring the spontaneous necrotic phenotype. *HRp1-Kr1N* and *HRp1-Kr1* homozygous genomic DNAs were therefore run on an agarose gel and DNA from the region corresponding to the novel fragment was gel purified and amplified with three conserved *rp1* primer pairs (3535F/3990R, P19/4890R, P22/4890R; Table 1) that span the region corresponding to the *rp1* LRR probe that hybridizes to the novel fragment. The three primer pairs amplified fragments of expected size from *HRp1-Kr1N* and *HRp1-Kr1*. Direct sequencing of the PCR products was then attempted, reasoning that the products from the *HRp1-Kr1N* line may be composed mainly of a single product (corresponding to the unique restriction fragment), while the product from the *HRp1-Kr1* DNA (without the fragment) would be derived from a mixture of contaminating genes. The P19-4890R primer pair generated a PCR fragment from *HRp1-Kr1N* that sequenced well indicating it was derived mainly from a single gene. In contrast, no useful sequence could be generated from the *HRp1-Kr1* DNA with any of the three primer pairs. Sequences obtained from the *HRp1-Kr1N* amplification product were aligned with sequences from 37 other genes from *HRp1-K* to design primers specific to the novel fragment. *HRp1-Kr1N* appears nearly identical to its parental *HRp1-Kr1* haplotype, which is essentially indistinguishable from its parental *HRp1-K* haplotype (Fig. 2). Consequently, *HRp1-Kr1* and *HRp1-K* should carry mainly the same genes. The *Rp1* genes in *HRp1-K* have been partially characterized, but the genes from *HRp1-Kr1* have not. For these reasons, *HRp1-Kr1N* sequences were compared to genes from its *HRp1-K* grandparent.

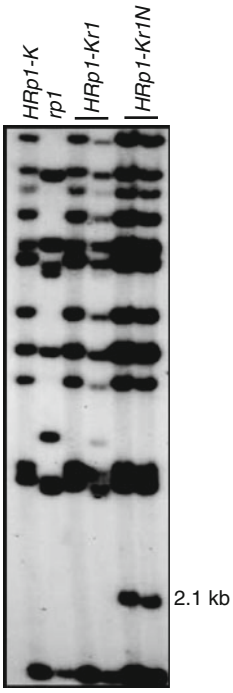

Fig. 2 Gel blot analysis of *HRp1-K*, *rp1*, *HRp1-Kr1* and *HRp1-Kr1N*. Homozygous DNA's were digested with *Hin*dIII and probed with an *Rp1* 3′ LRR probe. *HRp1-Kr1N* carries one gene (novel 2.1 kb *Hin*dIII fragment) distinguishable from those in the parental (*HRp1-Kr1*) and grandparental (*HRp1-K* and *rp1*) haplotypes

The primers Kr1N-1415F and Kr1N-2556R were designed to amplify sequences corresponding to the novel *Rp1-Kr1N* fragment, but none of the known sequences from *HRp1-K*. Genomic DNAs isolated from *HRp1-K*, *HRp1-Kr1, HRp1-Kr1N* and *Hrp1-H95* (maize inbred line) were PCR amplified with the gene-specific primers to ensure primer specificity. Primers specific for the novel fragment did not amplify in the parental (*HRp1-Kr1*) and grandparent haplotypes (*HRp1-K*, *Hrp1-h95*). However, the primer pair amplified a fragment of expected size in the recombinant haplotype (*HRp1-Kr1N*).

To identify a full-length gene from *HRp1-Kr1N* that carries a 2.1-kb *Hin*dIII fragment, a genomic *Lambda* library was constructed from DNA of seedlings homozygous for *HRp1-Kr1N*. Forty-seven *Lambda* clones were identified as carrying *rp1* sequences after probing with an *rp1* probe. Thirty-three of the clones hybridized to both 5′ (P6-1520R) and 3′ (P22-4890R) *Rp1-D* gene fragment probes indicating they potentially carried full-length genes. PCR positive clones were digested with *Hin*dIII to identify clones with a 2.1-kb *Hin*dIII fragment. Four of the 27 clones carried the fragment and generated fragments of the correct size when used as templates for PCR with the gene-specific primers. The four clones were partially digested with *Sau*3a and subcloned into pUC19 (*Bam*HI) to make plasmid templates for sequence analysis. Sequence analysis verified that the four *Rp1-Kr1N* clones were the same gene. One clone carrying the full predicted *Rp1-Kr1N* coding region (*Kr1N-35-2-60*) was fully sequenced and compared to genes from *HRp1-K*. Sequence comparisons demonstrated that the recombinant gene was identical to the *rp1-kp2* gene on the 5′ end and another gene from *HRp1-K* on the 3′ end (Fig. 3).

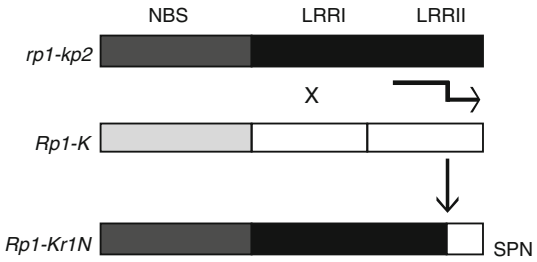

Fig. 3 Recombination events that created *Rp1-Kr1N* spontaneous necrotic variant. A recombination event between the *rp1-kp2* paralog and a gene from the *Rp1-K* haplotype generated *Rp1-Kr1N* recombinant gene, which confers a spontaneous necrotic phenotype. The recombinant contains the *rp1-kp2* NBS-LRRI and 577 bp of LRRII region. The remainder of LRRII is from a gene in the *Rp1-K* haplotype. *Arrows* indicate recombination exchange point. *SPN* indicates spontaneous necrotic phenotype

Phenotype verification of the *Rp1-Kr1N* gene

The *Rp1-Kr1N* spontaneous necrotic phenotype is expressed in the absence of a pathogen (Hu et al. 1996). To determine if the gene would cause cell death when delivered into a single cell, we conducted co-bombardment experiments with a construct expressing a GUS (pAHC27) reporter gene. Three treatments were used for six separate maize transient assay transformation experiments. Each experiment consisted of three separate bombardments each with equal amounts of two separate plasmids. The experimental treatment consisted of the co-bombardment of pAHC27 and Ubi-Kr1N plasmids and tested the function of the recombinant *Rp1-Kr1N* gene by determining the ability of this gene to cause cell death when delivered into a single plant cell. Two control treatments were utilized. One control treatment consisted of the co-bombardment of pAHC27and Kr1N plasmids. In this treatment, the *Kr1N* plasmid does not have a promoter so the bombardment should therefore demonstrate how plant cells would appear if expression of the *Rp1-Kr1N* gene had no effect on cell viability. A second control treatment consisted of the co-bombardment pAHC27 and Ubi-Rp1-D plasmids. The Ubi-Rp1-D plasmid carries the *Rp1-D* gene driven by the maize *ubiquitin* promoter and demonstrated how plant cells would appear when expressing a functional resistance gene other than the recombinant *Kr1N* gene. The results indicated that co-bombardment of maize leaf sections with the recombinant gene under the control of the *ubiquitin* promoter and a GUS reporter gene (pAHC27: Ubi- Kr1N) effected cell viability when compared to the control treatments (Fig. 4a). Statistical analysis of numerical data from six separate transient transformation assay experiments showed there were significantly ($P < 0.0001$) fewer GUS expressing cells in all experiments performed in maize (B73) when compared to the two control treatments (Fig. 5). The same three treatments were also performed in transient transformation experiments in rice (cv. Fanny), big blue stem, sorghum (line BTX623), and wheat (cv. Fielder).

No significant differences were observed in the number of GUS expressing cells in the experimental and control treatments in these species. Stable transgenic maize plants expressing the *HRp1-Kr1N* transgene have not yet been recovered. In addition, a very high frequency of callus tissue death was encountered during tissue culture, which was probably caused by the *Rp1-Kr1N* gene.

Identification of the recombinant *Rp1-MD*19* gene

The *Rp1-MD*19* variant was previously recovered from an *rp1-D*19/Rp1-M* x *rp1/rp1* testcross population (Hu et al. 1996). The *rp1-D*19* variant was originally identified as a susceptible recombinant from an *Rp1-D/Rp1-D* x *rp1/rp1* testcross population (Pryor 1987). A recombination event between *Rp1-D* and the *rp1-dp5* paralog gave rise to the r*p1-D*19* recombinant (Sun et al. 2001). The first 330 bp of the 5′ coding region was identical to *rp1-dp5,* while the remainder of the gene was from *Rp1-D*. It was therefore postulated that this gene was involved in the recombination event that gave rise to *Rp1-MD*19*, since the gene re-acquired the *Rp1-D* specificity. If the putative recombinant gene in *HRp1-MD*19* is oriented with the 3′ end closest to the telomere, like other *Rp1* genes examined (Sun et al. 2001), the flanking marker constitution of the recombinant indicates the gene would probably have the distal (3′) end of the *rp1-D*19* gene and the 5′ end of a gene from the *Rp1-M* parent haplotype. To amplify the putative recombinant gene, the *Rp1-D* specific reverse primers DR4 and DR5 were coupled with a nonspecific forward primer P6 from the 5′ region of the gene, and used to PCR amplify the gene using DNA from *Rp1-MD*19* as template. After cloning and sequencing the amplification product, the sequence data indicated that the recombinant *Rp1-MD*19* allele consists of the N-terminus of an *rp1* member from the *M* haplotype and C-terminus of the *Rp1-D* (from *rp1-D*19*) gene (Fig. 6). In the recombinant gene product, the entire LRR-encoding region is identical to the *Rp1-D* gene.

Construction of recombinant genes in vitro

To determine if we could reproduce the phenotype of the *Rp1-D*21* and *Rp1-MD*19* gene by making similar exchanges in vitro, two recombinant genes were made by swapping parts of the LRR region of the *rp1-dp2* gene with fragments of the *Rp1-D* gene LRR (Fig. 1). The Rp1-dp2-D-1 recombinant construct codes for the amino terminal 495 amino acids of the *rp1-dp2* gene, including the NBS-encoding region, and the *Rp1-D* 760 amino acid LRRI-LRRII region. The Rp1-dp2-D-2 construct codes for the amino terminal 864 amino acids of the *rp1-dp2* gene,

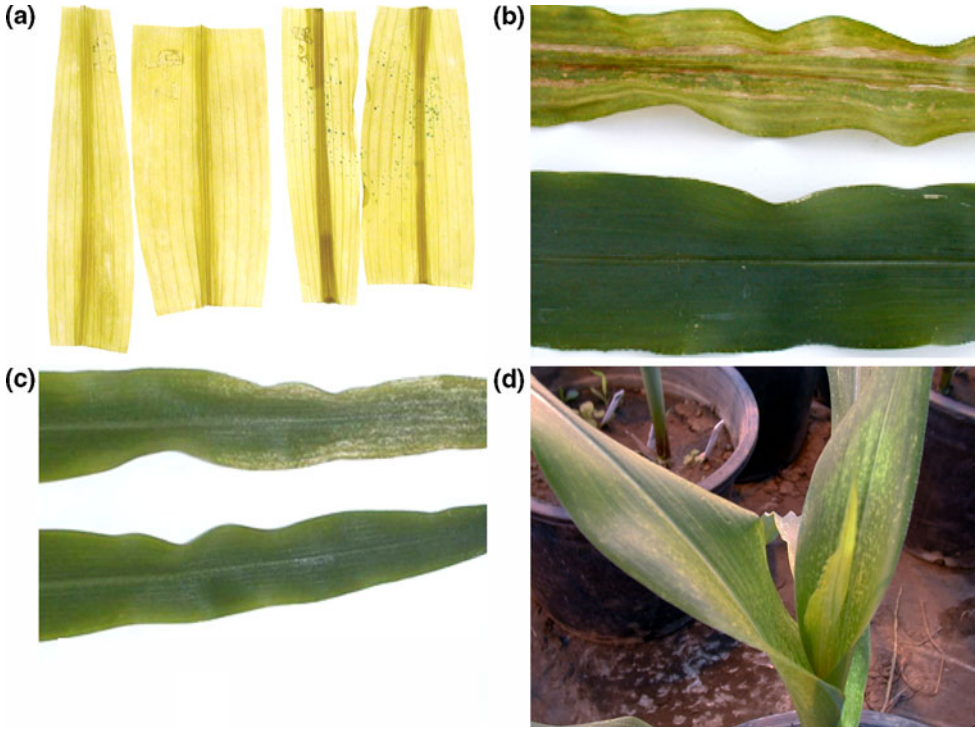

Fig. 4 *Rp1* recombinant gene phenotypes. **a** Maize Ubi-Kr1N transient assay. Co-bombardment of maize B73 seedlings with the GUS (*ß-glucuronidase*) reporter gene and the *Rp1-Kr1N* gene (Ubi-Kr1N) driven by the maize *ubiquitin* promoter (experimental; *two left leaves*) and *Rp1-Kr1N* with no promoter (control; *two right leaves*). *Blue spots* indicate healthy plant cells expressing the GUS reporter gene. **b** Rp1-D-dp2 transformation assay. *Rp1-D*21* confers a nonspecific resistance and a spontaneous necrotic phenotype. It arose by an unequal crossover from an *Rp1-D* homozygote. Two constructs (Rp1-D-dp2-D-2; *top leaf* and Rp1-dp2-D-1; *bottom leaf*) were created using different combinations of the LRR from the two parental genes (*Rp1-D* and *Rp1-dp2*) and tested in stable transgenic plants (HiIIA background). Constructs were co-bombarded with pAHC20 plasmid as a selectable marker. A line expressing the Rp1-dp2-D-2 construct showed the *Rp1-D*21* spontaneous necrotic phenotype without inoculation. **c** *HRp1-Kr1N* confers a spontaneous (uninoculated) necrosis. *HRp1-Kr1N* arose from an *HRp1-Kr1* homozygote. *HRp1-Kr1N* homozygotes are lethal, while heterozygotes (*Rp1-Kr1N*/*rp1*; *top leaf*) develop a diffuse necrosis on the tip of the leaves that expands to the base of the leaf as they become fully expanded. *rp1* homozygotes (*rp1*/*rp1*; *bottom leaf*) do not develop this phenotype. **d** T1 transgenic maize line expressing the Rp1-D*21 transgene showed a spontaneous necrotic phenotype on fully expanded leaves appearing first near the tip of seedling leaves and then extending down the leaf

including the regions coding for the NBS and LRRI regions, and the C-terminal 293 amino acids coding for the *Rp1-D* LRRII region. Therefore these constructs differ by how much of the *Rp1-D* LRR they retain. Comparatively, *Rp1-D*21* has the *rp1-dp2* NBS domain and first 280 amino acids of the LRR region. The DNA coding for the remaining 480 amino acids (LRRI-LRRII region) are from *Rp1-D*. The *Rp1-MD*19* recombinant has the NBS-encoding domain and the first 151 amino acids of the LRR region from a *HRp1-M* gene. The remaining 609 amino acids of the LRR domain is derived from *Rp1-D*.

The Rp1-dp2-D-1 and Rp1-dp2-D-2 recombinant constructs were tested in stable transgenic maize plants (HiII). Four transgenic maize lines were regenerated from callus carrying the Rp1-dp2-D-1 construct, and all four generated a fragment of expected size when amplified by RT-PCR with primers specific for the gene indicating it was transcribed in these lines. None of the four stable transgenic maize lines expressing the Rp1-dp2-D-1 construct conferred the *HRp1-D*21* spontaneous necrotic phenotype (Fig. 4b) and all were highly susceptible to rust isolate IN2, which is avirulent on lines expressing *Rp1-D*. In contrast, only one of four lines carrying the Rp1-dp2-D-2 construct expressed the transgene as determined by an RT-PCR assay. The three lines not expressing the transgene appeared normal, while all 14 plants regenerated from the callus genotype expressing the transgene showed a very severe spontaneous necrotic phenotype without inoculation (Fig. 4b). All 14 plants died before making viable pollen or seed. A second attempt at producing maize lines expressing the Rp1-dp2-D-2 transgene resulted in six maize lines carrying the transgene, but transcripts could not be detected in any of the six and all had normal phenotypes.

Rp1-D contributes to necrotic spotting phenotypes

Some *Rp1* haplotypes with apparently *normal* race-specific effects have been observed to control mild chlorotic or

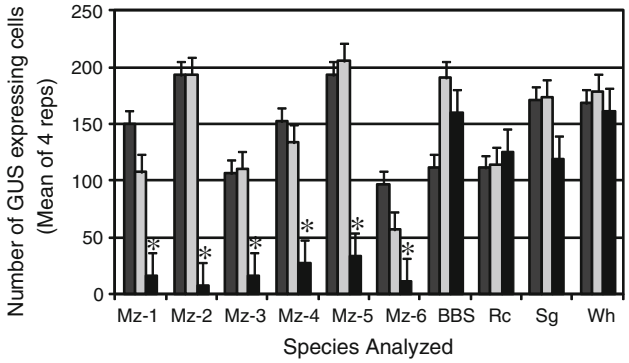

Fig. 5 Inhibition of reporter gene expression by co-bombardment with *Rp1-Kr1N*. The *X* and *Y* axis indicates the species analyzed and the number (mean of 4 replications) of GUS (*ß-glucuronidase*) expressing cells respectively. All treatments include the GUS expressing construct co-bombarded with a second plasmid. *Dark gray bars* denote the *Rp1-Kr1N* gene with (GUS: Kr1N) with no promoter; *light gray bars* indicate the *Rp1-D* gene under the control of the *ubiquitin* promoter (GUS: Ubi-Rp1-D); *black bars* denote the -Rp1-*Kr1N* gene under the control of the *ubiquitin* promoter (GUS: Ubi-Kr1N), all co-bombarded with GUS. An *asterisk* denotes a significant difference ($P < 0.0001$) in the number of GUS expressing cells between the bombardments with the Ubi-Kr1N and the controls. *Mz* maize, *BBS* big blue stem, *Rc* rice, *Sg* sorghum, *Wh* wheat

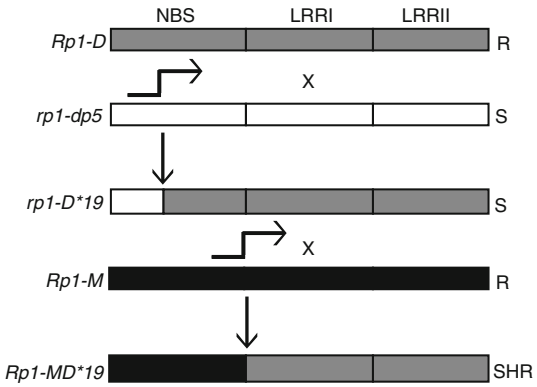

Fig. 6 Recombination events, that gave rise to *Rp1-MD*19* spontaneous necrotic variant. A recombination event between *Rp1-D* and *rp1-dp5* paralogs gave rise to the r*p1-D*19* recombinant, with no detectable resistance phenotype (Sun et al. 2001). A second recombination between *rp1-D*19* and a gene from the *Rp1-M* haplotype, gave rise to *Rp1-MD*19* recombinant gene, which confers a spontaneous necrotic phenotype. *Arrows* indicate recombination exchange points. *R* indicates haplotype confers a resistance phenotype. *S* indicates haplotype confers a susceptible phenotype. *SHR* indicates haplotype confers a spontaneous hypersensitive response

necrotic spotting phenotypes. This was first observed for an *Rp1-DJ* haplotype which carries both *Rp1-D* and *Rp1-J* in addition to other *rp1* homologs in the complex (Hu et al. 1997). This haplotype was associated with chlorotic spotting and partial nonspecific resistance to both common rust (*P. sorghi*) and southern rust (*P. polysora*). Additional crossing with this, and other haplotypes indicated these effects were dependent on the genetic background of the cross. In a field experiment planted on 14 April 2000 in Manhattan Kansas, two replications of 25-plant rows were planted for lines A188, H95 and four lines carrying *Rp1-A* and *Rp1-D* in the A188 and H95 backgrounds. Each of the genes had been backcrossed at least three times to the A188 and H95 parents. Plants in two rows carrying the *Rp1-D* haplotype in the A188 background (backcross three to A188) showed necrotic spots at the adult plant stage. When scored during pollen shed, all the plants were scored either as a three (heavy necrotic spotting on leaves below the ear) or a 4 (heavy spots on nearly all leaves). The average rating was 3.50. In contrast, little if any spotting was observed on A188 plants (*rp1-A188* haplotype) or plants homozygous for the *HRp1-A* haplotype in the A188 background. Most plants were rated 0 (no spotting) and several were rated 1 and had few chlorotic spots. Plants homozygous for the *HRp1-A* or *HRp1-D* haplotypes in the H95 inbred background were indistinguishable from the H95 parent (*Hrp1-H95* haplotype) and had no noticeable spotting phenotypes. The same trend was observed in other years in additional generations with these genes and with other haplotypes that carry the *Rp1-D* gene. The spotting phenotype with the *Rp1-D* gene in the A188 background seemed particularly consistent in early spring plantings (data not shown).

Observations made with *Rp1-D* transgenics indicate this gene, and not some other aspect of the *HRp1-D* haplotype, contributes to the spotting phenotype. After the original transgenics (HiII background; Ayliffe et al. 2004) were backcrossed twice to A188, a sample of the progeny were grown to maturity in the greenhouse. Five of ten plants that showed the *Rp1-D* rust resistance (heterozygotes) showed mild spotting phenotypes on the lower leaves at maturity (pollen shedding stage); Three were scored as 2 reactions and two as 1 reactions. The five plants not expressing the *Rp1-D* resistance did not show noticeable spotting (0 reactions).

Discussion

Three recombinant *Rp1* haplotypes have been characterized that confer severe necrotic reactions (Sun et al. 2001, Hu et al. 1996). One of these (*HRp1-Kr1N*) conferred a diffuse necrosis whose progression was not affected by rust inoculation and the other two (*HRp1-D*21* and *HRp1-MD*19)* conferred necrotic spots. The spots typically occur on fully expanded seedling leaves as they become fully expanded even in the absence of rust but can also be induced by rust infection, similar to the normal hypersensitive reaction, under the appropriate conditions. All three recombinants were associated with novel recombinant *Rp1* genes derived from crossovers in their LRR regions.

The recombinant genes in the *HRp1-D*21* and *HRp1-MD*19* haplotypes both carry part of their 3', LRR-encoding

regions from the *Rp1-D* gene. The *Rp1-D* gene itself can also cause a weak necrotic spotting phenotype on the mature leaves of adult plants in some genetic backgrounds. Individual *Rp1* genes can therefore have both race-specific effects and nonspecific effects associated with necrotic spotting and partial resistance. The LRR region of most *Rp1* genes consists of 14 very degenerate LRRs (LRR I region) followed by an approximately 100 amino acid region with no noticeable LRR homology and another 12 degenerate LRRs at the carboxy terminus (LRR II region). *Rp1-D*21* codes for a protein with the last two LRRs of LRR1 and the LRR2 region from the *Rp1-D* gene and does not confer the *Rp1-D* specificity since it does not confer any more resistance to rust isolates that are avirulent on *Rp1-D* than it does to *Rp1-D*-virulent isolates. In contrast, *Rp1-MD*19* does have the *Rp1-D* specificity in addition to the nonspecific necrotic reaction. The entire LRR-encoding region of this gene is identical to *Rp1-D* but the N terminal and NBS-encoding regions come from a gene from the Hrp1-M haplotype. The *rp1-dp1-D-1* gene was synthesized to carry the whole Rp1-D LRR but the N-terminus of *rp1-dp2*, the same N-terminus as the *Rp1-D*21* gene. This gene conferred no spontaneous necrosis or resistance when expressed in transgenic plants. The other gene synthesized in vitro (Rp1-dp2-D-2) carried LRR2 from Rp1-D and the N-terminus and LRR1 from rp1-dp2. This appeared to confer a very severe spontaneous necrotic phenotype similar to the *Rp1-D*21* gene but only a single transformation event was regenerated and every seedling from this event died before race specificity could be examined. Thus it appears the nonspecific spontaneous necrotic phenotype can be caused by a recombinant LRR-encoding region, or exchanging the whole *Rp1-D* LRR region onto a different N-terminus.

The LRR domains have been proposed to be responsible for the specific recognition functions in the NBS-LRR class of disease resistance genes and thus determine resistance specificities (Baker et al. 1997; Ellis et al. 1999; Dodds and Schwechheiner 2002, Ellis et al. 2007). The results with the *Rp1* recombinants are consistent with this idea. The recombination event that gave rise to Rp1-MD*19 restored an *Rp1-D* specificity and the recombinant gene in this haplotype included an intact *Rp1-D* LRR. Apparently, this LRR, and recombinant LRR with only the C-terminal half of it, are also able to confer a nonspecific spontaneous necrotic phenotype when combined with the appropriate N-terminus. The effect may be small, as with the *Rp1-D* gene whose nonspecific reactions are only noticeable in certain genetic backgrounds in adult plants, or they may be very strong. It is not clear if they function by actually interacting with a specific protein or compound made by the fungus or the fungal interaction because they can cause HR in the absence of the fungus. It is possible their ability to respond to fungal infection under the appropriate conditions is caused by priming cells towards defense reactions which then occurs when the cells are challenged by a pathogen.

HRp1-Kr1N also confers a spontaneous necrotic phenotype, but does not exhibit a classic lesion mimic, spotting phenotype. It is also unique in that the necrosis is not inducible by rust inoculation, but appears to be developmentally controlled (Fig. 4c). Rust resistance has also been assayed on *HRp1-Kr1N* seedlings prior to necrosis, and it is clear that the *HRp1-Kr1N* haplotype still confers the *Rp1-Kr1* parental resistance specificity (Hu et al 1996). Consequently, the recombinant gene in this haplotype is probably different from that causing the *Rp1-Kr1* specificity. The function of the Rp1-*Kr1N* recombinant gene was tested in transient and stable transformation experiments. Transient transformation experiments demonstrated that delivery of the gene into single cells interfered with cell viability sufficiently to inhibit reporter gene expression (Fig. 5). Therefore, the *Rp1-Kr1N* recombinant gene kills cells spontaneously and confers the necrotic phenotype in maize supporting the postulate that the change in the distal half of LRRII domain of the *Kr1N* recombinant gene is responsible for the spontaneous necrotic phenotype. Moreover, the inability of the two control constructs to kill cells demonstrated that the harmful effect on cell viability was due to the expression of the recombinant *Kr1N* gene and not the expression of any *rp1* resistance gene. There were no stable transgenic maize plants expressing the *Rp1-Kr1N* transgene recovered. The high frequency of callus tissue death during tissue culture were likely due to expression of the *Kr1N* gene. These two factors indicated that the recombinant confers a lethal phenotype in stable transgenic plants, making it difficult to recover transgenics expressing this gene. It is plausible that the change in the LRR domain of *Kr1N* allows it to inappropriately interact with plant signaling components to trigger activation of the defense response pathway in the absence of a pathogen.

Delivery of *Rp1-Kr1N* into cells of rice, big blue stem, sorghum and wheat had no noticeable effect on cell viability, as assayed by reporter gene expression. Therefore, the *Rp1-Kr1N* gene does not appear to function in these distantly related cereal species. Similarly, stable transgenic wheat plants expressing the *Rp1-D*21* gene showed no noticeable resistance or spontaneous necrosis. This may be due to a lack of necessary complementary components of a protein complex or signaling component required for activation of the defense response pathway and *Rp1-Kr1N* expression. Alternatively, Ayliffe et al. (2004) found many of the *Rp1-D* transcripts in stable wheat and barley transgenics were truncated, raising the possibility that other cereal species were not making *Rp1* proteins properly.

Characterization of the three *Rp1* variants provides evidence that some spontaneous necrotic variants are the result of an alteration of actual disease resistance genes. The hypothesis that similar types of resistance genes, or other

genes involved in defense responses, are affected in other spontaneous necrotic variants is also supported by the analysis of spontaneous necrotic variants in several different species (Walbot et al. 1983; Dietrich et al. 1994; Johal et al. 1995; Lorrain et al. 2003). One example is the Arabidopsis *dll1* (disease-like lesions1) spontaneous lesion variant. This variant produces lesions spontaneously that mimics bacterial speak disease and showed accumulation of genes related to defense. These spontaneous or 'autoactive' alleles have been best characterized at the *L* locus in flax (Howles et al. 2005). Several mutant and recombinant variants were created with alterations in the NBS and LRR regions that caused a gene dosage-dependent dwarf phenotype and constitutive expression of plant defense genes in the absence of the pathogen. Similarly, the autoactive *Rp1* genes described here were also created by alterations (intragenic recombination) in the LRR region. It is therefore plausible that regions of *Rp1* proteins function as negative regulators of defense signaling that are mediated by other regions of the R-protein. Once these interactions are hindered by recombination in the LRR region, constitutive signaling can occur activating plant defenses in the absence of the pathogen or the AVR protein.

One of the most interesting aspects of the *Rp1* alleles conferring spontaneous necrotic phenotypes is that their necrotic reaction was induced by inoculation with any rust biotype, and every rust species that was tested (Hu et al. 1996). Therefore, the race-nonspecific resistance of these genes has the greatest potential for durable disease control. The one disadvantage of using resistance genes to control disease is the fact that their resistance is race-specific and therefore not durable due to shifts in pathogen populations. Quantitatively inherited disease resistances are usually considered more durable, but they are very difficult to manipulate genetically (Hooker 1967). Race-nonspecific resistance that can be manipulated as single loci would be invaluable as a method for controlling disease. The variant *Rp1* genes provide evidence that a race-specific rust resistance gene can be engineered to confer race-nonspecific resistance and provides some insight as to the information needed for designing the types of changes that might be made to resistance genes to create genes with the desired nonspecific effects.

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