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Genome scanning for resistance-gene analogs in rice, barley, and wheat by high-resolution electrophoresis

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Abstract Genes cloned from diverse plants for resistance to different pathogens have sequence similarities in domains presumably involved in pathogen recognition and signal transduction in triggering the defense response. Primers based on the conserved regions of resistance genes often amplify multiple fragments that may not be separable in an agarose gel. We used denaturing polyacrylamide-gel electrophoresis to detect PCR products of plant genomic DNA amplified with primers based on conserved regions of resistance genes. Depending upon the primer pairs used, 30–130 bands were detected in wheat, rice, and barley. As high as 47%, 40%, and 27% of the polymorphic bands were detected in rice, barley, and wheat, respectively, and as high as 12.5% of the polymorphic bands were detected by certain primers in progeny from a cross of the wheat cultivars 'Stephens' and 'Michigan Amber'. Using F_6 recombinant inbred lines from the 'Stephens' × 'Michigan Amber' cross, we demonstrated that polymorphic bands amplified with primers based on leucine-rich repeats, nucleotide-binding sites and protein kinase genes, were inherited as single loci. Linkages between molecular markers and stripe rust resistance genes were detected. This technique provides a new way to develop molecular markers for assessing the genetic diversity of germplasm based upon potential candidate resistance genes in diverse species.

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Introduction

Genes for resistance to pathogens have been cloned from diverse plant species (Johal and Brigg 1992; Martin et al. 1993; Bent et al. 1994; Jones et al. 1994; Mindrinos et al. 1994; Whitman et al. 1994; Grant et al. 1995; Lawrence et al. 1995; Song et al. 1995; Dixon et al. 1996; Baker et al. 1997). Sequence comparisons among these genes reveal structural similarities. Although the overall sequence homology among the genes is low, and not sufficient to be detected by cross hybridization using RFLP, the conserved domains in the genes offer opportunities for PCR-amplification and the isolation of similar sequences in other plant species (Kanazin et al. 1996; Leister et al. 1996; Michelmore 1996; Yu et al. 1996).

The candidate resistance gene approach has been used to isolate plant resistance genes and develop molecular markers (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996; Feuillet et al. 1997). Using degenerate primers based on the conserved leucine-rich repeats (LRR) of the *RPS2* gene of *Arabidopsis thaliana* and the *N* gene of tobacco, Leister et al. (1996) obtained amplification products from potato that were homologous to known resistance genes and absolutely linked to the nematode resistance locus *Gro1* and the late blight resistance locus *R7*. Kanazin et al. (1996) designed primers based on regions of amino-acid identity in the *L6* gene from flax, the *N* gene and the *RPS2* gene, and mapped several resistance gene analog (RGA) loci near known resistance genes in soybean. Similarly, using primers for the nucleotide-binding site of *N* and *RPS2*, Yu et al. (1996) mapped the RGA to the vicinity of known soybean genes for resistance to potyviruses

(*Rsv1* and *Rpv*), *Phytophthora* root rot (*Rps1*, *Rps2*, and *Rps3*), and powdery mildew (*rmd*). Using the oligonucleotides corresponding to the conserved subdomains in serine/threonine protein kinases, Feuillet et al. (1997) successfully isolated the wheat leaf-rust resistance gene *Lr10*.

To-date, all reports of the cloning of RGAs have used agarose-gel electrophoresis to resolve PCR products, and RFLP to find polymorphisms among plant genotypes. Heterogeneity of DNA fragments from single bands in an agarose gel was observed by Kanazin et al. (1996), Leister et al. (1996), and Yu et al. (1996). In attempts to clone RGA fragments from agarose gels, we also noted that the PCR products were highly heterogeneous (unpublished data). We reasoned that these heterogeneous RGA fragments could be separated and polymorphic bands directly detected using high-resolution electrophoresis. This report deals with a genome scanning technique for RGAs in rice, barley, and wheat using high-resolution electrophoresis which increases the efficiency of detecting putative resistance genes and may be applied to assess the diversity of disease resitstance in crop germplasm.

Table 1 Rice near-isogenic lines used in the study and their single genes for resistance to *M. grisea*

O. sativa indica[a]			*O. sativa japonica*[b]		
No.	Line	Gene	No.	Line	Gen
1	C039		7	F80-1	*Pi-k*
2	C101A51	*Pi-2*	8	F987	*Pi-km*
3	C101LAC	*Pi-1*	9	F124-1	*Pi-ta*
4	C101PKT	*Pi-ta*	10	F128-1	*Pi-ta*^{2}
5	C104PKT	*Pi-3*	11	F129-1	*Pi-kp*
6	C105TTP	*Pi-4a*	12	F145-2	*Pi-b*

[a] The *indica* rice near-isogenic lines were developed by Inukai et al. (1994)
[b] The *japonica* rice near-isogenic lines were developed by Ling et al. (1995)

Materials and methods

Plant materials

Twelve rice (Table 1), 23 barley (Table 2), and 44 wheat cultivars and lines (Table 3), together with 101 F_6 recombinant inbred lines from the "Stephens" × "Michigan Amber" wheat cross, were used in this study. The rice lines consisted of six *O. sativa indica* and six *O. sativa japonica* near-isogenic lines (NIL) that have single race-specific genes for resistance to rice blast (*Magnaporthe grisea*), developed by Inukai et al. (1994) and Ling et al. (1995). The barley genotypes were selected based on our previous study of the reactions of barley selections to *Puccina striiformis* f. sp. *hordei* (Chen XM et al. 1995). The wheat genotypes consisted of commercial cultivars grown in the Pacific Northwest of the United States, and genotypes either resistant or susceptible to races of *P. striiformis* f. sp. *tritici*. The 101 F_6 recombinant inbred lines were developed by single-seed descent from the Stephens × Michigan Amber cross. This population was previously characterized for durable, high-temperature, adult-plant resistance to stripe rust (Chen and Line 1995a, b).

Disease evaluation

Resistance of the barley genotypes to 31 races (PSH-1 to PSH-31) of *P. striformis* f. sp. *hordei*, and resistance of the wheat genotypes and the F_6 recombinant inbred lines to races CDL-6, CDL-17, CDL-29, CDL-35, CDL-43, and CDL-45 of *P. striiformis* f. sp. *tritici*, was evaluated based on infection type under the controlled conditions described by Chen and Line (1992). Resistance of the rice near-isogenic lines was reported by Inukai et al. (1994) and Chen et al. (1995).

DNA Extraction and PCR amplification

Three grams of fresh leaves were ground in liquid nitrogen and genomic DNA was extracted from the leaf powder using the cetyltrimethylammonium bromide (CTAB) procedure described by Murray and Thompson (1980). The DNA was quantified by the minigel method (Maniatis et al. 1982). Each PCR reaction was performed in a 25-μl vol consisting of 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Sigma Chemical Co., St. Louis, Mo); 5 mM of $MgCl_2$; one unit of *Taq* DNA polymerase (Promega, Madison, Wis.), 4–8 μM of primer, depending on the specific primer (see Table 4); 30 ng of

Table 2 Reactions of barley (*H vulgare* L.) genotypes and lines to *P. striiformis* f. sp. *hordei*

No.	Genotype	Reaction[a]	No.	Genotype	Reaction[a]
1	Topper	Susceptible	13	P1548734	Resistant
2	Heils Franken	Differential	14	P1548747	Resistant
3	Emir	Differential	15	BBA 2890	Resistant
4	Astrix	Differential	16	Abyssinian 14	Resistant
5	Hiproly	Differential	17	Grannelose Zweizeilige	Resistant
6	Varunda	Differential	18	Steptoe	Susceptible
7	Abed Binder 12	Differential	19	Cambrinus	Differential
8	Trumpf	Differential	20	BBA 809	Differential
9	Mazurka	Differential	21	Zephyr	Differential
10	Bigo	Differential	22	Nakai Zumizairai	Differential
11	I 5	Resistant	23	Stauffers Obersulzer	Resistant
12	P1548708	Resistant			

[a] "Susceptible" indicates that the genotype is susceptible to, and "resistant" indicates that the genotype is resistant to, all detected races of *P. striiformis* f. sp. *hordei* in the United States. "Differential" indicates that the genotype is resistant to some races but susceptible to other races. Reactions of barley genotypes to races PSH-1 to PSH-14 were reported by Chen et al. (1995)

Table 3 Wheat cultivars and lines used in the study and their reactions to cereal disease laboratory (CDL) races of *P. striiformis* f. sp. *tritici*

Cultivar or line[a]		Reaction to CDL races[b]	Cultivar on line[a]		Reaction to CDL races[b]
No.	Name		No.	Name	
1	Barbee	6, 17, 35, 43, 45/29	23	Chinese 166	6, 29, 35, 43, 45/17
2	Crew	6, 17, 29, 35, 43, 45/	24	Chinese Spring	/6, 17, 29, 35, 43, 45
3	Hiller	6, 17, 29, 35, 43, 45/	25	Compair	Resistant
4	Hyak	6, 17, 29, 35, 43/45	26	Daws	/6, 17, 29, 35, 43, 45
5	Jacmar	6, 17, 35, 45/29, 43	27	Druchamp	17, 29, 35, 43, 45/6
6	Moro	6, 17, 35, 45/29, 43	28	Dusty	6, 17, 29, 35, 45/43
7	Omar	/6, 17, 29, 35, 43, 45	29	Fielder	17, 29, 35, 43, 45/6
8	Omar Mutant	/6, 17, 29, 35, 43, 45	30	Gaines	/6, 17, 29, 35, 43, 45
9	OR092007	6, 17, 29, 35, 43/45	31	Hatton	17, 35, 45/6, 29, 43
10	OR092049	/6, 17, 29, 35, 43, 45	32	Hill 81	6, 17, 29, 35, 43, 45
11	Paha	6, 17, 35, 45/29, 43	33	Lee	6, 29, 35, 43, 45/17
12	PS279	Susceptible	34	Lemhi	/6, 17, 29, 35, 43, 45
13	Rely	6, 17, 29, 35, 43, 45/	35	Luke	6/17, 29, 35, 43, 45
14	Rohde	6, 17, 29, 35, 43, 45/	36	Madsen	6, 17, 29, 35, 43, 45
15	Suwon 92/Omar 53	6, 17, 35, 45/29, 43	37	Michigan Amber	Susceptible
16	Tres	6, 17, 29, 35, 45/43	38	Nugaines	/6, 17, 29, 35, 43, 45
17	Tyee	6, 17, 29, 35, 43/45	39	Penawawa	6, 35, 43, 45/17, 29
18	WA 7622	6, 17, 29, 35, 43, 45/	40	Produra	17, 29, 35, 43, 45/6
19	WA 7752	6, 17, 29, 35, 43, 45/	41	Riebesel 47/51	Resistant
20	WA 7692	6, 43, 45/17, 29, 35	42	Stephens	6, 17, 29, 43, 45/35
21	WA 7697	6, 17, 29, 35, 43/45	43	*T. spelta album*	Resistant
22	Cashup	17, 35, 43, 45/6, 29	44	*T. spelta saharances*	Susceptible

[a] Cultivar no. 40 is durum wheat (*T. durum* Desf.); cultivar nos. 43 and 44 are spelta wheats (*T. spelta* L.), and the remaining cultivars are bread wheat (*T. aestivum* L.). Cultivar nos. 1–21 are club wheats. Cultivar nos. 24, 25, 29, 33, 34, 39, 40, and 44 are spring wheats, and the rest of the cultivars are winter wheats. Cultivar nos. 23 and 24, 25, 27, and 41 originated in China, the United Kingdom, France, and Germany, respectively, and all others originated in the USA, and primarily from the Pacific Northwest

[b] Reactions of plants to CDL races of *P. striiformis* f. sp. *tritici*; the *numbers* to the left of the slash (/) indicate the CDL race numbers that the cultivar or line was resistant to, whilst the CDL race numbers to the right of the slash indicate those that the cultivar or line was susceptible to. "Resistant" indicates the cultivar or line that is resistant to, and "susceptible" indicates the cultivar or line that susceptible to, all the detected North American races

template DNA; 2.5 μl of 10 × *Taq* polymerase buffer (Promega). For control reactions, template DNA was substituted by sterile distilled H_2O to ensure that there was no contamination. The PCR reaction was overlaid with mineral oil. Amplification was carried out in a Perkin-Elmer model 480 thermal cycler programmed for 5 min at 94°C for initial denaturation and 45 cycles consisting of 1 min at 94°C, 1 min at 45°C, and 2 min at 72°C, followed by a final 7-min extension at 72°C. A 2.5-min ramp time was used between the 94°C denaturation and the 45°C annealing steps; the fastest possible ramp for all other temperature transitions used. Primers corresponding to the conserved domains of the plant resistance genes are listed in Table 4.

Electrophoresis, silver staining, and photography

After amplification, a 5-μl PCR product was electrophoresed in a 1% agarose gel in 0.5 × TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA). A 1-kb DNA ladder (0.15 μg) (Gibco BRL, Bethesda, Md.) was used to estimate the size of each amplified DNA fragment. The gel was run for 90 min at 100 V, stained with ethidium bromide (0.5 μg/ml) and photographed under UV light. Detection of a low-molecular-weight (ranging from 30 bp to 2 kb) smear indicated a successful amplification. To the remaining 20-μl solution, 7 μl of formamide loading buffer [98% formamide; 10 mM EDTA, pH 8.0; 0.5% (w/v) bromophenol blue, and 0.5% (w/v) xylene cyanol] was added to prepare samples for polyacrylamide-gel electrophoresis.

Denaturing polyacrylamide-gel electrophoresis (PAGE) was used to separate amplified DNA fragments. A 5% polyacrylamide gel (398 mm × 338 mm × 0.4 mm) was prepared using Bind Silane (Promega), as recommended by the manufacturer. After polymerization, the gel was pre-run in 1 × TBE buffer for 40 min at 1600 V to reach a gel temperature of 50°C. Urea and gel debris were removed from the well area and a 0.4-mm-thick shark comb (Gibco, BRL, Gaithersburg, Md.) was inserted. Prior to loading, the samples and a 1-kb ladder DNA in formamide loading buffer were denatured for 5 min at 94°C and immediately put on ice. Five to ten microliters of each sample were loaded depending on the amplification intensity seen on the agarose gel. The polyacrylamide gel was run at 1400 V for 2–2.5 h. After electrophoresis, the gel was silver-stained according to the manufacturer's recommendation (Promega). A gel photograph was produced using a silver-sequence automatic processor-compatible film (Promega).

Data analysis

To infer the genetic relationships among the germplasms, dendrograms based on the banding patterns were constructed using the Numerical Taxonomy System (NTSYS-pc), version 1.80 (Rohlf 1993). All the amplified bands were treated as dominant genetic markers. The presence of a band in a polyacrylamide gel was coded as 1 and its absence was coded as 0. A similarity matrix based on simple matching was generated by the SIMQUAL program, and cluster analysis was performed with the unweighted pair group

arithmetic mean method (UPGMA) in the SAHN program. The dendrogram that best fit the similarity matrix based on co-phenetic value and matrix comparison (MXCOMP) was chosen. The statistical stability of the branches in the cluster was estimated by bootstrap analysis with 1000 replicates using the Winboot computer program (Nelson et al. 1994). Linkage analysis of both RGA markers and stripe rust resistance loci was performed using the computer program Mapmaker 3.0 (Lander et al. 1987), and independent linkage groups were obtained using the "group" command. The most probable linkage order and map distances within linkage groups were determined by the "compare" and "map" commands.

Results

High degree of polymorphism in RGAs detected by polyacrylamide-gel electrophoresis

Amplification of the genomic DNA of rice, barley, and wheat with RGA primers resulted in mostly one to a few bands in 1% and 1.5% agarose gels. Except for the rice lines amplified with the NLRR for/NLRR rev primers, polymorphism was not found within the three species when PCR products were separated by agarose-gel electrophoresis. However, cloning of single major bands from an agarose gel often resulted in several different clones. Therefore, we reasoned that a large number of bands and fragment-length polymorphisms could be detected by high-resolution electrophoresis. Using denaturing polyacrylamide-gel electrophoresis, 30 to >130 bands were detectable among the rice, barley, and wheat genotypes. The highest polymorphisms detected with a primer pair in rice, barley, and wheat were 47, 48, and 39%, respectively, and the mean polymorphisms were 31% in rice, 38% in barley, and 21% in wheat. The number of scored bands and polymorphic bands detected in rice, barley, and wheat using different primers are shown in Table 4. The results confirmed the observation that highly heterogeneous products are produced by PCR-amplification with RGA primers and showed that, compared to agarose-gel electrophoresis, the high-resolution of denaturing polyacrylamide-gel electrophoresis greatly increases the ability to detect polymorphism.

Polymorphism in rice near-isogenic lines

A total of 188 polymorphic bands were obtained in the 12 rice NILs using eight primer pairs (Table 4). Figure 1 shows the amplified fragments of the 12 rice NILs with the NLRR for/NLRR rev primers. Of the 188 polymorphic bands, 68 were only present in lines with *O. sativa indica* rice background (Co39 series) and 61 were present in lines with the *O. sativa japonica* background (LTH series). Thirteen bands were present in only one of the NILs, suggesting that these bands can differentiate the corresponding NILs and that they may be associated with each of the specific rice-blast resistance genes. Based on the consensus dendrogram obtained from the cluster and bootstrap analyses based on the polymorphic bands (Fig. 2), all of the branches had high bootstrap values, indicating the robustness of the branching pattern. The NILs with an *indica* background were clearly separated from those with a *japonica* background.

Polymorphism in barley genotypes

The 23 barley genotypes consisted of cultivars or lines that are either resistant or susceptible to races of *P. striiformis* f. sp. *hordei*. A total of 165 polymorphic bands were observed among the barley genotypes using six primer pairs (Table 4). Figure 3 illustrates the polymorphism detected in barley germplasm using the primer pair S2/AS3, which was derived from the conserved domain of the *RPS2* and *N* gene products. Unique bands were observed in Abyssinian 14, Bigo, Emir, Grannelose Zweizeilige (GZ), Hiproly, I.5, Steptoe, Mazurka, Nakai Zumizairai, Topper, and Trumpf.

Cluster analysis based on polymorphic RGA bands separated the barley genotypes into two groups (Fig. 4). Hiproly, GZ, PI 548708, PI 548734, PI 548747, and Abyssinian 14 formed one group (BG 2) and all the remaining genotypes formed another group (BG1). Except for Hiproly, all genotypes in BG2 are resistant to the races of *P. striiformis* f. sp. *hordei* that have been identified in the United States. Hiproly is resistant to some races and susceptible to others. Within BG2, Hiproly and GZ, the only hulless cultivars, are more closely related to each other than to the other genotypes. Three bands amplified with the RLK for/RLK rev primers were only present in Hiproly and GZ, and two bands amplified with the S2/AS3 primer pair were absent in Hiproly and GZ but present in all other genotypes.

Genotypes in BG1 are more heterogeneous than those in BG2, as indicated by the lower bootstrap values for the majority of the branches in BG1; however, sub-clusters appear to reflect biological relationship. Topper and Steptoe, which are susceptible to all identified races of *P. striiformis* f. sp. *hordei* in the United States, were more closely related to each other than to the other genotypes. The clustering of BBA 809 and BBA 2890 is consistent with the German origin of the two genotypes. Thus, clusters produced by RGA markers are consistent with the genetic relationships inferred by known phenotypic characteristics and geographic origins.

Polymorphism in wheat cultivars and lines

Figure 5 illustrates the polymorphism detected among wheat genotypes using the primers Pto kin1/Pto kin 2. A total of 95 polymorphic bands were observed using

Table 4 Numbers of the scored and polymorphic bands amplified in rice, barley, and wheat by primers corresponding to conserved domains of resistance genes, using denaturing polyacrylamide-gel electrophoresis

Primer[a]	Sequence (5′–3′)[b]	Number of scored bands				Number of polymorphic bands			
		Rice	Barley	Wheat	F_6 S/M[c]	Rice	Barley	Wheat	F_6 S/M[c]
Pto kin 1 Pto kin 2	GCATTGGAACAAGGTGAA AGGGGGACCACCACGTAG	90	73	97	72	32	35	38	9
RLRR for RLRR rev	CGCAACCACTAGAGTAAC ACACTGGTCCATGAGGTT	30	45	56	28	8	14	10	3
S2 AS3	GGIGGIGTIGGIAAIACIAC IAGIGCIAGIGGIAGICC	97	125	132	114	35	50	31	10
S2 AS1	GGIGGIGTIGGIAAIACIAC CAACGCTAGTGGCAATCC	89	–	–	–	30	–	–	–
XLRR for XLRR rev	CCGTTGGACAGGAAGGAG CCCATAGACCGGACTGTT	91	80	38	–	27	32	6	–
RLK for RLK rev	GAYGTNAARCCIGARAA TCYGGYGCRATRTANCCNGGITGICC	61	63	56	–	3	20	5	–
NLRR for NLRR rev	TAGGGCCTCTTGCATCGT TATAAAAAGTGCCGGACT	95	–	68	46	45	–	5	3
NBS-F1 NBS-R1	GGAATGGGNGGNGTNGGNAARAC YCTAGTTGTRAYDATDAYYYTRC	68	–	–	45	8	–	–	1
CLRR for CLRR rev	TTTTCGTGTTCAACGACG TAACGTCTATCGACTTCT	–	44	–	–	–	14	–	–
Total bands		621	430	445	305	188	165	95	26

[a] The primer pairs Pto kin1 and Pto kin2 were designed based on the DNA sequence encoding for protein kinase in the tomato *Pto* gene conferring resistance to *Pseudomonas syringae* pv *tomato*. The primers S2, AS1 and AS3 were designed by Leister et al. (1996) based on the resistance genes *RPS2* of *A. thaliana* and *N* of tobacco. The primer pairs RLK for and RLK rev were designed by Feuillet et al. (1997) to amplify serine/threonine kinase sequence subdomains II to VIII of the wheat *Lr10* gene conferring resistance to *Puccinia recondita*. The primer pairs NBS F1 and NBS R1 were designed by Yu et al. (1996) based on the amino-acid sequences of two highly conserved motifs of the nucleotide-binding site in tobacco *N* and *Arabidopsis RPS2* genes. The primer pairs RLRR for and RLRR rev, XLRR for and XLRR rev, NLRR for and NLRR rev, and CLRR for and CLRR rev were designed based on leucine-rich repeat regions of genes *RPS2*, *Xa21* in rice against *Xathomonas compestris* pv *oryzae*, *N*, and *Cf9* in tomato against *Cladosporium fulvum*, respectively (Naweed Naqvi, IRRI). All primers were made by Operon (Alameda, California, USA)

[b] Codes for mixed bases: Y = C/T, N = A/G/C/T, R = A/G, and D = A/G/T

[c] F_6 S/M signifies F_6 recombinant inbred lines from the Stephens × Michigan Amber wheat cross

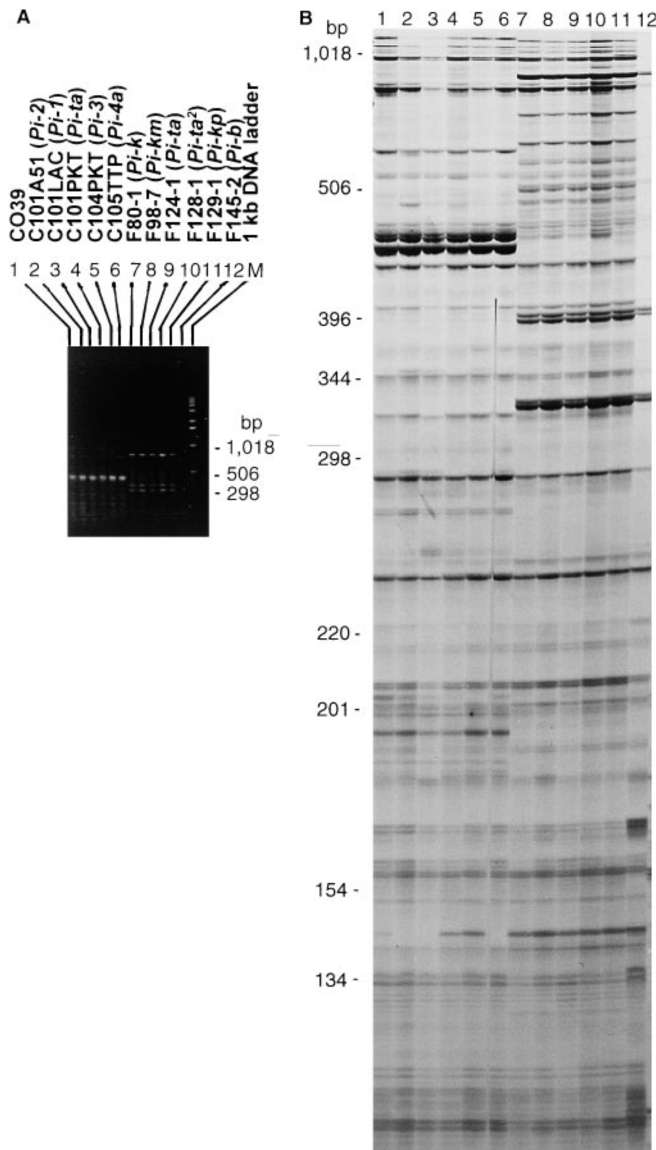

Fig. 1 Agarose gel (**A**) and silver-stained denaturing polyacrylamide gel (**B**) showing polymorphism among rice (*O. sativa*) near-isogenic lines. DNA was amplified with the NLRR for/NLRR rev primers. Note the relatively few bands resolved by agarose-gel electrophoresis compared to the denaturing polyacrylamide-gel electrophoresis

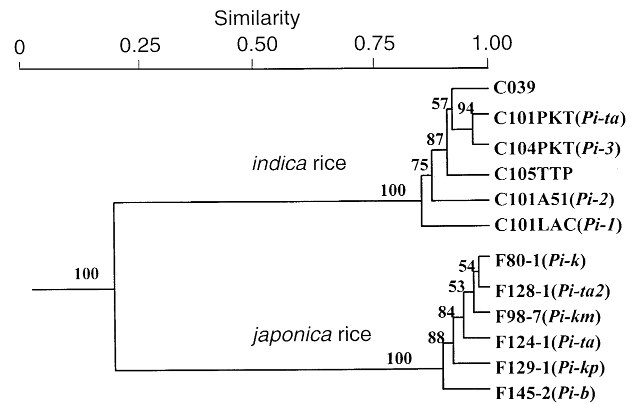

Fig. 2 Dendrogram of rice (*O. sativa*) near-isogenic lines with race-specific resistance genes to *M. grisea* based on 188 polymorphic bands amplified with primers corresponding to conserved sequences of cloned plant resistance genes. The *number* at each branch shows the percentage of times the group of lines in a branch occurred, based on 1000 cycles in a bootstrap analysis using the Winboot program

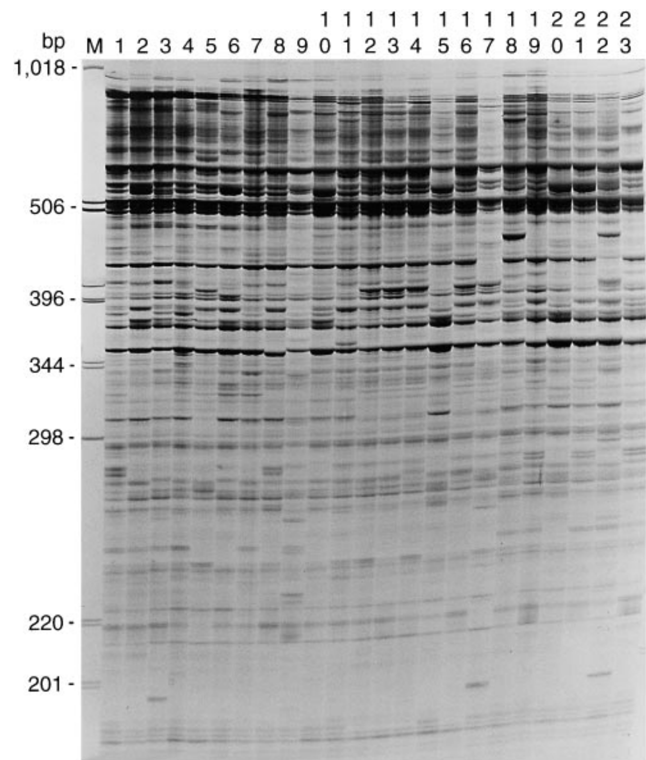

Fig. 3 Silver-stained denaturing polyacrylamide gel showing polymorphism among barley (*H. vulgare*) genotypes. DNA was amplified with the S2/AS3 primers

six primer pairs (Table 4). The highest number and highest percentage of polymorphic bands were observed with Pto kin1/Pto kin 2. Compair, Dusty, Omar, Omar Mutant, Riebesel 47/51, and Produra had unique bands. Some bands were absent in Produra, a durum wheat that has the A and B genomes but not the D genomes, but were present in the hexaploid cultivars, which have the A, B, and D genomes. This suggests that the bands absent in Produra may be on chromosomes of the D genome in the hexaploid wheats.

In contrast to the analyses of rice and barley germplasm, distinct clusters were not observed in wheat genotypes based on polymorphic RGAs (Fig. 6). These results are consistent with the fact that the wheat cultivars used in this study are closely related and have a relatively narrow genetic background. Nonetheless, some sub-clusters appeared to be correlated with

Fig. 4 Dendrogram of 23 barley (*H. vulgare*) genotypes based on 165 polymorphic bands amplified with primers corresponding to conserved sequences of plant resistance genes. The *number* at each branch shows the percentage of times the group of lines in a branch occurred, based on 1000 cycles in a bootstrap analysis using the Winboot program. *HF* = Heils Franken, *AB12* = Abed Binder 12, *SO* = Stauffers Obersulzer, *NZ* = Nakai Zumizairai, and *GZ* = Granelose Zweizeilige

Fig. 5 Silver-stained denaturing gel showing polymorphism among wheat (*T. aestivum*) cultivars and lines. DNA was amplified with the Pto kin1/Pto kin2 primers

known agronomic characteristics. WG1 and WG2 consisted of club-wheat cultivars. In WG2, Rely and Tres were tightly clustered; this is expected since the multiline cultivar Rely was developed using Tres as the recurrent parent. Groups WG 1 to WG 10 consisted of cultivars developed in the Pacific Northwest (PNW) of the United States and the stripe-rust-resistant lines used to develop PNW cultivars. In WG9, both Penawawa and Fielder spring wheats were more closely clustered to each other than to the rest. In WG 11, *T. spelta* var. *saharances* and Lemhi, which are spring wheats that are highly susceptible to *P. striiformis* f. sp. *tritici*, are clustered together. In WG 12, Chinese Spring and Compair, which was developed from Chinese Spring, were clustered together.

Segregation and linkage analyses of polymorphic markers in F_6 recombinant inbred lines from Stephens × Michigan Amber

Using five primer pairs, 26 polymorphic bands were observed in the F_6 recombinant inbred lines of the cross of Stephens with Michigan Amber (Table 4). Figure 7 shows two polymorphic bands (approximately 138 and 140 bp) amplified with the S2/AS3 primers. Among the 26 polymorphic bands, 22 (85%) segregated in a 1 : 1 ratio in the recombinant inbred population, indicating that the bands were inherited as a single

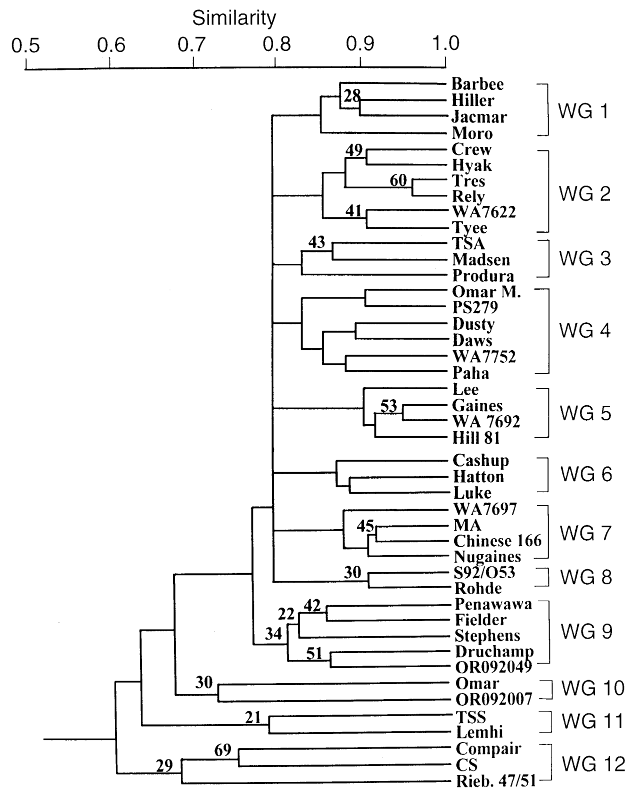

Fig. 6 Dendrogram of 44 wheat (*T. aestivum*) genotypes based on 89 polymorphic bands amplified with primers corresponding to conserved sequences of plant resistance genes. The *number* at each branch shows the percentage of times the group of lines in a branch occurred, based on 1000 cycles in a bootstrap analysis using the Winboot program (Nelson et al. 1994). *TSA* = *T. spelta* var. *album*, *Omar M.* = Omar Mutant, *MA* = Michigan Amber, *S92/O53* = Suwen 92/Omar 53, *TSS* = *T. spelta* var. *saharances*, *CS* = Chinese Spring, and *Rieb. 47/51* = Riebesel 47/51

locus (Table 5). The other four markers showed significant deviation from the expected 1 : 1 ratio, suggesting either segregation distortion or inaccurate recording. The segregation of resistance to races CDL-17, CDL-29, and CDL-6 of *P. striiformis* f. sp. *tritici* in the F_6 population also fits a 1 : 1 ratio ($P = 0.37$, 0.49, 0.67 for races CDL-17, CDL-29, and CDL-6, respectively). The segregation of resistance to race CDL-43 fits a 3 : 1 ratio ($P = 0.19$), suggesting two genes for the resistance.

To correlate the segregation of RGAs and stripe rust resistance in wheat, linkage analyses of the 26 RGA markers and the stripe rust resistance loci were conducted with Mapmaker. At a LOD value of 3.0, six linkage groups (LG) were detected. Five of the six LGs each consisted of two RGA markers without recombination. These RGA markers appeared to represent tightly linked loci or tandem repeats of the conserved domains. One linkage group consisted of two RGA markers, Ptokin1/Ptokin2-330 and Ptokin1/Ptokin2-328, and the loci conferring seedling resistance to races CD1-17, CDL-29 and CDL-43 of *P. striiformis* f. sp. *tritici*. The other 14 RGA markers were unlinked, and the locus for resistance to race CDL-6 was not linked to any RGA marker or any other loci for stripe resistance. As shown in Fig. 8, when LOD = 1.0 was used, six linkage groups were also detected but unlinked markers were reduced to 11 RGA markers and the locus for resistance to race CDL-6. However, LG 4 was the same when either LOD = 1 or LOD = 3 was used, indicating that the loci for resistance to races CDL-17, CDL-29 and CDL-43 are linked to the RGA markers Ptokin-328 and Ptokin-330.

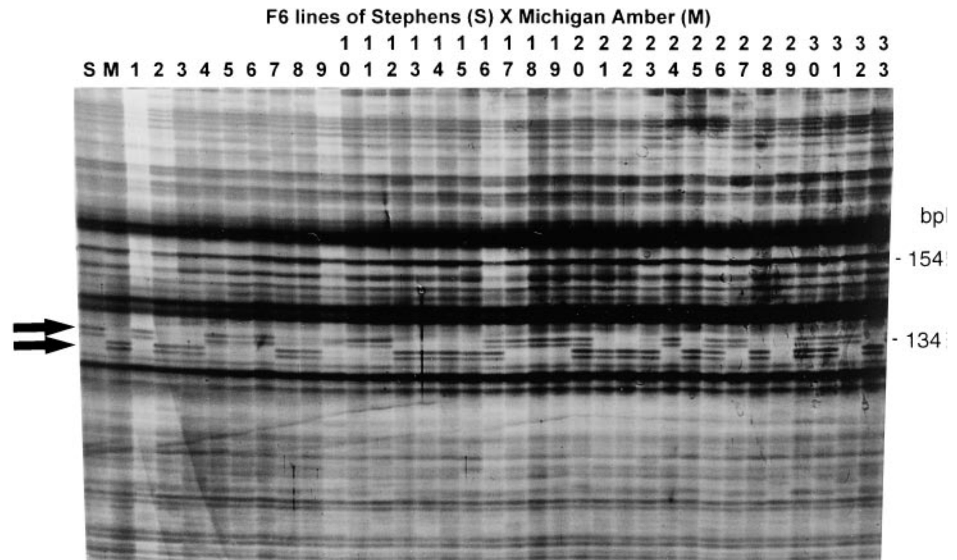

Fig. 7 Silver-stained denaturing gel showing the segregation of polymorphic bands, indicated by *arrows*, among F_6 recombinant lines from the wheat cross Stephens × Michigan Amber. DNA was amplified with the S2/AS3 primer pair

Table 5 Segregation of polymorphic amplified resistance gene analogs in the parents and F_6 recombinant inbred lines of the wheat cross Stephens × Michigan Amber

Marker number	Primer pair	Size (bp)	Stephens[a]	Michigan Amber[a]	No. of F_6 recombinant lines		P (ratio = 1 : 1)[b]
					Present	Absent	
1	RLRR for/RLRR rev	390	+	–	47	42	0.60
2	RLRR for/RLRRrev	270	+	–	27	58	< 0.01
3	RLRR for/RLRRrev	170	–	+	34	51	0.07
4	S2/AS3	530	–	+	55	34	0.03
5	S2/AS3	410	+	–	51	37	0.14
6	S2/AS3	230	+	–	42	47	0.60
7	S2/AS3	220	–	+	33	10	< 0.01
8	S2/AS3	140	+	–	52	37	0.11
9	S2/AS3	135	+	–	52	37	0.11
10	S2/AS3	130	–	+	50	39	0.24
11	S2/AS3	125	–	+	50	39	0.24
12	S2/AS3	50	–	+	39	45	0.51
13	S2/AS3	45	–	+	39	45	0.51
14	Pto kin1/Pto kin2	400	–	+	22	22	1.00
15	Pto kin1/Pto kin2	330	+	–	22	22	1.00
16	Pto kin1/Pto kin2	325	+	–	22	22	1.00
17	Pto kin1/Pto kin2	307	–	+	18	26	0.23
18	Pto kin1/Pto kin2	305	–	+	18	26	0.23
19	Pto kin1/Pto kin2	290	–	+	21	23	0.76
20	Pto kin1/Pto kin2	270	+	–	27	17	0.13
21	Pto kin1/Pto kin2	136	+	–	22	22	1.00
22	Pto kin1/Pto kin2	100	–	–	12	32	< 0.01
23	NLRR for/NLRR rev	277	+	–	23	23	1.00
24	NLRR for/NLRR rev	275	+	–	23	23	1.00
25	NLRR for/NLRR rev	130	+	–	13	33	< 0.01
26	NBS F1/NBS R1	270	–	+	23	23	1.00

[a] + = marker present and – = marker absent
[b] P = probability of chi-square test for goodness of fit of observed ratio to expected ratio

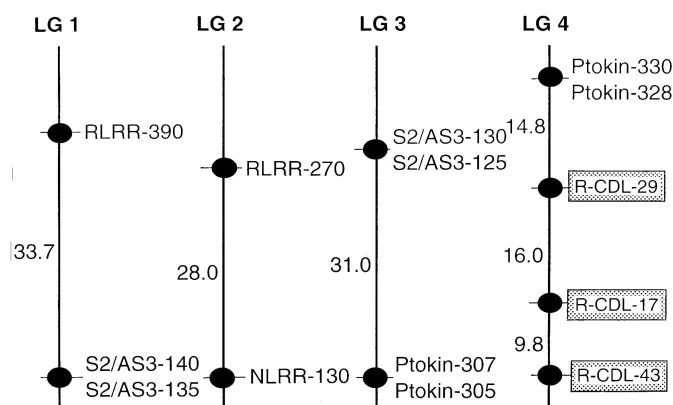

Fig. 8 Linkage groups (*LGs*) of amplified RGA markers, and loci for seedling resistance to races CDL-17, CDL-29, and CDL-43 of *P. striiformis* f. sp. *tritici* in F_6 recombinant lines from the wheat cross Stephens × Michigan Amber using Mapmaker (version 3.0) (Lander et al. 1987). RGA markers S2/AS3-50 and S2/AS3-45 were linked, while NLRR-277 and NLRR-275 were linked without recombination. Unlinked RGA markers RLRR-170, S2/AS3-530, S2/AS3-410, S2/AS3-230, S2/AS3-220, Ptokin1/Ptokin2-400, Ptokin1/Ptokin2-290, Ptokin1/Ptokin2-270, Ptokin1/Ptokin2-136, Ptokin1/Ptokin2-100, NLRR-130, NBS-270, and resistance to race CDL-6 of *P. striiformis* f. sp. tritici

Discussion

The structural similarity of cloned plant resistance genes offers an opportunity for the isolation of similar sequences from different plant species using PCR (Michelmore 1996). So far, RGA detection has been based on the separation of PCR-amplified products by agarose-gel electrophoresis (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996; Feuillet et al. 1997). In the present study, we have broadened the use of the conserved sequences for plant resistance genes to examine the germplasm diversity of three crop species. We showed that numerous PCR-amplified products can be detected by high-resolution electrophoresis, which greatly increases the power of detecting polymorphism. This approach is similar to that of Welsh and McClelland (1990, 1991) who used polyacrylamide-gel electrophoresis to resolve PCR products amplified with arbitrary primers and consensus rRNA gene primers. Similarly, Lee et al. (1996) used polyacrylamide gels to separate PCR products for the identification of wheat-rye chromosomal translocations. As compared to DNA fingerprinting with arbitrary markers, the RGA scanning

technique is highly reproducible due to the use of longer primers, and the amplified products may be correlated with biological functions.

Segregation analyses of recombinant inbred lines of Stephens × Michigan Amber wheat cross showed that most of the detected polymorphic bands were inherited as single loci. Linkage analysis of the 26 polymorphic RGA markers showed that the RGAs are distributed throughout the wheat genomes with some loose linkages detected. Three of the loci conferring seedling resistance to stripe rust were loosely linked to RGAs amplified by the conserved protein kinase primers. These results indicate that the amplified polymorphic RGAs resolved in the polyacrylamide gel can be used as reliable molecular markers in genome mapping. Although no perfect associations of RGAs to stripe rust resistance genes have been found, the abundance of the RGA markers makes it likely that functional resistance genes can be mapped using additional populations segregating for a variety of resistance genes.

The amount of RGA polymorphisms observed in rice, barley, and wheat compare favorably to commonly used marker techniques such as RFLP, RAPD, and AFLP. Mackill et al. (1996) reported a mean of 28% polymorphism for AFLP markers among both *indica* and *japonica* cultivars, and the mean percent polymorphism was 22% for AFLPs, 24% for RAPDs, and 36% for microsatellites within the *japonica* cultivars. Similarly, Maheswaran et al. (1997) found an average of 22% polymorphism in AFLP markers in a doubled-haploid rice population. Becker et al. (1995) reported 11% polymorphism in a barley mapping population using the AFLP technique. Nagaoka and Ogihara (1997) identified 17% polymorphism in three hexaploid wheats using the inter-simple sequence repeat polymorphic (ISSR) DNA technique. With the AFLP technique, 24% polymorphism was observed in hexaploid wheat germplasm (Z. Q. Ma, personal communication). Although it is difficult to compare the amount of RGA polymorphisms with these studies because of the different genotypes employed, RGA genome scanning may be especially useful for detecting genetic variation associated with disease resistance.

While the number of amplified bands varies among RGA primers, there is no obvious relationship between the kinds of primers and the detected level of polymorphism. Among the nine primer pairs, five were used in rice, barley, and wheat. Across species, the S2/AS3 primers, which were designed based on NBS and LRRs in *N* and *RPS2* (Leister et al. 1996), produced the highest number of scorable bands; whereas the primer pair Pto kin1/Pto kin2 produced higher polymorphism in one species but lower polymorphism in another species. For example, the NLRR for/NLRR rev primers produced the highest percentage of polymorphism (47%) in the rice NILs but the lowest polymorphism (7%) in wheat genotypes. For the barley germplasm, the highest number of polymorphic bands (50) was detected with S2/AS3, whereas the highest percentage of polymorphism (48%) was detected with Pto kin1/Pto kin2. For the wheat germplasm, both the highest number (38) and the highest percentage (39%) of polymorphic bands were detected with Pto kin1/Pto kin2. Overall, the average percentage of polymorphic bands was higher in rice and barley than in wheat.

Genome scanning for RGA is expected to have immediate applications for germplasm evaluation and disease-resistance breeding. First, as genomic markers, RGAs can be used to infer genetic relationships among germplasms. This was convincingly demonstrated in the rice NILs when the RGAs markers clearly separated the lines with the *O. sativa indica* from the *O. sativa japonica* background. Though less striking, the dendrograms generated for barley and wheat genotypes are consistent with known agronomic characteristics of the genotypes. These results support our conclusion that the RGA genome-scanning technique can be used both to determine the origin and relatedness among genotypes and to identify specific genotypes.

The most significant advantage of RGAs over arbitrary DNA markers is that they represent potentially useful genes. Although not all amplified products are functional disease resistance genes, they all contained the conserved sequences of LRR, kinase, and/or NBS. Therefore, there is a high probability that the amplification products are involved in signal transduction pathways in plants. For example, Tornero et al. (1996) reported that a novel extracellular matrix-associated protein from tomato belongs to the LRR family of protein. Based on the genomic organization and intron-exon arrangement of the gene, they hypothesized that the LRR domains present in the gene evolved by exon duplication and shuffling. The polymorphisms detected in rice, barley, and wheat may result from mutations due to duplications, frame shifts, and deletions in or between the repetitive sequences within the conserved domains. Regardless of the cause of the polymorphism, the amplified products may represent candidate genes for disease resistance or other important signal-transduction processes in plants.

In conclusion, the RGA fingerprinting we described offers a number of advantages for evaluating the diversity of host resistance when compared to existing molecular markers. The technique is reproducible and non-radioactive. The level of detected polymorphism is high and the versatility in scanning the genome can be further increased by varying the primer combinations. The RGA markers serve both as candidate genes for cellular recognition and as informative markers for inferring genetic relationships between germplasms. If the clustering of genotypes truly reflects the functional diversity of disease resistance, this information will be highly relevant for the selection of lines for the breeding and deployment of disease resistance genes. We have generated a large number of mapping populations segregating for resistance against diverse pathogens in

rice, wheat, and barley and are currently testing this hypothesis.

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