Theor Appl Genet (2005) 110: 1439–1444
DOI 10.1007/s00122-005-1974-z

ORIGINAL PAPER

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Identification and molecular tagging of a gene from PI 289824 conferring resistance to leaf rust (*Puccinia triticina*) in wheat

Received: 9 December 2004 / Accepted: 19 February 2005 / Published online: 7 April 2005

Abstract Host-plant resistance is the most economically viable and environmentally responsible method of control for *Puccinia triticina*, the causal agent of leaf rust in wheat (*Triticum aestivum* L.). The identification and utilization of new resistance sources is critical to the continued development of improved cultivars as shifts in pathogen races cause the effectiveness of widely deployed genes to be short lived. The objectives of this research were to identify and tag new leaf rust resistance genes. Forty landraces from Afghanistan and Iran were obtained from the National Plant Germplasm System and evaluated under field conditions at two locations in Texas. PI 289824, a landrace from Iran, was highly resistant under field infection. Further evaluation revealed that PI 289824 is highly resistant to a broad spectrum of leaf rust races, including the currently prevalent races of leaf rust in the Great Plains area of the USA. Eight F_1 plants, 176 F_2 individuals and 139 $F_{2:3}$ families of a cross between PI 289824 and T112 (susceptible) were evaluated for resistance to leaf rust at the seedling stage. Genetic analysis indicated resistance in PI 289824 is controlled by a single dominant gene. The AFLP analyses resulted in the identification of a marker (*P*39 *M*48-367) linked to resistance. The diagnostic AFLP band was sequenced and that sequence information was used to develop an STS marker (TXW_{200}) linked to the gene at a distance of 2.3 cM. The addition of microsatellite markers allowed the gene to be mapped to the short arm of Chromosome 5B. The only resistance gene to be assigned to Chr 5BS is *Lr52*. The *Lr52* gene was reported to be 16.5 cM distal to Xgwm443 while the gene in PI 289824 mapped 16.7 cM proximal to Xgwm443. Allelism tests are needed to determine the relationship between the gene in PI 289824 and *Lr52*. If the reported map positions are correct, the gene in PI 289824 is unique.

Keywords Wheat · Leaf rust · Resistance genes

Communicated by R. Bernardo

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Introduction

Wheat (*Triticum aestivum* L. em Thell) is one of the most important cereal grains worldwide, and a critical source of sustenance for a major portion of the world population. A major deterrent in realizing genetic potential for grain yield is the occurrence of leaf rust caused by *Puccinia triticina* (Roberge ex Desmaz. f. sp. *tritici*). Although yield loss can vary greatly on a yearly basis, significant yield loss can occur even in years when environmental conditions are not particularly conducive to disease development and spread. Yield losses due to leaf rust in highly susceptible cultivars grown in the U.S.

Central Plains in 2004 were estimated to be 30–40% (Long 2004). Although some level of control can be achieved with the application of fungicide, the additional economic cost, coupled with the relatively low value of wheat grain, usually renders this solution economically unfeasible. The most successful and economical approach to combat leaf rust is the use of resistant cultivars. Numerous resistance genes have been identified and introgressed into released cultivars (McIntosh et al. 1995), yet the continuous shifting of predominant races of *P. triticina* has constituted a substantial challenge to breeders attempting to produce cultivars with durable resistance. Although multiple sources of resistance occur, the incidence of loss due to *P. triticina* has not been reduced. Thus it is necessary to continue to identify novel sources of resistance, incorporate them into elite cultivars, and properly deploy these cultivars through resistance-gene management to minimize the ability of the pathogen to overcome the genetic resistance.

One approach to overcoming the loss of resistance due to pathogen race shifts is the incorporation of multiple genes into a single cultivar. Currently, numerous wheat cultivars are thought to contain multiple sources of resistance to leaf rust (McIntosh et al. 1995). However, the combining of these resistance genes typically has occurred after at least one of the resistance genes has been compromised by its prior release as the sole source of resistance in a variety. Additionally, the pyramiding of leaf rust genes was due more to the combining ability of parents producing superior derived lines than to a purposeful strategy of stacking multiple sources of leaf rust resistance. Pyramiding of undefeated genes is also hindered by an inability to easily determine the number of leaf rust resistance genes in a given plant of a segregating population. Efficient pyramiding requires the utilization of markers closely linked to the resistance genes. The use of marker assisted selection (MAS) to pyramid multiple resistance genes in rice (*Oryza sativa* L.) has been utilized by Huang et al. (1997) and Singh et al. (2001) to pyramid genes conferring resistance to *Xanthomonas oryzae* pv. *oryzae* (Xoo), and by Hittalmani et al. (2000) for resistance to *Magnaporthe grisea* (Herbert) Barr (anamorph *Pyricularia grisea* Sacc.). In both cases the genotypes containing multiple resistance genes had superior levels of resistance when compared to genotypes containing only one of the genes.

The goal of this research was the identification of novel sources of resistance to leaf rust for use in the development of multiple gene pyramids. Landraces from Afghanistan and Iran that have been reported to be resistant to leaf rust were obtained from the National Small Grains Collection and evaluated in the field at McGregor and College Station, Texas, in 1998. Individual PIs which were resistant to field races of leaf rust at both locations were utilized for further analysis. We report here on the identification and mapping of resistance to leaf rust from PI 289824, a winter landrace collected near Mazandaran, Iran. We developed a PCR based marker tightly linked to this resistant gene to facilitate its utilization in MAS. We plan to map additional resistance genes from other PIs in our attempt to pyramid multiple sources of leaf rust resistance into elite cultivars adapted to the Great Plains region. Our ultimate objective is the strategic deployment of pyramided sources of leaf rust resistance.

Materials and methods

Population development and rust screening

Forty landraces from Afghanistan and Iran were obtained from the National Plant Germplasm System. The landraces were planted in individual head rows and exposed to naturally occurring races of *P. triticina* at McGregor, Texas, and College Station, Texas, in 1998. Following the identification of PIs that were resistant, individual heads were selected and harvested. PI 289824 was selected for further evaluation because it had the highest level of resistance in the field. It was crossed with T112, an experimental line from Trio Research (Valley Center, Kansas) that is susceptible to prevalent races of leaf rust. Both parents and eight F_1 plants were planted in 10-cm^2 plastic pots filled with vermiculite and inoculated at the two-leaf stage with a field collection of leaf rust from Uvalde, Texas, as a suspension of urediospores in mineral oil following the method of Browder (1971). Inoculated seedlings were placed in a 16°C moist chamber overnight. Seedlings were then placed in a greenhouse and scored 10–14 days after inoculation following the scale of Stakman as modified by Roelfs et al. (1992). The F_1 plants were then vernalized for 6 weeks and grown to maturity in the greenhouse under a 16 h photoperiod and a 27°C/16°C temperature regime. The parents and 25 F_2 seeds from each F_1 plant were planted in flats and inoculated with *P. triticina* race MFBL 14 days after emergence following the previously described protocol. Seedlings were scored 8 days after inoculation as previously described. Individual plants were tagged to maintain their identity for later analysis. The final F_2 population consisted of 176 individuals, which were derived from the eight individual F_1 plants. Following vernalization for 6 weeks, the F_2 plants were transferred to individual pots and grown to maturity following the method described for the F_1 plants. One hundred and thirty-nine F_2 plants produced sufficient seed for progeny testing. The F_3 families, consisting of 25 seed from each F_2 plant, were planted, inoculated with *P. triticina* race MFBL, and scored for disease reaction following the previously described method. Additionally, selfed progeny from each parent were inoculated with eight other races of *P. triticina* in order to determine the spectrum of resistance of PI 289824. These isolates are virulent on the most widely grown wheat cultivars grown in the Great Plains. The virulence/avirulence formulae are shown in Table 1.

Table 1 Avirulence/virulence phenotypes of *P.triticina* races and corresponding disease reaction of wheat parents

Race	Avirulence/ Virulence	Infection type	
		PI289824	T112
MFBL	2a,2c,3ka,9,11,16,17,18,30/1,3a,10,24,26	0	4
PBJL	2a,9,10,11,16,19,24/1,2b,2c,2d,3a,15,17,18	0	4
PNMQ	2a,11,16,17,26/1,2c,3a,3ka,9,10,18,24,30	0	4
TFGL	3ka,9,16,17,18,30/1,2a,2c,3a,10,11,24,26	0	3
MCDL	2a,2c,3ka,9,11,16,18,24,30/1,3a,10,17,26	0	4
CBBQ	1,2a,2b,2c,2d,3ka,3c,9,11,16,17,19,24/3a,10,18	0	4
KDBL	1,3ka,9,11,16,17,18,26,30/2a,2c,3a,10,24	0	4
PNML	2a,11,16,17,18,26/1,2c,3a,3ka,9,10,24,30	0	4
TBGL	3ka,9,16,17,18,24,26,30/1,2a,2c,3a,10,11	0	4

DNA extraction and marker generation

A small amount (~5 cm^2) of leaf tissue from each F_2 plant was harvested prior to inoculation with rust spores and freeze-dried for 72 h. Leaf tissue was then vortexed for 10 min at full speed in a 1.5 ml tube containing three glass beads. The DNA was extracted following the PEX method described by Williams and Ronald (1994). Extracted DNA was quantified by fluoremetry using a Fluorometer TD-360 (Turner Designs) and diluted to a final concentration of 100 ng/µl for the initial AFLP analyses. DNA for further analyses was isolated by harvesting fresh tissue (~0.5 g) of 20 seedlings from each of the 139 F_3 families 7 days after emergence. The DNA was extracted using the Plant DNAzol Reagent protocol (Invitrogen Life Technologies, UK). Extracted DNA was quantified by fluorimetry and diluted to 100 ng/µl for AFLP analyses.

A total of 500 ng of genomic DNA from each of the 176 F_2 plants was digested with *Mse*I (*M*) and *Pst*I (*P*) (New England Biolabs) and utilized for AFLP analyses following the protocol as described by Menz et al. (2002). Pre-amplification primers utilized were *P*+0 (GACTGCGTAGGTGCAG) and *M*+C (ACGATGAGTCCTGAGTAA+C). The selective amplification utilized primers labeled to fluoresce at either 700 or 800 nm, and were visualized using the LI-COR model 4200L-2 dual dye automated DNA sequencing system (LI-COR, Lincoln, Neb., USA). For the selective amplification six fluorescently labeled *Pst*I (*P*+3) primers (*P*+AGA, *P*+CAA, *P*+CTC, *P*+CGT, *P*+GAT, and *P*+TAG) were used in all possible combinations with 16 *M*+C+2 primers. Initially, each primer combination was run on the parental cultivars, 12 resistant and 12 susceptible F_2 plants. Primer combinations which resulted in markers showing potential association with a specific disease class were then utilized on the complete F_2 population.

Cloning and sequencing of target sequences

Polymerase chain reaction products from the AFLP primers *P*+AGA (*P*39) and *M*+CAC (*M*48) were separated on 7% acrylamide gels, visualized by silver staining, and extracted from the gel following the protocol of Fritz et al. (1999). The target band was re-amplified following the original PCR protocol for the AFLP selective amplification. Presence of the target PCR product was confirmed on a 7% acrylamide gel and visualized with silver stain. The PCR product was also run on a 1.5% low-melting point agarose gel to extract the band for cloning. Following band excision, cloning was carried out following the recommendations of the manufacturer of the TOPO-TA kit (Invitrogen Life Technologies). Twelve colonies (four from each of three original fragments PI 289824, F_2-152, F_2-160) were selected and grown overnight in Luria-Bertani (LB) medium. Following plasmid extraction utilizing the QiaPrep spin mini kit (Qiagen, Crawley, UK), the 12 samples were sequenced in both forward and reverse reactions at the Institute for Plant Genomics and Biotechnology at Texas A&M University. Sequencher (Gene Code Corporation) was utilized to align the fragments into a single contig.

Primer synthesis and analysis

Sequence-tagged site (STS) primers were designed using Primer3 (Whitehead Institute and Howard Hughes Medical Institute) and synthesized by the Gene Technology Laboratory at Texas A&M University. One STS primer amplified a 200 bp band contained within the original P39M48-367 bp AFLP fragment. The sequences of those primers were 5′-TGGAGCCTCTTGTCTCTCGT and 5′-GCAGAAGGCAAATTCTGTGC. Selective reactions utilizing the 20-mer STS primers were performed in 139 F_3 families in volumes of 15 µl containing one time PCR buffer, 2.5 mM $MgCl_2$, 3.0 mM each dNTP (Gibco-BRL), 15 ng each STS primer, 50 ng genomic DNA, and 0.9 U *Taq* DNA polymerase (Promega, Madison, Wisc., USA). Samples were amplified in a GeneAmp PCR System (Model 9700 Perkin-Elmer). PCR was performed as one cycle of 94°C for 4 min, followed by 27 cycles of 94°C for 30 s, 58°C for 60 s, and 72°C for 30 s, followed by a 5 min hold at 72°C. Reaction product was then run on a 1.5% agarose gel containing 0.2 µg/ml ethidium bromide and visualized with UV light.

Microsatellites and linkage analysis

A set of 400 SSRs with known map locations (Somers et al. 2004) were surveyed for parental polymorphisms. Polymorphic markers were further tested on a subset of the F_3 population to assess potential linkage with the resistant gene. Those markers that appeared to be associated with resistance were tested on the entire F_3 population for linkage analysis. The PCR amplification conditions were as described by Somers et al. (2004). Forward primers were labeled either with one of the IR fluorescent dyes (LI-COR), or with one of the three phosphoramidite fluorescent dyes (6-FAM, NED, HEX) for use with a LI-COR 4200 system or with the ABI 3100 DNA sequencing system (Applied Biosystems, Foster City, Calif., USA), respectively.

Linkage analysis was done with MapMaker v3.0 using a LOD threshold of 3.0, a maximum recombination fraction of 50 cM, and the Kosambi mapping function (Kosambi 1944).

Results

Disease screening

PI 289824 was inoculated with a diverse set of leaf rust races and proved to be resistant to all races with which it was tested (Table 1). When inoculated with race MFBL, the eight F_1 plants of the PI 289824 × T112 cross were all scored as 0 (resistant). PI 289824 was given a rating of 0 while T112 was rated 4 (susceptible). Screening of the F_2 progeny resulted in distinct classes. The F_2 segregation ratio was 126R:50S, which fits the ratio expected for a single dominant gene ($\chi^2 = 1.09$). In addition, 136 F_3 families segregated 36R:71H:29S, which is also consistent with the expected segregation at a single locus ($\chi^2 = 0.985$). Three F_3 families were not included in this ratio due to unclear segregation patterns, likely resulting from escape.

Marker analysis and mapping

In the AFLP analysis, the combination of 6 $P+3$ with all 16 $M+C+2$ primers resulted in 253 polymorphic bands, of which 17 were associated with either resistance or susceptibility more frequently than what was expected by chance. Primer combination $P+$AGA ($P39$) and $M+$CAC ($M48$) resulted in the amplification of a 367 bp band ($P39M48$-367) that distinguished the resistant and susceptible classes (Fig. 1). $P39M48$-367 amplified in all 11 resistant plants and none of the 12 susceptible plants that were sampled. When this primer combination was run on the entire F_2 population it was present in 87/126 resistant plants and 0/50 susceptible plants. The $P39M48$-367 band was sequenced and utilized to design STS primers, which were tested in the F_3 families. One of these primers selectively amplified a 200 bp fragment (TXW_{200}) that was present in 109/110 resistant (99.1%) and 1/27 (3.7%) susceptible F_3 families (Table 2).

Among the microsatellite markers tested, barc21, gwm443, gwm234 and gwm544 all showed linkage to the gene. Because these markers map to the short arm of Chromosome 5B (Somers et al. 2004), we can place the gene from PI 289824 on Chr 5BS. The gene is flanked by SSR marker Xgwm234 at 7.8 cM and the STS marker TXW_{200} at 2.3 cM (Fig. 3). The TXW_{200} marker was polymorphic on the International Triticeae Mapping Initiative (ITMI) mapping population and confirmed the location of this marker on Chr 5BS (data not shown).

Discussion

One unexpected result was the different marker data recorded for the original AFLP marker and the STS

Table 2 Test of independence, goodness of fit, and occurrence of markers $P39$ $M48$-367 in F_2 progeny and TXW_{200} in F_3 families from the cross PI289824/T112

Marker	Disease score	Present	Absent	Chi-square (χ^2)	
				Test of independence	Goodness of fit
$P39M48$-367	Resistant	87	39	75.4*	12.1*
	Susceptible	0	50		
TXW_{200}	Resistant	109	1	136.4*	2.0
	Susceptible	1	26		

* Significantly different from zero at $P = 0.001$

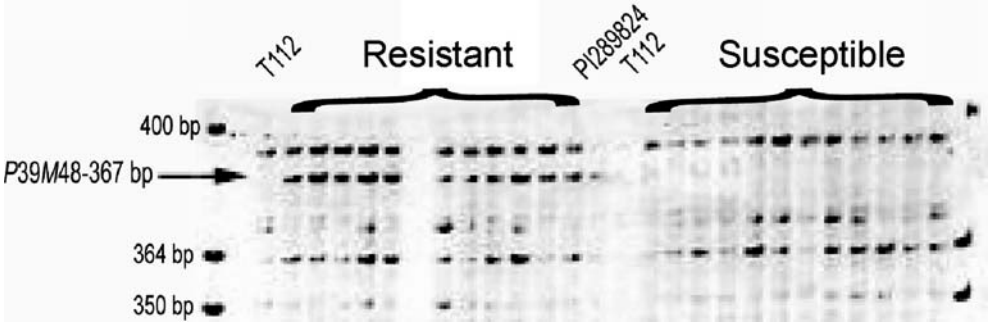

Fig. 1 Amplification of $P39M48$-367 in PI289824, T112, 12 resistant and 12 susceptible F_2 PI289824/T112 plants

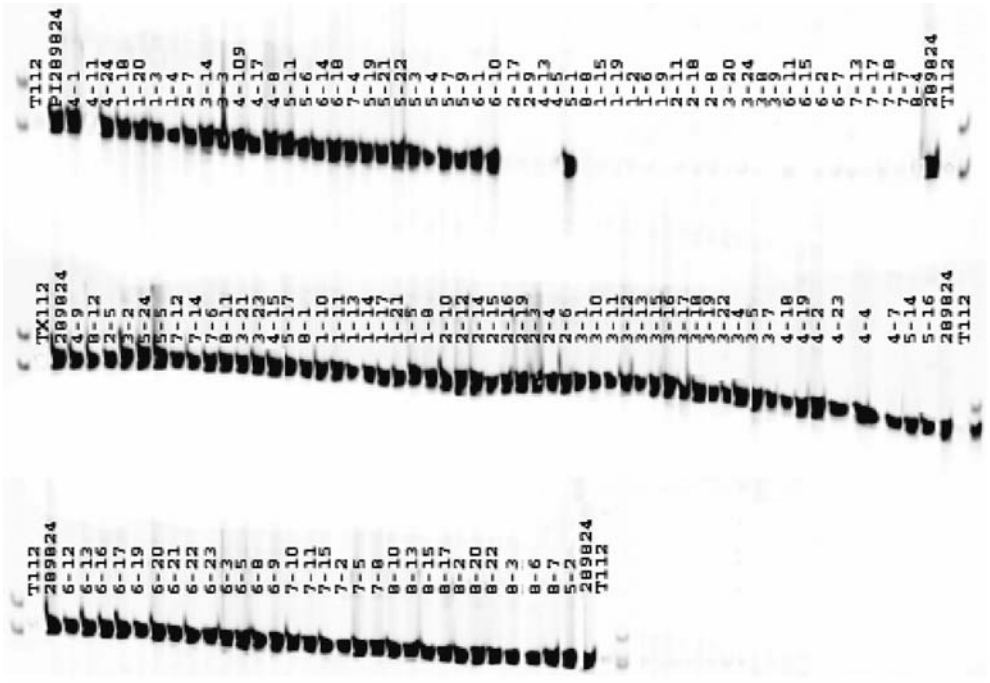

Fig. 2 Amplification of TXW_{200} in PI289824, T112, and 139 F_3 PI289824/T112 families. PCR products for the resistant families are in wells 4–1 to 6–10 and 4–9 to 5–2; those for the susceptible families in wells 2–17 to 8–4

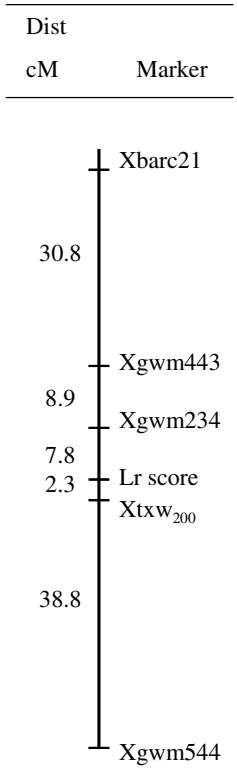

Fig. 3 Position of the *Lr* gene on the genetic map of wheat Chromosome 5BS

marker derived from the AFLP sequence. The most plausible explanation for this difference is that differential methylation of DNA led to incomplete restriction of the target region by *Pst*I, a methylation sensitive restriction enzyme. In the initial AFLP analysis, this would have resulted in the lack of marker amplification from some plants with the proper sequence to amplify the 367 bp band. Another possibility is differences in DNA quality among the samples. The AFLP technique is very efficient for the identification of linked DNA sequences, mainly due to its high multiplex ratio, but it requires very high quality DNA to be reproducible. Robust STS markers designed to produce a single amplification product are less demanding.

In this study, we mapped a gene from PI 289824 on the short arm of Chr 5B. The only other genes for leaf rust resistance that have been assigned to this chromosome are *Lr18* (http://www.cdl.umn.edu/res_gene/chrm-loc.html) and *Lr52* (Hiebert et al. 2005). It is unlikely that the gene in PI 289824 is *Lr18*. Three races (PNMQ, PBJL and CBBQ) that were used for screening are virulent on *Lr18*, but the landrace was resistant to all three races (Table 1). In addition, *Lr18* has been mapped to the long arm of Chr 5B (McIntosh 1983), whereas the gene in PI 289824 is on the short arm. The *Lr52* is the gene formerly designated *LrW* (Dyck and Jedel 1989) and was mapped to the short arm of Chr 5B by Hiebert et al. (2005). They placed *Lr52* 16.5 cM distal of Xgwm443. We mapped the gene from PI 289824 16.7 cM proximal of Xgwm443. We were also able to map fragments amplified by barc21 and gwm234 in the region. The marker order and distances we found for these microsatellites are in agreement with the consensus map of Chr 5BS (Somers et al. 2004). The different map positions reported for *Lr52* and the

gene in PI 289824 would suggest the genes are different. An alternative possibility is that the orientation of one of the maps is in error. We did not design allelism tests with this gene because it had previously been reported to reside on Chromosome 4A (Hiebert et al. 2002). We are currently conducting allelism tests between the gene in PI 289824 and *Lr52* to clarify the relationship between them.

Utilization for marker assisted selection

One of the reasons for pursuing the characterization of this gene is to build effective pyramids of leaf rust resistance genes in Great Plains adapted cultivars. This strategy has been frequently discussed, but has been difficult to implement. One of the challenges of pyramiding is the identification of effective genes that are linked to useful markers. Another challenge is that many of the new genes are widely distributed to various breeding programs. Inclusion of these genes in a pyramid can be undermined when another program releases a cultivar carrying any of the targeted genes on an individual basis. The gene in PI 289824 has the potential to be useful in pyramiding schemes because it is effective against a wide range of races, it is tightly linked to PCR based flanking markers suitable for use in MAS and it is not widely distributed in the winter wheat germplasm pool. We are currently pursuing the utilization of this resistance gene, in combination with other putative novel resistance genes, to develop pyramid(s) of multiple undefeated genes. Unlike most previous cultivars, which were only one mutation from becoming susceptible (due to the presence of only one undefeated gene), this strategy will potentially allow for the development of cultivars with more durable resistance to leaf rust.

Acknowledgements The authors would like to acknowledge financial support from The Texas Higher Education Coordinating Board through the Advanced Technology Program.

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