Lr39 + *Pm21*: a New Effective Combination of Resistance Genes for Leaf Rust and Powdery Mildew in Wheat

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Abstract

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Two effective resistance genes were introduced, one for leaf rust (*Lr39*) and the other for powdery mildew (*Pm21*), into the susceptible German wheat cv. Lexus. Molecular selection of plant material was carried out using closely linked markers to the introduced genes (foreground selection). In addition, for the BC_1F_1 population, background selection was carried out using AFLP markers that were distributed randomly throughout the wheat genome. Moreover, resistance tests were conducted using natural pathogen populations of *Puccinia triticina* and *Blumeria graminis*. The use of molecular markers for foreground selection in combination with pathology tests enabled 66 homozygous lines to be obtained that were simultaneously resistant to leaf rust and powdery mildew.

Keywords: *Blumeria graminis*; gene pyramiding; *Puccinia triticina*

Despite the knowledge of resistance genes, it is still important to look for new and effective sources of resistance to fungal diseases, especially because the resistance of cultivars can be overcome by new virulent pathogen races. One of the best strategies to obtain effective and durable resistance in cultivars is gene pyramiding, namely the selection of multiple resistance genes. Since the expression of individual resistance genes is difficult to monitor in offspring populations, marker-assisted selection (MAS) became a powerful alternative. There are several published examples of resistance breeding in common wheat using gene pyramiding, which include: *Pm2* + *Pm4a*, *Pm2* + *Pm21*, *Pm4a* + *Pm21* in the Chinese cv. Yang 158 (Liu *et al*. 2000), *Lr16* + *Lr34* and *Lr22a* + *Lr52* in wheat populations (Hiebert *et al.* 2010).

The ultimate aim of our work was to increase wheat resistance to powdery mildew and leaf rust by pyramiding two highly effective resistance genes *Lr39* and *Pm21.* The 6VS/6AL translocation line of Yangmai 5, which carries the *Pm21* gene derived from the wild wheat relative *Dasypyrum villosum*, was used as the donor of resistance to powdery mildew. The line KS90WGRC10, which carries the *Lr39* (formerly *Lr41*) gene derived from the diploid wild wheat *Triticum tauschii*, was used as the donor of resistance to leaf rust (Singh *et al.* 2004).

MATERIAL AND METHODS

Plant materials

The wheat line KS90WGRC10, which carries the *Lr39* gene, was used as the source of resistance to leaf rust. This line was developed at the

Wheat Genetic and Genomic Resources Center at Kansas State University, USA. The translocation line 6VS/6AL Yangmai5, which carries the *Pm21* gene, was used as the donor of resistance to powdery mildew. The Yangmai5 line was developed at the Cytogenetics Institute, Nanjing Agricultural University (CINAU) (Cao *et al.* 2006). The German wheat cv. Lexus, which was provided by Dr Andreas Jacobi from W. von Borries-Eckendorf (Leopoldshöhe, Germany), was used as the recurrent parent susceptible to both diseases.

Experimental design

To introduce the two resistance genes, *Lr39* and *Pm21*, into the cv. Lexus, the breeding scheme shown in Figure 1 was used.

Screening for leaf rust and powdery mildew

Screening for resistance to leaf rust and powdery mildew was carried out in a greenhouse on plants at the three-leaf stage. The greenhouse conditions were 16–22°C with a light:dark cycle of 16:8 h. The inoculum sources were natural populations of *P. triticina* and *B. graminis*. The chosen inocula for both pathogens were avirulent to line KS90WGRC10 and virulent to Lexus. Plants were inoculated initially with *P. triticina* and then after 3–4 days the same plants were inoculated with the Bgt28 *B. graminis*. Reactions to infection were assessed 8–10 days later using a scale in which 0–2 indicates resistant plants and 3–4 indicates susceptible plants, with the latter displaying symptoms of leaf rust (Levine & Cherewick 1952) or powdery mildew (Mains & Dietz 1930).

DNA extraction

For the detection of resistance genes in plant materials, DNA was extracted from fresh leaves of plants using the method described by Higgins *et al*. (2000). For background selection, total DNA was isolated using a spin column-based method (DNeasy Plant Mini Kit, Qiagen, Valencia, USA).

Foreground selection (FS)

***Lr39*, a leaf rust resistance gene**. In foreground selection to detect the *Lr39* gene, five microsatellites (Simple Sequence Repeats, SSRs) were used: *Gdm35* (170 bp), *Barc124* (250 bp), *Gwm261* (160 to 200 bp), *Gwm296* (135 bp), and *Gwm210* (182 bp) (Röder *et al.* 1998; Pestsova *et al.* 2000; Singh *et al.* 2004; Somers *et al.* 2004; Song *et al.* 2005). Polymerase chain reactions (PCRs) were performed in low-profile PCR tube strips (BIO-RAD, Hercules, USA) in a Mastercycler ep thermocycler (Eppendorf, Hamburg, Germany). PCRs were carried out under the following conditions when the total reaction volume of 8 µl contained: 1.5 µl of DNA solution, 1× buffer (MBI Fermentas, Vilnius, Latvia), 2.5mM $MgCl_2$, 0.2mM dNTPs, 0.5µM primer, and 1 U of *Taq* DNA polymerase (MBI Fermentas, Vilnius, Latvia). Amplification products were separated on denaturing polyacrylamide gels (Long Ranger Gel Solution, Rockland, USA) on an ABI Prism 377 automated DNA sequencer for 2 h.

***Pm21*, a powdery mildew resistance gene**. In foreground selection to detect the *Pm21* gene in plant material, three molecular markers were used: $SCAR_{1250}$ (1250 bp), $SCAR_{1400}$ (1400 bp) (Liu *et al.* 1999; Yildirim *et al.* 2004), and *NAU/xibao* (902 bp) (Cao *et al*. 2006). The 10-µl reaction mixture contained the following components: 4 µl of DNA solution, 1 × buffer (MBI Fermentas), 2.5mM $MgCl_2$, 0.2mM dNTPs, 0.5µM primer, and 1 U of *Taq* DNA polymerase (MBI Fermentas). Amplification products were separated on 1.6% agarose gels in 1 × TBE buffer for 4 h, and visualised under UV light after the addition of ethidium bromide.

AFLP analysis and estimation of the percentage of recurrent parent genome

Amplified fragment length polymorphism (AFLP) analysis was performed in accordance with the protocol described by Vos *et al.* (1995) at KeyGene (Wageningen, the Netherlands). After a prescreening of the parental lines, the 19 most informative *Pst*I+3/*Taq*I+3 primer combinations were chosen: *P38/T43*, *P41/T41*, *P39/T41*, *P35/T43*, *P36/T46*, *P38/T44*, *P41/T42*, *P36/T45*, *P41/T40*, *P42/T41*, *P39/T42*, *P35/T44*, *P39/T40*, *P44/T40*, *P37/T44*, *P37/T45*, *P35/T46*, *P36/T44* and *P40/T40*. On the basis of visual inspection of the fingerprints obtained in the prescreening, the five best *PstI-TaqI* AFLP primer combinations (*P38/T43*, *P41/T41*, *P39/T41*, *P35/T43*, *P36/T46*) were selected for the background analysis of BC_1F_1 plants to detect the AFLP fragments on a MegaBace 1000 capillary platform (GE Healthcare, Pittsburgh, USA). The percentage of the recurrent parent genome (% RP) in the BC samples was estimated

using the formula developed by KeyGene (Wageningen, the Netherlands).

Genetic map construction.

Genetic maps for the BC_1F_3 generation were constructed with 5 microsatellites that flanked the *Lr39* gene using JoinMap 4.0 (Kyazma B.V., Wageningen, the Netherlands) at LOD > 3.0 (Lander *et al.* 1987) and the Kosambi mapping function (Kosambi 1943).

RESULTS AND DISCUSSION

First, two crosses were carried out between Lexus and the line KS90WGRC10, and between Lexus and the translocation line 6VS/6AL Yangmai5. Next, we performed crosses between (Lexus × *Lr39*) and (Lexus × *Pm21*) and analysed 39 plants (Table 1). Three molecular markers were used to detect the *Lr39* gene in the F_1 plants: *Gdm35*, *Barc124*, and *Gwm210*. A 170 bp allele for the *Gdm35* marker was amplified in 24 plants, whereas in 13 plants both a 250 bp allele for *Barc124* and a 182 bp allele for *Gwm210* were amplified (Table 1). Two specific molecular markers were used to detect the *Pm21* gene in the F_1 plants: $SCAR_{1250}$ (1250 bp) and $SCAR_{1400}$ (1400 bp). Seventeen plants yielded the expected 1250 bp band for $SCAR_{1250}$ and 1400 bp band for $SCAR_{1400}$. When the results were compared, 15 plants carried both resistance genes (*Lr39* + *Pm21*) (Table 1).

The plants that carried both *Lr39* and *Pm21* were used in backcrosses with the Lexus cultivar as the recurrent parent to obtain a BC_1F_1 generation. Ninety-four BC_1F_1 plants were tested for the presence or absence of the resistance genes by foreground selection (Table 1). To detect the *Lr39* gene in the BC_1F_1 plants, the same 3 microsatellites described above were used. With the primers for *Gdm35*, a 170 bp fragment was amplified in 53 plants, whereas the primers for *Barc124* amplified a 250 bp fragment in 46 plants, and the primers for *Gwm210* amplified a 182 bp fragment in 12 plants (Table 1). This marker *NAU/xibao* was used to detect the presence of the *Pm21* gene in the BC_1F_1 generation, and the expected 902 bp fragment was amplified in 44 plants (Table 1). Overall, 24 plants showed the presence of both (*Lr39* + *Pm21*).

Next, these 24 plants were subjected for BS analysis using 5 combinations of AFLP primers: *P38/*

Table 1. Molecular analysis (foreground and background selection) and resistance of the ((Lexus ×*Lr39*) × (Lexus × *Pm21*)) × Lexus population to leaf rust and powdery mildew

Generation	Population size	Foreground selection								Background selection	Resistance test No. of resistant plants		No. of plants selected
		No. of plants with *Lr39*					No. of plants with *Pm21*						
		Gdm35 170 bp	*Barc124* 250 bp	*Gwm210* 182 bp	*Gwm261* 165 bp	*Gwm296* 135 bp	$SCAR_{1250}$ 1250 bp	$SCAR_{1400}$ 1400 bp	*NAU/xibao* 902 bp	range % RP	*P. triticina*	*B. graminis*	
F_1	39	24	13	13	–	–	17	17	–	–	–	–	15
BC_1F_1	94	53	46	12	–	–	–	–	44	32.1–75.0	–	–	15
BC_1F_2	188	47	42	27	76	15	–	–	111	–	151	162	45
BC_1F_3	742	184	200	135	150	165	–	–	262	–	471	642	66

– not tested

T43, *P41/T41*, *P39/T41*, *P35/T43*, and *P36/T46*. These primers generated a total of 28 polymorphic AFLP markers. On the basis of the results, the % RP for the BC_1F_1 was estimated. The % RP was in the range of 32.1–75% (Table 1). Fifteen plants which were in the range from 50 to 75% were used to produce the BC_1F_2 generation by self-pollination.

In total, 188 BC_1F_2 plants were analysed by foreground selection and phytopathology tests (Table 1). To detect the *Lr39* gene in the BC_1F_2 plants, 5 molecular markers were used: *Gdm35*, *Barc124*, *Gwm261*, *Gwm296*, and *Gwm210*. With the primers for marker *Gdm35*, a 170 bp DNA fragment from the line KS90WGRC10 was amplified in 47 plants (Table 1). The primers for *Barc124* amplified a 250 bp fragment in 42 plants, whereas the primers for *Gwm261* amplified a 165 bp fragment in 76 BC_1F_2 plants. The primers for *Gwm296* amplified a 135 bp fragment in 15 plants, and *Gwm210* a 182 bp fragment in 27 plants (*Lr39* + *Pm21*) (Table 1). The marker for the *Pm21* gene, *NAU/xibao*, was detected in 111 plants (Table 1). Phytopathology tests using a natural population of *P. triticina* revealed that 151 plants were resistant to this pathogen, whereas 162 BC_1F_2 plants were resistant to *B. graminis*. On the basis of the results from the foreground selection and phytopathology tests, 45 plants that carried both *Lr39* and *Pm21* were selected for production of the BC_1F_3 generation by self-pollination (Table 1).

In total, 742 BC_1F_3 plants were analysed by phytopathology tests using natural populations of *P. triticina* and *B. graminis* (Table 1). Four hundred and seventy-one BC_1F_3 plants were found to be resistant to leaf rust. In addition, 642 plants were found to be resistant to powdery mildew (Table 1). A total of 382 plants were analysed by foreground selection to detect the *Lr39* and *Pm21* genes. The primers for the marker *Barc124* amplified a 250 bp fragment in 200 plants, those for *Gdm35* amplified a 170 bp fragment in 184 plants, whereas the primers for *Gwm261* amplified a 165 bp fragment in 150 plants (Table 1). In addition, the primers for *Gwm296* amplified a 135 bp fragment in 165 plants and a 182 bp fragment for *Gwm210* was amplified in 135 plants (Table 1). Furthermore, the primers for the marker *NAU/xibao* amplified a 902 bp fragment in 262 plants (Table 1). On the basis of the molecular and phenotypic characteristics, a total of 66 homozygous plants carried both *Lr39* and *Pm21* in a single genotype, and were selected for field evaluation (Table 1).

Linkage analysis showed that the *Lr39* gene was linked to the 5 microsatellite markers *Gdm35*, *Barc124*, *Gwm261*, *Gwm296*, and *Gwm210*. Mapping of these markers showed that *Barc124* was linked most closely to *Lr39*, at a distance of 9.7 cM. The order of the other markers on the 2DS chromosome was *Gdm35*, *Gwm261*, *Gwm296*, and *Gwm210* at 14.8 cM, 20.6 cM, 26.0 cM, and 33.2 cM, respectively, from the locus of resistance *Lr39* (Figure 2).

The results described herein demonstrate that the success of gene pyramiding depends on the combination of molecular selection on the basis of DNA markers with phenotypic selection, both of which contribute significantly to the efficiency of the selection process. During foreground selection to detect the *Lr39* gene in the composite F_1 generation (crosses 2) (Figure 1), 3 microsatellites were used: *Gdm35*, *Barc124*, and *Gwm210*. On the basis of the results obtained for the composite F_1 generation, we applied additional polymorphic markers that are linked more closely to locus *Lr39*, according to the microsatellite consensus map constructed by Somers *et al.* (2004), namely *Gwm261* and *Gwm296*. Therefore, subsequently, 5 microsatellite markers were used for foreground selection: *Gdm35*, *Barc124*, *Gwm210*, *Gwm261*, and *Gwm296*. The use of these specific molecular markers to detect *Lr39* in breeding material was based on previous reports. Singh *et al.* (2004) used only 3 markers (*Gdm35*, *Barc124*, *Gwm210*) to perform successful foreground selection for *Lr39*. On the basis of the findings of Sun *et al.* (2009), other combinations of microsatellites could be used potentially for marker-assisted selection (MAS), such as *Barc124*, *Gwm210*, *Gdm35*, and *Cfd36*. From these earlier reports of the identification of *Lr39* resistance genes in breeding material, it can be concluded that there might be no universal marker that is suitable for MAS of *Lr39* in breeding programs.

The effectiveness of MAS and the suitability of individual markers depend on the parents used in the breeding program. Sun *et al.* (2009) proposed two SSR markers that can be used in MAS separately, namely *Gdm35* and *Barc124*. When the American cultivar Thunderbolt or line KS93U62 (Century*3/TA2460) is used as the source of the *Lr39* resistance gene, both above-mentioned markers can be applied, but when the line TX01V5719 is used, only *Barc124* can be applied. If the *Lr39* donor is another cultivar, such as Cardinal, Duster

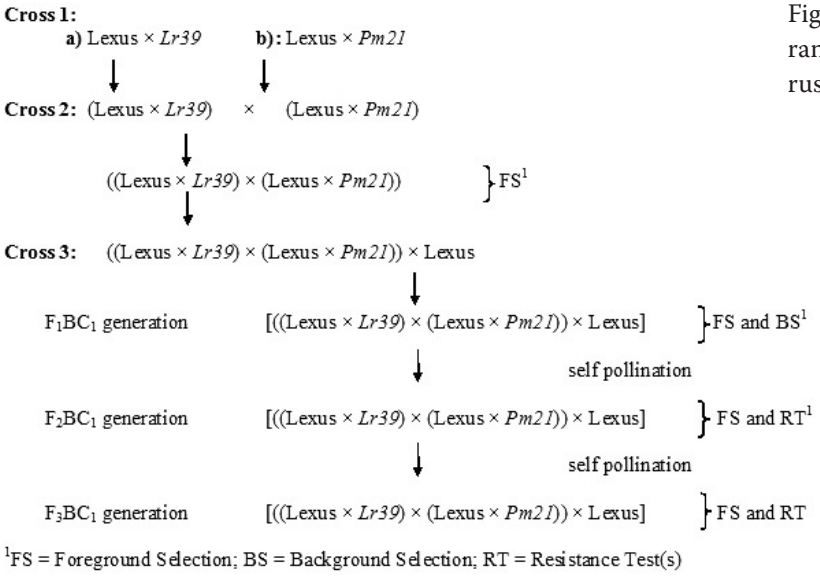

Figure 1. Experimental design for the pyramiding of two resistance genes for leaf rust and powdery mildew

or Foster etc., *Barc124* is not recommended for MAS (Sun *et al.* 2009). *Gdm35* is also particularly useful for MAS for *Lr39* when the KS90WGRC10/TAM 107 population is used as the donor of *Lr39* (Singh *et al.* 2004).

In the study reported herein, to interpret the results for the BC_1F_2 generation better (Figure 2), a genetic map was constructed using 5 microsatellites. This linkage map was compared with the genetic maps obtained by Somers *et al.* (2004) (map A), Singh *et al.* (2004) (map C), and Sun *et al.* (2009) (map D) (Figure 2). The locations of markers relative to the locus *Lr39* differed among the maps. For the map obtained with the BC_1F_2 generation, the order of the molecular markers on chromosome 2DS was as follows: *Barc124* (9.7 cM to *Lr39*), *Gdm35* (14.8 cM), *Gwm261* (20.6 cM), *Gwm296* (26.0 cM), and *Gwm210* (33.2 cM). According to the map of Singh *et al.* (2004), the closest marker to the resistance locus was *Gdm35* (1.9 cM to *Lr39*), followed by *Barc124* (5.8 cM) and *Gwm210* (7.9 cM). For the map constructed by Sun *et al.* (2009), the closest marker to *Lr39* was *Barc124* (1.0 cM to *Lr39*), followed by *Gwm210* (1.5 cM), *Gdm35* (2.8 cM), and *Cfd36* (4.1 cM). The differences in the locations of the molecular markers among the different maps suggest that the resistance gene *Lr39* might sometimes be difficult or problematic to detect in breeding material. In MAS, selection for the presence of the *Lr39* gene using only three microsatellites might not be sufficient for plant breeding. As a consequence, to increase the efficiency of selection of the *Lr39* gene in this study, 5 microsatellite markers were used: *Gdm35*, *Barc124*, *Gwm210*, *Gwm261*, and *Gwm296*. On the basis of our results, it is not possible to recommend one universal marker that is suitable for detection of the *Lr39* gene in large-scale breeding programs.

In this study, a total of 3 molecular markers were used to detect *Pm21* in breeding material, $SCAR_{1250}$, $SCAR_{1400}$, and co-dominant PCR marker *NAU/xibao*, all of which appeared to be suitable for

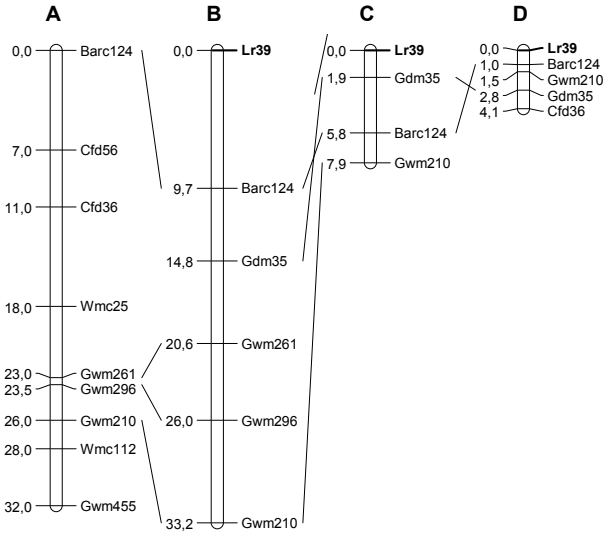

Figure 2. Partial map of wheat chromosome 2DS (B) including resistance gene *Lr39* based on the analysis of the BC_1F_3 population Lexus × KS90WGRC10; the order of the markers is compared with reference maps from (A) Somers *et al.* 2004, (C) Singh *et al.* 2004, and (D) Sun *et al.* 2009

MAS. Similar results were reported by Liu *et al.* (1999), Yildirim *et al.* (2004), and Cao *et al.* (2006). Other markers were previously used to detect *Pm21* and identify homozygous and heterozygous translocation in breeding materials, these include specific RAPD markers, such as *OPH17-1900* and *OPH17-1000* (Qi *et al.* 1996). In addition, Liu *et al.* (2000) used the RFLP marker *Psr113* to select *Pm21*. The most frequently encountered obstacles for MAS are the high cost and time requirements of RFLP technologies, and the low repeatability of RAPD markers. As a consequence, these types of marker are no longer used commonly for MAS.

One of the benefits of using molecular markers in breeding programs is the potential to estimate the proportion of alleles from the parental lines in the genomes of the offspring. Such information is particularly important in backcross breeding programs. Compared with conventional backcrossing, selection based on DNA markers (such as microsatellites and AFLPs) can speed up recovery of the recurrent parent (RP) genome (Ribaut & Hoisington 1998; Servin & Hospital 2002). This strategy is highly profitable after the first backcrosses in BC_1F_1 generations, when the widest variation for the RP genome content is observed and the individuals with the highest RP genome content can be selected, as was demonstrated in this study. Microsatellite and AFLP markers are used commonly to estimate genetic similarity, and they are effective tools for backcross breeding programs. In the present study, a set of 5 AFLP primer combinations was used to estimate genetic similarity among the backcross lines and it was estimated to be in the range of 32.1–70.8%. Chen *et al.* (2001) estimated genetic similarity using a set of 10 AFLP primer combinations, and found the recovery percentages for the recipient genome to be approximately 92.25% (BC_1F_1), 97.67% (BC_2F_1), and 98.8% (BC_3F_1). In a similar study, which was based on 35 microsatellites and 12 AFLP primer combinations, the range of % RP in BC_1F_3 was found to be 94.3–97% (Gupta *et al.* 2008).

In summary, gene pyramiding is a breeding strategy that enables multiple resistance genes to be introduced into a single genotype, which results in cultivars that are resistant to one or more diseases. Using foreground and background selection to introduce resistance genes considerably reduces the time required to breed new cultivars. The use of molecular selection in conjunction with resistance tests confers many benefits for MAS, especially when the foreground selection is difficult to interpret. In this study, we obtained new homozygous lines that were resistant to leaf rust and powdery mildew. These lines could be used as a source of resistance to these pathogens in wheat breeding programs.

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