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Development of SCAR markers linked to the *Pm21* gene conferring resistance to powdery mildew in common wheat

Z. LIU[1], Q. SUN[1,2], Z. NI[1] and T. YANG[1]
1 Department of Plant Genetics and Breeding, China Agricultural University, Beijing 100094, P. R. China;
2 Corresponding author. Email: *qxsun62@public.bta.net.cn*
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Abstract

Powdery mildew is an important disease in most of the wheat production areas of the world. The resistance gene *Pm21* (6AL/6VS translocation) derived from *Haynaldia villosa* confers resistance to all available isolates of *Erysiphe (Blumeria) graminis* f. sp. *tritici* in China and Europe. The objective of this study was to develop fast and reliable sequence characterized amplified region (SCAR) markers linked to the *Pm21* gene. A RAPD marker for *Pm21*, OPH171400, was converted to SCAR markers after sequencing the two ends of the polymorphic DNA fragment. Two SCAR markers, SCAR1265 and SCAR1400, were developed to detect the *Pm21* gene in different genetic backgrounds. The specific SCAR1265 marker enable large scale accurate screening for the presence/absence of *Pm21* allele.

Key words: *Erysiphe* (*Blumeria*) *graminis*, *Haynaldia villosa*, *Triticum aestivum*, Marker-assisted selection, *Pm21* gene, Powdery mildew resistance, RAPD, SCAR,

Powdery mildew caused by *Erysiphe (Blumeria) graminis* f. sp. *tritici* is one of the most important wheat diseases in many regions of the world. Breeding for resistance has proved to be the most economical and effective way for controlling wheat diseases. So far, twenty-five genes for resistance to powdery mildew pathogen (*Pm1* - *Pm25*) have been identified and assigned to specific chromosomes or chromosome arms (McIntosh et al 1998, Shi et al 1998). The *Pm21* gene (6AL/6VS), originating from *Haynaldia villosa*, is one of the most effective resistance genes both in China (Duan et al 1998) and Europe (Huang et al 1997).

Pyramiding of resistance genes has been suggested as a strategy to provide durable resistance to pathogens and molecular marker technology could facilitate such effort (Michelmore 1995). Both restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNAs (RAPD) (Williams et al 1990) have been used to tag disease resistance genes in wheat. To date, RFLP and RAPD markers linked to the powdery mildew resistance genes, *Pm1*, *Pm2*, *Pm3*, *Pm4*, *Pm12*, *Pm13*, *Pm21*, and*Pm25*, have been identified (Hartl et al 1993, 1995; Ma et al 1994, Jia et al 1996, Cenci et al 1998, Qi et al 1996, Shi et al 1998). However, RFLP analysis is costly and time-consuming, and RAPD technology has the shortcoming of relatively lower reproducibility and reliability, which limits its use in breeding programs. Paran and Michelmore (1993) described a reliable PCR assay defined as sequence characterized amplified regions (SCARs) by sequencing the two ends of the polymorphic DNA fragment and synthesizing two longer primers (24mer) homologous to each end.

It was reported that a RAPD marker, OPH171900, could be used to tag *Pm21* gene (Qi et al 1996). However, due to the limitations of the RAPD method, fast and reliable PCR based markers for the detection of *Pm21* are needed for marker-assisted selection (MAS). The aim of this study was to develop SCAR markers for molecular identification of the *Pm21* gene in wheat breeding programs.

Materials and Methods

Plant materials: *Haynaldia villosa* L. (2n = 2X = 14, VV), the donor of the *Pm21* gene, was kindly provided by Dr. Hongjie Li, Hebei Academy of Agricultural and Forestry Sciences, Shijiazhuang, China. *Haynaldtriticum* 'Haynald-146' (2n = 8X = 56, AABBDDVV) was kindly provided by Prof. Junming Li, China Agricultural University, Beijing, China. Several wheat-*Haynaldia villosa* substitution and translocation lines, e.g. 'R089', 'R137', 'R139', 'R178', 'R149-10' and 'Yangmai 5/Sub6V', were obtained from Prof. Dajun Liu and Prof. Peidu Chen, Cytogenetics Institute, Nanjing Agricultural University, Nanjing, China. All tested breeding lines were selected from the wheat-breeding program of China Agricultural University.

Powdery mildew test: Seeds of each breeding population and the highly susceptible cultivar 'Yanda1817' were planted in pots of 8-10 cm diameter. Seedlings were artificially inoculated, when the first leaf was fully expanded, with race No. 15, a local isolate of *E. graminis* f. sp. *tritici*, avirulent to the 6AL/6VS stocks and their derivatives. Inoculations were performed by dusting or brushing conidia from neighboring sporulating susceptible seedlings onto the seedlings to be tested. Reactions were scored 15 days after inoculation when the susceptible check, 'Yanda 1817', was heavily infected, using a 0, 0;, and 1 to 4 scale, with 0 representing no visible symptoms, 0; for necrotic flecks, and 1, 2, 3, 4 for highly resistant, resistant, susceptible and highly susceptible reactions, respectively.

DNA isolation: Total DNA was extracted from the second or third health leaf of each resistant or susceptible plants using the cetyltrimethylammonium bromide (CTAB) method (Saghai-Maroof et al 1984) with minor modification. Following a single chloroform/isoamyl alcohol (24:1) extraction, the precipitated DNA was resuspended in sterile distilled H_2O for amplification by PCR.

Polymerase chain reaction: All PCR reactions were performed in 25 µl volumes in a Perkin-Elmer (Norwalk, CT, USA) 480 Thermocycler. RAPD conditions were the same as that of Williams et al (1990). PCR products were separated on 1.4% agarose gels and visualized under UV light after ethidium

bromide staining. SCAR reactions contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 150 µM of each dNTP, 50 ng of each pair of primers, 50 - 100 ng total DNA and 1 U Taq DNA polymerase. The amplification was programmed at 94 C for 3 min, followed by 40 cycles at 94 C for 1 min, 55 C for 1 min and 72 C for 2 min, with a final extension at 72 C for 5 min. PCR products were separated on a 1.0% agarose gel.

Cloning and sequencing of the polymorphic RAPD fragment: The 1400 bp polymorphic RAPD fragment amplified from *Haynaldia villosa* using random primer OPH17 was cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) as recommended by the manufacturer. The recombinant vectors were used to transform *E. coli* competent cells DH5a. Two different clones, p1400-8 and p1400-9, were sequenced on an ABI Prism 377 DNA Sequencer (Applied Biosystems Inc., Foster City, CA, USA) after the correct insert size was confirmed by EcoRI digestion. Double strand sequencing was done by the dideoxy-chain termination method using SP6 and T7 universal primers. The sequence data were submitted to GenBank (accession numbers AF133433, AF133434, AF133435 and AF133436).

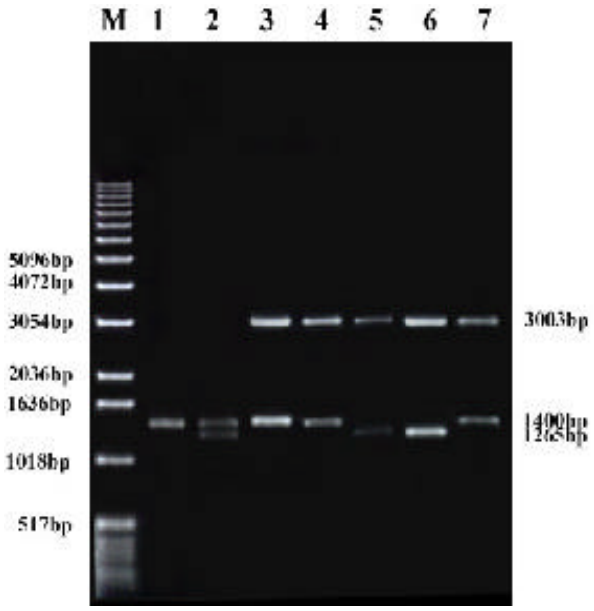

Fig. 2 EcoRI digestion of the recombinant clones and EcoRI digested, undigested reamplified PCR products of OPH171400 DNA fragment using primer OPH17, separated on 0.7% agarose gel. Lane 1, undigested reamplified PCR products. Lane 2, EcoRI digested reamplified PCR products. Lanes 3-7, EcoRI digested recombinant clones p1400-5, p1400-8, p1400-9, p1400-11 and p1400-12, respectively.

Results

Tagging *Pm21* using primer OPH17

RAPD analysis was performed using random primer OPH17 and DNA samples of the resistant germplasm *Haynaldia villosa*, the 6AL/6VS translocation line 'R137', the backcross breeding line '8866'*4//'87-1'/'R149-10', the susceptible wheat line '8866', and derivatives of *Pm21*. Instead of a 1900 bp polymorphic DNA fragment (Qi et al 1996), a 1400 bp polymorphic DNA fragment (OPH171400) was found to be linked to *Pm21*. (Fig. 1).

Cloning and sequencing of $OPH17_{1400}$

In order to develop a reliable SCAR marker for the detection of $OPH17_{1400}$, the 1400 bp polymorphic DNA fragment amplified from *Haynaldia villosa* was cloned into the pGEM-T easy vector. When the insert DNA from different clones was digested with EcoRI (Fig. 2), it was found that the 1400 bp fragment actually contained two different DNA sequences, one of which had a EcoRI restriction site at about 135 bp from the 5' end of the sequence whereas the other had no EcoRI restriction site. Both are the same size. Two different clones, p1400-8 and p1400-9, were sequenced after the correct insert size was confirmed by EcoRI digestion. The sequencing result (GenBank accession AF133435) confirmed that clone p1400-9 has an EcoRI restriction site.

Development of SCAR markers linked to *Pm21* gene

Based on the sequences of clones p1400-8 and p1400-9, two pairs of primers of 22-23 bases in length, *Pm21*A and *Pm21*B, *Pm21*C and *Pm21*D, which include the original 10 bases of random primer OPH17, were designed:

*Pm21*A: 5'- CACTCTCCTCCCCAACCTATAT-3';
*Pm21*B: 5'-CACTCTCCTCTTCGGGGAACAC-3';
*Pm21*C: 5'-CACTCTCCTCAAACCTTGCAAG-3';
*Pm21*D: 5'-CACTCTCCTCCACTAACAGAGG-3'.

Primer combination *Pm21*A and *Pm21*B amplified a 1400 bp DNA fragment only in *Haynaldia villosa* but not in translocation line 'R137', '8866'*4//'87-1'/'R149-10' and common wheat line '8866', suggesting that the amplified DNA fragment was not linked to *Pm21* and was therefore amplified from a chromosome other than 6VS. Primer combination *Pm21*C and *Pm21*D amplified a 1400 bp polymorphic DNA fragment (SCAR1400) in *Haynaldia villosa*, translocation line 'R137', '8866'*4//'87-1'/'R149-10', but not in susceptible line '8866' (Fig. 3). Further investigation was conducted using primers *Pm21*C and *Pm21*D to detect *Pm21* in derivatives of different *Pm21* stocks. The SCAR1400 was detected in all resistant plants but in none of the

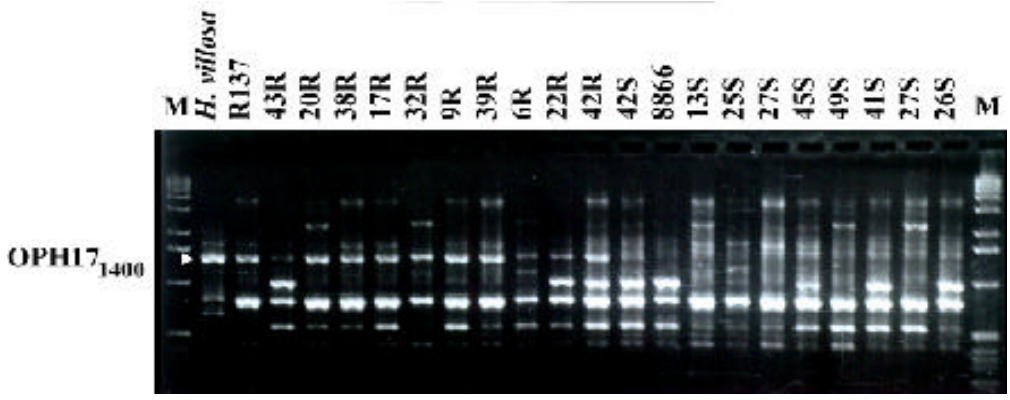

Fig. 1 Polymorphic RAPD marker, OPH171400, detected by primer OPH17 in resistant (R) and susceptible (S) plants of derivatives of different *Pm21* stocks, separated on 1.4% agarose gel. Arrow shows the OPH171400. M, 1 kb DNA ladder (Gibco, BRL, Life Technologies, USA)

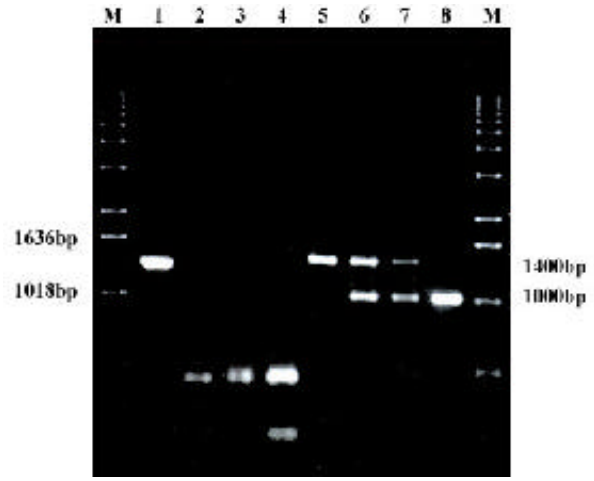

Fig. 3 PCR products amplified by primers *Pm21*A and *Pm21*B (Lanes 1 - 4) and primers *Pm21*C and *Pm21*D (Lanes 5 - 8) in *Haynaldia villosa* (Lanes 1, 5), 6AL/6VS translocation line 'R137' (Lanes 2, 6), backcross breeding line '8866*4'//'87-1'/'R149-10' (Lanes 3, 7) and susceptible wheat line '8866' (Lanes 4, 8).

susceptible plants (Fig. 4a), indicating that SCAR1400 is linked to *Pm21*. In addition to the SCAR1400, primers *Pm21*C and *Pm21*D yielded an additional non-polymorphic 1000 bp DNA fragment in all wheat lines irrespective of the presence of *Pm21* and annealing temperature (55º C to 65º C). In order to convert the 1400 bp polymorphic DNA band into a single locus presence/absence SCAR marker, another specific primer, *Pm21*E (5'-GTTTGTTCACGTTGAATGAATTC-3'), was designed according to the EcoRI restriction site in the sequences of clone p1400-9, since there is no EcoRI restriction site in the 1000 bp DNA fragment (Fig. 5). The primer combination *Pm21*E and *Pm21*D amplified a 1265 bp band only in resistant plants, but not in susceptible plants, indicating that this pair of primers could be used as a single locus presence/absence SCAR marker for the detection of *Pm21* in wheat (Fig. 4b).

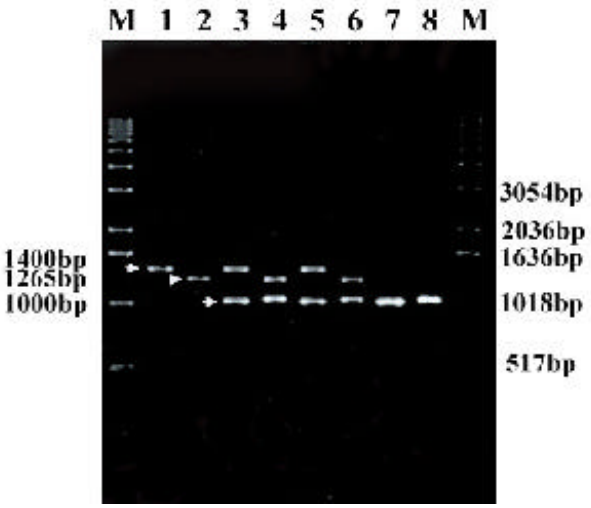

Fig. 5 Undigested and EcoRI digested PCR products amplified in *Haynaldia villosa* (Lanes 1, 2), 'R137' (Lanes 3, 4), '8866*4'//'87-1'/'R149-10' (Lanes 5, 6), '8866' (Lanes 7, 8) using primers *Pm21*C and *Pm21*D. Arrow indicates the 1265 bp DNA fragment digested from the SCAR1400 DNA fragment by EcoRI.

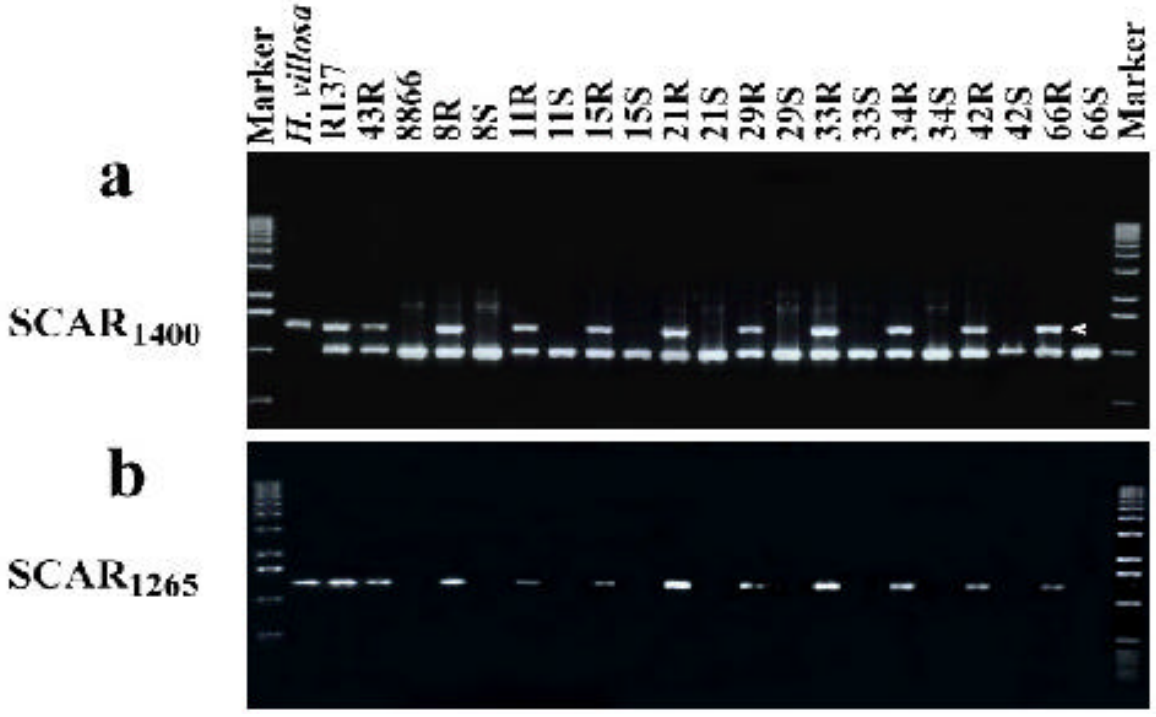

Fig. 4: PCR products from resistant (R) and susceptible (S) plants in derivatives of different *Pm21* stocks, amplified by primers *Pm21*C and *Pm21*D (see Fig. 4a) and primers *Pm21*E and *Pm21*D (see Fig. 4b). Arrows indicate the polymorphic markers SCAR1265 and SCAR1400.

Table 1 Comparison of RAPD, $SCAR_{1265}$ and $SCAR_{1400}$ markers for the detection of *Pm21*

Type of tests	Number of PCR amplification	Expected result (%)	Difficult to interpret (%)	No amplification (%)
RAPD	303	260 (84.81)	18 (6.94)	25 (8.25)
$SCAR_{1265}$	194	194 (100)	0	0
$SCAR_{1400}$	213	213 (100)	0	0

Table 2 Evaluation of SCAR markers for *Pm21* in wheat lines containing different *Pm* genes

Wheat lines	*Pm Gene*	IT*	SCAR1400	SCAR1265
'Ulka'/'8*Cc'	*Pm2*	0;	-	-
'Khapli'/'8*Cc'	*Pm4a*	0-1	-	-
'Armada'	*Pm4b*	0-1	-	-
'Coker 747'	*Pm6*	0;	-	-
'Coker 983'	*Pm5+Pm6*	0;	-	-
'Brock'	*Pm2+Talent*	0;	-	-
'Maris Dove'	*Pm2+mld*	0;	-	-
'CI 12632'	*Pm2+Pm6*	0;	-	-
'Maris Huntsman'	*Pm2+Pm6*	0;	-	-
'Kenquia'	*Pm1+Pm4a+Pm8*	1	-	-
'Baimian 3'	*Pm4a+Pm8*	0;	-	-
'Song'/'Line 31' F_4	*Pm12*	0;	-	-
'Song'/'*Pm*13'//'101'/3/'781'/4/'158' F_3	*Pm13*	0;	-	-
'BRG 3N'/'76-F2-205'	*Pm16*	0;	-	-
'Amigo'	*Pm17*	2-3	-	-
'Helami 105' (1B/1R)	*Pm17*	1-2	-	-
'6BS/6RL'	*Pm20*	1-2	-	-
'Yangmai 5/Sub.6V' (6AL/6VS)	*Pm21*	0	+	+
'8866*4'//'87-1'/Wild Emmer 'C20'	Unknown	0;	-	-
'Chinese Spring'		4	-	-

* IT: Infection type

Evaluation of the RAPD and SCAR markers of *Pm21* gene in different genetic backgrounds

To test the advantage of the SCAR markers over the RAPD marker for the detection of *Pm21* in breeding programs, 56 derivatives of resistant stocks ('R089', 'R137', 'R139', 'R149-10', 'R178', 'Yangmai5/Sub6V'), resistant parents, and susceptible parental wheat lines ('87-1', '8866', '411', '158', '101') were tested for reaction to powdery mildew and were subjected to marker analysis. Both resistant and susceptible progenies of different *Pm21* stocks were tested 3 to 5 times using the random primer OPH17, $SCAR_{1400}$ and $SCAR_{1265}$ (Table1). In a total of 303 RAPD reactions, 25 (8.25%) yielded no OPH171400 products, and 18 (6.94%) were difficult to interpret. In all tests with clear results, OPH171400 co-segregated with resistance. The SCAR1400 and SCAR1265 primer combinations amplified reproducible and reliable markers linked to *Pm21*and no recombinations between these markers and *Pm21* were found. Both markers were successfully amplified in all 407 PCR reactions conducted at the higher annealing temperature (55-60 C).

The SCAR1400 and SCAR1265 markers were also evaluated in some cultivars (lines) containing other powdery mildew resistance genes (Table 2) which were proved resistant to powdery mildew in China. The two SCAR markers consistently detected polymorphism only between resistant plants containing *Pm21* and all other wheat lines.

Discussion

Molecular markers are useful in plant breeding because the presence of a gene can be detected without waiting for the phenotypic expression of the gene. Furthermore, molecular markers can be used to pyramid genes (Huang et al 1997) that are effective against available pathogen populations and therefore cannot be selected using standard biological assays.

In this study, RAPD marker OPH171400 and SCAR markers SCAR1400 and SCAR1265 co-segregated with *Pm21*. No recombination was observed between the markers and the gene. As *Pm21* was located on the translocated chromosome arm, the polymorphic locus could be anywhere on 6VS and not necessarily physically close to *Pm21*. The RAPD test was sensitive to the reaction conditions, resulting in about 15% failure in amplification or detection. On the other hand, SCAR markers derived from OPH171400 yielded products that detected the polymorphism between resistant and susceptible plants in all PCR reactions.

No virulence for *Pm21* has yet been found in China (Duan et al 1998) and Europe (Huang et al 1997). To provide a more durable resistance and to prevent a rapid breakdown of *Pm21*, this gene should be used in combination with other effective powdery mildew resistance genes. As more PCR-based DNA markers of powdery mildew resistance genes are developed, several powdery mildew resistance genes can be combined in the same genetic background through marker-assisted selection. Since the

6AL/6VS translocation carries stripe rust resistance gene *Yr26* (McIntosh et al 1998), the SCAR markers developed in this research could be useful for stripe rust resistance breeding.

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