Identification of RAPD Markers and Development of SCAR Markers Linked to a Powdery Mildew Resistance Gene, and their Location on Chromosome in Wheat Cultivar Brock

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Abstract: Wheat cultivar Jing 411 which is susceptible to powdery mildew, and wheat cultivar Brock and NILs of Jing 411, which are resistant to powdery mildew were analyzed for polymorphisms using 213 random amplified polymorphic DNA primers. Only one primer (S2092) stably produced a polymorphic band between the resistant and susceptible plants. Linkage analysis of this marker (S2092 972) revealed that the polymorphism existed in a 131 F2 segregating population. S2092 972 was closely linked to a powdery mildew resistance gene in wheat cultivar Brock, at a linkage distance was 4.9 cM. S2092 972 was converted to sequence characterized amplified region (SCAR) markers SCAR860 and SCAR200. The two SCAR markers were used for detecting F2 segregating population. SCAR860 and SCAR200 existed in resistant plants but were absent in the susceptible plants. We concluded that S2092 972 was located on the chromosome 3BL. These markers will be useful for marker-assisted selection and gene pyramiding in wheat resistance breeding.

Key words: Linkage analysis, Powdery mildew, RAPD, SCAR, Wheat.

Powdery mildew, caused by Blumenia graminis (DC.) Speer f. sp. tritici, is one of the most serious diseases of common wheat in China and in many countries of the world (Rong et al., 2000). In China, powdery mildew occurs only in the high rainfall area in southern China in the past. In the recent 20 years, however, powdery mildew has become more prevalent in northern China with improvement of irrigation and usage of nitrogenous fertilizer. It causes severe damage in wheat production. Several genes conferring powdery mildew resistance have been transferred from Secale cereale, Triticum monococcum and T. dicoccum (Nevo, 1995), but most of the genes have already been overcome by new virulent B. graminis strains owing to the single resistance gene source and its repeated use (Xie et al., 2001). Therefore we need to search for new sources of genetic resistance to powdery mildew for cultivated wheat and the wild relatives of cultivated wheats.

Molecular marker technology is widely used to find markers linked to target genes (Hartl et al., 1995, Schachermayr et al., 1995, Hu et al., 2001). Molecular marker analysis in wheat appears to be particularly suited for identifying markers linked to the powdery mildew resistance gene (Devos and Gale, 1992; Wang et al., 2000) and has been used for marker-assisted selection and gene pyramiding in wheat resistance breeding (Liu et al., 1998; Myburg, et al., 1998; Hu et al., 2000; Liu and Liu, 2000). Brock is a cultivar of wheat highly resistant to powdery mildew pathogen in northern China. Brock carrying the Pm2 gene was reported as resistant to powdery mildew (Paillard et al., 2000). Aegilops tauschii is the original source of Pm2, which is located on chromosome 5DS. However, we found that Pm2 has completely lost resistance to powdery mildew pathogen in China and identified a molecular marker (OPP15 917) linked to a powdery mildew resistance gene in Brock with a genetic distance of 6.0 cM (Wang et al., 2001). We concluded the presence of a new powdery mildew resistance gene in Brock (Wang et al., 2004). In this paper, we report the identification and chromosomal localization of random amplified polymorphic DNA (RAPD) and sequence characterized amplified region (SCAR) molecular markers tightly linked to the powdery mildew resistance gene in the common wheat cultivar Brock, wheat fine Line 015, recurrent parent Jing 411 and individuals derived from resistant nearisogenic lines (NILs) of Jing 411. The aim of the present work was the selection of molecular markers tightly linked to the powdery mildew resistance gene, for use in wheat resistance breeding.

Materials and method

1. Plant Materials

The following materials were used for identification of RAPD molecular markers. The wheat cultivar Brock,
the donor of the resistance gene, was kindly provided by Dr. Ray Johnson. Line 015 is a fine wheat line, and Jing 411 is a fine wheat variety, which has been used in agricultural production. Line 015 and Jing 411 are susceptible to powdery mildew pathogen. Line 015 was crossed with Brock to obtain F1 seed. F1 individuals were artificially inoculated with powdery mildew race No. 15. Selected resistant individuals were crossed with Jing 411 seven times and selfed to produce progenies (resistant NILs of Jing 411). Resistant parent Brock, recurrent parent Jing 411 and the resistant NILs of Jing 411 were used for molecular marker identification.

2. Powdery mildew infection and tissue sampling

Powdery mildew of race No. 015 (Duan et al., 1998; Zhou et al., 2002) was artificially inoculated to seedlings when the first leaf was fully expanded, and reactions were scored 15 days after inoculation. Jing 411 and Line 015 were heavily infected. The response type of the plants to powdery mildew was evaluated according to Liu et al. (1999) and Wolfe (1965), using a scale of 0, 0+, 1, 2, 3 and 4 at the three-leaf stage: 0 represents no visible symptoms, 0+ necrotic flecks, and 1, 2, 3 and 4 highly resistant, resistant, susceptible and highly susceptible, respectively. Individuals scored 0, 0+ and 1-2 were identified as resistant to powdery mildew, and used as resistant materials; but those scored 3-4 were identified as susceptible to powdery mildew, and used as susceptible materials (Liu et al., 1999).

3. DNA extraction and RAPD analysis

Genomic DNA was extracted as previously described (McDonald M. B., 1994). RAPD analysis was performed according to the method of Williams et al. (1990) and Welsh and McClelland (1990) with a modified procedure. For the reaction, we used a total volume of 25µl containing 1× PCR buffer, 20ng genomic DNA, 300µM MgCl₂, 1µM 10 mer primer, 200µM of each deoxynucleotide and 1 unit Taq DNA polymerase. The amplification was performed in a Minicycler PTC-150-25, according to the procedure of Wang et al. (2000) with the program of the first 3 cycles at 96°C for 1min, 37°C for 1min and 72°C for 2min, followed by 45 cycles at 94°C for 45 sec, 37°C for 1min and 72°C for 90 sec, with a final extension at 72°C for 10 min. PCR products were separated on 1.4% agarose gel (containing 0.5µg/µl ethidium bromide) and visualized under UV light.

4. Identification of the polymorphic molecular marker for resistance and linkage analysis

A preliminary screening for polymorphism of the molecular markers among the resistance parent Brock, susceptible parent Jing 411 and Line 015 and their progenies, was carried out using 213 random 10 mer primers. Linkage relationships among the resistance gene, RAPD markers, and SCAR marker were analyzed with MAPMAKER (Lander et al., 1987). The mapping population consisted of 151 BC1F2 plants, including 101 resistant individuals and 30 susceptible individuals. Recombination values were transformed to

| Varieites and crosses | Resistance | Susceptibility | Number of F2 plants* |
|-----------------------|------------|---------------|---------------------|
| Brock                 | + (0)      |               |                     |
| Line 015              |            | − (3.4)       |                     |
| Jing 411              |            | − (3.4)       |                     |
| Brock × Jing 411      |            | + (1)         | 101                 |
| NILs of Jing 411      |            | + (1)         | 30                  |

+ : resistant. − : susceptible. Number in brackets is the response of plants to powdery mildew.

*(χ²=0.3078, P>0.05)
linkage distances in centiMorgans (cM) according to Kosambi(1994). ALOD score of 3 was set to determine the significance of linkage.

5. Cloning and sequencing of RAPD products

Using the standard cloning method (Lu, 1993), we excised PCR bands from the agarose gel and ligated them into the pGEM-T® Easy Vector (Promega) according to the manufacturer’s specifications and then transformed to the Escherichia coli JM109. The correspondence of the cloned fragments to the original polymorphic amplification products was verified by amplification with special primer T7, and SP6 and by comparing the size of the digested plasmids using the corresponding enzymes. DNA sequence analysis was carried out at Sangon Company. The results were analyzed with the software of Biology Information/SMS, and homology searches of sequences in databases were conducted by using the BLAST program (Altschul et al., 1994).

6. Design of primers and analysis of SCAR markers

The cloned fragment was sequenced from both ends. According to the sequence, three 20 mer oligonucleotide primers were designed for use as SCAR primers synthesized by Sangon Company. Amplification was performed according to the method of Venter et al. (2000) and Myburg (1998), with the use of a modified procedure. The reaction according to the procedure of Huang et al. (2000) was conducted in a total volume of 25 µl containing 1× PCR buffer, 20 ng genomic DNA, 1.6 mM MgCl2, 150 µM each deoxynucleotide, 5 pmol of SCAR forward and reverse primers and 1.25 unit Taq DNA polymerase. The amplification was performed in a Minicycler PTC-150-25 with the program of 30 cycles of 94°C for 1 min, 64°C for 2 min and 72°C for 2 min, with a final extension at 72°C for 5 min. PCR products were separated on 1.0% agarose gel (containing 0.5 µg/µl ethidium bromide) and visualized under UV light.

7. SSR analyses

SSR markers were amplified in a total volume of 20 µl containing 1× PCR buffer, 500 ng genomic DNA, 200 µ
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M each deoxynucleotide and 1U Taq DNA polymerase and 50ng wheat microsatellite primers synthesized according to the sequence published (Röder et al., 1998b). The amplification was performed in a Minicycler PTC-150-25 with the program at 94°C for 3 min, followed by 45 cycles at 94°C for 1 min, 50°C (55°C or 60°C) for 1 min, 72°C for 2 min, and final extension at 72°C for 10 min. The PCR products were separated on an 8% non-denaturing polyacrylamide gel and detected by silver staining.

Results

1. Response type of plants to powdery mildew

Brock. Line 015. Jing 411, 131 individuals obtained from hybrid F2 population (Brock × Jing 411), and resistant NILs of Jing 411 were artificially inoculated with powdery mildew race No. 015. Table 1 shows the response type of plants to powdery mildew.

Cultivated wheat Brock was highly resistant to powdery mildew race No. 15, and common wheat cultivar Jing 411 and Line 015 were highly susceptible. NILs were highly resistant. NILs containing the resistance gene of Brock were still resistant to powdery mildew through backcrossing with the susceptible parent Jing 411 for 7 generations. This result revealed that NILs, which were selected under stress of race No. 015, a local isolate of _B. graminis_ f. sp. _tritici_, were resistant to powdery mildew. F1 (Brock × Jing 411) was resistant to powdery mildew. Among 131 F2 plants, 101 individuals were resistant and 30 individuals were susceptible, which fitted to the expected Mendelian

Fig. 3. Linkage map of S2092972 molecular marker and powdery mildew resistance gene (Pm), OPP15900 and Xgwm114120 identified previously by Wang et al. (2004) are shown in italics.

Fig. 4. Nucleotide sequence of molecular marker S2092972.

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GGTTGTTTTCAAAAGAGGTCTAGCATATTGAGGTATAATGAGGCGCAACAA 50
TAATCTTCTAACTTTGGTGTGGAATCCCTCTCATAGGGTGATGGGAAACGC 100
GGGCATCTGATGTGAAATGCTTCTGTGCCTTTGATTGGAAGG 150
GTGATCCATCAACATGGGGTGGTACAGAGATGGCGTGTCCCTCCAGATTG 200
AGGAACACATGGGAAGAGTGGAGATGGTGATACTGACGAGACAGAGATGAA 250
GTTCGCCTATGTTAATGTTATGAGCAACAAAATGGAACATAAGAGG 300
TTTATGGGAGATGTTGTACGTTTATTGGAGACAAAAATGGAACAATAAGG 350
CAGAAATTTGCCACCCGGGAGAATGTTTAGTTTCCTAGAGAACAAAACTTA 400
CTCTAATGTCGTGAGTACCTACCCACTGGAAGTACAAATGATGTTTGAGT 450
GTGAGAACAAATCTAGTTGGAAGAGAAGATGTTTGATTATCTTTGAGG 500
AGTAAAATAATACAAAATAGCAAGAATTTTTGTGAAATCTATGTCGGA 550
TCAATCTTCTAAATCTCTAGATGGAGGACAATAATGATGGGAAACAA 600
TAATCTGATGTCAGCTGTTCAAAAATATTTTTGAAATCCAGATCTC 650
ACAAACAAAACTGTGACATCGTCTTCCAGCCTCCCTCCTGAAAAATTC 700
CAAAACATTCTAGCTAGCAGAGACACATTGATGGGAACACATTA 750
GTTACCTTCAACAGAGTTTTCCACTACAAAGTATGTTGTGTTTTGTG 800
TGTGGAGCAACTCTTGGTGTATAGCCCATAGGCGCTGCACTC 850
TTCAAACATGTAGTACTGATATGTATGCTATTTTTCAGG 900
CAATACTGAGAATCTTGTAAATTAATTAAAAAACTGGGCAAATCTCTT 950
GAATCTATTAAGGGAACACA 972
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ratio of 3:1 ($x^2_{2,1}=0.3078$, $P>0.05$).

2. Identification of RAPD markers linked to the powdery mildew resistant gene in Brock

A total of 213 primers were screened to identify polymorphisms between the resistant parent Brock, susceptible parent Jing 411 and the NILs of Jing 411 by using RAPD techniques. Eight primers produced a polymorphic band between the resistant and susceptible plants. The polymorphic frequency was 3.8%. Of these primers, only one primer (S2092) generated reproducible polymorphic amplification patterns between Brock, Jing 411 and resistant NILs. The reproducibility of the RAPD experiments was checked and S2092 was shown to generate a stable polymorphic product of 900bp in resistant parent Brock and resistant NILs, absent in susceptible parent Jing 411 (Fig. 1).

3. Linkage analysis of the specific fragment S2092900 to the powdery mildew resistance gene in Brock

In order to test whether the molecular marker S2092 is linked to the powdery mildew resistant gene, we tested 131 F2 plants, including 101 resistant and 30 susceptible individuals, by primer S2092.

Ninety-five of 101 resistant individuals had the polymorphic fragment S2092900, but, the remaining 6 resistant individuals did not. In the 30 susceptible individuals, we observed no amplification product of S2092900 (Fig.2). This result suggested that molecular marker S2092900 is linked to a resistance gene of powdery mildew in Brock.

Linkage map of S2092900 molecular marker and powdery mildew resistance gene is shown in Fig. 3.

4. Cloning, sequencing of RAPD marker S2092900 and converting to SCARs

Since RAPD technology has the shortcoming of relatively lower reproducibility and reliability, which limits its use in breeding programmes, we cloned this molecular marker and sequenced and converted to SCARs. The DNA fragment was purified and cloned into pGEM-T Easy Vector. After sequencing from both ends, we obtained the nucleotide sequence of molecular marker S2092900 (Fig. 4).

The sequencing result showed that the RAPD marker S2092900 has 972bp. There is no homology of this sequence to any gene appearing in GenBank, and we did not find the signal sequences. So it is a new sequence accepted by GenBank (accession number: AY324385).

Based on the sequence of clone S2092972, 3 primers, 20 bases long (S2092972A, S2092972B, S2092972C) were designed:

S2092972A, 5'GGTTGTTCCCAAAGAGGTCA3';

S2092972B, 5'ATGTTTGAAGCAGGGTGCAG3';

S2092972C, 5'TCCTCAATCTGGAGGGACAC3'.

S2092972A contained the original 10 bases of the RAPD primer and the first 10 internal bases adjacent to the RAPD primer. S2092972B and S2092972C the reverse primers, were designed based on the sequence of the fragment using PRIMER3.0 software without the RAPD primer. The results indicate that the 3 primers produced amplification fragment of 860 bp, and 200bp, respectively, that were present in Brock and resistant NILs of Jing 411, but absent in Jing 411(Fig. 5). In order to test whether the two SCAR markers are linked to the powdery mildew resistance gene,
101 resistant and 30 susceptible individuals that had been tested for powdery mildew infection, were tested by them. It was shown that 95 out of 101 resistant individuals have SCAR860, and SCAR200 markers. However, in the susceptible individuals no SCAR markers were detected. Linkage distances from Pm gene to SCAR860, and SCAR200 markers are 4.9cM (Fig. 3, Fig. 6).

5. Chromosomal location of a new powdery mildew resistance gene in Brock determined by using molecular markers

Because microsatellite markers are chromosome-specific, recently, two new powdery mildew resistance genes Pm30 (Liu et al., 2002) and Pm31 (Xie et al., 2003), were located on the chromosomes by this strategy. On the wheat microsatellite map constructed by Röder et al. (1998), Xgwm114 was mapped on the long arm of chromosome 3B and short arm of chromosome 3D. We selected two SSR primers, Xgwm229 which were mapped on 3BL and Xgwm71 on 3DS, that are near Xgwm114, and tested their polymorphism among Brock, Jing 411 and resistant NILs of Jing 411. The result indicated that Xgwm229 amplified a stable polymorphic product of 130bp in resistant parent Brock and resistant NILs of Jing 411, but susceptible parent Jing 411 did not show this fragment (Fig. 7). However primer Xgwm71 did not amplify the polymorphic product in Brock, Jing 411 and resistant NILs of Jing 411. Based on the analysis, we concluded that a new resistance gene is located on the long arm of chromosome 3B.

Discussion

Brock carrying the Pm2 gene is resistant to powdery mildew (Liu et al., 1999), but, cultivated wheat plants carrying Pm2 have already lost resistance to powdery mildew in China (Wang et al., 2001). We screened 11 cultivated wheat varieties containing Pm2, including Brock, for resistance to powdery mildew using the isolate race No. 15 (the prevailing race in China) in order to test whether the powdery mildew resistance gene was Pm2 or the new gene in Brock. Results showed that only Brock was resistant and other cultivated wheat varieties carrying Pm2 were susceptible to the race No.15. We conclude that a new powdery mildew resistance gene exists in Brock. The same result was reported by Wang et al. (2004). To further clarify
if S2092972 was a molecular marker of Pm2 in Brock, we tested 10 different wheat varieties with Pm2 gene using S2092 primer, but polymorphic fragment of S2092972 has not been identified. We conclude that S2092972 in this research is a molecular marker associated with the new powdery mildew resistance gene in Brock. It was reported that Pm2 was located in 5DS (McIntosh et al., 1970). In the previous paper (Wang et al., 2004), we reported the two molecular markers (Opp15 and Xgwm114) that yielded reproducible polymorphic amplification patterns in Brock, Jing 411 and resistance NILs of Jing 411, were associated with a new resistance gene. In the present study, a RAPD marker S2092972 and two SCAR markers, SCARopp and SCARxgwm which were developed from S2092972, and a SSR marker (Xgwm229) also showed the polymorphism among Brock, Jing 411 and resistance NILs of Jing 411. Linkage analysis showed that S2092, SCARopp, and SCARxgwm were associated with the new resistance gene in Brock. This new resistance gene was located on the long arm of chromosome 3B. Therefore, these markers (Opp15, Xgwm114, S2092, SCARopp and SCARxgwm) can be located on the same linkage map (Fig. 3).

In this study, we searched for polymorphic markers between resistant and susceptible plants using a total of 213 RAPD primers. As a result the polymorphic fragment of S2092972 were identified in the resistant parent Brock and most of the resistant NILs of Jing 411. Linkage analysis, using 101 resistant individuals and 30 susceptible individuals, obtained from the F2 segregation population (Brock × Jing 411), showed that 95 of the 101 resistant individuals amplified fragment of S2092972 but 30 susceptible individuals and the 6 resistant individuals did not. This molecular marker was tightly linked to a new powdery mildew resistance gene in resistant cultivated Brock at a genetic distance of 4.9cM.

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 (Liu et al., 1999). S2092972 is a molecular marker linked to powdery mildew resistance gene in Brock. SCARopp and SCARxgwm were converted from RAPD marker S2092972. They are reliable reproducible molecular markers, and are very useful for facilitating the selection of resistance genes in wheat breeding.

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