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Identification of molecular markers for resistance to *Septoria nodorum* blotch in durum wheat

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Abstract The development of *Septoria nodorum* blotch-resistant cultivars has become a high priority objective for durum wheat breeding programs. Marker-assisted selection enables breeders to improve selection efficiency. In order to develop markers for resistance to *Septoria nodorum* blotch, a set of F_5 recombinant inbred lines, derived from the crosses Sceptre/3–6, Sceptre/S9–10 and Sceptre/S12–1, was developed based on the F_2-derived family method. Two RAPD markers, designated $UBC521_{650}$ and $RC37_{510}$, were detected by bulked segregant analysis and located approximately 15 and 13.1 centiMorgans (cM) from the resistance gene *snbTM*, respectively. A SCAR marker was also successfully developed for marker-assisted selection in breeding programs based on the sequence of the RAPD marker $UBC521_{650}$. This is the first report of DNA-based markers linked to resistance for *Septoria nodorum* blotch in durum wheat.

Keywords RAPD · SCAR · Durum wheat ·*Stagonospora nodorum*

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Introduction

Septoria nodorum blotch (SNB), a foliar, glume, and stem disease of bread wheat (*Triticum aestivum* L.) and durum wheat (*Triticum turgidum* L.), is caused by the fungus *Stagonospora nodorum* Berk. (Nelson and Gates 1982). The disease occurs in most countries where bread wheat and durum wheat are grown (Tomerlin et al. 1984), resulting in significant losses in grain yield and quality (Eyal 1981). Frecha (1973) reported that a single dominant gene is involved for resistance in the winter wheat cultivar Atlas 66, but in most cases the inheritance of SNB resistance appears quantitative (Nelson and Gates 1982; Ecker et al. 1989; Wilkinson et al. 1990; Bostwick et al. 1993; Wicki et al. 1999). An additive effect seems to be the most-important genetic component in the inheritance of SNB quantitative resistance (Bruno and Nelson 1990; Du et al. 1999). Nass and Johnson (1985) applied a foliar fungicide in order to identify spring wheat genotypes with resistance to SNB. Application of the foliar fungicide resulted in little or no increase in grain yield or kernel weight in the more-resistant genotypes relative to the large increase in susceptible genotypes. These authors claimed that grain yield and kernel weight were good indicators of resistance to SNB. However, grain yield is also readily affected by environmental factors. In addition, the environmental factors could result in misclassification of the disease reaction in plant-breeding programs.

An alternative approach for the plant breeder is to use marker-assisted selection (MAS) in a conventional breeding program since molecular markers have several advantages over traditional phenotypic markers. The use of molecular markers can improve the efficiency of conventional plant breeding by selecting a marker, or markers, linked to the trait of interest (e.g., disease resistance) because the molecular markers are not modified by the environment. In addition, these markers can been detected at all stages of plant growth, whereas phenotypic markers can only be identified in a given plant-growth stage. Random amplified polymorphic DNA (RAPD) is

a simple and inexpensive technique compared to restriction fragment length polymorphism (RFLP). RAPD markers are also more-rapidly and more-easily detected than RFLP markers (Welsh and McClelland 1990; Williams et al. 1990). The RAPD technique, combined with bulked segregant analysis (BSA), has been used to identify markers closely linked to economically important traits (Michelmore et al. 1991). Sequence-characterized amplified region (SCAR) markers have been converted from RAPD markers and have also proven useful (Paran and Michelmore 1993; Maisonneuve et al. 1994). More recently, a new technique, called amplified fragment length polymorphism (AFLP), has been developed and is a very powerful tool for DNA fingerprinting since it increases the frequency of detectable polymorphisms in polyploid wheat (Vos et al. 1995) and rice (Mackill et al. 1996). However, the development of specific sequence primers for SCAR markers from AFLP markers is not an efficient process in wheat (Shan et al. 1999). The objective of the present study was to develop RAPD and SCAR markers linked to the gene for resistance to SNB in durum wheat.

Materials and methods

Plant material

Durum wheat lines S3–6, S9–10 and S12–1, derived from *Triticum timopheevii* (PI290518), are resistant to SNB and have the same resistance gene *snbTM* (Ma and Hughes 1995). The durum wheat cultivar Sceptre is currently registered for durum production in western Canada, but is susceptible to SNB. The susceptible cultivar Sceptre was crossed with the three resistant lines S3–6, S9–10 and S12–1. The F_2-derived family method was used to advance these populations to the F_5. Bulked segregant analysis, based on a bulked DNA sample from resistant lines vs a bulked DNA sample from susceptible lines, was used to screen the primers. Since S3–6, S9–10 and S12–1 have the same resistance gene derived from *T. timopheevii*, 107 recombinant inbred lines, derived from the crosses Sceptre/S3–6, Sceptre/9–10 and Sceptre/12–1, were used for genetic-linkage analysis with MAPMAKER/EXP 3.0 (Lander et al. 1987). The procedures for the disease test, including pathogen culture, inoculation and disease rating, were the same as described by Ma and Hughes (1995).

DNA extraction

Fresh seedling leaf tissue was ground to a powder in liquid nitrogen in a 1.5-ml centrifuge tube. Five hundred microliters of hot (65°C) 2× CTAB buffer (2% CTAB w/v, 20 mM EDTA, 2.8 M NaCl, 1% PVP, 100 mM Tris pH 8.0) were added. The mixture was placed in a water bath (65°C) for 15 min, then 1 vol of chloroform/isoamyl alcohol (24:1) was added and mixed thoroughly to form an emulsion. After centrifugation (300× g, 20 min), the supernatant (about 450 µl) was transferred to a fresh tube and the waste discarded. A 1/10 vol of hot (65°C) 10% CTAB solution was added to the supernatant. The mixture was placed in a water bath (65°C) for 20 min. Subsequently, the chloroform/isoamyl alcohol and centrifugation steps were repeated. DNA was precipitated from the supernatant with 2 vol of cold (–20°C) 95% ethanol by inverting the capped tube several times and placing it on ice for 20 min. DNA was pelleted by centrifugation (300× *g*, 20 min). To the DNA pellet, 500 µl of cold (–20°C) 70% ethanol were added and the tube with the DNA pellet was placed on ice for 20 min to remove excess salts from the DNA. Following centrifugation (300× *g*, 20 min) the ethanol was poured off. The DNA pellet was air-dried at room temperature and re-suspended in water. The quality of the genomic DNA was examined by agarose-gel (1.0% w/v) electrophoresis. All extracted DNA was quantified by spectrophotometric measurement of UV absorption at 260 nm (GeneQuant, Pharmacia LKB Biochrom Ltd). DNA stock solutions were prepared for PCR at a final concentration of 25 ng/µl and stored at –20°C.

Oligonucleotide primers

Oligonucleotide primers (10-mers) were purchased from the Biotechnology Laboratory, University of British Columbia, whereas 9–mer primers were synthesized on an Applied Biosystems Model 394 DNA synthesizer. Each primer (10-mer and 9-mer) was dissolved separately in water to yield a DNA concentration of 5 µM and stored at –20°C.

DNA amplification for RAPD analysis

A 25-µl reaction mixture consisting of 10 mM Tris–HCl (pH 8.8 at 25°C), 50 mM KCl, 2.0 mM $MgCl_2$, nucleotides dATP, dTTP, dCTP, and dGTP (150 µM each), 0.4 µM of primer, 25 ng of template DNA, and 1.0 unit of Ampli*Taq* DNA polymerase (GIBCO BRL) was overlain with 25 µl of light mineral oil. Amplifications were carried out in a Robocycler (Gradient 40, Stratagene) programmed for one cycle at 94°C for 4 min, followed by 40 cycles of 1 min at 94°C, 1 min at 35°C and 1 min at 72°C. After the last cycle, the samples were kept at 72°C for 5 min and then cooled to 6°C.

Cloning and sequencing of RAPD products

PCR bands were excised from the agarose gel and extracted using a QIAquick Gel Extraction Kit (QIAGEN Inc., 1995). The recovered DNA fragments were cloned in the PCR II vector (TA cloning kit, Invitrogen Corporation). This vector (3.9 kb) was designed for direct cloning of PCR products. It has a 3′-thymidine overhang that binds to adenine residues naturally added at the 5′ ends of PCR products by thermostable polymerases. It carries a *Lac Z* gene for blue/white colour-selection of transformed bacteria, ampicillin and kanamycin resistance genes, and a polylinker. Plasmid DNA was extracted using Wizard Minipreps from overnight cultures of the transformed bacteria (Promega) and purified by ethanol-precipitation. Both strands of the cloned fragments were sequenced using the *Taq* Dyedeoxy Terminator cycle sequencing kit (Applied Biosystems) on a 370 A sequencer (Applied Biosystems) at the Plant Biotechnology Institute (PBI, Saskatoon).

Design of specific primers and amplification of the target fragments

A pair of specific 20-mer oligonucleotide primers (Primer 1: 5′ CAC TAT AAC CAT ATA CGG AA 3′; Primer 2: 5′ TGA TAG GTC TGC TAA GTG TC 3′) were designed based on the sequences of a RAPD fragment using Primer (3.0) software and synthesized with a DNA synthesizer (Oligo 1000, Beckman) using standard reagents. PCR was performed as described in the DNA-amplification of RAPD analysis, except for the use of a 44°C annealing temperature instead of 35°C.

Gel electrophoresis

The PCR products (20 µl) with 5 µl of loading buffer were fractionated by electrophoresis using a 1.5% (w/v) agarose gel in Tris-acetate (TAE) buffer. Gels were stained with ethidium bro-

mide (10 mg/ml). DNA fragments were then visualized under UV light and photographed using a gel documentation system (DiaMed Lab Supplies Inc). Sizes of amplified products were estimated by comparing with a 1-kb DNA ladder (BRL, Bethesda, Md., USA).

Results and discussion

The SNB-resistant bulk (SNBRB), from the SSD-F_5 population of the cross Sceptre/S12–1, contained resistant lines 18, 22, 26, 27 and 28, whereas the SNB-susceptible bulk (SNBSB) consisted of the susceptible lines 16, 19, 20, 24 and 42. Both SNRB and SNSB were screened with random primers to detect polymorphisms. Three hundred and forty primers (9- and 10-mer) were screened, and seven primers detected polymorphisms between SNRB and SNSB. However, only two primers, UBC521 (sequence 5′ CCG CCC CAC T 3′) and RC37 (sequence 5′ ACC GCC GTT 3′), generated reproducible DNA fragments, which were present in the resistant bulk and absent in the susceptible bulk. The primer UBC 521 produced a fragment of 510 bp, designated $UBC521_{650}$. The RAPD marker was associated with resistance to SNB by multiple bands (Fig. 1). The primer RC37 produced a fragment of 510 bp, designated $RC37_{510}$. This RAPD marker was also linked to the gene for SNB by multiple bands (Fig. 2).

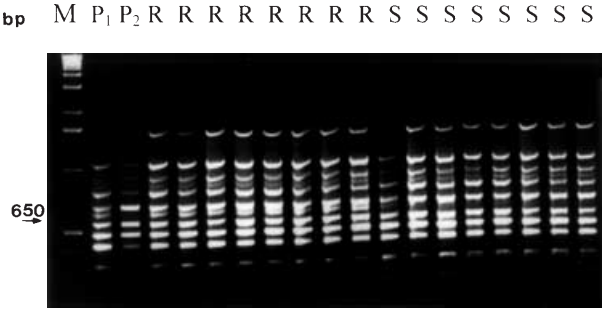

Fig. 1 A RAPD marker $UBC521_{650}$ (*arrow*) was linked to the gene for resistance to *Septoria nodorum* blotch. *M* molecular-weight marker, *P1* resistant parent S12–1, *P2* susceptible parent Sceptre, *R* resistant line, *S* susceptible line

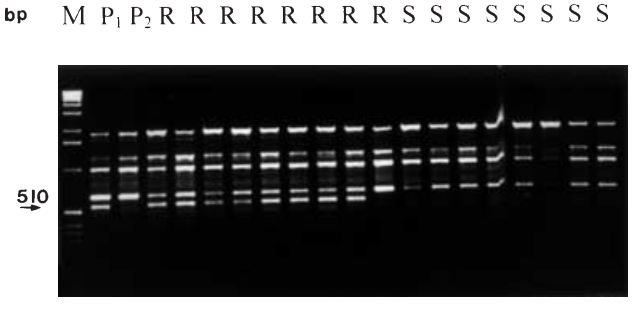

Fig. 2 A RAPD marker $RC37_{510}$ (*arrow*) was linked to the gene for resistance to *Septoria nodorum* blotch. *M* molecular-weight marker, *P1* resistant parent S12–1, *P2* susceptible parent Sceptre, *R* resistant line, *S* susceptible line

Short random primers (9- and 10-mer) used in RAPD analysis usually anneal with multiple sites in different regions of the genome so that several genetic loci are amplified. Moreover, poor reproducibility can occur in RAPD analysis. To overcome these problems, and to improve the utility of RAPD markers in MAS, RAPD markers need to be converted into SCAR markers. The RAPD marker $UBC521_{650}$ was cut from an agarose gel, cloned and sequenced. Two specific primers (20-mer) were designed based on the nucleotide sequence of the marker $UBC521_{650}$ and a SCAR marker was successfully developed using these primers (Fig. 3), indicating that the use of longer and specific SCAR primers can eliminate a multiple banding pattern. The SCAR marker was the same size as RAPD marker $UBC521_{650}$, but was expressed more intensely and was easier to recognize. Usually, one pair of specific primers generates a single SCAR marker; however, two bands appeared in Fig. 3. The two homoeologous loci on the A and B genomes of durum wheat probably annealed with the specific primers, so that two fragments were amplified.

Resistant durum wheat lines S12–1, S9–10 and S3–6 were derived from the *T. timopheevii* accession PI 290518 and have the same resistance gene *snbTM* (Ma and Hughes 1995). We tested the three resistant durum wheat lines and the *T. timopheevii* accession PI 290518 using the SCAR marker and random primers. The result showed that all the resistant lines and the *T. timopheevii* accession PI 290518 produced the SCAR marker and RAPD marker, respectively (Figs. 4 and 5), indicating that the same resistance gene of these three lines was derived from *T. timopheevii* accession PI290518. Therefore, the marker $UBC521_{650}$ was used to test 107 recombinant inbred lines from the crosses Sceptre/S3–6, Sceptre/S9–10 and Sceptre/S12–1. The data were analyzed using the computer program MAPMAKER/EXP 3.0 (Lander et al. 1987). The RAPD markers $UBC521_{650}$ and $RC37_{510}$ flanked the resistance gene by 15 and 13 cM, respectively. RAPD marker $RC37_{510}$ was also linked (37.1 cM) to the gene for red seed colour. This supported the finding that the resistance gene is located on chromosome 3 A in durum wheat (Ma and Hughes 1995), since

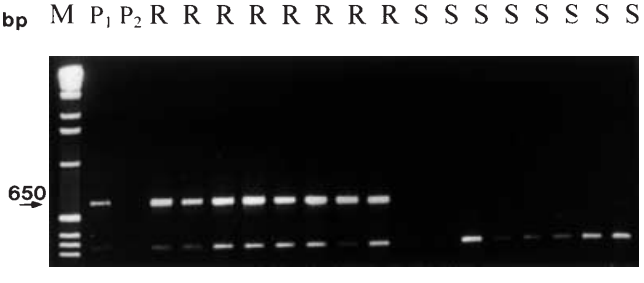

Fig. 3 A SCAR marker for resistance to SNB was developed from the RAPD marker $UBC521_{650}$. *M* molecular-weight marker, *P1* resistant parent S12–1, *P2* susceptible parent Sceptre, *R* resistant line, *S* susceptible line

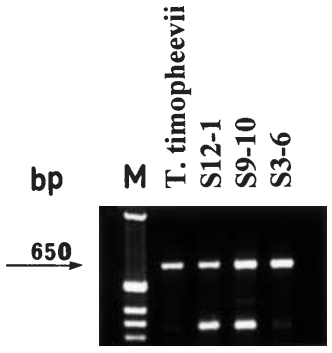

Fig. 4 The expression of a SCAR marker in resistant lines S3–6, S9–10, S12–1 and *T. timopheevii* accession PI 290518. *M* molecular-weight marker

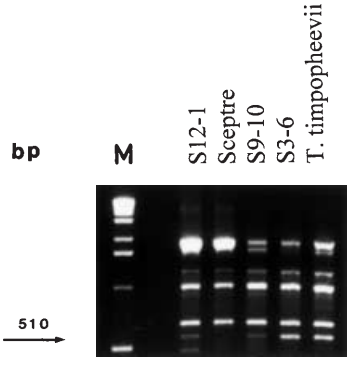

Fig. 5 The RAPD marker $RC37_{510}$ was present in resistant lines S3–6, S9–10, S12–1 and *T. timopheevii* accession PI 290518, but absent in the cultivar Sceptre. *M* molecular-weight marker

the genes for red seed colour are located on homoeologous group 3 in wheat (Allan and Vogel 1965).

The development of SNB-resistant cultivars has become an objective of high priority in a durum breeding program. MAS can facilitate the pyramiding of different resistant genes and eliminate the time-consuming progeny disease testing of individual plants. The precision of MAS is dependent on the genetic distance between the marker and the gene of interest. If a marker used for selection is at a distance away from the gene of interest, cross-overs will occur between the marker and the gene of interest. This would lead to a high percentage of false-positive/negative selection in screening the process. However, the simultaneous use of two flanking markers can reduce the selection error due to cross-over (Procunier et al. 1997). Therefore, another SCAR marker is needed to be developed for RAPD marker $RC37_{510}$ so that simultaneous use of the two flanking SCAR markers can be tested as a means of reducing selection error resulting from the loose linkage between *SnbTM* and the RAPD markers.

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