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PCR-based molecular marker for the Bdv2 *Thinopyrum intermedium* source of barley yellow dwarf virus resistance in wheat

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Abstract. Because of the importance of BYDV in wheat production worldwide, and given the difficulties of bioassaying for resistance, a molecular marker was developed for the resistance known as *Bdv2* that originates on the long arm of chromosome 7Ai1 of *Thinopyrum intermedium*. This resistance was identified in a partial amphiploid line TAF46, a disomic addition line to wheat (L1), a telosomic addition line (7Ai1 L), and a series of recombinants and translocations. A RAPD (random amplified polymeric DNA) marker for the resistant germplasm was cloned and sequenced, and primers were designed against that sequence to produce a sequence characterised amplified region (SCAR) marker. A single PCR product is produced only with genotypes carrying the resistance from any of the available recombinants. The cloned sequence, recommended primers, and PCR protocols are described. The usefulness of the marker has been demonstrated for following *Bdv2* in segregating wheat breeding germplasm, with the imminent release of a BYDV-resistant cultivar.

Additional keywords: cereal yellow dwarf, SCAR, sequence characterised amplified region, marker assisted breeding, *Agropyron*, luteovirus.

Introduction

Barley yellow dwarf virus (BYDV) is a group of luteoviruses of considerable economic significance worldwide, particularly in higher rainfall regions where the aphid vector itself is problematic. The group of viruses regarded as the BYDV complex together is recognised as the most important virus group of small grain cereals (Pike 1990). Yield losses in wheat have been reported to be as high as 43% (El Yamani 1990), 48% (McKirdy and Jones 1997), 34% (Herbert *et al.* 1999), and 46% (Riedell *et al.* 1999). Banks *et al.* (1995*a*) showed increasing loss of yield with increasing disease incidence over 6 genotypes up to over 50% losses at high rates of infection.

Thinopyrum intermedium (host) Barkworth and Dewey, also known as *Agropyron intermedium*, *Elytrigia intermedia*, or intermediate wheatgrass, has proved a source of BYDV resistance genes. One of these was identified in a 56 chromosome partial amphiploid TAF46 and a $2n = 44$ disomic addition line called L1 derived from TAF 46 (Brettell *et al.* 1988). The alien chromosome in L1 belongs to Group 7 and was called 7Ai1 by Friebe *et al.* (1992); the BYDV resistance is located on the long arm (Brettell *et al.* 1988). GISH analysis strongly suggests that *Th. intermedium* consists of 3 distinguishable groups of chromosomes given genomic names J, J^s, and S (Chen *et al.* 1998). However, it remains disputed whether the L1 additional chromosome (7Ai1) carrying the BYDV resistance is 7J (Chen *et al.* 1999) or 7S (Wang and Zhang 1996). We utilised cell culture of the monosomic addition tissues to induce recombination or translocation between the alien and wheat chromosomes (Banks *et al.* 1995*b*). A series of 7D–7Ai1 recombinants (TC5, TC6, TC8, TC9, TC10, and TC14) and one 1BS.7Ai1 L translocation (TC7) have been identified (Banks *et al.* 1995*b*; Banks and Larkin 1995; Hohmann *et al.* 1996). Some of these translocations may be useful for wheat improvement; we and others are currently introgressing them into advanced breeding lines.

There are, however, practical difficulties in following BYDV resistance in a breeding population. The virus is transmitted only by aphids and therefore a bioassay requires maintaining the infrastructure of a clean population of aphids, a pure source of virus of known serotype, antisera against that virus, and prepared ELISA plates. It furthermore takes about 8 weeks and considerable labour to raise viruliferous aphids, infest individual plants with aphids, grow the plants after inoculation in appropriate controlled environments, extract leaf saps, and conduct ELISA determinations of virus titres. Our experience was that plant breeders were generally reluctant to devote the resources needed for such bioassays. The molecular marker reported here for the TC series introgressions of the *Th. intermedium* 7Ai1 L source of resistance, overcomes these difficulties and

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should permit efficient introgression of this resistance-conferring chromatin.

Materials and methods

RAPD marker

The primer used for RAPD PCR reactions was OPA07 (Operon technologies) with sequence 5′-GAAACGGGTG. The PCR reaction mix included 200 µM dNTPs, 1.5 mM MgCl2, 0.5 mM primer, 50 ng genomic DNA template, and 0.5 unit Taq polymerase in 25 µL reaction. Cycler conditions were 94°C 3 min, 37°C 30 s, 72°C 60 s, 40× (94°C 45 s, 37°C 30 s, 72°C 60 s).

Cloning and sequencing of RAPD marker band

Two hundred µL of an OPA07-primed PCR reaction of *T. intermedium* was precipitated (0.3 M Na acetate pH 6.1, 66% ethanol), washed with 70% ethanol, resuspended in H_2O, and electrophoresed in 1% low melting point agarose. After size separation the BYDV diagnostic band (~650 bp) was excised and purified using a Wizard prep PCR column (Promega). The resultant fragment was cloned into pGEM-T Easy (Promega). White colonies were selected for inserts of appropriate size (i.e. ~700 bp) by PCR screening and then grown in LB medium overnight. Following extraction of the plasmids (Qiagen Miniprep Kit), 2 plasmids with inserts of about 700 bp were sequenced using a BigDye terminator cycle sequencing kit and an ABI Prism 377 DNA sequencer (Applied Biosystems).

SCAR primers and PCR conditions

Sequence characterised amplified region (SCAR) primers were developed from the sequence avoiding primer candidates that had internal repeated motifs or stretches of complementary sequence. Several such primer combinations were synthesised, but only one of the combinations was extensively tested and developed. The recommended primer sequences were BYAgi Forward (5′ CATGGATAATTCAGGGAGCATTCTG) and BYAgi Reverse (5′ CTGAACACGAATTTGCTGAGGTTG). The PCR conditions used for these primers were: HotStar Taq polymerase (Qiagen) (includes buffer and dNTPs); 0.5 µM of each primer; 40 ng template DNA in a 20 µL reaction; 95°C 15 min; 30× (94°C 45 s, 56°C 30 s, 72°C 45 s); and finally 72°C 10 min.

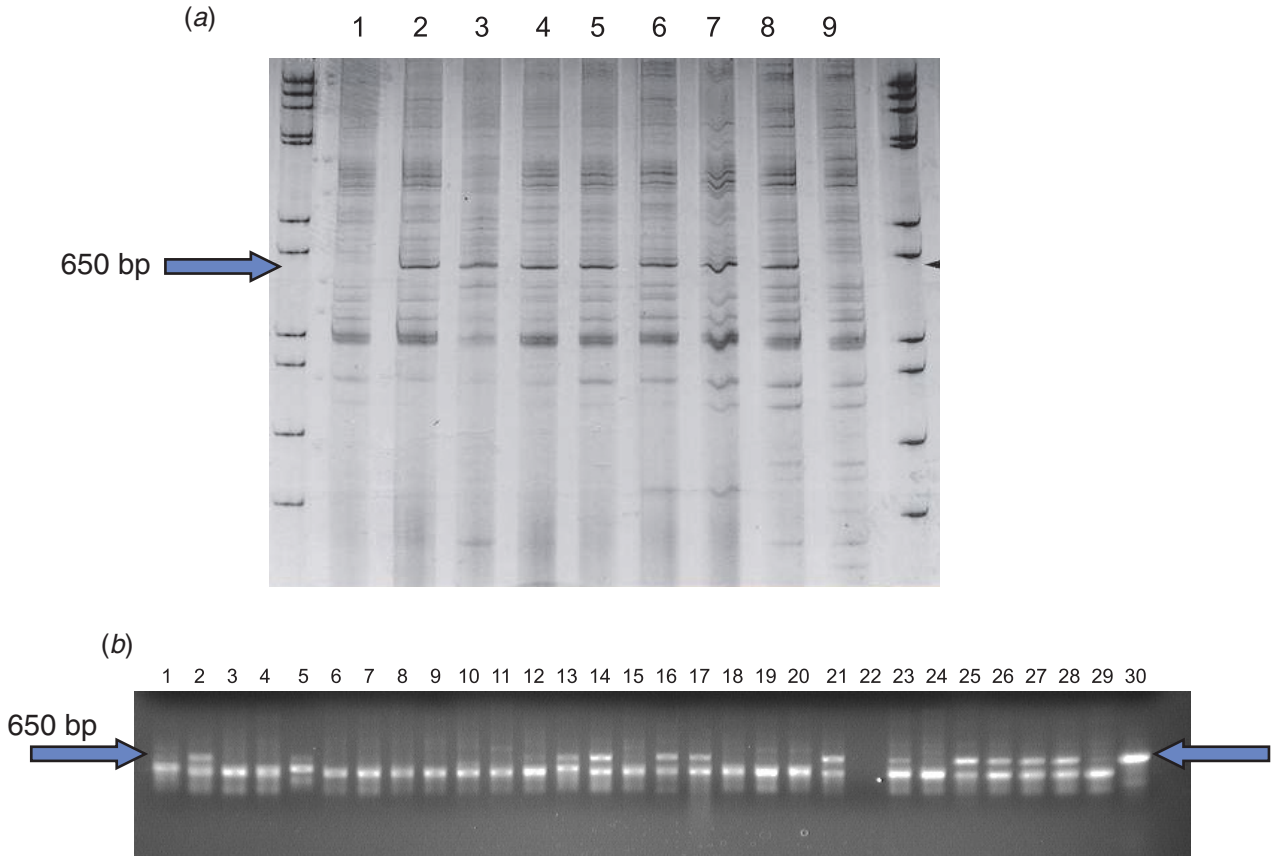

Fig. 1. RAPD PCR products of various wheat lines using OPA 07 10-mer primer (5′-GAAACGGGTG). The PCR reaction mix included 200 µM dNTPs, 1.5 mM $MgCl_2$, 0.5 mM primer, 50 ng genomic DNA template, and 0.5 unit Taq polymerase in 25 µL reaction. The products could be (*a*) separated on polyacrylamide gels and silver stained, or (*b*) separated on agarose gels and detected with ethidium bromide fluorescence. The samples shown on PAGE in (*a*) are: tracks 1 and 9, susceptible recurrent parent wheat Hartog; tracks 2–4, resistant TC9 recombinants BC4 F4 into Hartog; tracks 5–7, resistant TC10 recombinants BC4F4 into Hartog; track 8, a TC14 recombinant BC1F4 into Hartog. The diagnostic RAPD marker band (indicated with the arrow) at about 650 bp is evident in each of the recombinants. Molecular weight markers are on both sides. The samples shown separated on agarose in (*b*) are: tracks 1–27, breeding lines segregating for the resistance (no product developed in track 22); track 28, TC5; track 29, susceptible wheat Hartog; track 30, *Th. intermedium*. The arrow shows the marker RAPD band.

Results

A RAPD marker for the TC series recombinants carrying BYDV resistance was identified through screening a range of commercial RAPD primers (Operon). Fig. 1 gives an example of the RAPD marker using the OPA07 primer when separated on polyacrylamide gels and silver stained. Clearly, many other products develop and it has been our experience that the diagnostic RAPD band was not always easily visible, especially when RAPD reaction products were separated on agarose gels and visualised with ethidium bromide and UV illumination. Attempts at using this RAPD marker for screening BYDV resistance in breeding lines proved inconsistent and problematic. These inconsistencies may have been due to competing target sequences and products of the PCR reaction. A sequence-specific PCR product (SCAR marker) should be and has proved more robust for higher throughput screening of segregating breeding material.

The RAPD marker band was cut out and cloned as described in the Materials and methods. Two independent clones were sequenced and both contained an identical 641 bp insert that was bordered by 10-nucleotide sequences that corresponded exactly to the OPA07 primer sequence. The sequence was characterised by an A/T content of 52.4%, and a Blast search of the GenBank nucleotide database revealed no significant homology to any described sequence. One of these plasmids was designated pPS001. The sequence of the cloned fragment in plasmid pPS001 is shown in Fig. 2.

The fragment was excised from plasmid pPS001 by *Eco*RI digestion and used to synthesise radioactive probes (Megaprime, Amersham Pharmacia). An OPA07 primed RAPD reaction of both BYDV resistant (*Bdv2*) and susceptible wheat and *T. intermedium* DNA was run out on 1% agarose and transferred to Hybond N+ (Amersham) nylon membrane. The probe was hybridised to the membrane overnight in a Southern hybridisation buffer consisting of 50% formamide, 6 × SSC, 0.5% SDS, 5 × Denhardt's solution, and 120 µg/mL of denatured salmon sperm DNA at 42°C. After hybridisation the filter was washed 3 times in 0.5 × SSC, 0.1% SDS at 55°C. The hybridisation pattern was analysed using a PhosphoImager (Molecular Dynamics).

The probe only hybridised to the *T. intermedium* lane, the BYDV-resistant wheat lane, and the positive controls (Fig. 3). The absence of hybridisation to the BYDV-susceptible wheat lane suggested that the cloned probe was hybridising to the OPA07-derived diagnostic RAPD band alone.

Forward and reverse SCAR primers were designed to the ends of the cloned sequence: BYAgi Forward (5′ CATGGATAATTCAGGGAGCATTCTG) and BYAgi Reverse (5′ CTGAACACGAATTTGCTGAGGTTG) and are underlined in Fig. 2. This primer combination gave a single band of the expected size (566 bp) only when the germplasm was carrying the *Bdv2* resistance segment from *T. intermedium*. A gradient PCR reaction was used with annealing temperature varying from 54 to 65°C. Only a single product developed at annealing temperatures from 54 to 61°C. A temperature between 56 and 58°C is recommended.

Because it is hoped that this marker will be of use to breeders working with larger numbers of samples, several DNA extraction techniques were tested that might lend themselves to high throughput methods. Initially, a sap extractor was used to crush young leaves. The sap was collected in microcentrifuge tubes and a CTAB [hexadecyltrimethylammonium bromide] DNA extraction method (Lodhi *et al.* 1994) was used to purify DNA. Later, DNA was extracted from young leaves using a FastDNA kit (Quantum Biotechnologies Inc.). Our results indicated that the sap extraction method gave DNA that yielded PCR bands with variable signal intensity. On the other hand, DNA extracted with the FastDNA kit gave strong PCR bands with uniform intensity.

In an attempt to streamline the screening process, DNA was extracted from seed using the FastDNA kit. This method involved halving the seed with a scalpel, crushing the endosperm half with a hammer in the fold of a weighing paper, and storing the embryo half for later germination. DNA was then extracted from the crushed half seed using the FastDNA method beginning with 18 s agitation with 2 ceramic beads on the FastDNA shaker (speed setting 4.5).

A<u>CATGGATAA TTCAGGGAGC ATTCTG</u>CTGC ATGACGATGC AGGAGCCCAT
GTAACGGCTT CGTTCAAAAA GTCAAACGAT GGCAGGCTCG TCGTACAAGA
AGGTTTTCGG AAGTACGTCG TACAACTCGA ACTGAAGGAA GATGATCTTG
TGGTCAGCAC GTTCCACAGG CGAGCCGGGA TCTGCATCCA TAACCGTCCG
GATGTGTATC CTGTCTTAAA ATTCCTAGAG TATGTCTACT ATATAGGCAG
GGCACTAGCT ATGTATACCT TCAATGCCAT TTTACATGTG GGTGCAGCTC
TGGAATGCTT GCTCTTTTGT GAACGTAGAC ATTTAAGGAC TTGTAGCGTT
CTCGGTGTGT TCCGTGACTT TGTCTAACTT TCGACCTATG TCGCTCCAGT
GACTATCAAA ATTTGGTTGG AATAATTTTA CTAATGGTCG TCTGCTCCCG
CCTTGCAACG TCTGCTATAA TTTGTGTGTA GTGGTTATTG TTGGGAATTT
TACCTGAACC AATATCCTGA GTTGAAAAAT GAACACCCAA CCT<u>CAACCTC</u>
<u>AGCAAATTCG TGTTCAG</u>GAT CACATAACAT GAGCTTGGTT TCGCCATTGA
TAGATCCCAC CACCGCCACC CAATCCCCTT A

Fig. 2. The sequence of pPS001. The regions used for the SCAR primers are underlined.

Fig. 3. Southern hybridisation using the insert sequence from pPS001 as a probe. Track 1, the insert band from pPS001; 2–3, plasmid pPS001 and an independently cloned version of the same; 4, reamplification from pPS001 using primer OPA07; 5, amplification from *Th. intermedium* using primer OPA07; 6, amplification from susceptible Hartog wheat using primer OPA07; 7, amplification from resistant TC14 wheat using primer OPA07.

PCR reactions using seed-extracted DNA consistently detected the presence of *Bvd2*-resistant chromatin (Fig. 4).

The marker has proved reliable not only with the original chromosome addition lines and recombinants but with advanced breeding material in our winter wheat breeding program. Hundreds of lines from both the conventional breeding and from a doubled haploid project have been screened using BYDV-PAV bioassays and the SCAR assay. In addition, these lines have been grown in the field during natural BYDV infections to confirm their BYDV resistant status. The utility of the marker as a predictor of resistance has been confirmed in these breeding materials derived from *Bdv2* recombinant lines TC5, TC6, and TC14 as illustrated in the companion paper Zhang *et al.* (2001, this issue).

Discussion

The TC series of recombinants for BYDV resistance has been distributed to various groups worldwide. Reports of its utility against local BYDV serotypes and complexes have been generally but not universally positive. CIMMYT reported its resistance to PAV-Mex, MAV-Mex, RPV-Mex, and RMV-Mex but not to PAV-Canada (Henry 1997; Ayala *et al.* 2001). Resistance was observed in France to PAV and MAV serotypes (J. Jahier, pers. comm.). Resistance has also been reported in Romania (N. Saulescu, pers. comm.), Italy (Loi *et al.* 1997), Syria (K. Makkouk, ICARDA, pers. comm.), China (Nie *et al.* 1996), New Zealand (W. Griffin, pers. comm.), and in the US (S. Haley, pers. comm.; H. Ohm,

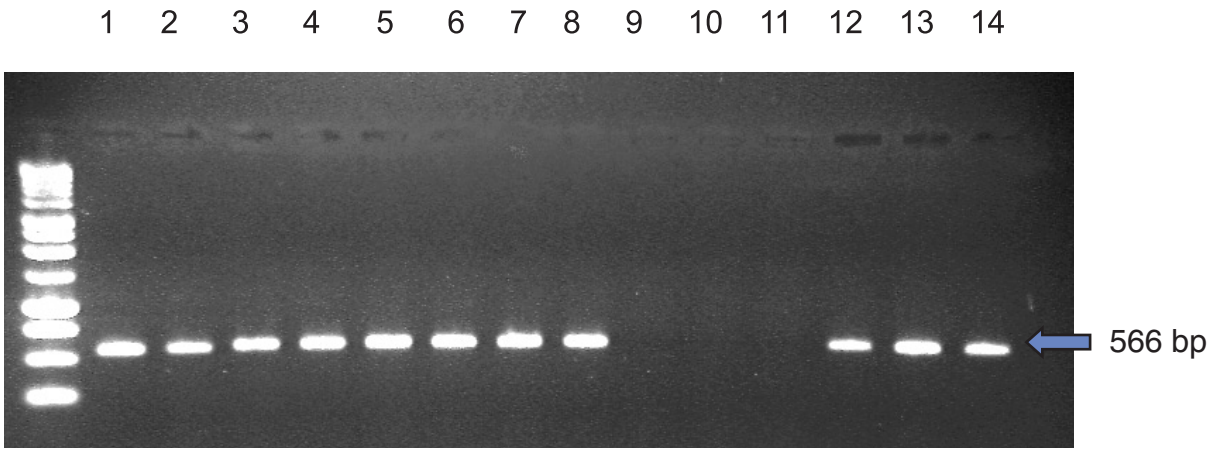

Fig. 4. The SCAR reaction products. Track 1, BYDV-resistant recombinant, TC5; 2, BYDV-resistant recombinant, TC6; 3, BYDV resistant recombinant, TC14; 4–8, BYDV-resistant doubled haploid breeding lines (derived from TC6); 9, BYDV-susceptible doubled haploid wheat; 10, susceptible background wheat, Hartog; 11, susceptible wheat, Lawson; 12, *Thinopyrum intermedium*; 13, L1 chromosome addition line; 14, Telosomic addition line 7Agi1 L.

pers. comm.). W. Huth (pers. comm.) did not observe resistance in Germany in the field. It continues to be effective against BYDV (predominantly PAV, RPV, and MAV) in the Australian Southern Tablelands and in Western Australia, and has been incorporated successfully into advanced breeding lines of dual purpose (grazing and grain) winter wheats bred for this higher rainfall zone (S. Kleven, pers. comm.). *Bdv*2 has also proved effective against a number of isolates of RPV in Australia including an isolate from Tasmania for which the Yd2 resistance of barley proved ineffective (Banks *et al.* 1992).

Ayala *et al.* (2001) demonstrate that TC14 has no negative effects in 3 wheat backgrounds to yield, 1000-grain weight, biomass, harvest index, protein percentage, grain hardness, and flour SDS sedimentation. Furthermore, they used an SSR marker (gwm37) to determine the zygosity of the TC14 translocation. They demonstrated in all wheat backgrounds that the yield losses associated with BYDV infection (both PAV and MAV serotypes) were least for the homozygous TC14 lines (8.7–12.2%), intermediate for the hemizygous TC14 lines (16.9–21.0%), and greatest for the nullizygous lines (21.8–34%).

The SSR marker used by Ayala *et al.* (2001) has the advantage of being able to distinguish hemizygous from homozygous plants. However, the SCAR marker reported here, being only one PCR product, has the advantage of being converted to high throughput colour reaction formats. A companion paper in this issue (Zhang *et al.* 2001, this issue) reports the conversion of the SCAR marker into a solid phase PCR reaction in which the presence of the *Bdv2* chromatin results in colour formation in the well of a microtitre plate, obviating the need to run agarose electrophoresis and permitting higher throughput screening.

Khan (2000) has described an excellent set of recombinant chromotypes between 7A and 7Ai1. It will be informative to test these lines for the presence of the present SCAR marker and to characterise them for the BYDV resistance. This may give some further indication of how close to the resistance the SCAR marker is. It should be noted, however, that the TC translocations represent alien chromatin that does not appear to recombine meiotically with wheat chromosomes when in hemizygous condition. Generally, therefore, it is not anticipated that there will be further recombination within the alien translocation in breeding lines. However, those using the marker should be alert to the possibility that such a recombination to separate the resistance from the marker may be a rare event and lines destined for commercial release should have virus resistance confirmed against the predominant serotypes of the target regions.

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