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The development of a STS marker linked to a yellow rust resistance derived from the wheat cultivar Moro

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Abstract A sequence-tagged-site (STS) marker has been developed for a gene conferring yellow rust resistance originating from the wheat cultivar Moro. The single, dominant, seedling yellow rust resistance gene, designated *YrMoro*, was mapped to the group 1 chromosomes. The STS marker was developed from an AFLP band which cosegregated with the *YrMoro* gene. Sequence-specific primers were made which incorporated the selective bases of the AFLP primers, plus 16 and 17 additional bases extending into the AFLP band. This simple, PCR-based marker will allow wheat breeders to pyramid this resistance gene, along with other resistance genes, into a single wheat genotype.

Keywords AFLP · Disease resistance · Sequence-tagged-site · Wheat · Yellow rust

Introduction

Yellow rust is a major foliar disease of wheat in many parts of the world. Caused by the biotrophic fungus *Puccinia stritiformis* f.sp. *tritici*, the disease is controlled through resistance breeding and fungicides. The most common form of resistance employed is race-specific, usually involving single, dominant genes. Alone, such forms of resistance often have a limited effective life, with virulence towards the resistance arising within the pathogen population.

One approach to prolong the life of race-specific resistance genes would be to combine a number of resistance genes within a single genotype. By pyramiding resistance genes which alone may be ineffective, but together provide resistance to the current pathogen population, the useful life of these genes can be extended. Pyramiding of resistance genes is only possible by conventional breeding methods if suitable races of the pathogen are available to test for the presence of each resistance gene. In the absence of isolates carrying the correct combination of virulence and avirulence factors, molecular markers, closely linked to each resistance gene, can be used to select for given combinations of genes. For use as an effective tool in marker-assisted selection, a marker screen must be both time- and cost-effective. Therefore, markers based on the polymerase chain reaction (PCR), where the product can be easily and rapidly detected, is the system of choice.

AFLPs are a reproducible marker system with the ability to analyse a large number of polymorphic loci simultaneously (Ridout and Donini 1999). However, AFLPs are too costly and time-consuming to be used directly for marker-assisted selection. By converting a polymorphic AFLP band linked to the character of interest into a simple PCR-based marker this can then be used to select for the gene/s of interest.

Two dominant, seedling resistances for yellow rust have been identified in the wheat cultivar Moro (Chen and Line 1992). One gene, *Yr10*, has been assigned to chromosome 1B (Metzger and Silbaugh 1970) and a second gene, *YrMor*, has tentatively been assigned to chromosome 4B (Chen et al. 1995). In this study we used a NIL (BC_7) of Lemhi, where one of the Moro resistances had been introgressed (R. Johnson, personal communication), to develop a BC_8F_2 mapping population. The Moro resistance in this population was mapped using RFLP and AFLP markers and a specific sequence-tagged-site (STS) marker developed from a cosegregating AFLP band.

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Materials and methods

Plant material and mapping population

Wheat cultivars Lemhi and Moro and a NIL, Lemhi*7/*YrMoro*, derived from the cross Lemhi × Moro and containing a yellow rust resistance gene derived from Moro, were used as controls. A

BC_8F_2 mapping population was obtained by crossing the NIL to Lemhi and selfing the resulting progeny. The BC_8F_2 mapping population consisted of 88 individuals.

Mutants deficient in an active *YrMoro* resistance were obtained using the method of Koebner and Hadfield (2001). The NIL Lemhi*7/*YrMoro* was crossed to the Chinese Spring line monosomic for chromosome 1B. The F_2 seed from this cross was irradiated with 3Gy of fast neutrons. The F_2 seedlings were then tested for *YrMoro* resistance. Putative *YrMoro* susceptible mutants were checked for the origin of the 1B chromosome (i.e. from Lemhi*7/*YrMoro* or Chinese Spring) using SSR markers for chromosome 1B (Koebner and Hadfield 2001).

Pathogen isolate and yellow rust disease tests

The *P.s*. f.sp. *tritici* isolate WYR69-10 (race104E137) was used in all yellow rust disease tests (John Innes Rust Collection). Isolate WYR69-10 is virulent on Lemhi (Infection Type – IT4) and avirulent on Moro and Lemhi*7/*YrMoro* (IT0/;). Isolate WYR69-10 was bulked on cultivar Lemhi in a spore-free greenhouse (Boyd and Minchin 2001).

All yellow rust disease tests were carried out on 12–14 day old seedlings grown under spore-free conditions. The inoculation procedure is as described in Boyd and Minchin (2001).

RFLP and AFLP mapping

Plant DNA was extracted from uninfected leaf material from 21 day old seedlings using the CTAB protocol (Saghai-Maroof et al. 1984). For RFLP analysis 20 µg of genomic DNA was cut with *Bam*HI, *Dra*I or *Pst*I and transferred to a Hybond N+ membrane (Amersham) as described in Sambrook et al. (1989). A 1.3-kb fragment of the Lrk10 clone (Feuillet et al. 1997), from the 5′ extracellular domain between restriction enzyme sites *Hind*III and *Pst*I, was used to map the *Lrk10* locus. All probes were labeled with ^{32}P by random priming. Washes were performed at 65 °C in 2 × SSC and 0.1% SDS.

AFLP markers were identified by bulk segregant analysis (Michelmore et al. 1991) using the procedure of Vos et al. (1995). Two resistant and two susceptible bulks were made from equal amounts of DNA from four resistant and four susceptible BC_8F_2 plants. *Sda*I was used as the rare-cutting restriction enzyme and *Mse*I as the frequent cutter. *Sda*I and *Mse*I double-stranded adapters were ligated:

The *Sda*I adapter; 5′-CTCGTAGACTGCGTACATGCA-3′, 3′—CATCTGACGCATGT—5′.

The *Mse*I adapter is as described by Vos et al. (1995).

The pools were screened using *Mse*I primers with two or three selective bases and *Sda*I primers with two selective bases (Ridout and Donini 1999).

The selective PCR program included 13 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min, followed by 23 cycles in which the annealing temperature was reduced to 56 °C. AFLP markers were recorded by primer combination and band molecular weight.

The RFLP and AFLP markers were mapped using JoinMap (version 2.0: Stam 1993; Stam and Ooijen 1995). The map was constructed using a LOD of 3.0 and the Kosambi mapping function. AFLP primer combination S26M47 gave two bands of similar size in Moro and the resistant bulks, fragment S26M47-150. The S26M47-150 bands in Moro were excised together from the polyacrylamide gel, re-amplified using primers S26 and M47, and cloned into the vector, pGEM-T ^{Easy} (Qu et al. 1998). Bands S26M47-150 were cut from the gel, placed in 100 µl of 1 × $T_{0.1}$ buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and left overnight at 4 °C. The gel slice was then heated at 50 °C for 10 min. The supernatant was used directly as the source of DNA template for re-amplification with primers S26 and M47.

Characterisation of cloned AFLP fragments

Fifty white and ten blue control colonies were selected for analysis. The size of the plasmid insert was checked by PCR using the T7 and SP6 primers. Plasmids were then characterised by restriction enzyme digestion using the enzymes *Bfa*I and *Hin*fI and by SSCP analysis (Hayashi 1991).

For SSCP analysis the insert was amplified by PCR using the primers T7 and SP6. The PCR product was run on a 0.5 × MDE (FMC Bioproducts) denaturing polyacrylamide gel at 8 W for 16 h, in 0.6 TBE running buffer. The DNA bands were visualised by silver nitrate staining (Promega Inc.).

Plasmid inserts and PCR products were sequenced using ABI PRISM Big Dye reactions following the manufacturer's protocol. Sequence database searches were done using TBLASTX and BLASTX algorithms (Altschul et al. 1997).

STS marker development and amplification conditions

Primers were synthesised which complemented the sequence at each end of the cloned AFLP band. The primers were designated S26M47Forward (5′ TTTACAGGTTGGAATCTA) and S26M47-Reverse (5′ AACTTCTTTTCCATATAAG). PCR amplification using this STS primer pair was carried out in a 50-µl reaction, containing 5–10 ng of genomic DNA, 50 pmol of each primer, 0.25 mM of dNTPs, and 0.5 µ of *Taq* polymerase. Reaction conditions were 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 42 °C for 30 s and 72 °C for 30 s.

Results

Yellow rust disease test

Individual seedlings of the BC_8F_2 population were tested for resistance to yellow rust following inoculation with *P.stritiformis* f.sp. *tritici* isolate WYR69-10. The population segregated 65 resistant (IT0/;) to 23 susceptible (IT4) giving a good χ^2 fit to a 3:1 ratio ($\chi^2 = 0.06$, 1 *df*, probability > 0.70) and indicating the presence of a single, dominant gene for yellow rust resistance, designated *YrMoro*.

RFLP and AFLP mapping

The Lrk10 clone mapped 3.8 cM from *YrMoro* (Fig. 1). Lrk10 is linked to the leaf rust resistance gene *Lr10* (Feuillet et al. 1997) which has been assigned to chromosome 1A (McIntosh et al. 1995). Nullisomic analysis placed the Lrk10 clone on the group 1 chromosomes (data not shown), identifying homologs of Lrk10 on chromosomes 1B and 1D.

AFLPs were identified by bulked segregant analysis (Michelmore et al. 1991). In total, 746 primer combinations were screened, generating an estimated 75,000 bands which ranged in size from 130 to 600 bps. Fifty polymorphic bands were identified in the resistant bulks, but absent in the susceptible bulks. Many of these produced faintly amplified bands and therefore only 20 were selected for mapping. Of the 20 bands only five gave reproducible bands in the individuals of the mapping population, two of which gave distorted segregation ratios. The three remaining polymorphic AFLP bands mapped

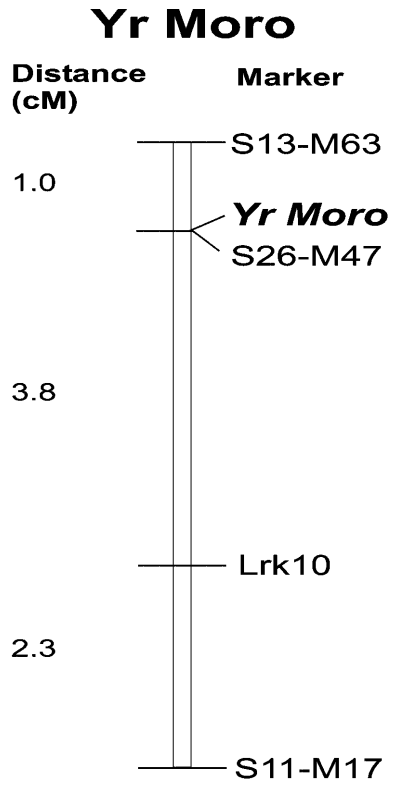

Fig. 1 DNA markers linked to a yellow rust resistance gene, *YrMoro*, derived from the wheat cultivar Moro. One RFLP (Lrk10) and three AFLP markers are shown

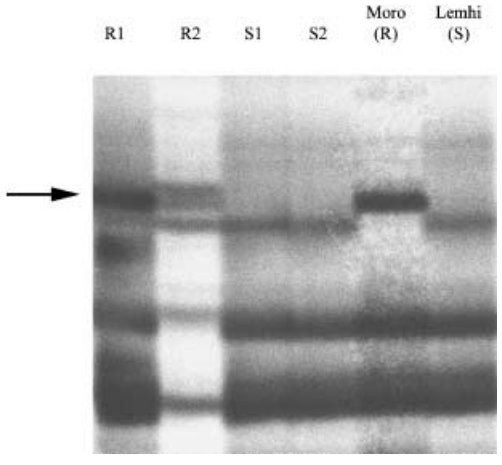

Fig. 2 The AFLP primer combination S26M47 generates two bands that cosegregate with the yellow rust resistance gene, *YrMoro*. The bands (indicated by the *arrow*) are present in resistant bulks (*R1* and *R2*) and the resistant cultivar Moro (*M*), but absent in the susceptible bulks (*S1* and *S2*) and the susceptible cultivar Lemhi (*L*)

near to *YrMoro* in the BC_8F_2 population (Fig. 1). The AFLP primer combination S26M47 produced two bands of almost identical size, approximately 150 bp (band S26M47-150), that completely cosegregated with *YrMoro* (Fig. 2). These cosegregating AFLP bands were isolated together and cloned for further analysis.

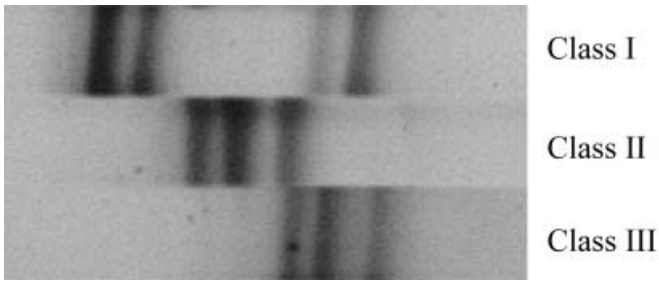

Fig. 3 SSCP analysis of the cloned AFLP band S26M47-150. The SSCP analysis divided the clones into three classes, I, II and III

Conversion of AFLP bands into a STS marker

Sixty clones were checked for an insert by PCR. Fifty one contained an insert of a size similar to the S26M47 - 150 AFLP bands. Two clones were selected at random for sequencing. The sequence of the two clones was totally different (data not shown). The sequences were analysed for suitable restriction enzyme sites with which to characterise the 51 clones. Restriction digestion with *Hinf*I and *Bfa*I divided the 51 clones into two classes. Seven clones in class I had one *Bfa*I and two *Hinf*I sites, while the 39 clones in class II had no *Bfa*I and one *Hinf*I site. Five clones did not give clear results and were excluded from further analysis.

The clones were also characterised by SSCP analysis (Fig. 3). The seven clones in class I gave identical SSCP profiles, but two of the 39 clones in class II gave a different SSCP profile, generating a third class.

Ten clones were selected for sequence analysis, three from class I, six from class II, and one from class III. Three independent batches of each clone were sequenced. The three repeat sequences of each clone were found to be identical. The three classes gave different DNA sequences, but were almost identical between clones from the same class. The size of the sequenced inserts differed, class I being 142-bp, class II 147-bp and class III 150-bp long. AFLP primers S26 and M47 were used to PCR the insert of each of the ten clones. When run on a denaturing polyacrylamide gel, the six class-II clones all produced two bands identical to those observed with genomic DNA.

Of the six clones sequenced from the predominant class-II group of clones, four had identical nucleotide sequences. In one clone, two adenosine nucleotides were substituted with guanosine, and in the sixth clone a further adenosine to guanosine substitution was observed (Fig. 4). The predominant class-II group of clones was therefore considered to be the AFLP band S26M47-150 seen to cosegregate with the resistance gene *YrMoro*. A search for open reading frames did not identify any extensive stretch of coding sequence within the S26M47-150 sequence. Therefore, a search of the databases for amino-acid homology was considered to be uninformative.

Sequence-specific primers were made to the class-II DNA sequence that incorporated the selective bases of

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                                                    G
  1   ttTACAGGTT GGAATCTAAG TTCTAACATA CTCCCCAAGT
      TTTACAGGTT GGAATCTA
      S26M47Forward
           G
 41   TTTGGAAATT TCAATATAGA CTATGGACTA CATATATGGA

                                              G
 81   TTGAAATGAT CCAGTGAGCA AACACACTAA AACGGCTTAT


121   ATTTGTGAAC TACTTATATG GAAAAGAAGttg
                GAATATAC CTTTTCTTCAA
                      S26M47Reverse
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Fig. 4 DNA sequence of the cloned AFLP band S26M47-150. The sequence of six clones of the AFLP band differed by the substitution of three adenosine (A) nucleotides with guanosine (G). The selective bases of the S26 and M47 primers are shown in *lower case*. The sequence of the STS primers, S26M47Forward and Reverse, are shown in *bold type*

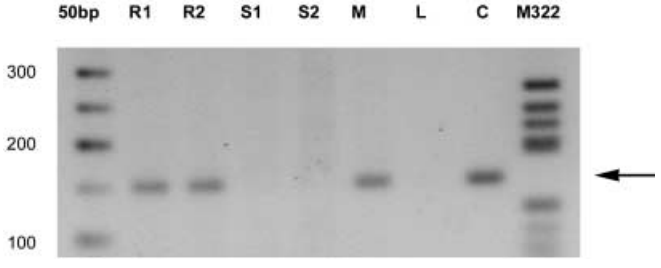

Fig. 5 The STS primers S26M47 Forward and Reverse amplify a single band of 151 bp (*arrow*) in cultivar Moro (*M*) and the resistant bulks (*R1 and R2*) carrying the *YrMoro* resistance gene, while no band is amplified in cultivar Lemhi or the susceptible bulks (*S1 and S2*). The cloned S26M47-150 AFLP band (*C*) was used as a positive control. *M322* and *50 bp* represent DNA size markers. The PCR products are run on a 2% agarose gel

the AFLP primers S26 and M47, plus 16 and 17 additional bases from the cloned AFLP band (Fig. 4). These sequence-specific primers, S26M47 Forward and Reverse, were tested against genomic DNA from cultivars Lemhi and Moro, NIL Lemhi*7/*YrMoro* and all 88 individuals of the BC_8F_2 population. Using an annealing temperature of 42 °C, and running the PCR products on a 2% agarose gel, a single band of approximately 150 bp was amplified in all lines carrying the *YrMoro* gene, but not in susceptible plants (Fig. 5). When the PCR products were run on a denaturing polyacrylamide gel two bands were observed.

The PCR product generated from genomic DNA of cultivar Moro using the STS primers was sequenced. The DNA sequence was identical to that shown in Fig. 4. This PCR product was used as a probe against genomic DNA from the resistant cultivar Moro and the susceptible cultivar Lemhi. All the restriction enzymes used produced the same banding pattern in both cultivars (data not shown). Therefore, the DNA polymorphism cosegregating with *YrMoro* does not involve a detectable deletion or insertion of DNA.

Twenty four mutants of *YrMoro*, generated using the NIL Lemhi*7/*YrMoro* by fast neutron bombardment (Koebner and Hadfield 2001), were screened with the STS marker primers S26M47 Forward and Reverse. The STS marker was absent from all 24 mutants, supporting the close association between the STS marker and the *YrMoro* locus.

Discussion

Yellow rust is a serious foliar pathogen of wheat in the more temperate growing regions of the world. In the UK epidemics followed the breakdown of *Yr9* in 1987/88 in the cultivar Sleijpner (Bayles et al. 1989) and again in 1995/96 when *Yr17* resistance in the cultivar Brigadier was overcome (Bayles and Stigwood 1996). Race-specific resistance genes may be used more effectively if two or more genes are combined into one genotype. This should be especially effective for yellow rust resistance, as the pathogen has no known sexual cycle, preventing the occurrence of new virulence combinations by genetic recombination.

Pyramiding effective resistance genes, to which no virulence exists in the pathogen population, is only possible with the aid of markers linked to the resistance gene. Resistance genes to both fungal blast and bacterial leaf blight infection have been successfully pyramided into new rice cultivars using molecular markers (Hittalmani et al. 2000). To optimise the efficiency of marker-assisted selection it is important that the recombination frequency between the target gene and the marker be as low as possible. By using a mapping population derived from a cross between a BC_7 NIL, Lemhi*7/*YrMoro*, and the recurrent parent we aimed to target markers closely linked to *YrMoro*. As the levels of polymorphism between the resistant and susceptible bulks were therefore expected to be low, AFLPs were used to search for polymorphic loci. From 75,000 bands generated by 746 primer combinations only 50 proved polymorphic, giving one polymorphic band for every 15 primer pairs tested. This is much lower than found in wheat in general, a comparison of 11 wheat cultivars giving an average of 24.7 polymorphic bands per primer pair (Ma and Lapitan 1998).

The AFLP primer combination S26M47 produced two bands, S26M47-150, in resistant plants of approximately the same size when run on a denaturing polyacrylamide gel. Cloning and subsequent testing showed the two bands to represent a single, double-stranded fragment of 147 bps when run on an agarose gel. The STS marker primers developed from S26M47-150 amplified a band only in plants carrying the *YrMoro* resistance gene.

To-date no virulence to the yellow rust resistance in the cultivar Moro has been detected in UK *P. striiformis* f.sp. *tritici* isolates, making this an effective source of resistance. The linkage to marker Lrk10 assigns *YrMoro* to the group 1 chromosomes. Using races of *P. striiformis* from North America two yellow rust resistance genes

have previously been identified in Moro, *Yr10* on chromosome 1B and *YrMor* on chromosome 4B (Chen et al. 1995). The yellow rust resistance gene identified in this study, *YrMoro*, may therefore be the same gene as *Yr10*.

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