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Identification of a STS marker linked to the *Aegilops speltoides*-derived leaf rust resistance gene *Lr28* in wheat

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Abstract A sequence-tagged-site (STS) marker is reported linked to *Lr28*, a leaf rust resistance gene in wheat. RAPD (random amplified polymorphic DNA) analysis of near-isogenic lines (NILs) of *Lr28* in eight varietal backgrounds was carried out using random primers. Genomic DNA enriched for low-copy sequences was used for RAPD analysis to overcome the lack of reproducibility due to the highly repetitive DNA sequences present in wheat. Of 80 random primers tested on the enriched DNA, one RAPD marker distinguished the NILs and the donor parent from the susceptible recurrent parents. The additional band present in resistant lines was cloned, sequenced, and STS primers specific for *Lr28* were designed. The STS marker (Indian patent pending: 380 Del98) was further confirmed by bulk segregation analysis of F_3 families. It was consistently present in the NILs, the resistant F_3 bulk and the resistant F_3 lines, but was absent in recurrent parents, the susceptible F_3 bulk and the susceptible F_3 lines.

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Introduction

The most common wheat rust, called leaf rust or brown rust, is caused by *Puccinia recondita* Rob. ex Desm. f. sp. *tritici* Ericks. & Henn, and occurs on the leaf blade and leaf sheath. Successful wheat production in areas of the world predisposed to rust continues to depend on the use of rust-resistant cultivars (Roelfs et al. 1992). During the last 2–3 decades much progress has been made in breeding for resistance to the disease. However, due to a narrow genetic base and continuously evolving pathogen races, resistant varieties become susceptible to the disease when grown in vast areas. One promising approach to overcome this problem is to develop a germplasm carrying combinations of several effective genes, especially those from wild relatives and related species, which can be successfully crossed with bread wheat (Jiang et al. 1994). So far, over 40 leaf rust (*Lr*) resistance genes have been identified in wheat and related species and some of them have been introgressed into wheat (McIntosh et al. 1995). Bringing more than one gene together into a single elite variety by conventional means is very laborious and time consuming. In some cases it is not achievable because screening for one resistance gene interferes with the ability to screen for another, a frequent problem in disease resistance breeding, while in certain cases the virulent isolates for the resistant genes are not available. In recent years, DNA-based markers have shown great promise in expediting plant breeding procedures. The identification of molecular markers for resistance genes can efficiently facilitate the pyramiding of major genes into a valuable background in less time, making it more cost effective (Tanksley et al. 1989).

Restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) have been used to tag a variety of resistance genes in wheat (Hartl et al. 1993; Ma et al. 1993; Dweikat et al. 1994; Eastwood et al. 1994; Schachermayr et al. 1994, 1995, 1997; Autrique et al. 1995; Procunier et al. 1995; Feuillet et al. 1995; Talbert et al. 1996; Dedryver et al. 1996; Procunier et al. 1997; Hu et al. 1997; William et al. 1997). It has been proposed that RAPDs may also be useful in monitoring the introgression of single chromosome segments. Near-isogenic lines (NILs) differing in a specific trait have been successfully used to isolate genetic markers for the gene determining that trait (Martin et al. 1991; Hartl et al. 1993). NILs are thus a valuable tool for identifying polymorphisms close to the gene of interest.

The leaf rust resistance gene *Lr28* was transferred into bread wheat from *Aegilops speltoides* (McIntosh et al. 1982) while attempting to transfer *Yr8*, a gene for stripe rust resistance from *Aegilops comosa* (Riley et al. 1968a, b). In that experiment, *Ae. speltoides* was used to induce homoeologous recombination. The transferred segment, along with *Lr28*, was derived from the short arm of the *Ae. speltoides* chromosome 7S#2, resulting in the translocation chromosome T4AS.4AL-7S#2S, and was assigned to chromosome 4AL in wheat (Friebe et al. 1996). This gene is not associated with deleterious characters and is present in the cultivar 'Sunland' released in Australia. It is also widely effective in South Asia, and Europe (McIntosh et al. 1995) and has been found to be effective against a wide spectrum of leaf rust races prevalent in India. Its presence, in combination with other resistance genes, is therefore desirable in the release of new cultivars and this strategy will have potential in decreasing yield losses caused by the rust pathogen.

Molecular markers have been reported that are closely linked to *Lr* genes either of alien origin – *Lr9*, *Lr19*, *Lr24*, *Lr25* and *Lr29* (Schachermayr et al. 1994, 1995; Procunier et al. 1995; Dedryver et al. 1996), or of wheat origin—*Lr1*, *Lr*10 and *Lr34* (Feuillet et al. 1995; Schachermayr et al. 1997; William et al. 1997). For more-efficient identification, the conversion of RAPD markers into sequence tagged sites (STS) has been proposed as a more reliable, reproducible and convenient approach (Olson et al. 1989). This strategy is especially advantageous in reducing the unwanted background signals produced in RAPD gels by giving rise to a locus-specific amplification product. For wheat, which has a large genome and a low level of polymorphism, enriching DNA for low-copy sequences can be beneficial. This has not only increased the level of detectable polymorphism by RAPD, but has also ensured the reproducibility of the results (Eastwood et al. 1994; William et al. 1997). The present study describes the use of similar approaches for the identification of a molecular marker linked to the *Lr28* gene in wheat.

Materials and methods

Plant material

Seven resistant NILs in different Indian varietal backgrounds (Table 1, kindly supplied by Dr. M.K. Menon, Head, IARI Regional Station, Wellington, The Nilgiris, TN, India), along with the donor source stock (CS 2A/2M 4/2) from which *Lr28* was transferred into the Indian varieties, were used to identify RAPD markers linked to the resistance gene *Lr28*. To confirm the linkage of the marker with the rust resistance gene, F_3 families developed as described below were tested. NIL HW-2035 was crossed to the recurrent parent NI-5439, F_2 plants were generated from individual F_1 seeds and about 50 F_2s were advanced to the F_3 generation. Of these, 15 progeny rows were classified for resistance/susceptibility using a mixture of leaf rust pathotypes applied as a suspension at the seedling stage.

DNA isolation and enrichment for low-copy DNA

DNA was isolated from 7–10-day-old glass-house-grown seedlings by the CTAB method as described by Rogers and Bendich (1988). DNAs were enriched for low-copy sequences essentially by following the procedure described by Eastwood et al. (1994). The genomic DNA was first sonicated for about 10 s to obtain a size range of 0.6–6.0 kb. The sonicated DNA was then re-suspended in phosphate buffer, denatured at 100°C and re-annealed at 60°C for about 20 h to a C_0t value of >100. The DNA samples were loaded onto hydroxyapatite (Bio-Rad, DNA grade) columns maintained at 60°C and the single-stranded DNA was eluted with 0.15 M phosphate buffer at 60°C. The low-copy DNA in phosphate buffer was dialyzed against TE buffer (pH 8.0) overnight at 4°C and then used for DNA amplification.

RAPD analysis

Random primers (Operon Technologies, Alameda, USA) were used to screen wheat NILs for *Lr28*. PCR was performed in a 25-µl reaction mixture containing 30–50 ng of enriched template DNA, 1.0 unit of *Taq* DNA polymerase (Bangalore Genei, India), 15 ng of primer, 100 µM each of dNTPs, and 1 × reaction buffer containing

Table 1 List of near-isogenic lines and corresponding recurrent parents for the leaf rust resistant gene *Lr28*

NILs	Recurrent parents	Backcross numbers
HW-2031	Sonalika	8
HW-2032	Lok-1	7
HW-2033	WH-147	7
HW-2034	C-306	9
HW-2035	NI-5439	7
HW-2036	J-24	7
HW-2037	HD-2329	7
HW-2038	HD-2285	7

1.5 mM $MgCl_2$. The reaction mixture was loaded into 500-µl Eppendorf tubes, overlaid with 30 µl of mineral oil (Sigma), and run on a thermocycler (Perkin Elmer, DNA Thermal Cycler-480) essentially by following the conditions described by Eastwood et al. (1994). The PCR cycle included: initial denaturation of 94°C for 5 min followed by five cycles of denaturation at 92°C for 30 s, annealing at 35°C for 2 min, and extension at 72°C for 1.5 min; 35 cycles of denaturation at 92°C for 5 s, annealing at 40°C for 20 s, and extension at 72°C for 1.5 min; one cycle of denaturation at 92°C for 10 s, annealing at 40°C for 20 s, and extension at 72°C for 5 min. All NILs (Table 1) and their respective recurrent parents were tested for the presence of polymorphism along with the *Lr28* donor stock. The amplified products were separated on a 2% agarose gel and viewed on a UV transilluminator after staining with ethidium bromide.

Cloning and sequencing of the RAPD product

The polymorphic band corresponding to the resistant phenotype (of the *Lr28* donor) was eluted from an agarose gel slice by the freeze-thaw method and part of the eluted DNA was re-amplified in a 100 µl reaction. The amplified reaction mixture was then extracted once each with phenol, phenol:chloroform:isoamyl alcohol (25:24:1), and chloroform:isoamyl alcohol (24:1). The DNA was finally precipitated with 2 vol of ethanol.

A portion of the re-amplified DNA was ligated to the pMOSBlue T-vector (Amersham, Inc.) and transformed into the *Escherichia coli* strain XL-1 Blue (Stratagene, Switzerland). The resulting white colonies on X gal-IPTG-LB agar plates were screened for the presence of an insert by restricting the recombinant plasmid using appropriate enzymes as described by Sambrook et al. (1989). A colony positive for the insert was chosen, grown in a large culture, and the plasmid DNA isolated.

For sequencing, the plasmid DNA was first purified using polyethylene glycol as described by Sambrook et al. (1989). It was then sequenced by Sanger's dideoxy chain-termination method (Sanger et al. 1977) using a Sequenase® version 2.0 sequencing kit (USB).

STS design and analysis

The sequence information of the cloned product $OPJ\text{-}01_{378}$ was used to design two STS primers, a forward primer of 20 bp *Lr28*-01, 5′CCCGGCATAAGTCTATGGTT3′ having the original 10 bp of the RAPD primer along with the next ten internal bases, and a reverse primer of 20 bp *Lr28*-02, 5′CAATGAATGAGATACGTGAA3′ having an entirely internal sequence next to the RAPD primer. The two STS primers were synthesized by the phosphoramidite method on a Pharmacia (Uppsala, Sweden) oligonucleotide synthesizer. PCR reactions were performed in a 25-µl reaction mixture containing 30–50 ng of non-enriched template DNA, 1.0 unit of *Taq* DNA polymerase, 40 nM of each primer, 100 µM of each dNTP, and 1 × reaction buffer containing 1.5 mM $MgCl_2$. The reaction mixture was loaded into 500-µl Eppendorf tubes, overlaid with 30 µl of mineral oil and run on a thermocycler where it was incubated at 94°C for 6 min for initial denaturation, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. The amplification reaction was concluded by a final extension of 72°C for 5 min. The amplification products were separated on a 2% agarose gel and viewed on a UV transilluminator after ethidium bromide staining.

Southern analysis

The gel of amplification products obtained with the random primer was Southern blotted and hybridization was carried out using a cloned polymorphic band as the probe. The hybridization conditions employed were essentially similar to those previously described by Gill and Gill (1991) and Gill et al. (1991).

Results and discussion

A total of 80 primers were screened to identify polymorphisms between seven resistant NILs and their respective recurrent parents along with the donor source stock. Primers that gave clear and distinguishable patterns were considered for further analysis. In addition, the reproducibility of polymorphism was confirmed by at least six repetitions to ensure the repeatability of the RAPD results. Of the tested primers, 17 gave no amplified product, while others amplified about 5–7 fragments each with a size ranging from 200 bp to 2500 bp. Most of the primers produced monomorphic RAPD profiles. Some primers produced polymorphic bands with few NILs (OPK-03, 07, 11 and 13, data not shown). Only one primer, OPJ-01, showed polymorphism corresponding to all the resistant genotypes, including the donor, as against the recurrent parents (Fig. 1 A). As shown in Fig. 1 A, very few prominent bands were obtained in the amplification products of OPJ-01 with enriched DNA. Band sizes ranged

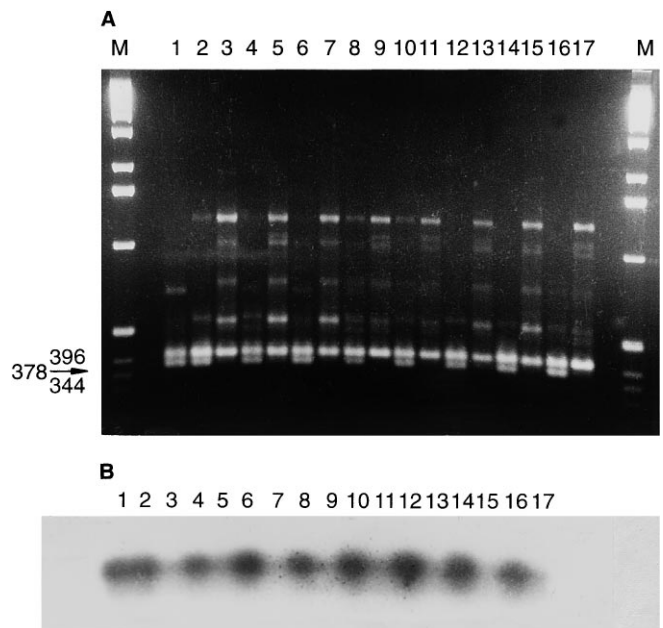

Fig. 1A RAPD profiles obtained by OPJ-01. A polymorphic band (RAPD marker) tightly linked to *Lr28* is indicated by the *arrow*. Products were separated on a 2% agarose gel. *1 Lr28* source stock (CS 2A/2M 4/2), *2* HW-2031, *3* Sonalika, *4* HW-2032, *5* Lok-1, *6* HW-2033, *7* WH-147, *8* HW-2034, *9* C-306, *10* HW-2035, *11* Nl 5439, *12* HW-2036, *13* J-24, *14* HW-2037, *15* HD-2329, *16* HW-2038, *17* HD-2285. *Lane M* DNA molecular-weight marker X (Boehringer Mannheim) **B** Autoradigram of the Southern blot prepared from the above gel and hybridized using a plasmid with the $OPJ\text{-}01_{378}$ insert as a probe. Only the 378-bp specific band in all the resistant genotypes shows intense hybridization

between 378 bp and 1300 bp and showed differences in intensity between the NILs and the recurrent parents. Compared to the recurrent parents, high-molecular-weight bands were less intense in the NILs. One band of 378 bp (OPJ-01_{378}) was present in the source stock and in all the NILs but was absent in the recurrent parents.

Subsequently, for STS development, this polymorphic band (OPJ-01_{378}) was eluted from the donor parent lane and cloned. The presence of the desired insert was confirmed by digesting the clone with *Hin*dIII/*Bam*HI restriction enzymes and then comparing the fragment size with the eluted polymorphic band and the RAPD profile of the donor parent (data not shown). The plasmid with the OPJ-01_{378} insert was used as a probe and hybridized to the Southern blot prepared from the gel of the RAPD profiles obtained with OPJ-01 (Fig. 1A). As seen in Fig. 1 B, only resistant genotypes specifically showed intense hybridization at a position corresponding to the band of 378 bp, thus verifying the identity of the cloned product and indicating the absence of sequence homology with other bands in the RAPD profiles obtained with primer OPJ-01.

When the STS primers were tested against non-enriched DNAs for all the NILs and the donor source, only one band of 378 bp was amplified in the resistant line and this band was absent in the recurrent parents (Fig. 2). The intensity differences of the bands between lanes were probably due to slight difference in the amounts of non-enriched template DNA. To investigate the segregation of the identified marker and *Lr28*, F_3 progeny of the cross HW 2035/NI 5439 were classified for resistance/susceptibility using a mixture of leaf rust isolates. Five resistant and five susceptible lines, along with the parents and resistant and susceptible DNA bulks, were analyzed for the presence of the STS marker. The marker was present in the resistant lines, the resistant bulk and HW 2035, but absent in the recurrent parent (NI 5439), the susceptible lines and the susceptible bulk (Fig. 3).

In wheat most of the genomic DNA is composed of highly repetitive DNA sequences (Smith and Flavell

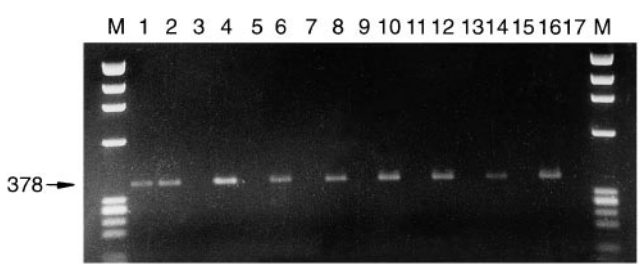

Fig. 2 Amplification products obtained by STS primers *Lr28*-01 and *Lr28*-02 showing a polymorphic band only in resistant lines possessing *Lr28*. Products were separated on a 2% agarose gel. The sequence of wheat genotypes is similar to that mentioned in Fig. 1: *lane M* is molecular-weight marker-ϕX-174/*Hae*lll

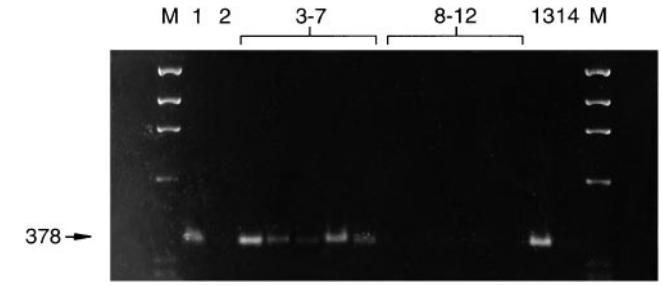

Fig. 3 Segregation of amplification products in F_3 lines. Amplifications were performed using primers *Lr28*-01 and *Lr28*-02. Products were separated on a 2% agarose gel. Resistant and susceptible lines were clearly distinguishable. *1* Resistant bulk, *2* Susceptible bulk, *3–7* Individual resistant lines, *8–12* Individual susceptible lines, *13* HW-2035, *14* NI-5439. *Lane M* is molecular-weight marker-ϕX-174/*Hae*III

1975). When the RAPD assay is used in wheat it often amplifies repetitive sequences. The repetitive sequences also lead to the formation of secondary structures in the template DNA, rendering many sites inaccessible to the primer. This could be the prime cause of the incongruous results obtained in wheat RAPDs. The removal of highly repetitive and abundant DNA from wheat genomic DNA, or the enrichment of low-copy DNA fractions, reduces the competition of highly repetitive sequences for RAPD primer sites. Thus the polymorphic RAPD-PCR products from low-copy DNA sequences can be more readily detected, including potential differences near the target region of the genome (Eastwood et al. 1994; William et al. 1997). The high reproducibility of our RAPD data is probably due to the enrichment of DNA for the low-copy sequences leading to the distinct polymorphic bands.

Compared to random primers, STS primers are longer, making them more specific to the site of interest. The identification of a desired phenotype also becomes much easier due to the production of a specific band. In addition, it provides a higher degree of repeatability compared to RAPDs. Therefore, we converted the RAPD marker to an STS marker which identified a known locus on the chromosome, representing a reliable and efficient assay. Moreover, we did not use enriched DNA when analyzing the STS marker, making it easier for application. STS markers have been used successfully to detect polymorphisms in wheat for traits such as resistance to rusts (Schachermayr et al. 1994, 1995, 1997; Feuillet et al. 1995; Dedryver et al. 1996), wheat streak mosaic virus (Talbert et al. 1996), loose smut (Procunier et al. 1997) and powdery mildew (Hu et al. 1997). The marker fragments amplified by these STS primers ranged from 282 bp (Schachemayr et al. 1997) to 1.1 kb (Schachermayr et al. 1994).

In order to determine the presence of a specific gene in a complex genetic background of other resistance genes, a genetic marker for the gene in question would

greatly facilitate the plant breeding procedure. A marker-assisted selection scheme could allow plant breeders to efficiently select for a resistance gene without waiting for the phenotypic expression of the specific gene in plants. Indirect selection using DNA markers would also be helpful in elucidating rarely occurring recombination between resistance genes, thus facilitating the combination of these closely linked resistance genes into cultivars. Although no virulence for *Lr28* has been found in India as of yet, glass-house cultures have produced virulent mutants in Australia (R. A. McIntosh, personal communication) indicating that the development of virulent strains in India cannot be ruled out. To prevent the rapid breakdown of seedling resistance genes once they are integrated into new wheat varieties, it has been suggested that such genes should be used in combination with other leaf rust resistant genes (Roelfs et al. 1992), preferably with adult plant resistance genes. However, there are no reports of attempts to pyramid rust resistance genes in wheat using molecular markers. In order to combine several genes in the same line, markers for all of these genes need to be identified. Our future work will, therefore, concentrate on defining markers for the additional leaf rust resistant genes that are still effective. Also, efforts will be made to identify markers for the adult-plant resistant genes to enable their identification in a background of seedling resistant genes, which is otherwise not possible.

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