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Use of RFLP markers for the identification of alleles of the *Pm3* locus conferring powdery mildew resistance in wheat (*Triticum aestivum* L.)

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Abstract. The objective of this study was to identify molecular markers linked to genes for resistance to powdery mildew (*Pm*) in wheat using a series of 'Chancellor' near-isogenic-lines (NILs), each having one powdery mildew resistance gene. A total of 210 probes were screened for their ability to detect polymorphism between the NILs and the recurrent parent. One of these restriction fragment length polymorphism (RFLP) markers (Xwhs179) revealed polymorphism not only between the NILs for the *Pm3* locus, but also among NILs possessing different alleles of the *Pm3* locus. The location of the marker Xwhs179 was confirmed to be on homoeologous chromosome group 1 with the help of nullitetrasomic wheat lines. The linkage relationship between this probe and the *Pm3* locus was estimated with double haploid lines derived from a cross between wheat cvs 'Club' and 'Chul' (*Pm3b*). The genetic distance was determined to be 3.3 ± 1.9 cM.

Key words: *Triticum aestivum* – Mildew resistance – *Pm3* locus – Near-isogenic lines – RFLP marker

Introduction

Powdery mildew caused by *Erysiphe graminis* DC. f. sp. *tritici* Em. Marchal is one of the most economically important wheat diseases in temperate climates. As the most effective way to control this disease is the introduction of resistance genes in present cultivars, several genes conferring powdery mildew resistance have been identified in cultivars, land varieties or wild relatives of wheat. At present, 17 loci have been localized to particular wheat chromosomes (McIntosh 1988), and 1 of these, the locus *Pm3*, located on chromosome 1A (Briggle and Sears 1966; McIntosh and Baker 1969), consists of at least 6 different alleles or very closely linked loci (Zeller et al. 1993). Based to Flor's (1955) gene-for-gene hypothesis Moseman (1959) has demonstrated that the presence of a specific gene for mildew resistance can be ascertained by an interaction with the mildew culture that lacks the corresponding gene for virulence. With the number of resistance genes which can be combined in a wheat cultivar increasing, the use of the gene-for-gene model for the identification of these resistance genes will become difficult, since the number of isolates needed for analysis will become prohibitive. Restriction fragment length polymorphism (RFLP) markers have been developed to determine genetic differentiation at the DNA level in order to improve the diagnosis of resistance genes.

Closely linked RFLP markers have been identified by various different strategies for several resistance genes in various crop plants. For example, Michelmore et al. (1991) used pooled DNA from susceptible and resistant plants to isolate markers linked to a gene for resistance to downy mildew in lettuce; Leonard-Shippers et al. (1992) employed an F_2 progeny segregating for late blight resistance caused by *Phytophthora infestans* in potato for the identification of closely linked RFLP markers to the *R1* resistance locus, and Jung et al. (1992) mapped DNA markers linked to nematode resistance genes using chromosome addition and translocation lines originating from wild beets. The use of near-isogenic lines (NILs), which are constructed by a recurrent backcross program, is the most effective way for identifying and isolating new closely linked RFLP markers. In tomato this has been shown for the

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I_2 locus for resistance against *Fusarium oxysporum* (Sarfatti et al. 1989), for the *Tm2a* locus for resistance against tomato mosaic virus (Young et al. 1988), for the *Mi* locus for resistance against root knot nematode *Meloidogyne* ssp. (Klein-Lankhorst et al. 1991; Ho et al. 1992) and for the *Pseudomonas* resistance gene using also random amplified polymorphic DNAs (RAPDs) (Martin et al. 1991); in maize, for resistance against maize dwarf mosaic virus (McMullen and Louie 1989); in rice, for the genes *Pi-2* and *Pi-4* for blast resistance (Yu et al. 1991) and in barley, for the *mlo* resistance (Hinze et al. 1991) and the *Mla* resistance (Schüller et al. 1992) against powdery mildew. Tightly linked RFLP markers will also offer an alternative for the identification and isolation of these genes using the methods of "reverse genetics" (Orkin 1986) when the products, of the resistance genes are unknown (Manners et al. 1985).

The present paper reports the development and use of RFLP markers in the wheat genome and the identification of a *Pm3*-linked RFLP marker using near-isogenic lines.

Material and methods

Plant material

The German spring wheat cv 'Ralle' and the synthetic wheat line 'XX85' (*T. dicoccoides* × *Ae. squarrosa* = ABD-11), kindly provided by Dr. S. Ohta, Kyoto, Japan, were used to determine the efficiency of probes revealing RFLPs. A set of six near-isogenic lines of wheat for mildew resistance genes, supplied by Dr. R. A. McIntosh, Cubbitty, Australia, were employed to find polymorphic markers linked to the genes conferring mildew resistance. These lines were developed by Briggle (1969) after seven backcross generations with 'Chancellor' (Cc) as recurrent parent and 'Axminster' (*Pm1*), 'Ulka' (*Pm2*), 'Asosan' (*Pm3a*), 'Chul' (*Pm3b*), 'Sonora' (*Pm3c*) and 'Khapli' (*Pm4a*) as donor lines. The chromosomal location of the sequences homoeologous to the polymorphic probes were determined using ditelosomic and nulli-tetrasomic lines developed by Sears (1966). Genetic distances between the polymorphic RFLP markers and the *Pm3* locus were determined on a set of double-haploid (DH) lines derived from anther culture progenies of 91 F_2 plants from a cross between the wheat cvs. 'Club' and 'Chul', kindly provided by Dr. H. Kempf, Saatzucht Schwaiger, Feldkirchen, Germany. The cultivars 'Norin 3' (from Dr. T. Yamada, Ibaraki, Japan), 'Norin 29' (from Dr. Y. Tosa, Kochi, Japan), 'Saluda' and 'Tyler' (from Dr. C. A. Griffey, Blacksburg, USA) containing the *Pm3a* allele were used in the present investigation to determine the allele-specific RFLP pattern. *Triticum turgidum* ssp. *sphaerococcum* var 'spicatum' line 'Cawnpore' (TA11) Weihenstephan and *T. sphaerococcum* var 'rubiginosum' 'Hindukush', Halle, line TA12, both containing the *Pm3a* allele (provided by Dr. Ch. Lehmann, Gatersleben, Germany), were examined to verify this probe. 'Tyler' and 'Saluda' both carry the mildew resistance gene from 'Asosan' (Starling et al. 1984, 1986).

Construction of the genomic library

The synthetic wheat line 'XX201' [*Triticum durum* var 'Maroccos 182' (from Dr. A. C. Zeven, Wageningen, Netherlands) × *Ae. squarrosa 3* (from Dr. J. Jahier, Le Rheu, France)] developed by Lutz (1991) was used to construct the genomic library. Genomic DNA was prepared as described by Steinmüller and Apel (1986) for cloning into plasmids. DNA (50 µg) was digested to completion with *Pst*I and size fractionated by ultracentrifugation on a 10–40% sucrose gradient (Maniatis et al. 1982). The fractions, 0.5–2.5 kb in size, were ligated into *Pst*I-digested, dephosphorylated Bluescript M13 + vector (Stratagene, San Diego Cal. f.) and transformed into *Escherichia coli* DH5α cells.

Plasmids containing highly repetitive DNA sequences were discarded after dot-blot hybridization with total genomic wheat DNA. Further screening of the clones was performed on previously selected clones that showed weak and undetectable signals. Mini-blots with one lane of *Bam*HI-digested wheat DNA were hybridized with each probe to eliminate multi-copy and low-repetitive sequences.

Southern analysis

Wheat DNA for Southern analysis was isolated from frozen leafs according to Saghai-Maroof et al. (1984) and was digested (10 µg DNA/lane) with the restriction enzymes *Bam*HI, *Hin*dIII, *Eco*RI, *Eco*RV and *Xba*I following the manufacturer's instructions (Amersham). Electrophoresis was conducted on 0.75% agarose gels (Seakem FMC) covered with TAE buffer containing 0.5 µg ethidium bromide per milliliter at 1 V/cm overnight. The DNA was then transferred onto Biodyne B nylon membrane (Pall, Portsmouth) under alkaline conditions (0.6 *M* NaCl, 0.4 *M* NaOH).

Plasmid DNA was prepared by the rapid lysis method of Homes was Quigley (1981). For labelling the probes, this DNA was digested with *Pst*I, and the resulting inserts were separated from vector DNA by electrophoresis using 1% low-melting agarose (NuSieve FMC). Labelling was performed by the random-primer method (Feinberg and Vogelstein 1983). Non-incorporated nucleotides were removed by gel filtration through Sephadex G-50 (Pharmacia, Uppsala). Hybridization was performed in a solution containing 0.9 *M* NaCl; 50 m*M* NaH_2PO_4, pH 7.4; 5 m*M* EDTA; 0.1% Polyvinylpyrrolidone; 0.1% BSA; 0.1% Ficoll; 0.2% SDS and 0.25 mg/ml herring sperm DNA at 65 °C. After hybridization, membranes were washed twice for 20 min at 50 °C in 0.5 × SSC, 0.1% SDS (Maniatis et al. 1982) and once for 20 min at 60 °C in 0.2 × SSC, 0.1% SDS. The DNA was removed from the filters with 0.2 *M* NaOH, 0.1% SDS, and the filters were reused up to ten times.

Data analysis

Linkage estimation based on the maximum likelihood method of Allard (1956) was conducted with MAPMAKER (Lander et al. 1987). This recombination fraction was transformed into cM according to Kosambi (1944).

Powdery mildew test

Powdery mildew tests were performed at the seedling stage using detached leaves on agar containing 50 mg/l benzimidazol and 50 µg/ml ampicillin. Seedlings were raised in a growth chamber in order to prevent contamination. Mildew infections were carried out according to the method described by Aslam and Schwarzbach (1980). The powdery mildew isolates used in this study originated from a collection of single-spore progenies that differ in virulence and are maintained at the Institute of Agronomy and Plant Breeding, TUM Weihenstephan. After inoculation, the agar plates were kept under controlled conditions for 9 days until disease readings were scored as described by Lutz (1991).

Results

Since the genome of the recurrent parent cv 'Chancellor' and the near-isogenic lines are supposed to be identical except for the regions flanking the gene of interest probes showing polymorphism between the recurrent parent cv 'Chancellor' and its NILs should be located near the gene of interest. A total of 1050 probe-enzyme combinations were screened to isolate RFLP markers showing polymorphism between the recurrent parent 'Chancellor' and six NILs. When DNA was digested with five restriction enzymes and hybridized to RFLP marker Xwhs179, polymorphism was revealed not only between the NILs for the *Pm3* locus and the recurrent parent, but also among the NILs possessing different alleles of the *Pm3* locus (Fig. 1). The location of this informative probe on the specific chromosome was confirmed by hybridization on DNA filters from nulli-tetrasomic and ditelosomic lines. The result of this analysis showed that probe Xwhs179 is located on wheat homoeologous chromosome group 1, to which the *Pm3* locus has been assigned (Fig. 2).

The genetic distance between this probe and the *Pm3* locus was estimated with a population of DH lines from the cross 'Club' × 'Chul'. These DH lines carry the *Pm3b* allele of the *Pm3* locus and were used instead of a F_2 generation, which does not clearly segregate into susceptible and resistant groups (Jahoor and Fischbeck 1987). For the DH population the segregation was confirmed upon inoculation with a powdery mildew culture virulent against cv 'Club' and avirulent against 'Chul'.

The RFLP patterns obtained from these lines were hybridized using the probe Xwhs179 (Fig. 3), and both data sets subjected to linkage estimation. With this probe only 3 out of 91 DH lines showed recombination between the mildew reaction of 'Club' and the RFLP pattern from 'Chul', and only 1 DH line showed the reverse. The genetic distance between this probe and the *Pm3* locus was determined to 3.3 ± 1.9 cM.

The relationship between the Xwhs179 RFLP marker and the alleles of the *Pm3* locus was investigated in a more detailed study. The marker was tested against 6 wheat lines carrying the *Pm3a* or the *Pm3c* alleles. Allele-specific RFLP patterns were again verified in all observations (Fig. 4). The results confirmed the close linkage between the Xwhs179 marker and the *Pm3* locus.

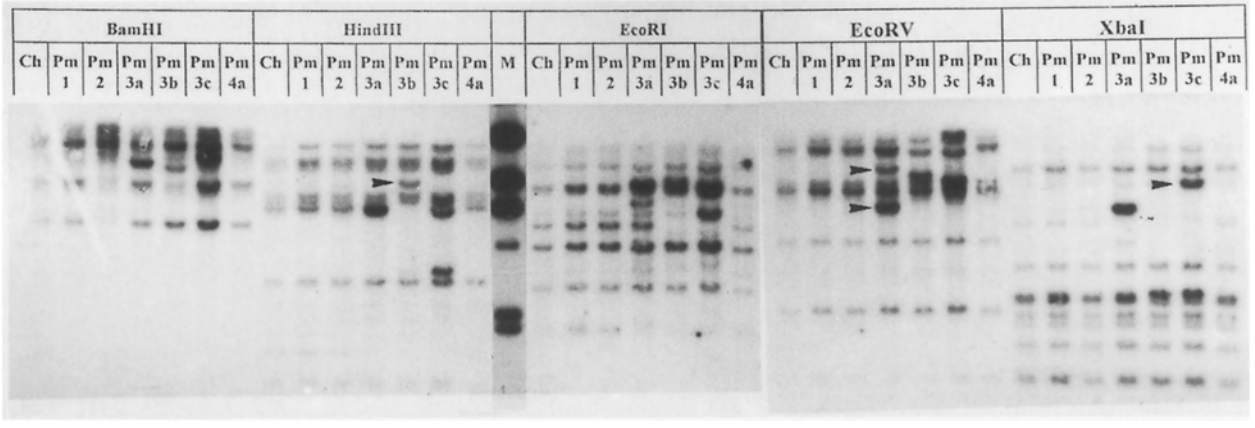

Fig. 1. Southern blot analysis of near-isogenic lines possessing different powdery mildew resistance genes (*Pm*). DNA was digested with five restriction enzymes and hybridized to clone Xwhs179. *Arrows* indicate the specific bands for the different *Pm3* alleles. *Ch* 'Chancellor'

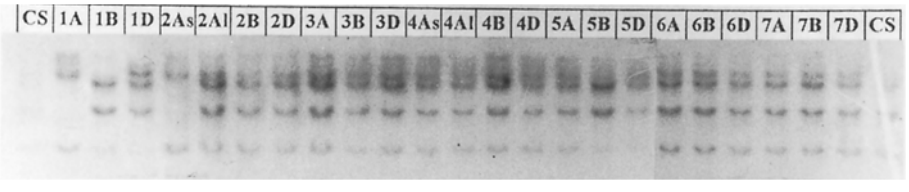

Fig. 2. Localization of the probe Xwhs179 by hybridization on a Southern blot of nulli-tetrasomic (e.g. *1A* = nulli 1A tetra 1B) and ditelosomic (e.g. *2Al* = ditelo 2AL) lines, respectively. DNA was digested with *Bam*HI. Based on the absence of different fragments in 1A, 1B and 1D probe Xwhs179 was assigned to the group 1 chromosomes (one band can be allocated to chromosome 2AL). *CS* 'Chinese Spring'

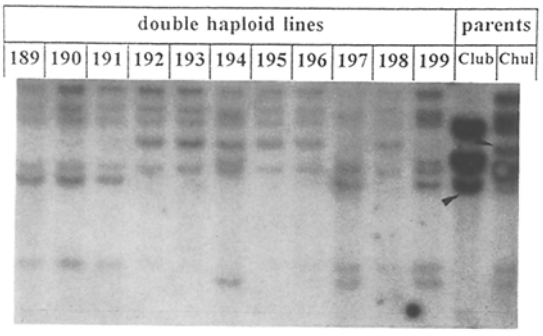

Fig. 3. RFLP pattern of the clone Xwhs179 on DH lines derived from a cross between wheat varieties 'Club' and 'Chul' segregating for the *Pm3b* gene. DNA was digested with *Hin*dIII. *Arrows* indicate the band that is linked to *Pm3b*

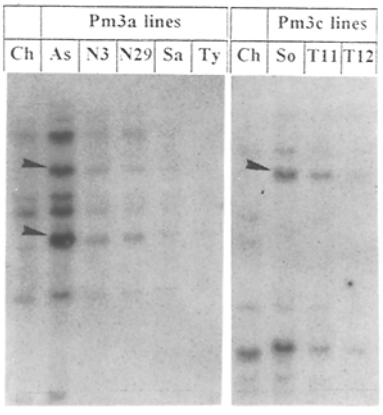

Fig. 4. RFLP's of lines carrying *Pm3a* and *Pm3c* alleles, respectively. DNA of the *Pm3a* lines was digested with *Eco*RV, whereas the DNA of the *Pm3c* lines was digested with *Xba*I. Marked bands (*arrows*) correspond to specific bands for *Pm3a* and *Pm3c*, respectively (see Fig. 1). *Ch* 'Chancellor', *As* 'Asosan', *N3* 'Norin3', *N29* 'Norin29', *Sa* 'Saluda', *Ty* 'Tyler', *So* 'Sonora', *T11* 'Cawnpore', *T12* 'Hindukush'

Discussion

Genes for disease resistance are not distributed randomly over the genome of a species, rather they occur frequently in groups on particular chromosomes (Islam et al. 1989). In the case of mildew resistance multiple allelism has been found on 3 loci, namely *Mla*, *Mlp* and *mlo* in barley (Jahoor and Fischbeck 1993; Jahoor et al. 1989; Hentrich 1979) and on the *Pm3* locus in wheat (Briggle and Sears 1966).

The *Pm3* locus in wheat, which carries at leat 6 different alleles (Zeller et al. 1993) and the *Mla* locus in barley, with 29 different alleles (Jahoor and Fischbeck 1993), are supposed to be homoeologous to each other. They are located on the short arm of chromosomes of the homoeologous group 1 of wheat and barley. The *Mla* locus in barley is flanked by the *HorB* and *HorC* loci (Siedler and Graner 1991; Sørensen 1989). A similar situation is found for the *Pm3* locus of wheat, which is located between the *Gli-1* and *LMW-Glu-3* loci on the homoeologous chromosome arm. In addition, parts of the nucleotide sequence of the barley B1 hordein (*HorB*) are identical with corresponding sequences of the wheat *Gli-1* protein (Kreis et al. 1985).

This specific region of homoeologous chromosomes consists of similar multigene families originating probably from a common ancestor. Either these multigene families can originate from the duplication of a particular DNA fragment within a region (Heidecker et al. 1991), or they are produced by an intralocus recombination like the one that has been shown for the *HorB* locus (Shewry et al. 1990) and the *Mla* locus (A. Jahoor, unpublished). Therefore, present knowledge seems to justify that complex loci are not randomly distributed but may occur in groups upon certain chromosomes. However, further studies are needed to verify this hypothesis. Since *Pm3a* and *Pm3b* have not been used at present in wheat breeding programs in Europe, they should be introduced into these programs in the future, because of the low virulence frequency of mildew population in Europe to these alleles (Felsenstein 1991). In contrast to barley, in which most of the resistance genes introduced into present cultivars are located on the *Mla* locus on chromosome 5 (1H), the genes conferring powdery mildew resistance in wheat cultivars are distributed all over the genome. Therefore, combinations of different genes conferring mildew resistance are generally used in breeding programs. The isolated probe in the present study provides a new protocol for the successful identification of alleles of the *Pm3* locus irrespective of genetic background and also independent of the codominance of different mildew resistance genes. Even at this stage of development, the probe Xwhs179 closely linked to *Pm3* locus provides favorable conditions for the molecular analysis and identification of the *Pm3* locus and its alleles.

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