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Identification and localization of molecular markers linked to the *Lr9* leaf rust resistance gene of wheat

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Abstract Near-isogenic lines (NILs) for the leaf rust resistance gene *Lr9* were screened for polymorphisms at the molecular level. RAPD (random amplified polymorphic DNA) primers as well as RFLP (restriction fragment length polymorphism) markers were used. Out of 395 RAPD primers tested, three showed polymorphisms between NILs, i.e., an additional band was found in resistant lines. One of these polymorphic bands was cloned and sequenced. Specific primers were synthesized, and after amplification only resistant lines showed an amplified product. Thus, these primers define a sequence-tagged site that is specific for the translocated fragment carrying the *Lr9* gene. A cross between a resistant NIL and the spelt (*Triticum spelta*) variety 'Oberkulmer' was made, and F_2 plants were analyzed for genetic linkage. All three polymorphisms detected by the PCR (polymerase chain reaction) and one RFLP marker (cMWG684) showed complete linkage to the *Lr9* gene in 156 and 133 plants analyzed, respectively. A second RFLP marker (PSR546) was closely linked (8 ± 2.4 cM) to the *Lr9* gene and the other four DNA markers. As this marker maps to the distal part of the long arm of chromosome 6B of wheat, *Lr9* and the other DNA markers also map to the distal region of 6BL. All three PCR markers detected the *Lr9* gene in independently derived breeding lines and varieties, thus proving their general applicability in wheat breeding programs.

Key words Leaf rust · RAPD · RFLP · *Triticum aestivum* · *Triticum spelta*

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Introduction

Leaf rust is one of the most important diseases of wheat. Specific resistance genes are known and have been used to breed resistant varieties. Many of these resistance genes were introgressed from wild relatives of wheat by wide crosses (Baum et al. 1992); for example, the *Lr9* resistance gene, localized on the long arm of chromosome 6B (Sears 1961), was translocated into wheat from *Aegilops umbellulata* (Sears 1956). In several European countries, including Switzerland, no virulence has yet been detected for this gene (Denissen and van der Putten 1991; Poinso and Ollivier 1988; von Kröcher et al. 1992; our unpublished data). In addition, no undesirable traits have been associated with the leaf rust resistance of *Aegilops umbellulata* (Soliman et al. 1963). Therefore, its presence, in combination with other resistance genes, is desirable in new cultivars to be released. In order to determine the presence of *Lr9* in a complex genetic background of other leaf rust resistance genes a genetic marker for *Lr9* is needed.

Both restriction fragment length polymorphism (RFLP) (Botstein et al. 1980) and random amplified polymorphic DNA (RAPD) (Williams et al. 1990) have been used as molecular markers in wheat. While genetic maps based on RFLP markers are known for several chromosomes (Chao et al. 1989; Liu and Tsunewaki 1991; Devos et al. 1992, 1993), RAPD markers have been found to be of limited use in the construction of linkage maps in wheat (Devos and Gale 1992). However, it has been proposed that the latter may be useful in the characterization of introgressed 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. This approach has given tightly linked RFLP markers for several important disease resistance genes, e.g., the *Tm-2a* viral resistance gene in tomato (Young and Tanksley 1989), the *ml-o* resistance locus in barley (Hinze et al. 1991), *Ml-a* in barley (Schüller et al. 1992), the *Ht1* gene in maize (Bentolila et al. 1991) and *Pm3* in wheat (Hartl et al. 1993). NIL-based markers have also been used to identify other agronomically important genes, such as an aroma gene in rice (Ahn et al. 1992). In the present study a similar ap-

proach for the identification of molecular markers for the wheat *Lr9* gene was used. As the genetic map of wheat is not very dense as yet, both RFLP and RAPD markers were used. A comparison of NILs resulted in three RAPD and one RFLP markers that are all completely or tightly linked to *Lr9*. One of the RAPD markers was converted into a sequence-tagged site (STS, Olson et al. 1989) for easy identification.

Materials and methods

Plant material

Two resistant (P11 and P15) and two susceptible (P10 and P14) F_8 NILs of *Lr9*/7* 'Arina' (i.e., seven times backcrossed to 'Arina' and afterwards in the F_8 generation) were used to identify RAPD and RFLP markers linked to the *Lr9* resistance gene. These lines have been described by Fried and Winzeler (1990). In addition ten F_3 plants of the ninth backcross generation (five resistant and five susceptible BC_9 F_3), the *Lr9* donor ('Transfer'/6* 'Thatcher'), and cvs 'Thatcher', 'Arina', and 'Transfer' were tested for the presence of the markers. A stock of seeds from *Lr9*-containing varieties was kindly provided by Dr. A. P. Roelfs (Cereal Rust Laboratory, University of Minnesota). A F_2 population of 162 plants from a cross between a resistant NIL (P11) and the susceptible cv 'Oberkulmer' (*Triticum spelta*) was used to map the *Lr9* gene and the linked markers. Segregation of the *Lr9* gene was evaluated by artificial infection at the one- to two-leaf stage with a mixture of eight isolates of leaf rust avirulent on *Lr9*. Spores were sprayed as a suspension with mineral oil Soltrol 170 (Phillips Petroleum, Paris). The aneuploid line nullisomic 6B – tetrasomic 6A (N6BT6A) of 'Chinese Spring' (CS) (Sears 1966) and a set of wheat-barley addition lines containing disomic additions of entire chromosomes of *Hordeum vulgare* cv 'Betzes' in CS (Islam et al. 1981) were used to verify the homoeologous group and the chromosome.

DNA techniques

Isolation of genomic DNA, Southern analysis and probe labelling were performed essentially as described by Graner et al. (1990). Five restriction endonucleases (*Eco*RI, *Hin*dIII, *Xba*I, *Eco*RV, and *Dra*I) were chosen for RFLP analysis.

RAPD reactions similar to those described by Williams et al. (1990) were performed with some minor modifications. Amplification reactions contained 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 2 m*M* $MgCl_2$, 0.001% gelatine, 100 µ*M* of each dNTP, 15 ng 10-mer primer (Operon Technologies, Calif.), 5 ng genomic DNA, and 0.5 Units *Taq*DNA polymerase (Perkin Elmer Cetus, Switzerland) in a volume of 25 µl, overlaid with one drop mineral oil (Sigma, Ill.). Amplifications were performed in a Perkin Elmer Cetus DNA Thermal Cycler programmed for 6 min at 94 °C, 45 cycles of 1 min at 92 °C, 1 min at 35 °C, 2 min at 72 °C, and after the last cycle a final extension of 72 °C for 4 min. The amplification products were separated in 1.2% agarose gels and visualized by ethidium bromide staining.

RFLP probes

A set of mapped wheat probes was kindly provided by Dr. M. D. Gale (Cambridge Laboratory, Norwich) and Dr. P. Gay (Ciba-Geigy, Basel). In addition, NILs were probed with barley cDNAs (Graner et al. 1991).

Cloning and sequencing of a RAPD product

The amplification products of primer OPJ-13 were phosphorylated according to Maniatis et al. (1982). After separation by agarose gel electrophoresis the polymorphic fragment was excised from the gel and blunt-end ligated into the dephosphorylated vector pIBI24 (Dente et al. 1983) that had been linearized with *Sma*I. Transformation of *E. coli* host DH5α resulted in the recombinant clones pGS1 and pGS3 containing the fragment in both orientations. The identity of the cloned RAPD product was verified by a digest with the restriction enzymes *Eco*RI and *Hin*dIII.

Double-strand sequencing (^{T7}Sequencing ^{TM} kit Pharmacia, Switzerland) was done by the dideoxy-chain termination method using M13 universal primer.

STS design and analysis

For the cloned amplification product OPJ-13_{1100} two 21-bp oligonucleotides were designed to be used as STS primers. Primer J13/1 contained the original 10 bases of the RAPD primer plus the next 11 internal bases. Primer J13/2 was a 21-bp sequence with a GC content equal to that of J13/1. Primers were synthesized by Microsynth (Windisch, Switzerland). Amplification of genomic DNA was done in 25 µl reaction volume containing 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 2 m*M* $MgCl_2$, 0.001% gelatine, 100 µ*M* of each dNTP, 40 n*M* of each primer, 5 ng genomic DNA, and 0.5 Units *Taq*DNA polymerase, overlaid with one drop of mineral oil (Sigma). The reactions were performed in a Perkin Elmer Cetus DNA Thermo Cycler programmed for 6 min at 94 °C, 45 cycles of 1 min at 94 °C, 1 min at 62 °C, 2 min at 72 °C, and after the last cycle a final extension of 72 °C for 4 min.

Linkage analysis

Linkage estimation was based on the maximum likelihood method using the appropriate formulas of Allard (1956). This recombination fraction was transformed in centiMorgans (cM) according to Kosambi (1944).

Results

Analysis of near-isogenic lines with RAPDs

A total of 395 random primers were screened to identify polymorphisms between two resistant (P11 and P15) and two susceptible (P10 and P14) F_8 NILs of *Lr9*/7* 'Arina'. Of these, 44 gave no amplification products at all, while the others amplified about five fragments each on average that ranged from 400 bp to 2000 bp. For isogenic lines P10 and P11, 16 out of the 395 primers tested gave a polymorphism, whereas for P14 and P15 only 4 primers showed polymorphic bands. Three of these polymorphisms were detected in both pairs of isogenic lines. Primers OPA-07, OPJ-13 and OPR-15 resulted in one additional band in the resistant line, whereas several other bands generated by the same primer were identical in both the resistant and susceptible lines. To investigate whether these polymorphisms might be linked to the *Lr9* resistance gene, ten F_3 plants of the ninth backcross generation were tested for the presence of the markers. In these ten plants, the three markers segregated with the resistance gene. A pedigree analysis with these primers revealed the presence of the additional bands in the *Lr9* donor ('Transfer'/6* 'Thatcher') of our set of NILs as well as in cv 'Transfer'. None of these specific bands were amplified with the recurrent parents cvs 'Arina' and 'Thatcher' (Fig. 1a, b). The specific product OPJ-13_{1100} was difficult to determine, as a weak band of similar size was present in all NILs (Fig. 1b).

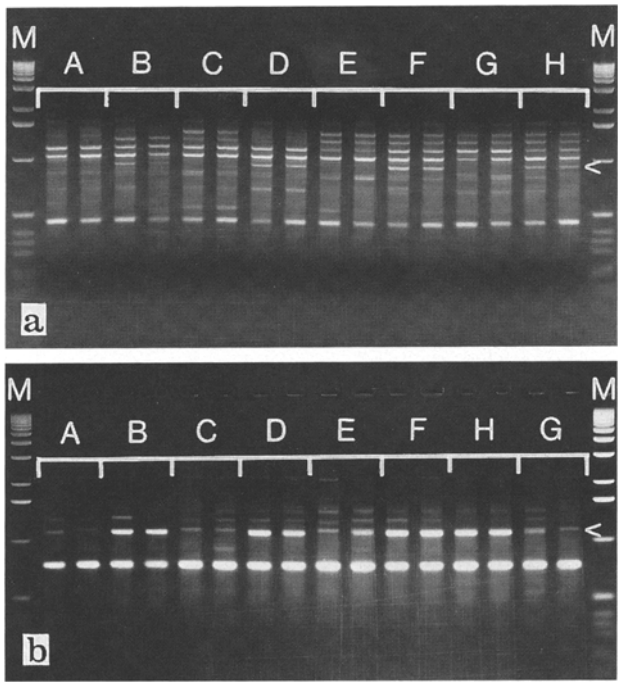


Fig. 1a, b **a** Amplification of a polymorphic DNA band (RAPD marker) produced by primer OPR-15. Amplification products are shown after agarose gel electrophoresis. *A* cv 'Thatcher', *B* cv 'Transfer', *C* the recurrent parent of the NILs = 'Arina', *D* the *Lr9* donor = 'Transfer'/6* 'Thatcher', *E* susceptible F_8 NIL of *Lr9*/7* 'Arina' = P10, *F* resistant F_8 NIL of *Lr9*/7* 'Arina' = P11, *G* susceptible F_3 plant of the 9th backcross generation. *H* resistant F_3 plant of the 9th backcross generation. *Arrows* indicate position of the polymorphic DNA band. *Lane M* is a 1-kb molecular-weight marker. **b** Amplification of a polymorphic DNA band (RAPD marker) produced by primer OPJ-13. Plant lines are identical to those in Fig. 1a

Conversion of a RAPD to a STS

In order to get a reliable assay for the polymorphism detected by primer J-13, a pair of 21-mer primers was synthesized (Table 1) using the sequence of the amplification product OPJ-13_{1100}. To optimize the reaction conditions for the specific primers we used genomic DNA from the parents of the mapping population and plasmid DNA from pGS1 as the template. Different concentrations of primer, ranging from 1 μ*M* to 0.04 μ*M* for each primer, resulted in only a single band of the expected size at the lowest primer concentration. The specifity of the reaction was also increased when the annealing temperature was raised from 60 °C to 62 °C. Under these conditions, only one DNA product was amplified in the resistant plants, and no amplification at all occurred in susceptible plants. Thus, the primers listed in Table 1 identify a sequence-tagged site (Olson et al. 1989) in wheat that is associated with the wheat *Lr9* gene. The STS J13/1 + 2 could be readily scored as a dominant marker (Fig. 2).

Table 1 Sequence of 21-mer oligonucleotide primers for the STS locus derived from a RAPD marker linked to the *Lr9* resistance gene

Primer[a]	Sequence[b]
J13/1	5′ CCACACTACCCCAAAGAGACG 3′
J13/2	5′ TCCTTTTATTCCGCACGCCGG 3′

[a] The first letter and the first number refers to the kit and primer number, respectively (Operon Technologies), used to identify the progenitor RAPD marker

[b] The underlined sequence represents the sequence of the progenitor RAPD primer

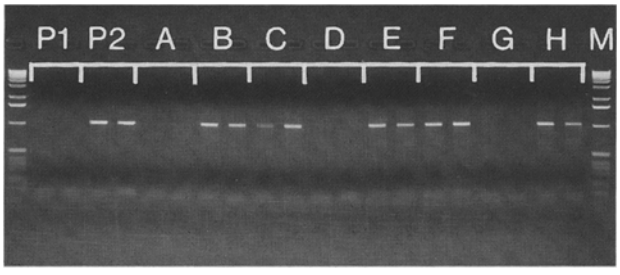
Localization of the markers

In addition to random primers, 21 RFLP probes with known map position on homoeologous chromosomes of group 6 were hybridized to the DNA of the NILs. One marker (PSR546) could distinguish the susceptible lines and the recurrent parent from the resistant lines and the donor. An *Eco*RV fragment of about 15.8 kb was present in the resistant lines and absent in the susceptible lines. This probe was also hybridized to Southern blots of N6BT6A and the wheat-barley addition lines (Fig. 3). CS and the addition lines showed the 15.8-kb *Eco*RV fragment, which was absent in the N6BT6A aneuploid. The addition line 6H showed two additional bands that comigrated with the fragments of the barley chromosome donor 'Betzes'. Therefore, we conclude that the 15.8-kb *Eco*RV fragment of probe PSR546 maps to chromosome 6B. Linkage results have demonstrated its position on the distal part of the long arm of chromosome 6B approximately 40 cM away from the centromere (unpublished data).

Linkage analysis at the *Lr9* region

Linkage studies were performed with a segregating F_2 population for the *Lr9* resistance gene and the DNA markers using P11 as the resistant parent and the spelt variety 'Oberkulmer' as the susceptible parent. In this population the resistance gene and the amplification products followed a monogenic three to one segregation. No preferential transmission of the alien segment took place. The probe PSR546 could be analyzed as a codominant marker with a 1:2:1 ratio in *Eco*RV

Fig. 2 Segregation of a PCR product that is completely linked to the *Lr9* resistance gene. Amplification was performed with the STS primers J13/1 + 2 on the F_2 progeny from a cross between the resistant NIL P11 (*P2*) and susceptible cv 'Oberkulmer' (*Triticum spelta*) (*P1*). Letters *A–H* indicate segregating F_2 plants. Resistant and susceptible genotypes are clearly distinguishable in the progeny. *Lane M* is a 1-kb molecular-weight marker



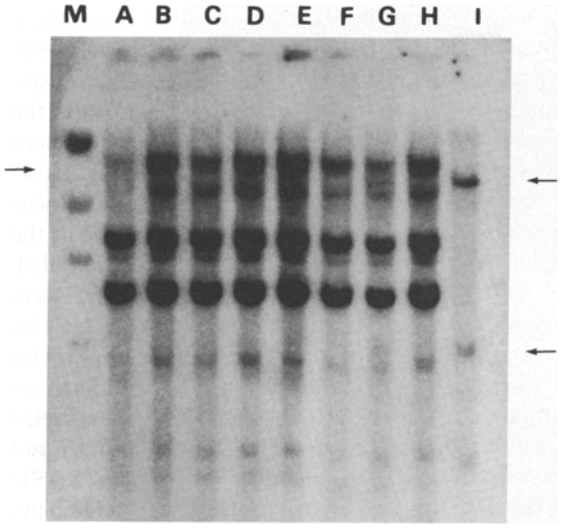


Fig. 3 Localization of probe PSR546 to the homoeologous group and the chromosome. The 15.8-kb *Eco*RV fragment is present in CS (*lane B*) and the wheat-barley addition lines (*lanes C–H*) and absent in the aneuploid N6BT6A (*lane A*) (*left arrow*). The addition line CS + 6H (*lane G*) shows two additional fragments identical to the fragments in the barley cv 'Betzes' (*lane I*) (*right arrows*). *M* Lambda *Hin*dIII

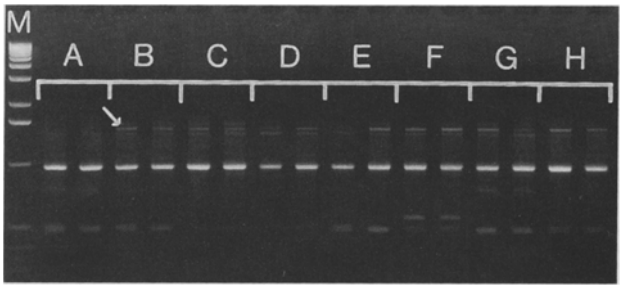


Fig. 4 Amplification of a polymorphic DNA band (RAPD marker) produced by primer OPA-07 in different genetic backgrounds: *A* indicates the susceptible cv 'Oberkulmer', *B* the resistant NIL P11, *C–H* cvs 'Transfer', 'Oasis', 'Abe', 'McNair', 'Coker 9877', 'Coker 762', respectively. *Arrow* indicates position of the polymorphic DNA band. *Lane M* is a 1-kb molecular-weight marker

digests. No recombination was found between the *Lr9* gene and the PCR products (Table 2). PSR546 mapped approximately 8 cM away from this locus. The alleles of the barley probe cMWG684 cosegregated also in the mapping population with the *Lr9* resistance gene. However, this marker could not differentiate between the NILs. All of these markers are therefore members of the linkage group 6BL.

Identification of DNA markers in different genetic backgrounds

To investigate whether the markers obtained are also indicative of the presence of the *Lr9* gene in other genetic backgrounds, 20 lines with assumed *Lr9* gene (A. P. Roelfs, personal communication) were tested (Table 3 and Fig. 4). With the exception of 'La Paz', all lines showed the products indicative of the presence of *Lr9*. In these 19 lines a separation of the resistance gene and the RAPD or STS markers did not occur. It may be that 'La Paz' is a recombinant line, but as none of the tested markers resulted in a specific amplification product it is possible that 'La Paz' does not possess the *Lr9* gene. It can be concluded that the RAPD and STS markers are of general applicability in the detection of the *Lr9* resistance gene.

Discussion

Wheat NILs (backcrossed seven times) carrying the *Lr9* resistance gene were very effective in defining molecular markers for this gene. Out of 395 RAPD primers that were tested, only 3 differentiated between the resistant NILs and 'Arina'. As each primer amplified about five fragments on the average, 3 out of approximately 2000 bands were different. Therefore, we estimate that between the lines analyzed and the recurrent parent only a small percentage of the genome is different.

All three RAPD markers showed complete linkage to the *Lr9* gene. It is possible that these markers are located physically close to the resistance gene, however, it is more likely that the recombination frequency in the translocated fragment is very low and that the markers are therefore completely linked. Two RFLP loci, one probe from wheat and one from barley, were also linked to *Lr9*. *Xpsr546* is located 8 cM away from the *Lr9* gene, whereas the alleles from the barley probe cMWG684 did not recombine with *Lr9*. As cMWG684 was

Table 2 Linkage analysis between DNA markers and the *Lr9* locus in the F_2 population of the cross P11 × 'Oberkulmer' (*R* Resistant phenotype, *s* susceptible genotype, *P* DNA marker allele of the parent P11, *O* DNA marker allele of the parent 'Oberkulmer')

DNA marker	Parent phenotype	*n*	Expected segregation	Segregation						Chi- square	Recombination fraction
				P_/R_	o/R_	P_/s	o/s				
J13/1 + 2	PR os	156	9:3:3:1	117	0	0	39			156	0.00
$OPR\text{-}15_{950}$	PR os	156	9:3:3:1	117	0	0	39			156	0.00
$OPA\text{-}7_{1500}$	PR os	156	9:3:3:1	117	0	0	39			156	0.00
				PP/R_	PO/R_	OO/R_	PP/s	PO/s	OO/s		
PSR546	PR Os	144	3:6:3:1:2:1	34	69	4	0	8	29	88	0.08 ± 0.02
cMWG 684	PR Os	133	3:6:3:1:2:1	36	63	0	0	0	34	133	0.00

Table 3 Usefulness of the DNA markers in different genetic backgrounds, tested on 20 wheat lines (+ Presence of the PCR products completely linked to the *Lr9* resistance gene, − absence of the PCR products completely linked to the *Lr9* resistance gene)

Variety/Line	Primer		
	OPA-07_{1500}[a]	J13/1 + 2[b]	OPR-15_{950}[a]
Abe	+	+	+
Oasis	+	+	+
McNair	+	+	+
Transfer	+	+	+
Precoz	+	+	+
Terraz	+	+	+
WiLr9	+	+	+
Mc201/Ramona	+	+	+
Coker 9877	+	+	+
Coker 762	+	+	+
Coker 97-66	+	+	+
Coker 86-25	+	+	+
Coker 93-23	+	+	+
CK 86-32	+	+	+
La Paz	−	−	−
WW75179	+	+	+
WW75395	+	+	+
WW75543	+	+	+
WW75387	+	+	+
WW75548	+	+	+

[a] These designations refer to the primers available commercially (Operon Technologies) and the approximate size of the polymorphic fragments
[b] These designations refer to the STS primers described in Table 1

not polymorphic within wheat lines, it cannot be used as a marker for the *Lr9* gene. However, it might be a starting point for a more detailed characterization of the chromosomal region containing the *Lr9* gene. This probe also shows that the RFLP map of barley can be used to improve mapping in wheat. The wide cross with the more polymorphic spelt enabled us to map this probe, confirming the usefulness of wheat × spelt crosses for genetic mapping, as has been previously demonstrated by Liu and Tsunewaki (1991).

Probe PSR546 was localized on chromosome 6B by analysis of nullisomic-tetrasomic wheat lines and wheat-barley addition lines, thus establishing the position of the markers on this particular chromosome. In addition, PSR546 was found to map to the distal part of the long arm of chromosome 6B (unpublished data). The linkage of *Xpsr546* and *Lr9* thus demonstrated the localization of *Lr9* in the same region of chromosome 6B. The combination of RAPD and RFLP techniques proved to be an efficient way to define markers. RAPD technology enables the screening of large numbers of genetic loci and differences between NILs can easily be defined, but there is no genetic map with RAPDs in wheat. The construction of a map based on RAPDs in wheat is probably not worthwile (Devos and Gale 1992). The sensitivity of the random amplification system can complicate the use of RAPD markers in different laboratories. In addition, chromosomal locations of RAPD products cannot be predicted, and the dominant phenotype devalues the use of RAPD as a generally applicable marker system in wheat. In combination with RFLP markers, however, we could map the RAPD markers and the completely linked *Lr9* gene.

We have converted one RAPD marker, OPJ-13_{1100}, into a sequence-tagged site (STS, Olson et al. 1989) using two specific 21-bp primers. We use the term sequence-tagged site here, although there are repetitive sequences within the amplified region (unpublished data) and such a fragment does not represent a probe useful for RFLP mapping. However, only one fragment representing only one genetic locus was amplified from the DNA of resistant plants, and thus the main criterium for the definition of a STS is fulfilled. Paran and Michelmore (1993) used alternatively the term sequence characterized amplified regions (SCARs) for this kind of markers. The amplification of a single band is useful for future automatization of the detection of *Lr9*. Such an automated method could be based on a simple quantitative detection of DNA after amplification. The use of longer and specific primers solves one of the major problems of RAPD markers in the wheat genome, i.e., problems of reproducibility with short primers and dependence on a particular PCR machine.

Up to now no virulence for *Lr9* has been found in Switzerland, the Netherlands, southern France, and Germany (Denissen and van der Putten 1991; Poinso and Ollivier 1988; von Kröcher et al. 1992; our unpublished data). To prevent a rapid breakdown of *Lr9* once it is integrated into new wheat varieties, this gene should be used in combination with other leaf rust resistance genes (Roelfs et al. 1992). In order to combine several genes in the same line, markers for all of these genes are needed. Future work will therefore concentrate on defining markers for the additional leaf rust resistance genes that are still effective.

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