Euphytica (2007) 155:329–336
DOI 10.1007/s10681-006-9334-x

Evaluation of leaf rust resistance genes *Lr1*, *Lr9*, *Lr24*, *Lr47* and their introgression into common wheat cultivars by marker-assisted selection

Francesca Nocente · Laura Gazza · Marina Pasquini

Received: 12 October 2006 / Accepted: 4 December 2006 / Published online: 30 December 2006

Abstract Epidemiological field controls in different Italian locations and seedling evaluations of the 'Thatcher' near-isogenic lines (NILs) carrying the leaf rust resistance genes *Lr1*, *Lr9*, *Lr24* and *Lr47* were conducted during 5 years of testing. These genes confirmed their effectiveness in both field and greenhouse conditions. Moreover a backcross program was carried out by using as recurrent parents the susceptible high-quality common wheat cvs 'Bolero', 'Colfiorito', 'Serio' and 'Spada' and the 'Thatcher' NILs carrying the above mentioned genes as donor parents. The progenies of different cross combinations were selected by both resistance tests and marker assisted selection using molecular markers (STS, SCAR, CAPS) closely linked to *Lr* genes: a complete cosegregation was observed between the resistance genes used and the corresponding molecular markers.

Keywords Common wheat · Leaf rust · *Lr* genes · Molecular markers · Resistance tests

F. Nocente · L. Gazza · M. Pasquini (✉)
Istituto Sperimentale per la Cerealicoltura,
C.R.A., Section of Applied Genetics,
Via Cassia 176, 00191 Rome, Italy
e-mail: pasquinimarina@mclink.it

Introduction

Leaf rust caused by the biotrophic fungus *Puccinia triticina* Eriks. (*Pt*) is one of the most important fungal disease affecting wheat cultivation in Italy. It reduces wheat yields in susceptible varieties and appears every year with disease severity depending on weather conditions (Casulli and Pasquini 1993, 1998; Pasquini and Zitelli 1984; Pasquini et al. 2003, 2005; Siniscalco et al. 1978).

Leaf rust needs to be constantly monitored to keep track of the fluctuation in the pathogen population. The ability of the pathogen to produce single-step mutations for virulence, makes breeding for resistance a never ending battle.

Pathotype monitoring is used extensively in many pathosystems and continues to provide timely information about the structure of pathogen populations that is rilevant to breeding programs and resistance deployment (Roelfs 1985a, b; Andrivon and De Vallavieille-Pope 1993).

The incorporation of effective and durable resistance is a valuable breeding strategy for wheat improvement. The 'pyramiding strategy', as to say the incorporation of more than one resistance gene to the same or different pathogens in a single genotype, could aid the breeder to maintain resistance any longer. Seedling resistance genes could be of little use when deployed alone in some regions, while they could be useful when deployed in combination with other genes.

Although this kind of resistance is considered to be more durable (Roelfs 1988), the introgression of different resistance genes is difficult to monitor by traditional phenotipic analysis because selection of genotypes carrying combinations of two or more genes is often prevented by the lack of pathotypes with virulence matching the corresponding seedling resistance gene(s). Specific molecular markers closely linked with resistance genes can facilitate expeditious pyramiding of major genes into elite background, making it more cost effective. Moreover, expression of molecular markers is not affected by environment, and they can be detected at all stages of plant growth (Gupta et al. 1999).

To date more than 50 leaf rust resistance loci have been identified and catalogued in wheat (McIntosh et al. 2003). Many of the leaf rust resistance genes were isolated from wild wheat relatives and maintained a good effectiveness for many years; on the other hand, they often have not been deployed in commercially grown wheat cultivars in Europe (Winzeler et al. 2000; Pathan and Park 2006).

The aim of this work was to evaluate leaf rust resistance genes *Lr1* (from common wheat), *Lr9* (from *Aegilops umbellulata*), *Lr24* (from *Thinopyrum ponticum*) and *Lr47* (from *Aegilops speltoides*) in the near-isogenic lines (NILs) of common wheat cv. Thatcher grown in several Italian locations during 5 years of testing. Moreover four molecular markers were analysed for their polymorfism and cosegregation with the *Lr1, Lr9, Lr24* and *Lr47* in the F_2 and F_1BC_1 progeny of the crosses between NILs and four susceptible bread wheat cultivars grown in Italy.

Materials and methods

Plant material

The common wheat cvs 'Bolero', 'Colfiorito', 'Serio' and 'Spada', grown in Italy and characterized by high yield and good quality but susceptible to leaf rust, were used as recipient parents for different cross combinations. 'Thatcher' NILs carrying the leaf rust resistance genes *Lr1* (Tc*6 × 'Centenario'), *Lr9* (Tc*6 × 'Transfer'), *Lr24* (Tc*6 × 'Agent') and their recurrent parent cv. Thatcher, kindly supplied by Prof. A. Mesterhàzy (Cereal Res. Inst., Szeged, Hungary) and the common wheat traslocation line T7AS-7S#1S-7AS · 7AL carrying *Lr47* resistance gene, kindly provided by Prof. J. Dubcovsky (University of California Davis, Calif., USA), were studied and used as resistant donor parents in the marker assisted selection. The common wheat variety Fortunato was used as susceptible check.

DNA extraction, PCR amplification and gel electrophoresis

Genomic DNA was isolated from leaves of 10-day-old seedling according with the procedure of Dellaporta et al. (1983). DNA was stored at −20°C until used.

Polymerase chain reaction (PCR) was performed in 25 µl reaction volume containing: 100 ng of genomic DNA, 1X *Taq* DNA polymerase buffer (50 mM KCl,10 mM Tris–HCl ph 8.8, 1.5 mM $MgCl_2$, 0.1% Triton X-100), 10 pmol of forward and reverse primers, 2.5 mM of each dNTP and 0.75 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA). The sequence for each primer set and PCR conditions are listed in Table 1. After amplification PCR products were separated on 1.5–2% agarose gels according to amplicon dimension, stained with ethidium bromide and visualized under UV light. To estimate the size of each amplified DNA fragment 50 or 100 bp ladder (Invitrogen) was used. The amplification product for the *Lr 47* CAPS marker was digested with *SacI* restriction enzyme (Promega, Madison, WI, USA) prior to electrophoresis.

Phytopathological analysis

Epidemiological field controls of the selected NILs and susceptible check cvs were conducted in different Italian locations from 2001 to 2005. The disease severity for each genotype was calculated as the average of severity values over different field sites, according to the modified Cobb's scale (0–100%) (Peterson et al. 1948) (Fig. 1).

The seedling behaviour of NILs and common wheat cultivars used as acceptor genotypes was tested in greenhouse by artificial inoculations

Table 1 Primer sequences, sizes of amplified marker fragments and references for leaf rust resistance gene markers used in the present work

Marker	Sequence of primers 5′-3′	PCR amplification conditions	Size of amplified marker fragments	References
Lr1	pTAG621-5: GGGTCA CGT ACT ACT ATA TA p TAG621-3: CCT TGC CAG CCC AAA AGA AG	94°C 5 min; 30 cycles (92°C—1 min., 55°C—1 min., 72°C—2 min); 72°C—10 min	560 bp	Feuillet et al. (1995)
Lr 9	J 13/1: TCC TTT TAT TCC GCA CGC CGG J 13/2: CCA CAC TAC CCC AAA GAG ACG	94°C 5 min; 35 cycles (92°C—1 min., 58°C—1 min., 72°C—2 min); 72°C—5 min	1,100 bp	Schachermayr et al. (1994)
Lr24	SC-H51: AGT CGT CCC CGA AGA CCC GCT GGA SC-H52: TCG TCC CCT GAT GCC ATG TAA TGT	94°C 3 min; 38 cycles (92°C—1 min., 68°C—2 min., 72°C—2 min); 72°C—5 min	700 bp	Dedryver et al. (1996)
Lr 47	PS10R: GCT GAT GAC CCT GAC CGG T PS10L: GGG CAG GCG TTT ATT CCA G	94°C 3 min.; 7 cyles of touchdown (94°C—30 s, 70°→ 64°C—30 s, 72°C–30 s); 35 cycles (94°C—30 s, 63°C—30 s, 72°C—30 s); 72°C—7 min	282 bp	Helguera et al. (2000)
Lr47 CAPS	PS10R: GCT GAT GAC CCT GAC CGG T PS10L2: GGG CAG GCG TTT ATT CCA G	94°C—3 min; 40 cycles (94°C—30 s; 55°C—30 s; 72°C—30 s); 72°C—7 min	450 bp 380 bp	Helguera et al. (2000)

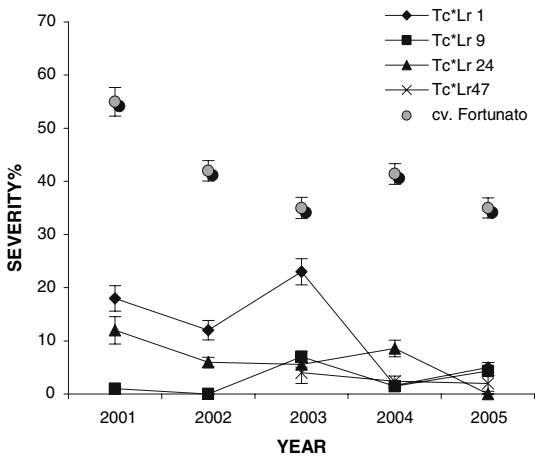

Fig. 1 Average leaf rust severity in the field on susceptible check variety 'Fortunato' and on 'Thatcher' NILs carrying resistance genes *Lr1, Lr9, Lr24* and *Lr47* in Italy from 2001 to 2005

with single pathotypes, annually identified within the *P. triticina* population. Out of these the 03766 pathotype, designed on the basis of a triplet code from the 15 differentials arranged in order of gene number (*Lr1*, *Lr2a*, *Lr2b*/*Lr2c*, *Lr3*, *Lr9*/*Lr11, Lr15*, *Lr17*/*Lr19*, *Lr21*, *Lr23/Lr24, Lr26, Lr28)* as described by Limpert and Muller (1994) and by Mesterhazy et al. (2000), was selected for the inoculation of parents and cross lines. This isolate, interesting for its virulence characteristics, resulted widely spread in Italy.

Seedlings, with the first leaf fully expanded, were inoculated with freshly collected urediospores of *P. triticina*, incubated at 100% relative humidity for 24 h in the dark and 24 h in the light at 23–24°C and placed in a growth chamber at 23–24°C with 14/10 h photoperiod.

Infection types at the seedling stage were recorded 12–14 days after inoculation, and followed the 0–4 infection type (IT) scoring system, in which ITs ≤ 2 were considered the expression of resistance and ITs from 3 to 4 were considered as host susceptibility.

Results and discussion

Epidemiological tests carried out in different Italian locations to control the behaviour of the potential donor parents, provided important evidence concerning the efficacy of the selected resistance genes. The lines remained substantially free of infection, compared with the susceptible

Table 2 Percentage of *Puccinia triticina* pathotypes virulent to the selected near-isogenic wheat lines at the seedling stage in Italy from 2001 to 2005

Gene	Year					Mean
	2001	2002	2003	2004	2005	
Lr1	14.3	11.8	20	15	4.9	13.2
Lr9	0	0	0	0	0	0
Lr24	0	4.4	0	3.3	2.4	2
Lr47	–	–	0	0	0	0
Number of pathotypes	70	68	55	60	41	

check cv. 'Fortunato', only a moderate percentage of infection being observed on some genotypes (Fig. 1).

The behaviour of the NILs at the seedling stage was tested in greenhouse conditions as well. On the basis of the virulence surveys carried out over a period of 5 years on a total of 294 *Pt* pathotypes, no virulence or low-virulence frequencies were found to the resistance genes *Lr9*, *Lr24* and, from 2003, *Lr47*; virulence to resistance gene *Lr1* fluctuated between locations and years (Table 2).

On the other hand, *Lr9* and *Lr47* genes have confirmed their effectiveness in large parts of Europe and in other continents; the virulence frequencies to *Lr1* showed a low increase in Europe in the last years, while gene *Lr24* resulted ineffective in North and South America and South Africa, but effective in Europe, Australia and in the Indian subcontinent (Dubcovsky et al. 1998; Martinez et al. 2005; Mesterhazy et al. 2000; Park and Felsenstein 1998). No deleterious effects on quality characters seem to be associated with these genes; for these reasons they are probably of greatest potential use to wheat breeders. Moreover, the *Lr24* gene is known to be linked to the *Sr24* gene for resistance to stem rust (McIntosh et al. 1995).

Molecular markers (STS, CAPS, SCAR), closely linked to the *Lr* genes used in this work, were tested for their presence/absence in NILs and recipient cultivars prior to their application into breeding program as suggested by Gupta et al. (1999) and Korzun (2002) (Table 3).

As observed previously (Chelkowski et al. 2003) markers linked to *Lr9*, *Lr24* or *Lr47* were found in the respective 'Thatcher' NILs, while no amplified products were detected in genotypes lacking *Lr9*, *Lr24* and, only by using PS10L and PS10R primers, in the genotypes lacking *Lr47*. The resistance tests performed at the seedling stage with leaf rust pathotype 03766, avirulent to all the resistance genes used, were compared with the molecular tests confirming the presence/absence of the corresponding gene (Table 3).

The SCAR marker for *Lr24* (see Table 1) was effective in marker assisted selection for the *Lr24* source line 'Agent' used in the present work. This

Table 3 Detection of leaf rust resistance genes *Lr1*, *Lr9*, *Lr24* and *Lr47* in the donor 'Thatcher' NILs and in the recipient varieties using molecular and host-pathogen interaction test

Genotypes		Host-pathogen interaction test leaf rust pathotype 03766[a]	Molecular test				
			STS *Lr1*	STS *Lr9*	SCAR *Lr24*	PCR marker for S genome *Lr47*	CAPS *Lr47*
Donor lines	Tc**Lr1*	*R*	+	–	–	–	+
	Tc**Lr9*	*R*	+	+	–	–	+
	Tc**Lr24*	*R*	+	–	+	–	+
	Tc**Lr47*	*R*	+	–	–	+	+
Recipient varieties	Bolero	*S*	+	–	–	–	+
	Colfiorito	*S*	+	–	–	–	+
	Serio	*S*	+	–	–	–	+
	Spada	*S*	+	–	–	–	+
	Thatcher	*S*	+	–	–	–	+

[a] Avirulence/Virulence formula on the 15 near-isogenic host differentials: *Lr1*, *Lr2a*, *Lr2b*, *Lr9*, *Lr19*, *Lr24* / *Lr2c*, *Lr3*, *Lr11*, *Lr15*, *Lr17*, *Lr21*, *Lr23*, *Lr26*, *Lr28*

R resistant; *S* susceptible

(+) presence of amplified product

(–) absence of amplified product

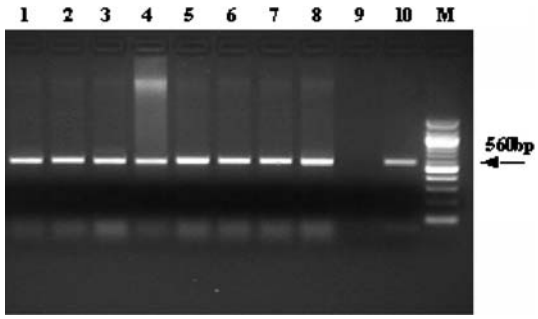

Fig. 2 PCR amplification using *Lr1* STS marker. *Lane* 1–Tc**Lr9*; *lane* 2–Tc**Lr24*; *lane* 3–T7AS-7S#1S-7AS·7AL *Lr47*; *lane* 4–cv. 'Bolero'; lane 5–cv. 'Colfiorito'; lane 6–cv. 'Serio; *lane* 7–cv. 'Spada'; *lane* 8–cv. 'Thatcher'; lane 9–no DNA control; *lane* 10–Tc**Lr1*; *lane* M is molecular size marker 100 bp ladder (Invitrogen)

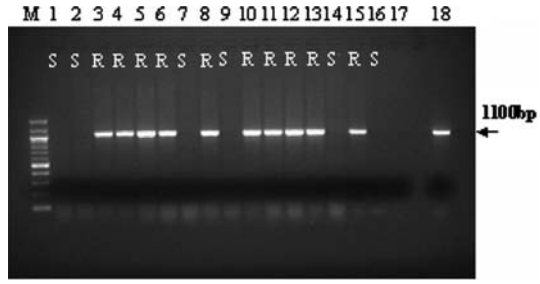

Fig. 3 STS marker-assisted screening of leaf rust resistance gene *Lr9* on segregating F_2 plants. *Lanes* 1–16–F_2 (Serio × Tc**Lr9*) plants; *lane* 17–cv. 'Serio'; *lane* 18–Tc**Lr9*; *lane* M is molecular size marker 100 bp (Invitrogen)

marker resulted non-polymorphic when utilized with the *Lr24* source line carrying a shorter portion of chromosome 3Ag (Gupta et al. 2006).

The STS marker for resistance gene *Lr1* (Feuillet et al. 1995) did not show polymorphism between resistant and susceptible individuals, likely because of its origin from common wheat. The amplification product of 560 bp specific to line Tc**Lr1* also occurred in 'Thatcher' NILs *Lr9, Lr24* and *Lr47* and in the recipient cultivars (Fig. 2). The presence of gene *Lr1* could be detected only by host-pathogen interaction test with leaf rust pathotype 03766 (Table 3).

Although a large number of molecular markers are now available, little has yet been done about their practical use in wheat breeding (Gupta et al. 1999). Moreover, being the genome of common wheat very complex some molecular markers (STS, SCAR) may give false-positive answers about the presence of the gene involved, especially considering the different genetic backgrounds of the cvs used either as donor or recipient parents (Blaszczyk et al. 2004). The expression of resistance genes is known to be modified by the genetic background of a cultivar (Gupta et al. 1984), expecially when these genes are transferred in common wheat from related species (Bai and Knott 1992; Friebe et al. 1996). The introgression of resistance genes should be confirmed by phytopathological tests also to verify their phenotypic expression in the new genetic background, discarding modifications for the presence of modifiers or suppressors.

Common wheat cvs 'Bolero', 'Colfiorito', 'Serio' and 'Spada' were used as recurrent parents in a backcross program with the 'Thatcher' NILs carrying *Lr1, Lr9, Lr24* or *Lr47* genes as donor parents.

The expected segregation ratio for the presence/absence of genes *Lr9*, *Lr24* and *Lr47* in F_2 and F_1BC_1 generations were confirmed by both resistance tests with pathotype 03766 and PCR amplifications of molecular markers (Table 4). The presence of *Lr1* gene in the F_1BC_1 and F_2 plants from the cross 'Colfiorito × Tc**Lr1*' was assessed by infection tests at the seedling stage with pathotype 03766 because all the 76 F_2 and 21 F_1BC_1 progeny analysed either resistant or susceptible showed the 560 bp marker (Table 4).

Generally, occasional recombination events could not be discarded in the progeny of different crosses: in the present work a complete cosegregation was observed between the resistance genes used and the corresponding molecular markers as already observed in other studies (Helguera et al. 2003).

Out of 108 F_2 plants from the cross 'Serio × Tc**Lr9*' and 24 F_1BC_1 plants from the cross [(Spada × Tc**Lr9*) × Spada] only 80 F_2 and 13 F_1BC_1 resistant plants showed the amplification of the expected fragment (Fig. 3; Table 4). The same result was obtained with the F_2 and F_1BC_1 progeny of the cross 'Bolero × Tc**Lr24*' and [(Serio × Tc**Lr24*) × Serio], respectively (Fig. 4; Table 4).

The STS/SCAR markers used for selection were dominant markers. Plant homozygous in

Table 4 Segregation for resistance to leaf rust pathotype 03766 in F_1BC_1 and F_2 plants from different cross combinations

	Number of F_1BC_1 plants	R^{+a}	R^-	S^+	S^-	χ^{2b}	Number of F_2 plants	R^+	R^-	S^+	S^-	χ^2
Colfiorito × Tc*_Lr1_	21	12	0	0	9	0.18 ns[c]	76	60	0	0	16	0.43 ns
Spada × Tc*_Lr9_	24	13	0	0	11	0.04 ns	–	–	–	–	–	–
Serio × Tc*_Lr9_	–	–	–	–	–	–	108	80	0	0	28	0.012 ns
Bolero × Tc*_Lr24_	–	–	–	–	–	–	98	77	0	0	21	0.49 ns
Serio × Tc* _Lr24_	18	8	0	0	10	0.05 ns	–	–	–	–	–	–
Spada × T7AS-7S#1S-7AS · 7AL _Lr47_	24	13	0	0	11	0.04 ns	–	–	–	–	–	–
Colfiorito × T7AS-7S#1S-7AS · 7AL _Lr47_	–	–	–	–	–	–	58	43	0	0	15	0.05 ns

[a] R^+ resistant, presence of marker; R^- resistant, absence of marker; S^+ = susceptible, presence of marker; S^- susceptible, absence of marker

[b] Yates correction for χ^2 test was applied when needed

[c] ns = not significant

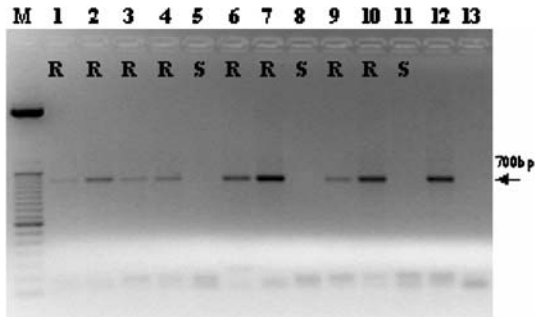

Fig. 4 STS marker-assisted screening of leaf rust resistance gene *Lr24* on segregeting F_2 plants. *Lanes* 1–11–F_2 (Bolero × Tc**Lr24*) plants; *lane* 12–Tc**Lr24*; *lane* 13–cv. 'Bolero'; lane M is molecular size marker 50 bp (Invitrogen)

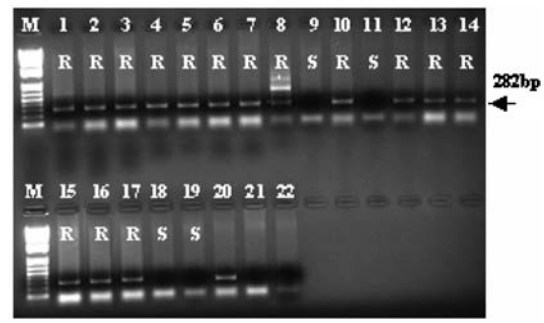

Fig. 5 STS marker-assisted screening of leaf rust resistance gene *Lr47* on segregeting F_2 plants using *T. speltoides* specific primers PS10R and PS10L. *Lanes* 1–19– F_2 (Colfiorito × T7AS-7S#1S-7AS·7AL *Lr47*) plants; *lane* 20–T7AS-7S#1S-7AS·7AL *Lr47*; *lane* 21–cv. 'Colfiorito'; lane 22 cv. 'Thatcher'; lane M is molecular size marker 50 bp (Invitrogen)

Lr9 and *Lr24* loci will be selected in further generations.

The PCR-specific primers PS10R and PS10L for the *T. speltoides* S genome allele of *Xabc465* (see Table 1) were used to determine the presence of the whole segment containing gene *Lr47* in individuals of earlier cross generations. This gene, widely effective in Italy, is located within an interstitial segment of *T. speltoides* chromosome 7S#1 transferred to the short arm of chromosome 7A of bread wheat translocation line T7AS-7S#1S-7AS·7AL (Friebe et al. 1996; Dubcovsky et al. 1998).

In total 58 F_2 plants from the cross (Colfiorito × T7AS-7S#1S-7AS·7AL *Lr47*) and 24 F_1BC_1 plants from the cross [(Spada × T7AS-7S#1S-7AS·7AL *Lr47*) × Spada] were tested by molecular and greenhouse tests. PCR-amplification of DNA using primers PS10R/PS10L showed a 282 bp fragment present in 43 F_2 and in 13 F_1BC_1 resistant plants; this fragment was absent in the susceptible plants (Fig. 5; Table 4).

Moreover a CAPS marker for *Lr47* gene was used to discriminate homozygous 7S7S from heterozygous 7A7S in F_2 individuals (Fig. 6). This marker is specific for the 7A allele of the *Xabc465* locus and it is detected by amplyfing genomic DNA using PS10R and PS10L2 PCR primers (Table 1). Two products of amplification were obtained: one of 450 bp (two products of amplification of the identical mobility from A and S genome allele) and the other of about 380 bp (B

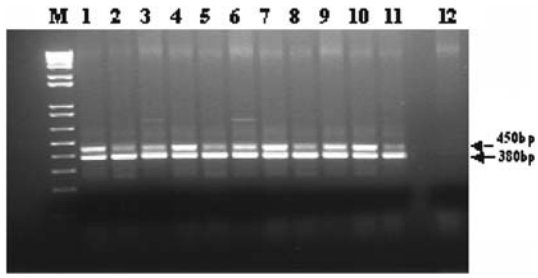

Fig. 6 PCR amplification on segregating F_2 plants using PS10R and PS10L2 primers. *Lanes* 1–9–F_2 (Colfiorito × T7AS-7S#1S-7AS·7AL *Lr47*) plants; *lane* 10–cv. 'Colfiorito'; *lane* 11–T7AS-7S#1S-7AS · 7AL *Lr47*; *lane* 12–no DNA control; *lane* M is molecular size marker 1 Kb plus (Invitrogen)

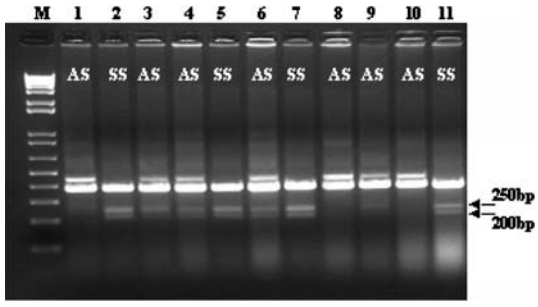

Fig. 7 Restriction analysis with *Sac*I on PCR amplification products obtained from F_2 (Colfiorito × T7AS-7S#1S-7AS·7AL *Lr47*) plants to discriminate homozygous 7S7S from heterozygous 7A7S individuals for *Lr47* gene

genome allele). The PCR products obtained were then digested with *Sac*I; in the heterozygous individuals a low intensity undigested 450 bp product (A genome allele) was still observed in addition to very faint 250 and 200 bp digestion products; on the other hand this 450 bp product was completely digested in homozygous plants for 7S, and only the 250 and 200 bp digestion fragments were detectable (Fig. 7).

The combination of different resistance genes is desirable in new cultivars to be released, but the 'pyramiding strategy' would only be effective when the virulence frequencies to each of the *Lr* gene are negligible. Up to now no or low virulence for *Lr1*, *Lr9*, *Lr24* and *Lr47* has yet been detected in Italy; besides no virulence has been reported on some combinations of these genes (Roelfs et al. 1992; Schachermayr et al. 1994).

To prevent a breakdown of single resistance genes when transferred into new wheat cultivars, plants from several cross combinations were intercrossed to pyramid more than one gene in the same background. Further work will concentrate on selecting the progenies by combined use of the markers found for each gene and by resistance tests.

As a whole, conventional cereal breeding is time consuming and depends on environmental conditions. The utilization of molecular markers in breeding programmes will allow to improve the efficiency and the earliness of selection, also by detecting a single resistance gene in a complex background of other resistance genes.

Novel selected genotypes will be available, useful as such as well as for further breeding work.

Acknowledgements Research supported by a grant from Mipaaf, Project AMIFRUGAM.

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