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Allele-specific amplification of polymorphic sites for the detection of powdery mildew resistance loci in cereals

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Abstract Primers for the polymerase chain reaction (PCR) were tailored to selectively amplify RFLP marker alleles associated with resistance and susceptibility for powdery mildew in cereals. The differentiation between marker alleles for susceptible and resistant genotypes is based on the discrimination of a single nucleotide by using allele-specific oligonucleotides as PCR primers. The PCR assays developed are diagnostic for RFLP alleles at the loci MWG097 in the barley genome and Whs350 in the wheat genome. The first marker locus is closely linked to *MlLa* resistance in barley, while the latter is linked to *Pm2* resistance locus in wheat. PCR analysis of 31 barley and 30 wheat cultivars, with some exceptions, verified the presence or absence of the resistance loci investigated. These rapid PCR-based approaches are proposed as an efficient alternative to conventional procedures for selecting powdery mildew-resistant genotypes in breeding programs.

Key words Allele-specific PCR (AS-PCR) · Powdery mildew · *Hordeum vulgare* · *Triticum aestivum* · Sequence-tagged site (STS)

Introduction

Tightly linked RFLP markers have been identified for many powdery mildew resistance loci in barley (Hinze et al. 1991; Hilbers et al. 1992; Schüller et al. 1992; Giese et al. 1993; Görg et al. 1993; Schönfeld et al. 1996) and wheat (Hartl et al. 1993, 1995; Ma et al. 1994).

Marker technology is slowly moving from hybridization-based RFLP markers to PCR-based markers, as the latter are more economically handled for high sample throughput and for the simultaneous analysis of multiple loci. Sequence-tagged sites (STSs, Olson et al. 1989) and random amplified polymorphic DNAs (RAPDs, Williams et al. 1991) are the most common PCR markers for gene diagnosis in plants. By the conversion of distinct RAPD fragments to STS markers, an increase in the reliability of detection of several leaf rust resistance genes in wheat has been achieved (Schachermayr et al. 1994, 1995). Furthermore, the genotyping of allelic variants of loci that result from both size differences and point mutations via allele-specific PCR has already been demonstrated for the *waxy* locus in maize (Shattuck-Eidens et al. 1991), the *Glu-D1* complex locus associated with bread-making quality in wheat (D'Ovidio and Anderson 1994), the *Lr1* leaf rust resistance locus in wheat (Feuillet et al. 1995), and the *Gro1* and *H1* alleles conferring resistance to the root cyst nematode *Globodera rostochiensis* in potato (Niewöhner et al. 1995).

The objective of the present study was to investigate the possibility of generating PCR-based markers from RFLP markers that are closely linked to the powdery mildew resistance genes *MlLa* of barley and *Pm2* of wheat.

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Materials and methods

Plant material

Wheat-barley addition lines (Islam et al. 1981), nullisomic-tetrasomic lines of 'Chinese Spring' (Sears 1966) (complete except for 2A and 4B), doubled-haploid progenies of barley crosses 'Alf' × 'Vogelsanger Gold' and 'Risø M1502' × 'Sultan' segregating for the *MlLa* resistance gene, and F_1 plants from the cross 'Gimpel' × 'Golf', were used for the evaluation of STS markers. Mapping of RFLP marker Whs350 was carried out in a population of 62 F_2 plants from the cross 'Axminster/8*Chancellor' (*Pm1*)×'Ulka/8*Chancellor' (*Pm2*). All other plant genotypes analysed are specified in the tables and figure legends.

Primer design, cloning of PCR products and sequencing

STS primer sets MWG097-R,L and Whs350-1,2 were derived from the DNA sequences of barley genomic clone MWG097 and wheat genomic clone Whs350, respectively (Table 1). The primer pairs

Table 1 Oligonucleotides used as primer sequences

Primer	Primer sequences
MWG097-R	5′-CGCCGGTACGTAGACTGCAT-3′
MWG097-L	5′-TGGACACACGGGGGTTCTCG-3′
MWG097-Res	5′-CTGCCATCTCCTCTTAGC-3′
MWG097-Sus	5′-CTGCCATCTCCTCTTAGT-3′
WHS350-1	5′-AGCTGTTTGGGTACAAGGTG-3′
WHS350-2	5′-TCCCCTGTGCTACTACTTCTC-3′
WHS350-Res	5′-GCCATCGTTTCTACTAG-3′
WHS350-Sus	5′-GCCATCGTTTCTACTAC-3′

were used to generate amplicons from DNA of barley and wheat cultivars differing in their powdery mildew responses. After polishing the amplicon termini with T4 Polynucleotide Kinase and T4 DNA Polymerase, PCR products were blunt-end ligated into the dephosphorylated phagemid pBluescript KSII+, which had been linearized with *Sma*I. The *E. coli* strain XL1-Blue was transformed and three independent clones of each genotype were sequenced due to the fact that *Taq* DNA polymerase lacks a proof-reading activity. Comparative sequence analysis of the cloned PCR products revealed DNA sequence variations that allowed the design of primers whose allele specificity was conferred only by their 3'-nucleotide (Table 1).

Cycle sequencing reactions were carried out using the DIG *Taq* DNA Sequencing Kit (Boehringer, Mannheim). DNA fragments were separated with a direct blotting electrophoresis device (GATC 1500, MWG Biotech) and detected colorimetrically with NBT/BCIP or with chemiluminescence.

PCR procedures

The STS-PCR procedure employed 50 ng of genomic DNA, 0.2 μM of each primer, 1.0 U of *Taq* DNA Polymerase (Pharmacia) in 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 9.0, and 100 μM of each dNTP in a total volume of 50 μl. DNA was amplified for 35 cycles [94°C 10 s, 58°C (barley primers) or 60°C (wheat primers) 10 s and 72°C 20 s] using an Omnigene temperature cycling system (Hybaid).

Allele-specific PCR amplifications were performed according to the protocol of Xu et al. (1994). To prepare template DNA, 50 ng of genomic DNA were added to a reaction mixture that contained 1.0 U of *Taq* DNA Polymerase (Pharmacia), 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 9.0, 100 μM of each dNTP, 0.25 μM of each of the allele-specific primers (MWG097-Res, MWG097-Sus and Whs350-Res, Whs350-Sus, respectively) plus 0.2 μM of the common primer (MWG097-L and Whs350-1, respectively) in a 50-μl reaction volume. Cycling conditions were for 20 cycles at 94°C for 10 s, 58°C (barley primers) or 60°C (wheat primers) for 10 s and 72°C for 20 s. For the second amplification round, 1 μl of template DNA was added to each of two mixtures, each of which contained the same reaction composition as above, except that Tube A included primers MWG097-Res and MWG097-L, while Tube B included MWG097-Sus and MWG097-L in equimolar amounts. For the *Pm2* assay the second amplification step was carried out only with the primer combination Whs350-Res and Whs350-1. The mixtures were then subjected to 14 cycles with the same temperature profile as above.

Results and discussion

MlLa

The primer set MWG097-R,L was obtained from full-length sequencing of the 420-bp-long barley genomic DNA clone MWG097, which is linked without recombination to the *MlLa* resistance locus located on the long arm of chromosome 2(2H) (Hilbers et al. 1992, unpublished data). PCR amplification of wheat-barley addition lines showed a 370-bp fragment only in wheat-barley addition line 2(2H), suggesting that the selected primers had amplified the expected sequence (Fig. 1). Furthermore, co-segregation of the allele-specific primer-generated amplicons with RFLP marker alleles of MWG097 was confirmed in two double-haploid progenies derived from plants of the crosses 'Alf'×'Vogelsanger Gold' and 'Risø M1502×Sultan'. The primer pair MWG097-R,L was assayed on DNA from the barley cultivars 'Igri' (susceptible) and 'Cerise' (resistant), but PCR products did not differ in size. Therefore, these amplicons were sequenced in order to detect single-base alterations. The comparative sequence analysis of the cloned amplicons from resistant and susceptible genotypes revealed a small deletion/insertion (3 bp) and also a G→A-transition at position 57 from the end tagged by primer MWG097-R. Oligonucleotides were constructed to match and mismatch the G and A alleles at this base-pair position. To test their potential for discrimination, the allele-specific primers MWG097-Res and MWG097-Sus were used in conjunction with their common primer on 31 barley cultivars (25 spring barley and 6 winter barley lines) for which the *MlLa* genotypes were known and, in each case, PCR confirmed that the expected base was present. Hence, amplification products are likely to be generated only from the perfectly matching primer sites (Fig. 2, Table 2). The amplification of genomic DNA from the susceptible cultivars 'Alexis', 'Pallas' and 'Koral' with primer MWG097-Res resulted in the detection of a 450-bp fragment which was greater than the expected size, and hence distinguishable from the resistant allele. The applicability of the primers in heterozygous plants carrying both MWG097 alleles was explored using F_1 plants from the cross 'Gimpel'×'Golf'. As expected, both MWG097 alleles were amplified when examining both primer sets

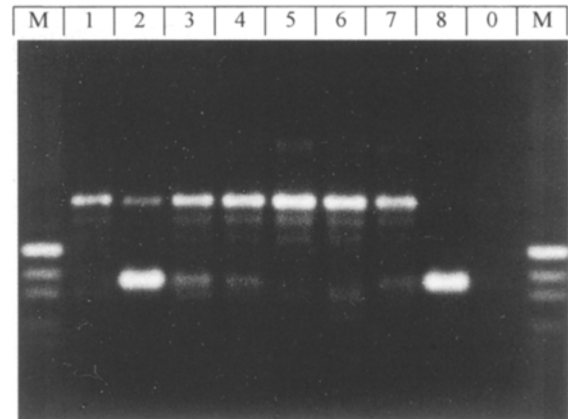

Fig. 1 Assignment of STS marker MWG97-R,L to barley chromosome 2(2H) by means of Wheat-barley addition lines. (*M*) pUC19/*Msp*I, (*0*) Control (-DNA), (*1*) DNA of 'Chinese Spring' (CS) + barley chromosome 1(7H), (*2*) CS + 2(2H), (*3*) CS + 3(3H), (*4*) CS + 4(4H), (*5*) CS, (*6*) CS + 6(6H), (*7*) CS + 7(5H), (*8*) 'Betzes'

Table 2 Allele-specific amplification of DNA from various barley cultivars (Jensen et al. 1992) depending on the PCR primer applied

Cultivar/line	Resistance genes	MWG097-Res	MWG097-Sus
Alexis	*mlo*	– (450 bp)	+
Arda	–	–	+
Aura	*Mla6, Mlg*	–	+
Elektra	–	–	+
Express	–	–	+
Gimpel	Heterogeneous	–	+
Gitte	*Mla1*	–	+
Igri	*Mlra*	–	+
Ingrid	*Mla8*	–	+
Pallas	*Mla8*	– (450 bp)	+
Puffin	*Mla12, Mlg, Mlh*	–	+
Sultan	*Mla12*	–	+
Union	*Mlg, Ml(CP)*	–	+
Vogelsanger Gold	*Mla6, Mlh, Mlra*	–	+
Koral	*Mla13, Mlg*	– (450 bp)	+
Welam	*Mla9*	–	+
Yriba	*Mla12, Mlg*	–	+
P23	***MlLa***	+	–
Alf	***MlLa***	+	–
Lami	***MlLa***	+	–
Risø M1502	***MlLa***	+	–
Vada	***MlLa***	+	–
Varunda	***MlLa***	+	–
Atem	*mlo*, ***MlLa***	+	–
Georgie	*Mlg*, ***MlLa***	+	–
Cerise	*Mlg, Ml(CP)*, ***MlLa***	+	–
Golf	*Mlg, Ml(CP)*, ***MlLa***	+	–
Hockey	*Mla12, Ml(CP)*, ***MlLa***	+	–
Claret	*Mla7, Mlk*, ***MlLa***	+	–
Klaxon	*Mla7, Mlk*, ***MlLa***	+	–
Menuet	*Mla12, Mlg, Ml(CP)*, ***MlLa***	+	–

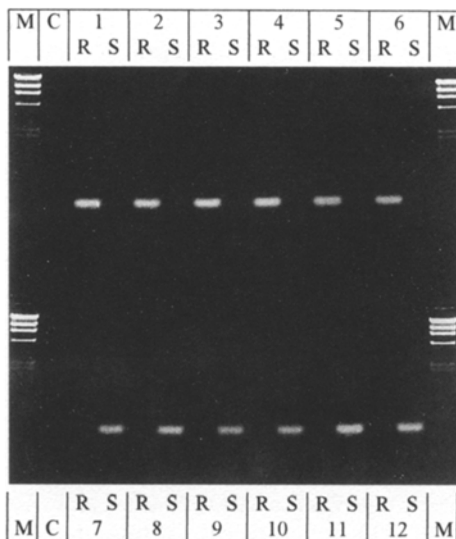

Fig. 2 Allele-specific amplification of RFLP marker alleles indicative for the presence or absence of the *MlLa* resistance locus. (*1*) 'Vada', (*2*) 'Cerise', (*3*) 'Golf', (*4*) 'Hockey', (*5*) 'Claret', (*6*) 'Georgie', (*7*) 'Igri', (*8*) 'Ingrid', (*9*) 'Express', (*10*) 'Union', (*11*) 'Yriba', (*12*) 'Arda', (*M*) λ/*Hin*dIII, (*C*) Control (-DNA), (*R*) resistant, (*S*) susceptible

on F_1 plants. In conclusion, these data indicate that the allele-specific primers could be used to predict the presence or absence of *MlLa* in a marker-assisted selection scheme, and by combining the scores of the amplification reactions from each of the allele-specific primers the assay becomes a co-dominant marker system.

Pm2

The RFLP band of marker Whs350, showing polymorphism between the near-isogenic line Ulka/8*Chancellor and its recurrent parent, was mapped at a genetic distance of 3.8 cM (LOD 8.97) from the *Pm2* locus on chromosome 5D using F_2 offspring from the cross 'Axminster/8*Chancellor' (*Pm1*)×'Ulka/8*Chancellor' (*Pm2*). PCR analysis of the Whs350 locus from nullisomic-tetrasomic lines of 'Chinese Spring' using primers Whs350-1,2 failed to assign this PCR marker to specific wheat chromosomes. This result may be due to the location of probe Whs350 on each of the homoeologous chromosomes of group 5 (with a *Pm2*-specific RFLP band on 5D) as determined by RFLP analysis of the aneuploid stocks (Hartl et al. 1995). The amplification of resistant and susceptible wheat lines

yielded a single monomorphic fragment. However, cleavage of the amplified 650-bp product with the restriction enzyme *Taq*I generated two smaller fragments while maintaining a band of the original size. This indicates that STS Whs350-1,2 is a mixture of at least two equal sized homoeologous fragments. For this reason, all fragments obtained from *Taq*I digestion of the susceptible cultivar 'Chinese Spring' and the resistant cultivars 'Ulka/8*Chancellor' (*Pm2*) and 'Nandu' were surveyed for point mutations by DNA sequencing. From the pool of point mutations that was found, a C→G-transversion was chosen for the design of an allele-specific oligonucleotide for the detection of the powdery mildew resistance locus *Pm2*. This primer, *Pm2*-Res, was used to amplify DNA from 17 susceptible and 13 resistant wheat cultivars. Data from this experiment demonstrated that the amplification reactions could be correlated with resistance and susceptibility for all but four of the wheat cultivars and lines tested (Fig. 3, Table 3).The wheat cultivars 'Normandie' and 'Sappo' were misclassified, as in the case of the study of Hartl et al. (1995), but recent studies have shown that at least cultivar 'Sappo' is known now not to contain *Pm2* (Høvemüller, personal communication). However, the use of this allele-specific PCR assay in wheat will be limited by its inability to detect heterozygotes since amplifications with primer *Pm2*-Sus confirmed that this allele also occurs in resistant cultivars.

The present study demonstrates that there is a useful level of variation within amplified DNA fragments in different barley and wheat genotypes and supports the use of RFLP markers as a source for the production of PCR-based markers. This finding is in agreement with the results of the evaluation of STS markers for genetic mapping in barley (Tragoonrung et al. 1992) and wheat (Talbert et al. 1994). By using primers recognizing different alleles of RFLP loci we were able to genotype the *MlLa* and *Pm2* alleles in barley and wheat, respectively. It has been shown that the nature of mismatch facilitates the specific discrimination of amplification (Kwok et al. 1990). In addition to a G:G and a C:A mismatch, we have also successfully used a G:T mismatch for PCR discrimination, although this type of mismatch should be avoided when designing allele-specific primers. Because sequence context can significantly alter the properties of mismatches (Huang et al. 1992), G:T mismatches can be also detrimental to amplification.

Allele-specific PCR analysis in wheat is technically more difficult to perform than in diploid barley. In wheat, many PCR primers amplify sequences from more than one genome. However, considerable differences are seen when homoeologous sequences from the different genomes are compared. This has enabled STS marker Whs350-1,2 to be converted to a genome-specific PCR marker. In general, a given allele has to be amplified from the background of the alternative allele(s). This is presumably due to the rarity of 'genuine' unique sequences in wheat which has three homoeologous genomes. This problem may be overcome by the application of microsatellite markers as they represent 'genuine' single-copy sequences in wheat (Röder et al. 1995). The speed and ease of PCR make the evalu-

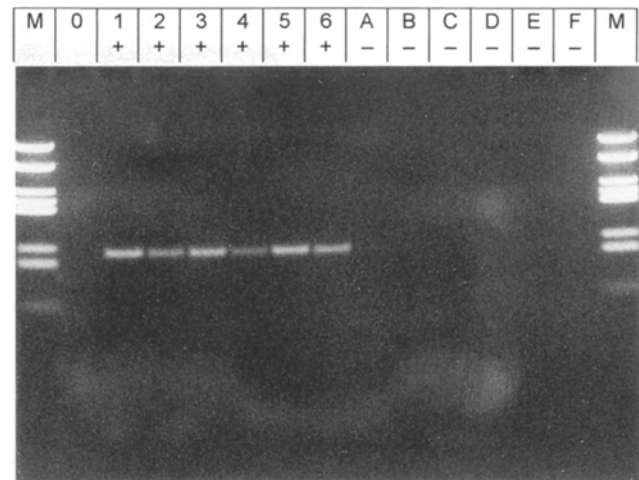

Fig. 3 Allele-specific amplification of a polymorphic site for the detection of *Pm2* resistance in wheat. (*1*) 'Nandu', (*2*) 'Knirps', (*3*) 'Planet', (*4*) 'Maris Dove', (*5*) 'Mephisto', (*6*) 'Troll', (*A*) 'Ares', (*B*) 'Turbo', (*C*) 'Herold', (*D*) 'Star', (*E*) 'Kolibri', (*F*) 'Chancellor', (*0*) Control (-DNA), (*M*) pBR322/*Alw*44I/*Mva*I

Table 3 Allele-specific amplification of DNA from various wheat lines (Anonymous 1995) with oligonucleotides indicative for the *Pm2* resistance gene

Cultivars/lines	Resistance genes	*Pm2*-Res
Chinese Spring	–	–
Chancellor	–	–
Kanzler	–	–
Ares	–	–
Club	–	–
Hanno	*Pm1*, *Pm4b*, *Pm9*	–
Chul/8*Cc	*Pm3b*	–
Sonora/8*Cc	*Pm3c*	–
Kolibri	*Pm3d*	–
Star	*Pm3d* (heterogeneous)	–
Herold	*Pm3d*	–
Turbo	*Pm3d*, *Pm4b*	–
Urban	*Pm5*	–
Ralle	*Pm3d*	–
Boheme	*Pm4b*	–
Rektor	*Pm5*	–
Clan	*Pm4b*, *Pm5*	–
Ulka/8*Cc	***Pm2***	+
Attis	*Pm1*, ***Pm2***, *Pm4b*, *Pm9*	+
Nandu	***Pm2***	+
Troll	*Pm1*, ***Pm2***, *Pm4b*	+
Mephisto	*Pm1*, ***Pm2***, *Pm9*	+
Maris Huntsman	***Pm2***, *Pm6*	+
Maris Dove	***Pm2***, *mld*	+
Planet	*Pm1*, ***Pm2***, *Pm4b*, *Pm9*	+
Knirps	***Pm2***, *Pm4b*, *Pm6*, *Pm8*	+
Sappo	*Pm1*, ***Pm2***, *Pm4b*, *Pm9*	–
Normandie	*Pm1*, ***Pm2***, *Pm9*	–
Halle 8810-47/7*Prins	***Pm2***	–
Axona	***Pm2***, *Pm3d*	–

ated markers valuable tools for selection in early generations, and will assist the pyramiding of resistance genes.

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References

Anonymous (1995) Beschreibende Sortenliste Getreide, Mais, Ölfrüchte, Leguminosen, Hackfrüchte. Bundessortenamt. Landbuchverlag, Hannover

D'Ovidio R, Anderson OD (1994) PCR analysis to distinguish between alleles of a member of a multigene family correlated with wheat bread-making quality. Theor Appl Genet 88:759–763

Feuillet C, Messmer M, Schachermayr G, Keller B (1995) Genetic and physical characterization of the *Lr1* leaf rust resistance locus in wheat (*Triticum aestivum* L.). Mol Gen Genet 248:553–562

Giese H, Holm-Jensen AG, Jensen HP, Jensen J (1993) Localization of the Laevigatum powdery mildew resistance gene to barley chromosome 2 by the use of RFLP markers. Theor Appl Genet 85:897–900

Görg R, Hollricher K, Schulze-Lefert P (1993) Functional analysis and RFLP-mediated mapping of the *Mlg* resistance locus in barley. Plant J 3:857–866

Hartl L, Weiss H, Zeller FJ, Jahoor A (1993) Use of RFLP markers for the identification of alleles of the *Pm3* locus conferring powdery mildew resistance in wheat (*Triticum aestivum* L.). Theor Appl Genet 86:959–963

Hartl L, Weiss H, Stephan U, Zeller FJ, Jahoor A (1995) Molecular identification of powdery mildew resistance genes in common wheat (*Triticum aestivum* L.). Theor Appl Genet 90:601–606

Hilbers S, Fischbeck G, Jahoor A (1992) Localization of the *Laevigatum* resistance gene *MlLa* against powdery mildew in the barley genome by the use of RFLP markers. Plant Breeding 109: 335–338

Hinze K, Thompson RD, Ritter E, Salamini F, Schulze-Lefert P (1991) Restriction fragment length polymorphism-mediated targeting of the *ml-o* resistance locus in barley (*Hordeum vulgare*). Proc Natl Acad Sci USA 88:3691–3695

Huang M, Arnheim N, Goodman MF (1992) Extension of base mispairs by *Taq* DNA polymerase: implications for single nucleotide discrimination in PCR. Nucleic Acids Res 20:4567–4573

Islam AKRM, Shepherd KW, Sparrow DHB (1981) Isolation and characterization of euplasmic wheat-barley addition lines. Heredity 46:161–174

Jensen HP, Christensen E, Jørgensen JH (1992) Powdery mildew resistance genes in 127 northwest european spring barley varieties. Plant Breed 108:210–228

Kwok S, Kellogg DE, McKinny N, Spasic D, Goda L, Sninsky JJ (1990) Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type-I model studies. Nucleic Acids Res 18:999–1005

Ma ZQ, Sorrells ME, Tanksley SD (1994) RFLP markers linked to powdery mildew resistance genes *Pm1*, *Pm2*, *Pm3* and *Pm4* in wheat. Genome 37:871–875

Niewöhner J, Salamini F, Gebhardt C (1995) Development of a PCR assay diagnostic for RFLP marker alleles closely linked to alleles *Gro1* and *H1*, conferring resistance to the root cyst nematode *Globodera rostochiensis* potato. Mol Breed 1:65–78

Olson M, Hood L, Cantor CH, Botstein D (1989) A common language for the physical mapping of the human genome. Science 24:1434–1435

Röder MS, Plaschke J, König SU, Börner A, Sorrells ME, Tanksley SD, Ganal MW (1995) Abundance, variability and chromosomal location of microsatellites in wheat. Mol Gen Genet 246:327–333

Schachermayr G, Siedler H, Gale MD, Winzeler H, Winzeler M, Keller B (1994) Identification and localization of molecular markers linked to the *Lr9* leaf rust resistance gene of wheat. Theor Appl Genet 88:110–115

Schachermayr GM, Messmer MM, Feuillet C, Winzeler H, Winzeler M, Keller B (1995) Identification of molecular markers linked to the *Agropyron elongatum*-derived leaf rust resistance gene *Lr24* in wheat. Theor Appl Genet 90:982–990

Schönfeld M, Ragni A, Fischbeck G, Jahoor A (1996) RFLP mapping of three new loci for resistance genes to powdery mildew (*Erysiphe graminis* f. sp. *hordei*) in barley. Theor Appl Genet 93:48–56

Schüller C, Backes G, Fischbeck G, Jahoor A (1992) RFLP markers to identify the alleles on the *Mla* locus conferring powdery mildew resistance in barley. Theor Appl Genet 84:330–338

Sears ER (1966) Nullisomic-tetrasomic combinations in hexaploid wheat. In: Riley R, Lewis KR (eds) Chromosome manipulation and plant genetics. Oliver and Boyd, Edinburgh, pp 29–45

Shattuck-Eidens DM, Bell RN, Mitchell JT, McWorther VC (1991) Rapid detection of maize DNA sequence variation. GATA 8:240–245

Talbert LE, Blake NK, Chee PW, Blake TK, Magyar GM (1994) Evaluation of 'sequence-tagged-site' PCR products as molecular markers in wheat. Theor Appl Genet 87:789–794

Tragoonrung S, Kanazin V, Hayes PM, Blake TK (1992) Sequence-tagged-site-facilitated PCR for barley genome mapping. Theor Appl Genet 84:1002–1008

Williams GK, Kubelik AR, Kenneth JL, Rafalski A, Scott VT (1991) DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535

Xu L, Hall BG (1994) SASA: a simplified, reliable method for allele-specific amplification of polymorphic sites. BioTechniques 6:44–45