Theor Appl Genet (1998) 97: 479–487

K. W. Pomper · A. N. Azarenko · N. Bassil
J. W. Davis · S. A. Mehlenbacher

Identification of random amplified polymorphic DNA (RAPD) markers for self-incompatibility alleles in *Corylus avellana* L.

Received: 10 January 1998 / Accepted: 26 January 1998

Abstract Random amplified polymorphic DNA (RAPD) markers were identified for self-incompatibility (SI) alleles that will allow marker-assisted selection of desired S-alleles in hazelnut (*Corylus avellana* L.). DNA was extracted from young leaves collected from field-planted parents and 26 progeny of the cross OSU 23.017 (S_1S_{12}) × VR6-28 (S_2S_{26}) (OSU23 × VR6). Screening of 10-base oligonucleotide RAPD primers was performed using bulked segregant analysis. DNA samples from 6 trees each were pooled into four 'bulks', one for each of the following: S_1 S_2, S_1 S_{26}, S_2 S_{12}, and S_{12} S_{26}. 'Super bulks' of 12 trees each for S_1, S_2, S_{12}, and S_{26} were then created for each allele by combining the appropriate bulks. The DNA from these four super bulks and from the parents was used as a template in the PCR assays. A total of 250 primers were screened, and one RAPD marker each was identified for alleles S_2 ($OPI07_{750}$) and S_1 ($OPJ14_{1700}$). $OPJ14_{1700}$ was identified in 13 of 14 S_1 individuals of the cross OSU23 × VR6 used in bulking and yielded a false positive in 1 non-S_1 individual. This same marker was not effective outside the original cross, identifying 4 of 5 S_1 progeny in another cross, 'Willamette' × VR6-28 ('Will' × VR6), but yielded false positives in 4 of 9 non-S_1 individuals from the cross 'Casina' × VR6-28 ('Cas' × VR6). $OPI07_{750}$ served as an excellent marker for the S_2 allele and was linked closely to this allele, identifying 12 of 13 S_2 individuals in the OSU23 × VR6 population with no false positives. $OPI07_{750}$ was found in 4 of 4 S_2 individuals from 'Will' × VR and 7 of 7 S_2 individuals of 'Cas' × VR6 with no false positives, as well as 10 of 10 S_2 individuals of the cross OSU 296.082 (S_1S_8) × VR8-32 (S_2S_{26}), with only 1 false positive individual out of 21 progeny. $OPI07_{750}$ was also present in 5 of 5 cultivars carrying the S_2 allele, with no false-positive bands in non-S_2 cultivars, and correctly identified all but 2 S_2 individuals in 57 additional selections in the breeding program. In the OSU23 × VR6 population, the recombination rate between the marker $OPJ14_{1700}$ and the S_1 allele was 7.6% and between the $OPI07_{750}$ marker and the S_2 allele was 3.8%. RAPD marker bands were excised from gels, cloned, and sequenced to enable the production of longer primers (18 or 24 bp) that were used to obtain sequence characterized amplified regions (SCARs). Both the S_1 and S_2 markers were successfully cloned and 18 bp primers yielded the sole $OPJ14_{1700}$ product, while 24-bp primers yielded $OPI07_{750}$ as well as an additional smaller product (700 bp) that was not polymorphic but was present in all of the S-genotypes examined.

Key words Hazelnut · Filbert · Sporophytic self-incompatibility (SSI) · Bulked segregant analysis (BSA) · Sequence characterized amplified regions (SCARs)

Oregon Agricultural Experiment Station Technical Paper No. 11299

Communicated by H. F. Linskens

K. W. Pomper (✉)[1] · A. N. Azarenko · N. Bassil
J. W. Davis · S. A. Mehlenbacher
Department of Horticulture, Oregon State University, Ag and Life Sciences 4017, Corvallis, OR 97331, USA

Present address:
[1] Atwood Research Facility, Kentucky State University, Frankfort, KY, W0601, USA

Introduction

The cultivated hazelnut (*Corylus avellana* L.) is monoecious, protandrous, and wind-pollinated (Lagerstedt 1975; Thompson 1979a). Most cultivars bloom in midwinter (December to February) in Oregon. The male inflorescences are catkins, and female inflorescences lack a perianth and appear as a tuft of red stigmatic styles protruding from the apex of compound buds borne on shoots or catkin peduncles.

Self-incompatibility is a genetic mechanism forcing cross-pollination in plants and is most frequently controlled by a single multi-allelic locus (the S-locus). Two main forms of self-incompatibility exist, gametophytic (GSI) and sporophytic (SSI) (Matton et al. 1994). SSI has been reported in the woody perennial *Corylus avellana* L. (hazelnut) of the *Betulaceae*. In SSI, if the same S-allele expressed in a pollen grain is also expressed in the recipient pistil, pollen-tube growth is arrested on the stigmatic surface (Hampson et al. 1993; Matton et al. 1994). Pairs of S-alleles are always codominant in the style and either dominant or codominant in the hazelnut pollen (Thompson 1979b; Mehlenbacher and Thompson 1988). To date, 25 unique S-alleles have been identified in hazelnut (Mehlenbacher 1997). SSI can dictate the direction of crosses and prevent many desirable ones in hazelnut breeding programs, therefore compatible pollinizer trees must also be planted in orchards to ensure good nut set. Molecular markers for S-alleles could be used in marker-assisted selection of young hazelnut seedlings years before the S-alleles of individual trees could be typed at flowering and thereby enable selection strategies for rare allelic combinations desirable in future cultivars or for the development of universal pollinizers.

Random amplified polymorphic DNA (RAPD) markers are generated via the polymerase chain reaction (PCR) using short primers (10 bases) of an arbitrary sequence and a lower annealing temperature than standard PCR reactions (Welsh and McClellan 1990; Williams et al. 1990). RAPD markers have been used in the detection of DNA polymorphisms in plants and been utilized in cultivar fingerprinting (Xu et al. 1995) and in the identification of markers for specific genes (Martin et al. 1991; Paran et al. 1991; Michelmore et al. 1991).

Many horticultural crops are clonally propagated, such as apple and hazelnut, and are highly heterozygous (Mehlenbacher 1995). Breeding of these crops can utilize the modified backcross method where a different recurrent parent is used in each backcross generation to avoid inbreeding. Since the parents are highly heterozygous, a tremendous amount of segregation can be observed in the F_1 progeny allowing linkage map construction. However, the development of near-isogenic lines of hazelnut is not feasible due to high heterozygosity and long generation times.

Bulked segregant analysis (BSA) utilizes a population segregating for a gene of interest (Michelmore et al. 1991; Giovannoni et al. 1991). The population is screened for the presence or absence of a phenotypic character. DNA samples from individuals are pooled to create bulks containing or devoid of the phenotype of interest. Any polymorphic products generated by RAPD analysis must arise from DNA linked closely to the target locus, since it is the only region in the DNA samples that differs significantly between the two bulks. This approach has been utilized in the identification of markers for several resistance genes in plants (Paran et al. 1991; Michelmore et al. 1991; Adam-Blondon et al. 1994). In hazelnut, F_1 progeny (considered to represent a segregating population due to the high heterozygosity of the parents) could be bulked according to S-allele type and used in a PCR-RAPD screening.

Map-based cloning has been successfully used to clone resistance genes in several plants (Martin et al. 1993, Mindrinos et al. 1994; Song et al. 1995; Dixon et al. 1996). Sequence-characterized amplified regions (SCARs) can be derived by cloning and sequencing the two ends of the amplified products of RAPD markers, which enables the synthesis of longer primers that offer highly reproducible amplification of single loci at high annealing temperatures (Paran and Michelmore 1993). SCARs could be used for screening genomic libraries [e.g., yeast artificial chromosomes (YAC) or bacterial artificial chromosome (BAC) libraries] for physical mapping of regions of chromosomes to obtain overlapping clones in the process of chromosome walking in order to clone a gene of interest (Debener et al. 1991; Martin et al. 1991; Paran and Michelmore 1993; Adam-Blondon et al. 1994; Frijters et al. 1997).

Our long-term goal is to understand the basic mechanism of SSI in hazelnut so that biotechnological approaches can be explored to manipulate SSI in present and future cultivars. The objective of the study reported here was to utilize the RAPD and BSA methodologies to identify RAPD and SCAR markers for hazelnut S-alleles that will allow marker-assisted selection of desired S-genotypes. Obtaining SCARs from the RAPD markers identified would also assist in efforts toward the eventual cloning of the locus responsible for SSI in hazelnut.

Materials and methods

Plant material

All hazelnut genotypes used in this study were planted at the Oregon State University (OSU) Vegetable Farm in Corvallis, Oregon, and all S-allelic combinations had been previously identified using the method of Thompson (1979a). Young leaf material that included an apical meristem was collected in mid-summer from the parents and 26 field-planted progeny of the cross OSU 23.017 (S_1S_{12}) × VR6-28 (S_2S_{26}) as well as from 21 field -planted progeny of the cross OSU 296.082 (S_1S_8) × VR8-32 (S_2S_{26}), 9 progeny from the cross 'Casina' ($S_{10}S_{21}$) × VR6-28, 7 progeny from the cross 'Willamette' (S_1S_3) × VR6-28, the cultivars 'Barcelona' (S_1S_2), 'Montebello' (S_1S_2), 'Ribet' (S_2S_{16}), 'Willamette' (S_1S_3), 'Gem' (S_2S_{14}), and 'Ennis' (S_1S_{11}), and 58 additional selections used in the OSU breeding program as found in Table 1.

DNA isolation

DNA was extracted basically using the method of Davis (1998). About 1 g of young leaf material was homogenized with a small mortar and pestle with 6 ml of cold extraction buffer [0.35 m*M* sucrose, 100 m*M* TRIS-HCl, 50 m*M* KCl, 5% polyvinylpyrrolidone (F.W. 40,000), 25 m*M* EDTA, 10 m*M* diethyldithiocarbamic acid, and 0.001% mercaptoethanol] and strained through cheesecloth into a chilled beaker. This homogenate was placed into 1.5-ml microfuge tubes (Fisher Scientific, Pittsburgh, Pa.) and centrifuged for 5 min at 4°C (10,000 *g*). For each tube, the supernatant was discarded, the pellet re-suspended in 750 µl lysing buffer (100 m*M* EDTA and 50 m*M* TRIS-HCl), 75 µl 25% Triton X-100, 75 µl 20% sarkosyl, and 5 µl proteinase K (Fisher Scientific, Pittsburgh, Pa.), vortexed briefly, and incubated for 1 h at 37°C. The mixture was occasionally mixed by inverting the tube every 20 min during incubation. After centrifugation for 5 min (10,000 *g*), isopropanol was added to the removed supernatant, and precipitated at −20°C for at least 1 h. The resulting pellet was re-suspended in 220 µl high-salt, 1 × TE buffer (10 m*M* TRIS-HCl, 1 m*M* EDTA, 2 *M* NaCl, pH 8.0) overnight at 4°C. After a phenol : chloroform : isoamyl alcohol (1 : 1 : 1/24) extraction, the nucleic acids were ethanol-precipitated. The pellet was subsequently rinsed with 70% ethanol and dried under a vacuum. The nucleic acid pellet was then re-suspended in

Table 1 Additional hazelnut selections examined from the OSU breeding program

OSU	
228.084 (S_1S_2)	440.105 (S_1S_2)
238.125 (S_1S_4)	443.107 (S_1S_2)
243.002 (S_3S_8)	446.017 (S_1S_2)
267.140 (S_2S_7)	446.018 (S_1S_2)
275.031 (S_2S_8)	446.106 (S_1S_2)
276.142 (S_3S_8)	447.031 (S_3S_{22})
278.113 (S_7S_8)	447.113 (S_1S_2)
287.008 (S_1S_2)	449.127 (S_2S_3)
309.074 (S_2S_{12})	450.033 (S_1S_2)
311.039 (S_1S_2)	468.090 (S_8S_{20})
312.030 (S_1S_{10})	469.038 (S_2S_3)
313.078 (S_2S_{12})	469.058 (S_8S_{17})
315.096 (S_1S_2)	470.009 (S_2S_3)[a]
325.091 (S_2S_7)	474.013 (S_1S_2)[a]
332.097 (S_2S_8)	475.091 (S_1S_2)
336.039 (S_3S_8)	486.001 (S_2S_{14})
350.091 (S_8S_{10})	487.049 ($S_{10}S_{26}$)
360.041 (S_8S_{20})	487.066 ($S_{10}S_{26}$)
369.066 (S_1S_2)	487.078 (S_2S_{10})
414.062 (S_1S_1)	488.022 (S_1S_{26})
436.070 (S_1S_{12})	494.087 (S_3S_2)
437.028 (S_1S_2)	504.065 (S_1S_2)
437.074 (S_1S_{26})	509.022 (S_3S_{12})
438.054 (S_2S_{12})	509.064 (S_3S_{15})
440.005 (S_2S_8)	510.041 (S_3S_{15})
440.007 (S_2S_3)	512.086 (S_2S_{23})
440.013 (S_3S_{26})	B-3 (S_2S_{25})
440.022 (S_2S_8)	VR17-19 (S_2S_3)
440.054 (S_2S_3)	Zimmerman (S_1S_3)

[a] Indicates that $OPI07_{750}$ was not present in these S_2 genotypes, with this marker being present in all other S_2 individuals in this table with no false positives

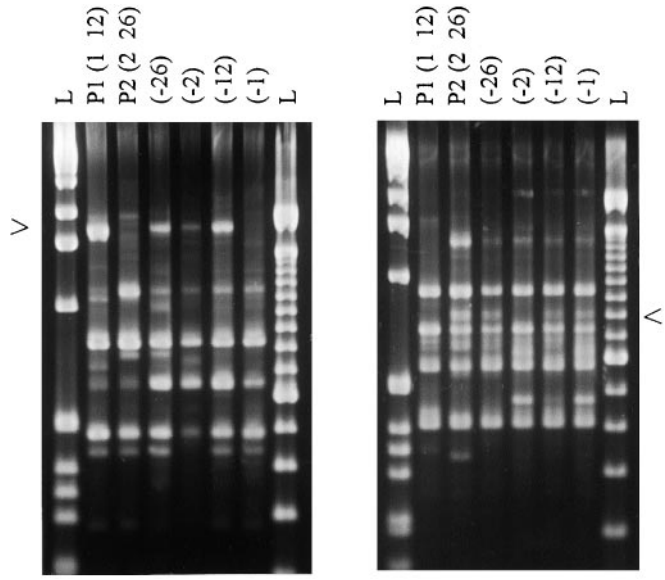

Fig. 1 Parents (OSU 23.017 × VR6-28) and super bulks containing progeny missing (-) either the S_{26}, S_2, S_{12}, or S_1 allele. *Left gel* The OPJ14 primer (10-mer) shows an amplification product at about 1.7 kb present only in OSU 23.017 (S_1S_{12}) and missing in lane 7 ($-S_1$), indicating this product is a potential marker for the S_1 allele. *Right gel* The OPI07 primer (10-mer), a potential S_2 marker, amplifies a product at about 0.75 kb in the parent VR6-28 (S_2S_{26}) and not in *lane 5* ($-S_2$). *L lane 1* is a 1 kb DNA ladder, while *lane 8* is a 100-bp DNA ladder

110 µl of 1 × TE buffer(10 m*M* TRIS-HCl, 1 m*M* EDTA, pH 8.0). Further purification of the DNA was achieved by digesting the RNA with RNase A (Sigma Chemical, St. Louis, Mo.) for 30 min at 37°C. The sample was again subjected to phenol : chloroform : isoamyl alcohol extraction and precipitation with 95% ethanol (with the addition of 0.1 vol. of 3 *M* NaOAc; pH 5.2). After centrifugation, the pellet was rinsed with 70% ethanol and dried under vacuum as described above. The DNA was reconstituted in 20 µl 1 × TE buffer and quantified using a Beckman Model 34 spectrophotometer.

In a BSA approach (Michelmore et al. 1991), 1 µg of DNA from each of 6 progeny trees of the cross OSU 23.017 × VR6-28 were pooled into four 'bulks', one for each of the following pairs of alleles: S_1S_2, S_1 S_{26}, S_2 S_{12}, and S_{12} S_{26}. Progeny of this cross were used in preparing DNA bulks for screening primers because it was the largest population available where S-alleles of the individuals had been identified. 'Super bulks' of 12 trees each were created by combining appropriate 'bulks' so that each super bulk contained progeny deficient in either the S_1, S_2, S_{12}, or S_{26} allele (See Fig. 1). The DNA from these four super bulks and the parents was used as a template in the PCR assays.

RAPD reactions

Reaction conditions for PCR amplification were those recommended by Williams et al. (1990). Reaction mixtures contained 10 m*M* TRIS-HCl, pH 8.3, 50 m*M* KCl, 2.0 m*M* MgCl, 0.1% Triton X-100, 100 m*M* each of dATP, dTTP, dCTP, and dGTP; 0.2 m*M* primer; 2.5 ng of genomic DNA template, and 0.5 U of *Taq* polymerase (Promega, Madison, Wis.) in a final reaction mixture of 15 µl. Two hundred and fifty 10-mer primers from Operon Technologies (OP; kits F, G, H, I, and J; Alameda, Calif.) and primers 101–200 and 300–350 from the University of British Columbia (UBC) were used to amplify DNA products. Reactions were overlaid with mineral oil. Amplifications were carried out using a Perkin Elmer Cetus DNA Thermal cycler programmed for 45 cycles of 1 min at 94°C, 1 min and 30 s at 37°C, 30 s at 54°C, 2 min at 72°C, followed by a final extension period of 15 min at 72°C and a hold at 4°C until recovery. Amplification products were separated by electrophoresis on 2% agarose gels and photographed using Polaroid 667 black and white film on a UV transilluminator after ethidium bromide staining. After identification of promising polymorphisms for each allele from the super bulks, individuals were tested to confirm a correlation with the alleles. A RAPD marker was considered to be a useful genetic marker if it was easily scorable and reproducible.

Cloning and sequencing RAPD markers

RAPD marker bands for S_1 and S_2 alleles derived from OSU 23.017 × VR6-28 were excised from 1% low-melting-point (LMP) agarose gels and the fragment DNA purified using a GELase™ Agarose Gel-Digesting Preparation kit (Epicentre Technologies, Madison, Wis.). After agarose gel digestion, the DNA was precipitated with 95% ethanol, rinsed in 70% ethanol, re-suspended in sterile water, and quantified via spectrophotometry. The excised RAPD bands were cloned using the pGEM-T Vector System (Promega, Madison Wis.) and introduced into competent *E. coli* JM109 High-Efficiency cells according to the supplier's instructions. Recombinants were identified as white colonies on LB plates with X-gal and IPTG. These colonies were further screened by taking a stab of a transformant colony, boiling it in 20 µl of sterile water

and then using 1.2 µl of the solution in an amplification reaction with the original primer. Amplification products were run on 2% agarose gels, stained with ethidium bromide, and photographed. Putative positive transformants, based on the size of the amplification product, were further characterized. Plasmid DNA was isolated from putative recombinants with the QIAprep Spin Plasmid Kit (QIAgen, Chatsworth, Calif.) using the standard protocol provided by the manufacturer. An aliquot of purified plasmid DNA was analyzed again by PCR, while another aliquot was digested by *Sal*I and *Nco*I restriction enzymes (Stratagene, Calif.). The resulting products were run on 2% agarose gels, stained with ethidium bromide, and photographed. Again putative positive transformants were evaluated based on the size of the amplification product and the size of the plasmid insert. Plasmid DNA from several promising putative transformants was sequenced by the Central Services Laboratory of the OSU Center for Gene Research and Biotechnology using T7 and SP6 sequencing primers.

Sequence-specific amplifications

Sequence-specific primers were synthesized by the Central Services Laboratory of the OSU Center for Gene Research and Biotechnology. Primers were designed by adding 8 or 14 bases to the 3′ end of the original 10-mer to make SCARs as described by Paran and Michelmore (1993). Amplifications were carried out using a Perkin Elmer Cetus DNA Thermal cycler programmed for 45 cycles of 1 min at 94°C, 1 min at 50°C (initially), 2 min at 72°C, a final extension period of 15 min at 72°C, and a hold at 4°C until recovery. A series of annealing temperatures were utilized from 50° to 68°C in 2°C increments in the PCR reaction conditions in an effort to identify an annealing temperature that would yield single, strongly amplified bands. Amplification products were electrophoresed on 2% agarose gels and photographed on a UV transilluminator after ethidium bromide staining.

Results

RAPD markers

A total of 250 primers were screened and one RAPD marker each was identified for alleles S_1 ($OPJ14_{1700}$) and S_2 ($OPI07_{750}$). Screening of parents and bulks with the OPJ14 primer showed an amplification product about 1.7 kb in length that was present only in OSU 23.017 (S_1S_{12}) and was missing in super bulk no. 4 ($-S_1$), indicating that this product was a potential marker for the S_1 allele (Fig. 1, left gel). $OPJ14_{1700}$ was identified in 13 of 14 progeny of OSU 23.017 × VR6-28 with 1 false positive (Fig. 2A, lane 29). The recombination rate between the marker $OPJ14_{1700}$ and the S_1 allele was 7.6%. The $OPJ14_{1700}$ RAPD marker was also present in 4 of 5 S_1 individuals from progeny of the cross 'Willamette' × VR6-28, where VR6-28 was a common parent of the bulked population used in initial primer screening (Fig. 3 A). In progeny of the cross of 'Casina' × VR6-28 there were no S_1 progeny in the population, and yet 4 of 9 individuals showed a RAPD band at the same position as the S_1 RAPD marker in the other two crosses discussed above (Fig. 3A). The primer $OPJ14_{1700}$ also identified the S_1 allele in 4 of 4 S_1 cultivars (Fig. 3A). However, there was a RAPD band indicating the presence of the S_1 allele in cv 'Ribet' and VR17-19, neither of which carries the S_1 allele. The amplification product $UBC180_{800}$ also showed promise as an additional marker for the S_1 allele, but amplification was much less consistent than with $OPJ14_{1700}$ (data not shown).

When the parents and bulks were screened with the OPI07 primer an amplification product of about 0.750 kb was present only in the parent VR6-28 (S_2 S_{26}) and was missing in super bulk no. 2 ($-S_2$) (Fig. 1; right gel) indicating that it is a potential S_2 marker. $OPI07_{750}$ was identified in 12 of 13 progeny of OSU 23.017 × VR6-28 with no false positives (Fig. 2B). The recombination rate between the $OPI07_{750}$ marker and the S_2 allele was 3.8%. $OPI07_{750}$ was subsequently identified in 4 of 4 S_2 individuals from the cross 'Willamette' × VR6-28 (Fig. 3B) and in 7 of 7 S_2 progeny from the cross 'Casina' × VR6-28. The primer $OPI07_{750}$ was also able to identify the S_2 allele in 5 of 5 S_2 cultivars. Furthermore, $OPI07_{750}$ was identified in 10 out of 10 S_2 progeny of the cross OSU 296.082 × VR8-32, with 1 false positive out of 21 progeny. $OPI07_{750}$ was also identified in 32 of 34 S_2 individuals from a collection of germplasm utilized in the OSU breeding program found in Table 1. $OPI07_{750}$ served as an excellent marker for the S_2 allele and appeared to be more closely linked to the S_2 allele than the $OPJ14_{1700}$ maker was to the S_1 allele because it was consistent in identifying the S_2 allele in the screening population and the other crosses and cultivars.

Cloning and sequencing RAPD markers

The amplification products of 35 recombinant colonies each for $OPJ14_{1700}$ and $OPI07_{750}$ were screened and four putative positive transformants for $OPJ14_{1700}$ and ten for $OPI07_{750}$ were identified based on the size of the amplification product (data not shown). Of these, two of each of the $OPJ14_{1700}$ and $OPI07_{750}$ putative transformants were sequenced approximately 450 base pairs from either end of the RAPD product. The two sequences of the putative $OPJ14_{1700}$ clones were very similar, with a 92% homology, while the two $OPI07_{750}$ clones had a 99% homology. Based on sequence information derived from all cloned RAPD bands, 18-mer and 24-mer oligonucleotide primer pairs for $OPJ14_{1700}$ and $OPI07_{750}$, respectively, were synthesized for use in PCR (Table 2).

Sequence-specific amplifications

Screening of parents and progeny of OSU 23.017 × VR6-26 with the $OPJ14A_{1700}$ (18-mer) primer pair (Table 2), derived from the cloned RAPD band, yielded a single amplification product at the same

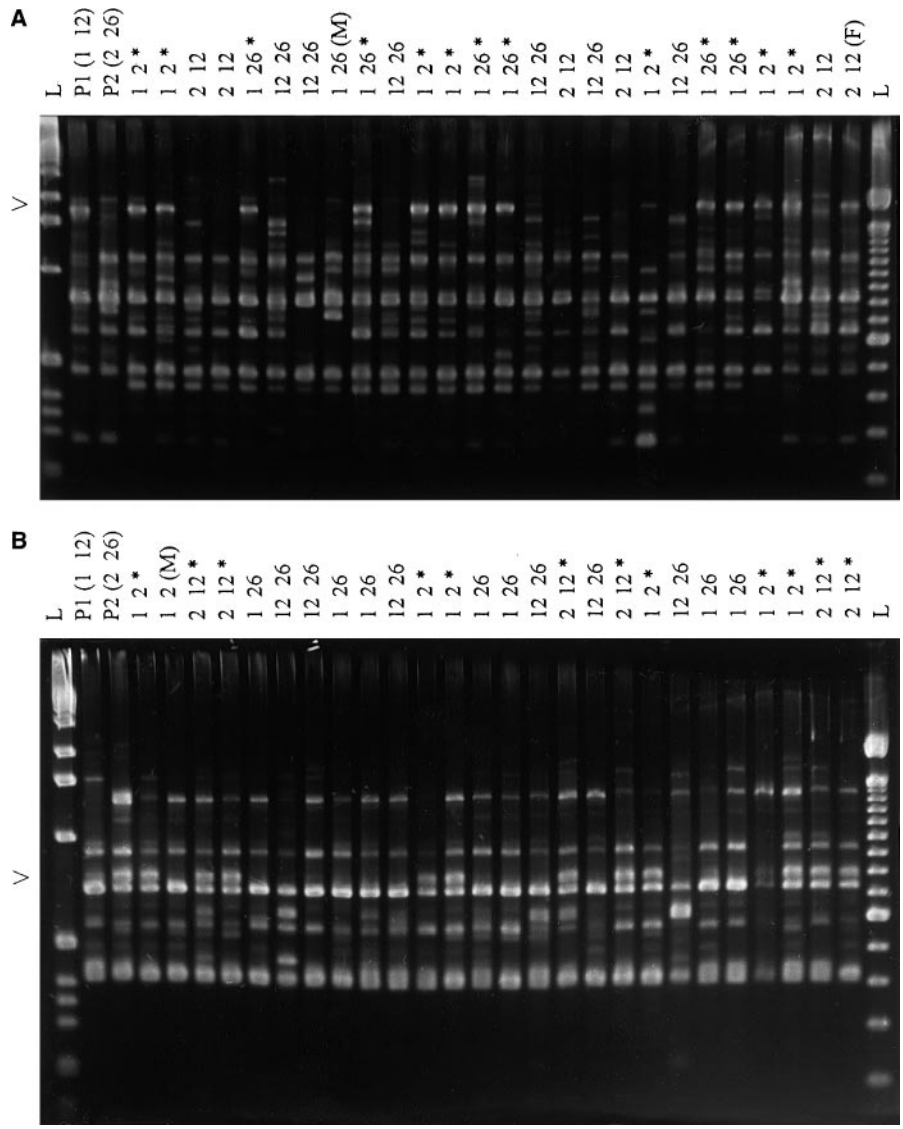

Fig. 2A,B Screening of the parents and F_1 progeny of OSU 23.017 × VR6-28 for $OPJ14_{1700}$ (**A**) and $OPI07_{750}$ (**B**). **A** This marker is present in the S_1 parent (*lane 2*) and in 13 progeny (indicated by *lanes* with an ∗); it is (*M*) from the individual in *lane 11*, and there is one false-positive (*F*) identification in *lane 29*. **B** This marker is present in the S_2 parent (*lane 3*) and 12 of 13 F_1 progeny, but missing (*M*) from the S_2 individual in *lane 5*, *L Lane 1* is a 1-kb DNA ladder, while *lane 30* is a 100-bp DNA ladder

position as the RAPD primer $OPJ14_{1700}$ (10-mer) (see Figs. 2 A and 3A) with 54°C being the optimal annealing temperature for producing a single, strongly amplified band. The $OPJ14B_{300}$ (18-mer) primer pair, which utilized the same 5′ end primer as $OPJ14A_{1700}$ and a 3′ end primer 300 bp internal to that end, yielded the same pattern as the RAPD primer $OPJ14_{1700}$ with a 300 bp amplification product (Figs. 2 A and 3B). When the $OPJ14B_{300}$ primer pair was used with individuals of 'Willamette' × VR6-28, 'Casina' × VR6-28, and selected cultivars, it yielded the same amplification pattern as the original rapid primer $OPJ14_{1700}$. Amplification products were still obtained in S_1 samples when templates from S_1 and non-S_1 individuals were mixed prior to PCR reactions, indicating that the polymorphic response was not due to substances inhibitory to the PCR reaction in the non-S_1 individuals. Interestingly, when the $OPJ14B_{300}$ primer pair was used with VR17-19, there were three distinct products generated: one was at the expected size of 300 bp and the two other bands were slightly larger. Three distinct bands were not distinguishable using the $OPJ14A_{1700}$ primer pair (Fig. 4). Based on the segregation pattern of the $OPJ14A_{1700}$ and $OPJ14B_{300}$ markers with the original RAPD primer $OPJ14_{1700}$, it appears we have successfully cloned the original $OPJ14_{1700}$ product.

The primer pair for the $OPI07_{750}$ product was used to screen all populations and selections previously examined, and in all cases yielded two products, a larger 750-bp product that segregated with the S_2 containing individuals and a 700-bp product that was present in all individuals irrespective of S-genotype (Fig. 5). A series of annealing temperatures was utilized from 50° to 68°C in 2°C increments for PCR reactions, but none eliminated the presence of the second 700-bp product.

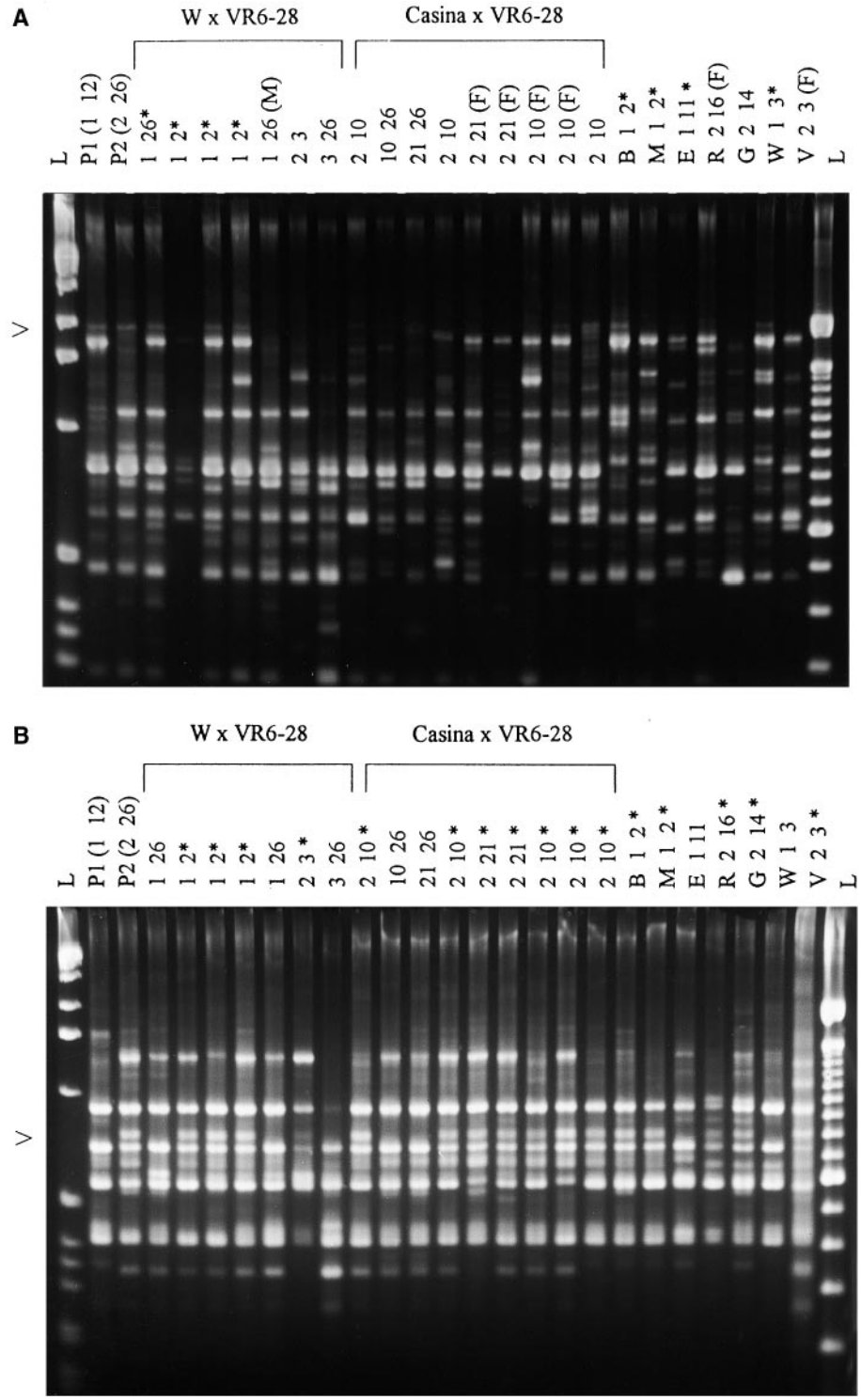

Fig. 3 **A** The RAPD marker $OPJ14_{1700}$ is present in 4 of 5 S_1 individuals (present in lanes denoted with an * but missing (*M*) in *lane 8* from the cross 'Willamette' (W) × VR6-28. In progeny of 'Casina' × VR6-28 there were no S_1 progeny and yet 4 of 9 individuals showed a false-positive (*F*) RAPD band (*lanes 15–18*) at the same position as in OSU 23.017 × VR6-26. $OPJ14_{1700}$ is also present in 4 of 4 S_1 cultivars, but yields false positives in 'Ribet' (*lane 21*) and VR17-19 (*lane 24*). **B** $OPI07_{750}$ is present (∗) in 4 of 4 S_2 individuals of W × VR6-28 and in 7 of 7 S_2 progeny of the cross 'Casina' × VR6-28. $OPI07_{750}$ also present in 5 of 5 S_2 cultivars with no false-positive bands present in any of the cultivars or progeny of the crosses examined. *L lane 1* is a 1-kb DNA ladder, while *lane 30* is a 100-bp DNA ladder. *B* 'Barcelona' · *M* 'Montebello' · *E* 'Ennis' · *R* 'Ribet' · *G* 'Gem' · *W* 'Willamette' · *V* VR17-19

Discussion

Molecular markers for the S_1 ($OPJ14_{1700}$) and S_2 ($OPI07_{750}$) alleles in hazelnut were successfully identified using RAPD methodology in conjunction with BSA. This study is the first report of the identification of RAPD markers that are specific for individual S-alleles in a plant. The use of heterozygous bulks appears to reduce the probability of detecting an unlinked polymorphism (Melchinger 1990). Benet et al. (1995), using a BSA approach with selfed progeny from highly heterozygous parents, found only 3 RAPD markers linked to black spot resistance in Chinese elm

Table 2 Sequence of 18-mer and 24-mer oligonuleotide primers pairs derived from cloned RAPD bands

Allele	Primer pair[a]	Sequence[b]	Polymorphism
S_1	$OPJ14AU_{1700}$	5′-CAC CCG GAT GTA GAG TTC-3′	Yes
	$OPJ14AL_{1700}$	5′-CAC CCG GAT GCT CAG AAC-3′	
	$OPJ14BU_{300}$	5′-CAC CCG GAT GTA GAG TTC-3′	Yes
	$OPJ14BL_{300}$	5′-CAC CCG GAT GAT ACG GTG-3′	
S_2	$OPI07U_{750}$	5′-CAG CGA CAA GAG TAG TTG TGT ACT-3′	Yes
	$OPI07L_{750}$	5′-CAG CGA CAA GTG ATT GTG ACG AAT-3′	

[a]The third letter and the first and second numbers refer to the kit and primer number (Operon Technologies), respectively, used to identify the progenitor RAPD marker. A and B are arbitrary letters to differentiate the primers sets. U = upstream 5′ end, L = lower 3′ end
[b]The underlined sequence represents the sequence of the progenitor RAPD primers

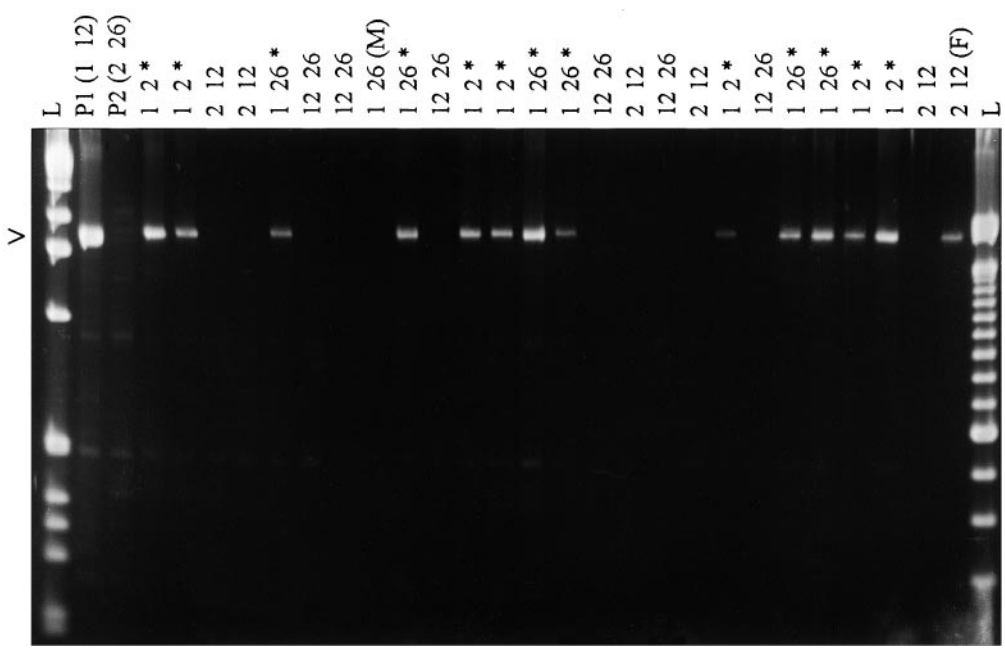

Fig. 4 Screening of the parents and progeny of OSU 23.017 × VR6-26 with the $OPJ14A_{1700}$ (18-mer) primer pair yields a single product at the same position as the $OPJ14_{1700}$ (10-mer) marker seen in Fig. 2A. *L* Lane 1 is a 1-kb DNA ladder, while *lane 30* is a 100-bp DNA ladder. *F* False positive, *M* missing

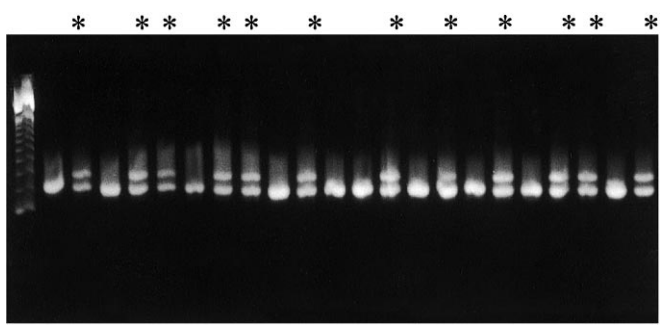

Fig. 5 Screening of the parents and progeny of the cross OSU 296.082 (S_1 S_8) × VR8-32 (S_2 S_{26}), OSU 23.017 × VR6-26 with the $OPI07_{750}$ (24-mer) primer pair yielded a single product at the same position as the $OPI07_{750}$ (10-mer) marker seen in Fig. 2B, with an additional 700-bp product in all genotypes. *Lanes 1* 100-bp DNA ladder, *2* 296.082 (S_1S_8), *3* VR8-32 (S_2S_{26}). F_1 progeny with the S_2 allele are found in *lanes 5, 6, 8, 9, 11, 14, 16, 18, 20, 21, 23,* and *24,* with all other lanes containing non-S_2 individuals. ∗ *above* lane = S_2 individual

after testing 220 primers. Koller et al. (1994) identified only 2 primers out 400 that showed polymorphic PCR products using bulks from cultivars resistant or susceptible to apple scab. In this study, we identified 2 primers out of 250 that yielded the desired polymorphic pattern, which is similar to that reported above. Screening of the four S-allele super bulks instead of two bulks, as is the case in studies identifying disease resistance markers using resistant and susceptible bulks, complicated the screening procedure and decreased the number of primers that could have been screened with each thermocycler run.

$OPI07_{750}$ was closely linked to the S_2 allele and can be utilized in marker-assisted screening for this S-genotype in hazelnut. This RAPD marker was able to identify the S_2 allele even in cultivars with genetic backgrounds very different from that of the bulking population (OSU × VR). Over 6,000 seedlings were screened using a SCAR marker for eastern filbert blight

(EFB) resistance in 1996 in the hazelnut breeding program at OSU. This demonstrates that mass screening of hazelnut seedlings can be accomplished and that screening could also be performed using either RAPD or SCAR markers for S-alleles. Template DNA from seedlings used in EFB resistance screening could also be utilized in PCR reactions to screen for desired S-alleles. Progeny of the cross of OSU 23.017 × VR6-28 displaying the S_1, S_2, S_{12}, and S_{26} alleles were used in this study in preparing DNA bulks for screening primers because it was the largest population available where S-alleles of the individuals had been identified. Traditional typing of other segregating populations with rare S-alleles will allow the identification of RAPD markers for these alleles.

The long-term goal of this laboratory is to identify and characterize the S-locus protein and encoding DNA and RNA sequences. SSI is displayed in species in the genus *Brassica*, and the mechanism of self-incompatibility is well-characterized (see Scutt et al. 1990; Stein et al. 1991; Trick and Heizman1992; Matton et al. 1994). Unfortunately, PCR-generated probes corresponding to the two conserved regions of SLG from *Brassica* do not hybridize with hazelnut DNA (Hampson et al. 1996). Therefore, it would appear that S-genes cloned from *Brassica* will not be useful for exploring SSI in hazelnut.

The original $OPJ14_{1700}$ RAPD product was successfully cloned, but unfortunately both the RAPD and SCAR marker were not tightly linked to the S_1 allele based on a recombination rate of 7.6%. The SCAR marker will not be useful for marker-assisted selection and is likely not close enough to the S-locus to assist in chromosome walking in efforts to clone the allele. The $OPJ14B_{300}$ primer pair yielded three distinct amplification products of about 300 bp in the selection VR17-19. It is not known whether there are multiple copies of homologous DNA of slightly larger sizes present in this selection or whether some other PCR event is leading to this series of products. The RAPD marker for the S_2 allele ($OPI07_{750}$) was also successfully cloned, however there was an additional smaller product that was amplified in all S-genotypes. RAPD polymorphism can be caused by differences in the nucleotide sequence at the priming sites, by structural rearrangements within the amplified sequence, or the presence/absence of introgressed DNA (Paran and Michelmore 1993). The additional smaller SCAR product that is present in all hazelnut genotypes may represent a DNA sequence similar to the marker sequence. If this is the case, this sequence could be a (pseudo)gene or a region of DNA either lacking an insertion or containing a deletion relative to the marker sequence. We are currently investigating this phenomenon. Based on our ability to use the $OPI07_{750}$ SCAR marker to detect the presence of the S_2 allele in widely diverse hazelnut genotypes, we believe that this marker is very closely linked to the self-incompatibility locus.

In conclusion, RAPD markers for the S_1 ($OPJ14_{1700}$) and S_2 ($OPI07_{750}$) alleles in hazelnut were identified with a screening protocol utilizing BSA. The marker $OPI07_{750}$ was found to be closely linked to the S_2 allele (recombination rate of 3.8%) and could be utilized in marker-assisted selection in identifying individuals with S_2 allele. The RAPD marker $OPJ14_{1700}$ was not as closely linked to the S_1 allele (recombination rate 7.6%), making it less suitable for marker-assisted selection. RAPD bands were excised from gels, cloned, and sequenced to allow the synthesis of longer primers (18 or 24 bp) for the production of SCARs. SCAR markers for both the S_1 ($OPJ14_{1700}$) and S_2 ($OPI07_{750}$) alleles were obtained, with $OPI07_{750}$ being closely enough linked to the S_2 allele to allow marker-assisted selection for this allele, as well as possibly assist in chromosome walking in efforts to clone the S-locus.

References

Adam-Blondon AF, Sevignac M, Bannerot H, Dorn M (1994) SCAR, RAPD, and RFLP markers linked to a dominant gene (*Are*) conferring resistance to anthracnose in common bean. Theor Appl Genet 88:865–870

Benet H, Guries RP, Boury S, Smally EB (1995) Identification of RAPD markers linked to a black leaf spot resistance gene in Chinese elm. Theor Appl Genet 90:1068–1073

Davis JW (1998) Identification and development of PCR-based markers linked to eastern filbert blight resistance in hazelnut. MS thesis, Department of Horticulture, Oregon State University. 57 pp

Debener T, Lehnacker H, Arnold M, Dang JL (1991) Identification and molecular mapping of a single *Arabidopsis* locus conferring resistance against a phytopathogenic *Pseudomonas* isolate. Plant J 1:289–302

Dixon MS, Jones DA, Keddie JS, Thomas CM, Harrison K, Jones JDG. (1996) The tomato *cf-2* disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. Cell 84:451–459

Frijters ACJ, Zhang Z, van Damme M, Wang G-L, Ronald PC, Michelmore MW (1997) Construction of bacterial artificial chromosome library containing large *Eco*RI and *Hin*dIII genomic fragments of lettuce. Theor Appl Genet 94:390–399

Giovannoni JJ, Wing RA, Ganal MW, Tanksley SD (1991) Isolation of molecular markers from specific chromosomal intervals using DNA pools from existing mapping populations. Nucleic Acids Res 19:6553–6558

Hampson CR, Azarenko AN, Soeldner A (1993) Pollen-stigma interactions following compatible and incompatible pollinations in hazelnut. J Am Soc Hortic Sci 118:814–819

Hampson CR, Coleman G, Azarenko AN (1996) Does the genome of *Corylus avellana* L. contain sequences homologous to the self-incompatibility gene of Brassica? Theor Appl Genet 93:759–764

Koller BL, Gianfranceschi F, Seglias N, McDermott J, Gessler C (1994) DNA markers linked to *Malus floribunda* 821 scab resistance. Plant Mol Biol 26:597–602

Lagerstedt HB (1975) Filberts. In: Janick J, Moore JN (eds) Advances in fruit breeding. Purdue University Press, West Lafayette, Ind., pp 456–489

Martin GB, Williams JGK, Tanksley SD (1991) Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato by using random primers and near-isogenic lines. Proc Natl Acad Sci USA 88:2336–2340

Martin GB, Brommonschenkel SH, Chuwongse J, Frary A, Ganal MW, Spivey R, Wu T, Earle ED, Tanksley SD (1993) Map-bases cloning of a protein kinase gene conferring disease resistance in tomato. Science 262 : 1432–1436

Matton DP, Nass N, Clarke AE, Newbegin E (1994) Self-incompatibility: how plants avoid illegitimate offspring. Proc Natl Acad Sci USA 91 : 1992–1997

Mehlenbacher SA (1995) Classical and molecular approaches to breeding fruit and nut crops for disease resistance. HortScience 30 : 466–477

Mehlenbacher SA (1997) Revised dominance hierarchy for S-alleles in *Corylus avellana* L. Theor Appl Genet 94 : 360–366

Mehlenbacher SA, Thompson MM (1988) Dominance relationships among S-alleles in *Corylus avellana* L. Theor Appl Genet 55 : 29–33

Mehlenbacher SA (1990) Use of molecular markers in breeding for oligogenic disease resistance Plant Breed 104 : 1–19

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions using segregating populations. Proc Natl Acad Sci USA 88 : 9828–9832

Mindrinos, M, Katagiri F, Yu G-L, Ausbel FM (1994) The *A. thaliana* disease resistance gene *RPS2* encodes a protein containing a nucleotide-binding site and leucine-rich repeats. Cell 78 : 1089–1099

Paran I, Kesseli RV, Michelmore RW (1991) Identification of RFLP and RAPD markers linked to downy mildew resistance genes in lettuce by using near-isogenic lines. Genome 34 : 1021–1027

Paran I, Michelmore RW (1993) Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor Appl Genet 85 : 985–993

Scutt LP, Gates PJ, Gatehouse JA, Boulter D, Croy RRD (1990) A cDNA encoding a S-locus-specific glycoprotein from *Brassica oleracea* plants containing the S5 self-incompatibility allele. Mol Gen Genet 220 : 409–413

Song WY, Wang G-L, Chen LL, Kim HS, Pi L-Y, Gardner J, Wang B, Holsten T, Zhai W-X, Zhu L-H, Fauquet C, Ronald P (1995) A receptor kinase-like protein encoded by the rice disease resistance gene *Xa21*. Science 270 : 1804–1806

Stein JC, Howlett B, Boyes DC, Nasrallah DC, Nasrallah JB (1991) Molecular cloning of a putative receptor protein kinase gene encoded at the self-incompatibility locus of *Brassica oleracea*. Proc Natl Acad Sci USA 88 : 8816–8820

Thompson MM (1979a) Genetics of incompatibility in *Corylus avellana* L. Theor Appl Genet 55 : 29–33

Thompson MM (1979b) Growth and development of the pistllate flower and nut in 'Barcelona' filbert. J Am Soc Hortic Sci 104 : 427–432

Trick M, Heizman P (1992) Sporophytic self-incompatibility systems: *Brassica* S gene family. Int Rev Cytol 140 : 485–524

Welsh J, McClellan M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res 18 : 7213–7218

Williams JKG, Kubelik ARK, Livak JL, Rafalski JA, Tingey SV (1990) DNA polymorphism amplified by random primers are useful as genetic markers. Nucleic Acids Res 18 : 757–763

Xu H, Wilson DJ, Arulsekar S, Bakalinsky AT (1995) Sequence-specific polymerase chain-reaction markers derived from randomly amplified polymorphic DNA markers for fingerprinting grape (*Vitis*) rootstocks. J Am Soc Hortic Sci 120 : 714–720