Theor Appl Genet (2004) 108:651–656
DOI 10.1007/s00122-003-1476-9

ORIGINAL PAPER

S. A. Mehlenbacher · R. N. Brown · J. W. Davis · H. Chen · N. V. Bassil · D. C. Smith · T. L. Kubisiak

RAPD markers linked to eastern filbert blight resistance in *Corylus avellana*

Received: 23 May 2003 / Accepted: 10 September 2003 / Published online: 21 October 2003

Abstract A total of 1,110 decamer primers were screened for RAPD markers linked to a dominant allele in hazelnut (*Corylus avellana*) that confers resistance to eastern filbert blight caused by *Anisogramma anomala*. Twenty RAPD markers linked in coupling, and five markers linked in repulsion, were found. A seedling population was used to construct a linkage map of the region flanking the resistance locus. The map spans 46.6 cM, with 14 markers on one side of the resistance locus and eight on the other side. Eleven markers showed less than 3% recombination with resistance, including three that showed no recombination. Seven of these 11 markers are sufficiently robust to allow their use in marker-assisted selection. These include $AA12_{850}$ which shows no recombination, and six markers on one side of the resistance locus: 173_{500}, 152_{800}, 122_{825}, 275_{1130}, $H19_{650}$ and $O16_{1250}$. Marker 268_{580}, which flanks the resistance locus on the other side, is also suitable for use in marker-assisted selection, but shows 5.8% recombination with resistance. Other markers are less suitable for marker-assisted selection because of sensitivity to changes in primer or $MgCl_2$ concentration, or the long time required for electrophoresis to separate bands of similar size. The 16 markers closest to the resistance locus were cloned and sequenced. The $W07_{365}$ marker, which showed no recombination with the resistance locus but is difficult to score, includes a CT microsatellite repeat. The sequence information will allow the design of SCAR primers and eventual map-based cloning of the resistance allele.

Communicated by D.B. Neale

S. A. Mehlenbacher (✉) · R. N. Brown · J. W. Davis · H. Chen · N. V. Bassil · D. C. Smith
Department of Horticulture, Oregon State University, 4017 ALS Building, Corvallis, OR 97331, USA
e-mail: mehlenbs@science.oregonstate.edu
Tel.: +1-541-7375467
Fax: +1-541-7373479

T. L. Kubisiak
Southern Institute of Forest Genetics, USDA Forest Service, 23332 Highway 67, Saucier, MS 39574, USA

Introduction

The European hazelnut (*Corylus avellana* L.) industry in Oregon's Willamette Valley is threatened by eastern filbert blight caused by the pyrenomycete *Anisogramma anomala* (Peck) E. Müller. Recent studies (Pinkerton et al. 1992, 1998a, 1998b, 2001; Stone et al. 1992; Johnson et al. 1996) have clarified the life cycle and dispersal of the pathogen. The causal fungus is endemic on the American hazel (*Corylus americana* Marsh.) native to the eastern United States where it is non-lethal and causes small cankers generally <10 cm in length. On the commercially important European hazelnut, perennial cankers expand up to 1 m per year. Ascospores are ejected from perithecia in winter and spring during periods of prolonged branch wetness. Trees become susceptible to the fungus shortly after budbreak in the spring, as tissues near the apical meristem of young shoots are susceptible to infection by germinating ascospores. Stromata become visible 12–14 months after infection, if an extended cold period is provided to the infected trees. An infected orchard of the moderately susceptible cultivar 'Barcelona' can remain productive for up to 12 years, but an orchard of the highly susceptible cultivar 'Ennis' can become unproductive in as little as 4 years. Hand pruning to remove infected branches, combined with fungicide applications (Johnson et al. 1993), can slow the spread of the disease, but these control measures are expensive.

Genetic resistance offers an alternative approach for disease control. The hazelnut breeding program at Oregon State University (OSU) is developing new cultivars that combine eastern filbert blight resistance with traits desired by the kernel market. Most resistant selections from the breeding program carry the single, dominant resistance allele originally found in the obsolete pollinizer 'Gasaway' (Mehlenbacher et al. 1991). When inoculated with an ascospore suspension under high humidity, susceptible trees become infected while siblings with the resistance allele remain free of the fungus. An enzyme-linked immunosorbent assay (ELISA) developed by Coyne et al. (1996) can detect the presence of the

fungus six months after infection, although cankers do not become visible until several months later.

Marker-assisted selection would allow the identification of seedlings likely to carry the resistance allele, and allow elimination of seedlings that lack the marker prior to planting trees in the field. This approach also allows selection of resistant seedlings in the absence of the pathogen. Random amplified polymorphic DNA (RAPD) markers, generated by the polymerase chain reaction (PCR), are one of the least expensive of the available molecular markers and are well-suited to the high-throughput required for routine use in applied breeding programs (Welsh and McClelland 1990; Williams et al. 1990). Some researchers have experienced difficulties with repeatability of some RAPD markers, as amplification is affected by primer concentration, $MgCl_2$ concentration, thermal cycler model, amplification program, and other factors that vary from one lab to another. Experience has shown that some RAPD markers are robust, giving repeatable results that are easy to score. Davis and Mehlenbacher (1997) used bulked segregant analysis (Michelmore et al. 1991) to identify five RAPD markers linked to the 'Gasaway' resistance gene in the cross 'Willamette' × VR 6–28. One of these markers, UBC 152_{800}, is robust and has been used in marker-assisted selection for several years at OSU. This study was undertaken to identify additional robust RAPD markers for the 'Gasaway' resistance allele.

Materials and methods

Plant materials

In 1993, a controlled cross of the susceptible selection OSU 252.146 and the heterozygous resistant selection OSU 414.062 was made, generating 144 seedlings in a progeny designated 93001 (Fig. 1). The resulting seeds were stratified, sown in flats in the greenhouse as they sprouted, and the seedlings were transplanted to 3.8 l pots when about 20 cm tall. They were grown in the greenhouse during the summer, and transplanted to the field in October 1994 at a spacing of 0.9 m within the row and 2.7 m between rows.

Disease inoculation

The fungus is not present in the field in Corvallis, and state quarantine regulations to slow the spread of the disease require that inoculated trees be kept in a locked greenhouse or grown in the northern Willamette Valley where the disease is present. Cankered shoots were collected from diseased orchards in the northern Willamette Valley in early December 2000 and stored at −20°C. Scions were collected from the seedling population in December 2000 and held at −1°C prior to grafting. Three scions of each of 129 seedlings were grafted onto *C. avellana* rootstocks in spring 2001. 'Gasaway' was included as a resistant control and 'Ennis' and 'Daviana' were included as susceptible controls. Grafted trees were placed in 5 l pots in a mix containing equal volumes of peat, pumice, and fine bark dust. Nine grams of Osmocote (Scotts, Marysville, Ohio) 3–4 month release fertilizer (17N-2.6P-10K with micronutrients) were added to each pot at planting time, and Peters' (20N-8.7P-16.6K) fertilizer (J.R. Peters, Allentown, Pa.) was applied as needed during the summer. The trees were grown in the glasshouse under 24°Cday/18°C night and natural light, and

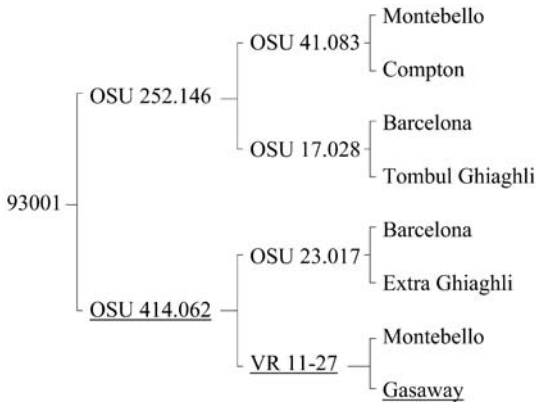

Fig. 1 Pedigree of mapping population. Heterozygous resistant genotypes are *underlined*

inoculation started approximately 4–7 weeks after grafting, when shoots had four to five nodes (Coyne et al. 1996). One or two shoots on each tree were marked with tape two to four nodes below the apical meristem to indicate the point of inoculation. A suspension of 1×10^6 spores/ml in distilled water was prepared and used to inoculate trees in a chamber in which high humidity was maintained (Lunde et al. 2000). The chambers were opened 5 days after inoculation, left open for 2 days, and then inoculations were repeated. Each tree was inoculated three times, and then grown in the glasshouse for at least six months prior to assaying for infection. Disease inoculation was repeated in the spring of 2002 if the results of the first test were inconclusive, and 11 seedlings not available for inoculation in spring 2001 were also inoculated at the same time. The spring 2002 inoculations were repeated at 3-day intervals and the chambers were not opened between inoculations. Four seedlings in the population had been inoculated in spring 2000 and the data were used in this study.

Disease susceptibility evaluation

Six or more months following inoculation, an enzyme-linked immunosorbent assay (ELISA) was performed (Coyne et al., 1996) on inoculated shoots using Nunc Maxisorp 96-well Microplates (Fisher Scientific, Pittsburgh). A genotype was scored as susceptible if infection was detected in one or more of the three trees. A genotype was scored as resistant if all three trees showed no infection. No score was assigned for the six genotypes whose tests gave conflicting or inconclusive results.

DNA extraction

DNA was extracted in the spring from young leaves of field-planted trees. In Corvallis, the method of Lunde et al. (2000) was used, which was modified from that of Davis et al. (1998). Fresh young leaves were sent to Saucier, Missouri, where a cetyltrimethylammonium bromide (CTAB)-based extraction method was used (Wagner et al., 1987), with a proteinase K (0.5 mg) digestion performed subsequent to the addition of *N*-lauroylsarcosine. An additional chloroform:octanol (24:1) extraction was also performed, and RNA was removed by incubation in the presence of RNase A. DNA extracted in Corvallis received no RNase treatment; the treatment had no effect on PCR results.

Primer screening

A total of 1,110 primers were screened: All primers in kits A-AE from Operon Technologies (Alameda, Calif.) and 490 primers in sets 1–800 from the Biotechnology Unit of the University of British

Columbia (Vancouver, Canada). Template DNA of eight genotypes was used for primer screening: the resistant parent (OSU 414.062), the susceptible parent (OSU 252.146), three resistant seedlings (688.012, 688.013, and 688.037), and three susceptible seedlings (688.022, 688.027, and 688.030). In Saucier, the PCR reactions were performed in a 24-μl volume containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.10% Triton X-100, 1.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, and dTTP, 0.5 μM of primer, 6.25 ng of template DNA, and 0.8 U of *Taq* polymerase (Promega, Madison, Wis.). Reactions were loaded into flexible microtitre plates and overlaid with 25 μl of mineral oil. The plates were placed in a preheated (85°C) programmable thermal cycler (PTC-100. MJ Research, Waltham, Mass.) and covered with Mylar film. The reaction were immediately subject to the following thermal profile: 5 s at 95°C, 1 min 55 s at 92°C; followed by 45 cycles of 5 s at 95°C, 55 s at 92°C, 1 min at 35°C, 2 min at 72°C; then 7 min at 72°C, ending with an indefinite hold at 4°C.

In Corvallis, the PCR reactions were performed in a volume of 15 μl containing 0.3 μM of primer, 3–25 ng of template DNA, and 0.75 U of *Taq* polymerase (Promega). Later PCR reactions used 0.4 U of Biolase DNA polymerase (Biolase USA, Randolph, Mass.) and the ammonium-based buffer supplied by the manufacturer. Ninety-six reactions were run simultaneously in microtitre plates using a Geneamp PCR System 9700 thermal cycler (Perkin-Elmer Corp., Foster City, Calif.) using the same thermal profile as described above. During the first five cycles the ramp time from 35°C to 72°C was reduced to 30% of maximum to reduce non-specific binding of primers. When necessary to improve repeatability of scoring, primer and $MgCl_2$ concentrations were adjusted (Table 1).

Amplification products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, destained, then visualized with a transilluminator and photographed. Primers that generated a band in the resistant parent and the three resistant seedlings but not in the susceptible parent nor in the three susceptible seedlings were investigated further. Primers that showed one recombinant among the six seedlings were also investigated further. Primers that generated promising bands were scored on the whole population of 144 seedlings. For some primers, electrophoresis was extended to as long as 11 hours to allow separation of bands of similar size.

Construction of a linkage map

Data were entered as either 1 for marker present or 0 for marker absent. Similarly, genotypes with no infection were scored as 1 for resistance gene present, and infected genotypes were scored as 0. The Mapmaker EXP program was used to construct a linkage map for the region surrounding the 'Gasaway' resistance locus (Lander et al. 1987). Double recombinants were reexamined, and PCR was repeated to fill in missing data points. PCR was also repeated to confirm data points on each side of a recombination event.

Marker cloning

The 16 markers closest to the resistance locus (Table 1) were excised from low melting-point agarose gels and the fragment cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, Calif.) and introduced into One Shot DH5aα-T1^R chemically competent *Escherichia coli* cells according to the supplier's instructions. Colonies (18 or 36 per marker) were streaked on agar plates and cultured overnight at 37°C. These colonies were then stabbed and amplified using PCR and the appropriate primer pair, and amplification products run on 2% agarose gels, stained with ethidium bromide, destained, and photographed. For markers $H04_{860}$ and $X02_{1140}$, Zero Blunt TOPO PCR Cloning Kits (Invitrogen) and blunt-end ligation were used.

Table 1 RAPD markers for 'Gasaway' resistance allele to eastern filbert blight. Markers are listed in map order (Fig 2)

Marker [a]	Primer concentration (μl/reaction) [b]	$MgCl_2$ concentration adjustment (%)	Electrophoresis time (h at 90 V) [c]	Fragment length (bp) [d]	GenBank accession no.
A08700	0.3	-	4	ND	-
$dV14_{650}$	0.8	-	4	ND	-
$d712_{775}$	0.3	-	5	ND	-
327_{925}	0.3	-	4	ND	-
521_{1200}	0.35	-	4	1200	CC875200
726_{665}	0.3–0.5	-	4	665	CC875201
268_{580}	0.3–0.6	-	2.5	580	CC875202
$H04_{860}$	0.3	+50%	4	860	CC875203
$AA12_{850}$	0.7	-	3.5	854	CC875204
$X01_{825}$	0.3	-	11	828	CC875205
$W07_{365}$	0.7	-	2.5	365	CC875206
173_{500}	0.3	-	2.5	497	CC875207
152_{800}	0.2	-	2.5	823	CC875208
122_{825}	0.4	-	2.5	828	CC875209
275_{1130}	0.3	-	4–8	1134	CC875210
$H19_{650}$	0.3	-	2.5	629	CC875211
$O16_{1250}$	0.4–0.6	-	2.5	1255	CC875212
$X02_{1140}$	0.3	-	11	1144	CC875213
$Y19_{540}$	0.4	-	2.5	542	CC875214
$A04_{510}$	0.3	-	2.5	506	CC875215
180_{250}	0.6–0.8	+25%	2	ND	-
557_{525}	0.3–0.8	+50%	4	ND	-
$dU12_{1125}$	0.3	-	4	ND	-
$d313_{950}$	0.3	-	6	ND	-
$d181_{1200}$	0.3	-	11	ND	-

[a] Designations for markers linked to resistance in coupling are the primer followed by the length of the polymorphic fragment in base pairs. Markers linked in repulsion are similarly designated, but begin with a lower case "d".
[b] Primer stock solutions were 10 μM.
[c] Agarose gel dimensions (2%) were 20 cm x 25 cm and thickness was 7 mm.
[d] ND = sequencing not performed and actual fragment length not determined for markers far from the resistance locus.

Marker sequencing

Plasmid DNA from one to three promising putative transformants was sequenced in both the forward and reverse directions by the Central Services Laboratory of the OSU Center for Gene Research and Biotechnology using appropriate primers (T3, T7, M13F, M13R, or SP6). The reverse complement of the reverse sequence was generated using the Reverse Complement program on the website of the University of Alberta (http://www.ualberta.ca/~stothard/javascript/rev_comp.html) and compared with the forward sequence using the program ClustalW (http://clustalw.genome.ad.jp/). The electrophoretograms were inspected by naked eye to resolve discrepancies in the two sequences, and a consensus sequence determined for each of the 16 markers.

Results

Twenty RAPD markers linked in coupling to the 'Gasaway' resistance gene were identified in the progeny OSU 252.146 × OSU 414.062 (Table 1), including four of the five markers previously identified by Davis and Mehlenbacher (1997) in the progeny 'Willamette' × VR 6–28. Their fifth marker, $H17_{1200}$, could not be scored in our population, nor could it be scored in a second population (OSU 23.017 × VR 17–19) examined in their study. Markers linked in coupling are designated by the primer followed by the size of the polymorphic fragment in base pairs. An additional five markers were linked in repulsion; their designations begin with a lower case "d". A map spanning 46.6 cM was constructed for the region around the 'Gasaway' gene (designated R in Fig. 2). Of the 25 markers, 14 are located on one side of the resistance locus and 8 on the other side. Three markers co-segregated with resistance, and eight others showed less than 3% recombination with resistance. Several of the markers near the resistance locus (268_{580}, $AA12_{850}$, 173_{500}, 152_{800}, 122_{825}, 275_{1130}, $H19_{650}$, $O16_{1250}$) are sufficiently robust to allow their direct use in marker-assisted selection, but other markers ($H04_{860}$, $W07_{365}$, $X01_{825}$) are less suitable as they are difficult to score. Three factors were adjusted to improve the accuracy of scoring of some markers: increasing the primer concentration, increasing the $MgCl_2$ concentration, and separation by electrophoresis for a longer time. Parent genotypes were included as checks in each gel when marker scoring was difficult. Although primers were initially screened at a concentration of 0.3 or 0.5 μM, higher concentrations improved the reliability of scoring of some markers and were used to amplify the population (Table 1). The marker $W07_{365}$ showed no recombination with resistance, but was more difficult to score than other markers. Accurate scoring required a higher primer concentration (0.7 μM per reaction), and many samples required re-amplification to verify accuracy of scoring. The marker $H04_{860}$ became easy to score only after the $MgCl_2$ concentration was increased to 2.25 mM. The marker $X01_{825}$ also showed no recombination with

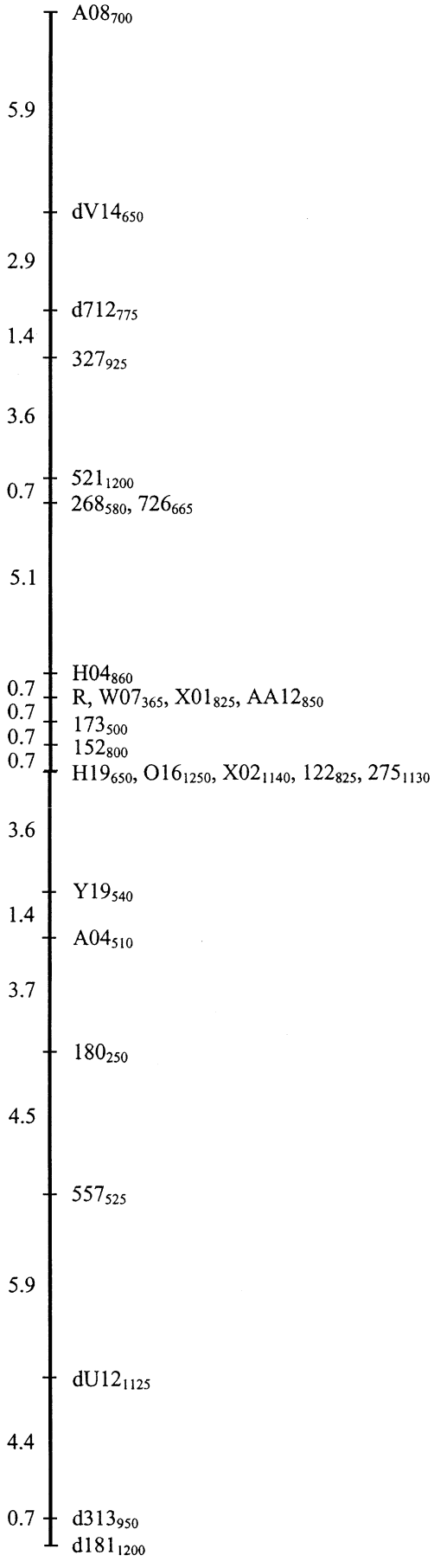

Fig. 2 Map of the chromosome of parent OSU 414.062 that shows the locus for resistance to eastern filbert blight (*R*) and 25 RAPD markers

resistance, but could not be reliably scored until gels were run overnight (11 h at 90 V) to separate many bands of similar size. The third marker that co-segregated with resistance, $AA12_{850}$, was easy to score when a higher primer concentration (0.7 μM) was used. The resistance locus and these three markers were flanked on one side by the difficult-to-score $H04_{860}$ and the robust marker 268_{580}. On the other side of the R locus there were six markers, all easy to score: 173_{500} and 152_{800} were closest to the resistance locus, while the more distant 122_{825}, 275_{1130}, $H19_{650}$ and $O16_{1250}$ were inherited as a block with no recombination.

The 16 markers closest to the resistance locus were cloned and sequenced (Table 1). The $W07_{365}$ marker, which showed no recombination with the resistance locus but which is difficult to score, includes a CT microsatellite repeat. The sequence of the $H04_{860}$ marker (that required a higher $MgCl_2$ concentration for accurate scoring) revealed a 2 bp mismatch within the primer binding site.

Discussion

Davis and Mehlenbacher (1997) identified five markers linked to the 'Gasaway' resistance gene in the cross 'Willamette' × VR 6–28. One of these markers (152_{800}) was found to be robust and has been used for marker-assisted selection for several years. However, direct use of the other markers has been limited by either their loose linkage relationship with the 'Gasaway' resistance gene, difficulty with scoring, or distorted segregation in some crosses. In this study, a total of 21 additional RAPD markers linked to the 'Gasaway' resistance gene were identified. Seven markers show less than 3% recombination with resistance and are sufficiently robust to allow their use in marker-assisted selection (Fig. 2). These include $AA12_{850}$, which shows no recombination with resistance. Six additional robust markers are located on one side of the resistance locus (173_{500}, 152_{800}, 122_{825}, 275_{1130}, $H19_{650}$ and $O16_{1250}$), the last four of which are inherited as a block. Recombination within this block would likely have been detected in a larger population. Marker 268_{580}, which flanks the resistance locus on the other side, is also suitable for use in marker-assisted selection, but shows 5.8% recombination with resistance. Selection based on marker $AA12_{850}$ alone or in combination with flanking markers 268_{580} and 173_{500} is suggested. Other markers are less suitable for marker-assisted selection because of sensitivity to changes in primer or $MgCl_2$ concentration, or the long time required for electrophoresis to separate bands of similar size. The marker sequence information will allow the design of sequence-characterized amplified region (SCAR) primers and eventual map-based cloning of the resistance allele.

These markers supplement and add precision to other methods for testing eastern filbert blight susceptibility. Exposure of trees to spores under field conditions is the ultimate evaluation method, but it is slow and laborious as cankers do not develop until 14–16 months after infection. Although the dominant allele from the 'Gasaway' gene is said to confer "complete resistance", we have noted some resistant trees that occasionally show small cankers. In most cases these small cankers fail to form perithecia, are walled off by the host plant and fail to develop further. Greenhouse inoculation followed by ELISA testing can reduce this time by several months, but space and time limit such tests to less than 300 genotypes per year. In greenhouse tests, different trees of the same genotype occasionally give conflicting results. Moreover, in some cases the ELISA values are on the threshold rather than clearly infected or clearly uninfected. Marker-assisted selection based on RAPD markers identifies trees that clearly lack the allele for resistance. Resistance using greenhouse or field inoculation can then be confirmed for the most promising selections.

Acknowledgements I thank Ruth Martin for considerable help in cloning and sequencing, and advice on other aspects of this study. This research was supported by the Oregon Hazelnut Commission and a Specific Cooperative Agreement with the United States Department of Agriculture, Agricultural Research Service.

References

Coyne CJ, Mehlenbacher SA, Hampton RO, Pinkerton JN, Johnson KB (1996) Use of ELISA to rapidly screen hazelnut for resistance to eastern filbert blight. Plant Dis 80:1327–1330

Davis JW, Mehlenbacher SA (1997) Identification of RAPD markers linked to eastern filbert blight resistance in hazelnut. Acta Hortic 445:553–556

Davis J, Henderson D, Kobayashi M, Clegg MT (1998) Genealogical relationships among cultivated avocado as revealed through RFLP analyses. J Hered 89:319–323

Johnson KB, Pscheidt JW, Pinkerton JN (1993) Evaluation of chlorothalonil, fenarimol, and flusilazole for control of eastern filbert blight. Plant Dis 77:831–837

Johnson KB, Mehlenbacher SA, Stone JK, Pscheidt JW, Pinkerton JN (1996) Eastern filbert blight of European hazelnut - it's becoming a manageable disease. Plant Dis 80:1308–1316

Lander ES, Green P, Abrahamson I, Barlow A, Daly MJ, Lincoln SL, Newbers L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental populations. Genomics 1:182–195

Lunde CF, Mehlenbacher SA, Smith DC (2000) Survey of hazelnut cultivars for response to eastern filbert blight inoculation. HortScience 35:729–731

Mehlenbacher SA, Thompson MM, Cameron HR (1991) Occurrence and inheritance of resistance to eastern filbert blight in 'Gasaway' hazelnut. HortScience 26:410–411

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 genome regions by using segregating populations. Proc Natl Acad Sci USA 88:9829–9832

Pinkerton JN, Johnson KB, Theiling KM, Griesbach JA (1992) Distribution and characteristics of the eastern filbert blight epidemic in western Oregon. Plant Dis 76:1179–1182

Pinkerton JN, Johnson KB, Stone JK, Ivors KL (1998a) Factors affecting the release of ascospores of *Anisogramma anomala*. Phytopathology 88:122–128

Pinkerton JN, Johnson KB, Stone JK, Ivors KL (1998b) Maturation and seasonal discharge pattern of ascospores of *Anisogramma anomala*. Phytopathology 88:1165–1173

Pinkerton JN, Johnson KB, Aylor DE, Stone JK (2001) Spatial and temporal increase of eastern filbert blight in European hazelnut orchards in the Pacific Northwest. Phytopathology 91:1214–1223

Stone JK, Johnson KB, Pinkerton JN, Pscheidt JW (1992) Natural infection period and susceptibility of vegetative seedlings of European hazelnut to *Anisogramma anomala*. Plant Dis 76:348–352

Wagner DB, Furnier GR, Saghai-Maroof MA, Williams SM, Dancik BP, Allard RW (1987) Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. Proc Natl Acad Sci USA 84:2097–2100

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

Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535