AN ABSTRACT OF THE THESIS OF

Joel W. Davis for the degree of Master of Science in Horticulture presented on March 3, 1998. Title: Identification and Development of PCR-based Markers Linked to Eastern Filbert Blight Resistance in Hazelnut.

Abstract approved: _____

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Using bulked segregant analysis, five RAPD markers were identified that were linked in coupling to a gene conferring complete resistance to eastern filbert blight caused by *Anisogramma anomala* (Peck) E. Muller in hazelnut (*Corylus avellana* L.). Two modified backcross populations were inoculated and scored for resistance to the pathogen and found to segregate in a 1:1 ratio. In the first population, the five markers were linked to the resistance gene at distances of 6.7, 0, 2.0, 2.0, and 2.0 cM. Three of these markers also were linked in coupling in the second population at distances of 6.0, 6.0, and 4.0 cM. Two of these markers were cloned and sequenced, and primers were designed based on the sequence information at the termini of the amplified marker fragments. These primers were used to amplify DNA from both populations and evaluated for their potential use in selecting for resistance among seedlings. One of these cloned markers ($SC152_{800}$) produced the same polymorphism among both populations and was consistent with the linkage exhibited by the original RAPD marker. These primers were also used to amplify DNA from a collection of germplasm accessions used in the hazelnut breeding program. A fragment of similar size was amplified in all of the resistant germplasm

tested, and also in some of the susceptible selections. This marker was shown to have potential for use in marker-assisted selection for breeding hazelnut cultivars resistant to eastern filbert blight.

Identification and Development of PCR-based Markers Linked to Eastern Filbert Blight Resistance in Hazelnut

by

Joel W. Davis

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented March 3, 1998
Commencement June 1998

Master of Science thesis of Joel W. Davis presented on March 3, 1998

APPROVED:

Major Professor, representing Horticulture

Chair of Department of Horticulture

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Joel W. Davis

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INTRODUCTION

Eastern filbert blight, caused by *Anisogramma anomala* (Peck) E. Muller, is a serious disease of European hazelnut (*Corylus avellana* L.) that threatens production in the major growing region in the Pacific Northwest. The disease is endemic to the eastern United States on *C. americana*, and was first detected in the state of Washington in 1970 (Davison and Davidson, Jr., 1973). It has since spread to 50% of the production areas in Oregon and Washington (Pinkerton et al., 1992).

The pathogen is an obligate biotrophic fungus restricted to several species of *Corylus* and has a two year life cycle (Pinkerton et al., 1995). Ascospores released from perithecia are dispersed by splashing rain or are forcibly ejected and infect young shoot tissues from spring to mid-summer. Germinating spores produce hyphae which colonize xylem, cambium, and phloem tissues during the first season. During the next year cankers are formed which eventually produce stromata bearing perithecia. Perennial cankers spread throughout the tree, girdling infected branches and resulting in death of mature trees in five to fifteen years (Gottwald and Cameron, 1979; Gottwald and Cameron, 1980; Pinkerton et al., 1995).

Current strategies for control of the disease include routine fungicide treatments, selective pruning of diseased branches, or removal of entire infected trees (Johnson et al., 1993; Johnson et al., 1994). Multiple fungicide applications are not considered

economical due to the low prices paid to growers for the crop. Pruning and tree removal are only partially effective and will only slow the spread of the disease in orchards (Johnson et al., 1993; Johnson et al., 1994.). The most desirable and economical means of controlling this disease is the use of resistant cultivars (Mehlenbacher, 1994). The cultivar 'Gasaway', an obsolete pollenizer, has been shown to be completely resistant to eastern filbert blight (Mehlenbacher et al., 1991). This genetic resistance is controlled by a single dominant gene. Crosses of susceptible genotypes with 'Gasaway' or its resistant progeny segregate 1:1 for resistance (Mehlenbacher et al., 1991). The exact mechanism of resistance is not yet known, but appears to be associated with the initial infection process (Pinkerton et al., 1995).

Molecular markers have the potential to facilitate the transfer of important traits, including disease resistance, to new varieties and cultivars. The most widely used markers to date are isozymes, restriction fragment length polymorphisms (RFLP's), and random amplified polymorphic DNA's (RAPD's). Isozyme methodology is relatively simple and efficient, however the number of polymorphic loci is limited in most breeding populations, and some enzymes can only be assayed in specific tissues or developmental stages. RFLP's can provide significantly greater numbers of polymorphic loci, are phenotypically neutral, and can be scored at any stage of plant development, but require labor-intensive and expensive techniques to generate markers and perform assays (Melchinger, 1990). The markers need to be maintained as clones in a genomic or cDNA library, and the assays typically require abundant amounts of high-quality DNA and the use of radioactive detection techniques.

The advent of the polymerase chain reaction (PCR) procedure (Saiki et al., 1988) led to the development of a new type of DNA marker, the random amplified polymorphic DNA (RAPD) marker (Welsh and McClelland, 1990; Williams et al., 1990). There are several advantages of RAPD markers over RFLP's (Kelly, 1995). RAPD's do not require the generation of cloned DNA probes or the use of hybridization techniques. Instead they rely on the use of a universal set of primers which can be quickly and efficiently screened for useful polymorphisms. The PCR reaction requires smaller amounts of DNA which can be of marginal quality and isolated using simple and rapid methods. RAPD's are also easier to assay and allow for more efficient processing of larger numbers of samples.

Linkage maps based on DNA markers should be useful for improving the efficiency of breeding programs, especially for tree crops which have long juvenile phases and require substantial investments in resources and land (Durham et al., 1992; Jarrell et al., 1992). Linkage and mapping studies have not been as common in clonally-propagated tree crops as in seed-propagated species. Most tree crops are cross-pollinated and therefore highly heterozygous and subject to inbreeding depression. This precludes the use of inbred lines for developing mapping populations. Other strategies have been devised to produce mapping populations compatible with dominant RAPD markers. In gymnosperms, the megagametophyte tissue of the seeds is haploid, and DNA extracted from this tissue can be used to map genetic markers (Devey et al., 1995). RAPD-based linkage maps have been constructed in this manner for slash pine (Nelson et al., 1993), maritime pine (Plomion et al., 1995), longleaf pine (Nelson et al., 1994), and Norway spruce (Binelli and Bucci, 1994). In many tree fruits and nuts exhibiting high heterozygosity, each variety can be treated as an

F_1. In less polymorphic crops these F_1 hybrids are produced by interspecific or even intergeneric crosses in order to increase heterozygosity (Mehlenbacher, 1995). The progeny produced from crossing these F_1 varieties will segregate for all loci heterozygous in the parents (Weeden et al., 1994). Using modified backcross or double-pseudotestcross configurations (Grattapaglia and Sederoff, 1994), linkage maps can be constructed for alleles segregating in a 1:1 ratio, where dominant RAPD markers are as equally suitable as codominant markers. This strategy has been used to generate linkage maps for apple (Hemmat et al., 1994), peach (Chaparro et al., 1994; Dirlewanger and Bodo, 1994; Rajapakse et al., 1994), almond (Arus et al., 1994), citrus (Cai et al., 1994), eucalyptus (Grattapaglia and Sederoff, 1994), blueberry (Rowland and Levi, 1994), and grape (Lodhi et al., 1995). Maps such as these may allow the identification of markers linked to disease resistance genes or other important traits.

Another technique known as bulked segregant analysis (Michelmore et al., 1991) has become widely used as a means of identifying markers linked to genes of interest without the need to develop saturated molecular marker maps. With this method DNA samples from individuals in a segregating population are pooled into two separate bulks: one bulk contains individuals having a specific trait, and the other bulk contains individuals lacking this trait. The two bulks along with the parental DNA samples can be rapidly screened with large numbers of random decamer primers to identify polymorphisms genetically linked to a desired trait. These pooled DNA samples differ genetically at the target locus but are random at all loci unlinked to this region. The amplification products

would exhibit a polymorphic band for the marker linked to the target locus, but all other bands would appear monomorphic.

Michelmore et al. (1991) describes the use of this method to identify three RAPD markers linked to a downy mildew resistance gene in lettuce using bulks of 14-20 individuals each. These markers were found to be 6, 8, and 12 cM from the resistance gene. Bulked segregant analysis has been used in conjunction with near isogenic lines (NIL's) to identify RAPD markers linked to disease resistance genes in many seed-propagated crops. In common bean, markers have been found for genes for resistance to rust (Haley et al., 1993; Miklas et al., 1993; Johnson et al., 1995), anthracnose (Young and Kelly, 1996), and a potyvirus (Haley et al., 1994a). RAPD markers have also been identified for enation mosaic virus resistance in pea (Yu et al., 1995), stem rust, leaf rust, and leaf blotch resistance in barley (Barua et al., 1993; Borovkova et al., 1995; Poulsen et al., 1995), and nematode resistance in tomato (Yaghoobi et al., 1995).

This method can also be successfully applied to tree crops, where inbreeding depression prevents the use of NIL's and traditional backcrossing. Many disease resistances are monogenic and typically segregate in a 1:1 (resistant:susceptible) ratio in modified backcross or pseudotestcross configurations, allowing for the screening of phenotypic pools with RAPD primers and identification of linked markers. Yang and Kruger (1994) applied this method to screen 170 random decamer primers to find a marker linked to the *Vf* locus from *Malus floribunda* 821 conferring resistance to scab (*Venturia inaequalis*) in apple. Koller et al. (1994) screened 400 primers and found two markers linked (10.6% and 19.7% recombination frequencies) to the *Vf* locus in another

segregating cross of apple. Seven RAPD markers were identified by Markussen et al. (1995) to be linked to the powdery mildew resistance gene Pl_1 in apple. The two most tightly linked were found to be 4.5 and 5 cM from the Pl_1 gene. Ling et al. (1994) found five markers linked to citrus nematode resistance after screening bulks from an intergeneric backcross population with 320 primers. Benet et al. (1995) screened 220 primers and found three RAPD markers linked to a black leaf spot resistance gene in Chinese elm, although this population was comprised of seedlings from self-pollination of a single tree and segregated in a 3:1 ratio of resistant to susceptible individuals. Devey et al. (1995) used bulked samples of megagametophyte DNA to screen 800 random decamer primers, identifying ten markers linked to a gene for resistance to white pine blister rust in sugar pine. Five of these markers were found to be within 5 cM of the gene. Davis and Mehlenbacher (1997) reported the preliminary results of an effort to find RAPD markers linked to a gene for resistance to eastern filbert blight in hazelnut. Five markers were found to be tightly linked (recomb. freq. < .07) in a modified backcross population segregating for resistance.

Linkage to other single traits have been reported in recent studies of tree crop genetics. Using bulking techniques to screen 700 primers, a RAPD marker was found linked to sex determination in pistachio by Hormaza et al. (1994). Lehner et al. (1995) screened 400 primers to find a marker linked to the single dominant gene controlling the pendula phenotype in Norway spruce. A marker linked to the expression of an incompatibility allele (S-allele) in hazelnut was reported by Pomper et al. (1996) in a preliminary screening of 100 random decamer primers. RAPD markers have also been

found for the yellow-flesh locus in peach (Warburton and Bliss, 1994) and for the red/yellow skin color locus in apple (Cheng et al., 1996) using bulked segregant analysis.

While these types of linkage studies may eventually be useful for map-based cloning of specific genes, the most immediate application to plant breeding is the development and implementation of marker-assisted selection (MAS) strategies. MAS can be used as a valuable supplement to existing disease resistance breeding programs. The availability of tightly linked markers can provide a means of improving the efficiency of pyramiding resistance genes where epistasis may mask expression of some of these genes. MAS would be most useful for increasing the efficiency of selection for disease resistance when accurate phenotyping is difficult or expensive to perform, or when selection must be carried out in the absence of the pathogen due to quarantine restrictions (Kelly, 1995; Mehlenbacher, 1995). MAS can also be advantageous under conditions which may hinder the effectiveness of conventional selection methods, including unreliable inoculum, environmental effects, and developmental regulation of resistance (Melchinger, 1990).

The success of MAS techniques depends on first accurately phenotyping the segregating population and identifying markers tightly linked to the gene of interest, then developing reliable marker assays and a cost-efficient method of preparing DNA samples. Targeted mapping and bulked segregant analysis are likely to provide tightly linked RAPD markers for single-gene traits if sufficient numbers of primers can be screened. The need for a reliable marker assay system stems from the inherent problems of reproducibility in RAPD's (Weeden et al., 1992), especially variation among

thermocyclers and primer mismatching at low annealing temperatures. The development of sequence-characterized amplified regions (SCAR's) by Kesseli et al. (1992) and Paran and Michelmore (1993) provided one solution to this problem. A SCAR is produced by isolating DNA from the polymorphic RAPD band and cloning it in a suitable vector/host system. The cloned RAPD fragment can then be sequenced for the purpose of designing and synthesizing primers homologous to each end of the fragment. The newly synthesized primers are typically 17-24 base pairs long, each containing the original 10-mer primer sequence. These longer primers are annealed at higher temperatures (50-65° C) and generally produce a single amplified fragment corresponding to the original polymorphic RAPD band. Using longer, sequence-specific primers the amplification of the SCAR marker is often more reliable than the original RAPD marker, even when using crude DNA samples as templates (Markussen et al., 1995; Paran and Michelmore, 1993). Finally, the utility of a MAS system relies on the ability to assay samples in a cost-efficient manner. DNA extraction protocols can be cumbersome and labor intensive. PCR assays generally can be performed using DNA templates from procedures simplified to accommodate large numbers of samples. Gu et al. (1995) described a rapid DNA isolation method for large-scale screening of plant material for MAS applications. The single amplification product typically generated by SCAR primers may also be assayed by direct ethidium bromide staining without the need for gel electrophoresis.

The current methods required to test seedlings for resistance to eastern filbert blight (EFB) are slow and laborious. Due to quarantine restrictions, field screening with the EFB pathogen is not possible where seedlings are currently grown and evaluated, as

this would spread the disease and threaten nearby commercial orchards. To test for resistance, scions are collected from trees established in the field, grafted to rootstocks, and grown in the greenhouse. When actively growing shoots have reached a suitable stage they are inoculated by spraying the apical growth with a spore suspension. Susceptibility is indicated by the development of cankers approximately 16 months later, or by detection of the fungus using either microscopic examination or ELISA tests 6 months after inoculation (Coyne et al., 1996).

These seedling trees are typically 4-5 years old when they are tested for susceptibility to EFB. By this time the trees have already been evaluated for several important traits, including resistance to big bud mite, precocity, nut shape, yield, kernel defects, % kernel, maturity date, free-falling nuts, and blanching ability. Only the most promising selections are kept and tested for resistance to EFB. At this point considerable time and labor have been expended on the culturing, planting, and evaluation of seedlings, 50% of which are susceptible (1:1 segregation ratios) and will eventually be eliminated. This represents an inefficient utilization of resources for the selection of new resistant genotypes.

The availability of a genetic marker tightly linked to the 'Gasaway' gene for resistance to eastern filbert blight could provide a means of identifying genotypes likely to be resistant at the early seedling stage, thus eliminating susceptible seedlings before they are planted in the field. Pre-selected populations would be greatly enriched for resistant types, increasing the likelihood of selecting material that is resistant as well as horticulturally superior. The identification of tightly-linked RAPD markers represents

the first step in developing a suitable MAS system for eastern filbert blight resistance breeding in hazelnut. With sequence-specific primers and efficient protocols for extraction and assay, MAS should greatly facilitate the breeding of new resistant cultivars.

MATERIALS AND METHODS

Plant material

Field-planted seedling trees from controlled pollinations growing at the Oregon State University Smith Horticulture Research Farm were used in this study. Scion wood was collected in the winter from 103 trees of the cross 'Willamette' × 'VR 6-28' and from 50 trees of the cross 'OSU 23-17' × 'VR 17-19' and placed in cold storage (0° C) for two to three months. The scion wood was grafted onto rootstocks (three grafted trees per seedling) in early spring and maintained in greenhouses at 24-27° C until ready for inoculation. Fresh leaf material consisting of juvenile foliage and apical meristems was collected in the field from these same seedling trees and the four parent trees throughout the spring and early summer and stored at 4° C for up to one week prior to DNA extraction.

Inoculations

Infected hazelnut twigs containing stromata were collected from a diseased orchard in Boring, Oregon, during the late fall in 1993 and 1994 and placed in cold storage (0° C) for six to eight months. Inoculum was prepared by excising stromata from the stem tissue and grinding them in distilled water with a mortar and pestle. After filtering through cheesecloth, the spore concentration was determined with a hemacytometer and diluted with distilled water to a concentration of 1×10^6 spores per ml. Two to three actively growing shoots on each grafted tree were labeled with tape three to four internodes below the shoot tip to mark the site of inoculation. The spore

suspension was applied to the apical growth of each shoot using a spray bottle. The trees were immediately placed in a humidity chamber or enclosed in polyethylene bags under shadecloth and maintained at 21-24° C in the greenhouse for five days. The trees were removed from the chamber for two days before repeating the inoculation with freshly prepared inoculum. All trees received a total of three inoculations using a concentration of 1×10^6 spores/ml. After the final inoculation, the trees were kept in the greenhouse at 24-27° C for four to six months before assaying for infection.

Infection assays

Grafted trees were tested for the presence of fungal hyphae using microscopic examination, ELISA tests, or a combination of the two methods. Vascular tissue was prepared for microscopic examination by hand sectioning stem tissue at the internodes surrounding the inoculation sites, placing thinly sliced sections in 0.1% trypan blue:lactoglycerol, and allowing them to soak overnight at room temperature. The stained sections were examined the following day at 100X magnification for the presence of hyphal filaments. ELISA tests were performed according to the method used by Coyne et al. (1996). For ELISA detection, sliced stem sections from the same internodal regions were ground in liquid nitrogen and lyophilized overnight. Ten mg of lyophilized tissue were placed in 1 ml of PBS buffer (137mM NaCl, 3mM KCl, 10mM Na_2PO_4, 2mM KH_2PO_4, pH 7.4), vortexed, and stored overnight at 4° C. A 100 µl aliquot of each sample was placed in a separate well in a Corning 96-well microtiter plate using three replicates of each sample per plate, including infected and non-infected controls. The plates were incubated at 37° C for two hours. The wells were rinsed three times with

PBS and 200 µl of blocking buffer (5% powdered milk in PBS) were added to each well. The plates were stored overnight at 4° C. The wells were washed twice with PBS and 50 µl of fungal-specific rabbit antibody diluted 1:1,000 in blocking buffer were added to each well. The plates were incubated at room temperature (21° C) for two hours, the wells rinsed four times with PBS, and 50 µl of anti-rabbit IgG alkaline phosphatase conjugate antibody (Sigma Chemical Co.) were added to each well. The plates were incubated for two hours at room temperature, washed three times with PBS and once with PNPP buffer (10mM diethanolamine pH 9.4, 50m $MgCl_2$). Fifty µl of p-nitrophenyl phosphate substrate (0.33mg/ml PNPP buffer) were added to each well and the plates incubated overnight at room temperature. Optical density readings were taken the next morning using a Tek Microplate Reader set at a wavelength of 405A. Positive (infected) threshold values were set at the average negative control plus 3.5× the standard deviation. Individuals testing positive for the presence of fungal hyphae in stained tissue sections were classified as susceptible phenotypes. Trees that showed no presence of hyphae the first year were regrafted the following year, reinoculated, and tested with both ELISA and microscopic examination methods. Individuals confirmed as testing negative for the presence of hyphae were classified as resistant phenotypes.

DNA extraction

Fresh leaf material was collected in the spring from field-planted trees. Homogenization of leaf tissue was performed using a modification of the method used by Davis et al. (1998). Approximately 5 g of tissue consisting of apical meristems and young expanding foliage were placed in a 250 ml stainless steel blender with 50 ml of

cold extraction buffer consisting of 0.35 M sucrose, 100 mM Tris, 50 mM potassium chloride, 5% polyvinylpyrrolidone (F.W. = 40,000), 25 mM EDTA, 10 mM diethyldithiocarbamic acid, and 0.1% mercaptoethanol. The tissue was ground at high speed with four to five pulses of approximately 5 seconds each, with a few seconds pause between pulses. The homogenate was filtered through four layers of cheesecloth into a 50 ml centrifuge tube on ice and centrifuged for 20 minutes at 16,000×g at 4° C. The supernatant was discarded and the remaining pellet was resuspended in 5 ml of lysing buffer (100 mM EDTA, 50 mM Tris pH 8.0) containing 2% n-lauroylsarcosine, 2.5% Triton X-100, and 50 μg/μl proteinase-K (Fisher Scientific). The tubes were capped and incubated for one hour at 37° C in a shaking incubator set at 200 rpm. The tubes were centrifuged for 10 minutes at 16,000×g (4° C) and the supernatant was transferred to a fresh centrifuge tube. An equal volume of cold isopropanol was mixed with the sample and the tube stored at -20° C for a minimum of 30 minutes. After centrifuging for 15 minutes at 20,000×g (4° C) the supernatant was removed and the pellet resuspended in 0.5 ml of high salt TE buffer (10 mM Tris, 1 mM EDTA, 2 M NaCl, pH 8.0) overnight at 4° C. The samples were transferred to 1.5 ml microcentrifuge tubes, extracted with an equal volume of phenol/chloroform/isoamyl alcohol ($1:1:^{1}/_{24}$), and centrifuged 10 minutes at 16,000×g (room temperature). The aqueous phase was transferred to a new microfuge tube and precipitated with 95% ethanol. The tubes were centrifuged briefly and the supernatant discarded. The pellet was rinsed once in 70% ethanol and dried under vacuum. After resuspending the pellet in 100 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) the sample was treated with RNase A (Sigma Chemical Co.) for 30

minutes at 37° C, extracted once with an equal volume of phenol/chloroform/isoamyl alcohol, and centrifuged for 10 minutes at 16,000×g. The aqueous phase was transferred to a new microfuge tube and precipitated with 0.1 volume 3 M sodium acetate pH 5.2 and 2 volumes 95% ethanol, and then centrifuged briefly. The supernatant was removed and the pellet rinsed once in 70% ethanol, dried under vacuum, and reconstituted in 50 µl of TE buffer. The DNA was quantified using a Beckman Model 34 spectrophotometer, and 10 ng/µl dilutions were prepared as template DNA for subsequent PCR amplification.

DNA bulks and primer screening

Two bulks of template DNA were made by pooling samples from ten susceptible individuals in one bulk and ten resistant individuals in the other bulk for the 'Willamette' × 'VR 6-28' progeny. Decamer primers obtained from Operon Technology and the University of British Columbia were used to amplify DNA from the two bulked samples and the two parents of this cross. Reaction conditions for PCR amplification were modified from those recommended by Williams et al. (1990). A 25 µl reaction volume was used that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 100 µM each of dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim), 0.2µM primer, 20 ng of DNA template, and 0.5 units *Taq* DNA polymerase (Promega). Amplification was carried out using a Perkin Elmer Cetus DNA Thermal Cycler programmed for 45 cycles of 1 min at 94° C, 1 min 30 sec at 37° C, 30 sec at 54° C, 2 min at 72° C, followed by a final extension period of 15 min at 72° C and held at 4° C until recovery. Amplification products were separated by electrophoresis on 2% agarose

gels, stained with ethidium bromide, and photographed on a UV transilluminator using Polaroid 667 black and white film. Primers that generated polymorphisms unique to the resistant bulk and resistant parent were tested for reproducibility by repeating the amplification at least two more times.

Linkage analysis

Goodness-of-fit between observed and expected segregation ratios for the resistance phenotype was tested using chi-square analysis. Primers that consistently generated polymorphic bands between the two bulks were used to amplify DNA templates from the individual progenies and a chi-square test was used to check for a 1:1 ratio (presence:absence) of the marker among the progeny. Linkage estimates were performed with MAPMAKER 3.0 (Lander et al., 1987; Lincoln et al., 1992) using the backcross function and default linkage criteria of LOD = 3.0. The most likely map order was determined by using the 'compare' command of MAPMAKER 3.0.

Development of sequence characterized amplified regions

Twenty-five microliters of amplification reaction products using primers OPH-17 and UBC-152 were separated by electrophoresis on 1% low melting agarose (Ultrapure) gels and stained with ethidium bromide. Bands containing the specific marker DNA fragments for each primer were visualized under UV illumination and excised from the gel. DNA was extracted from the agarose sections by digestion with Gelase™ enzyme followed by phenol-chloroform extraction and ethanol precipitation. The DNA was

reconstituted in 20 µl TE buffer and a 2 µl aliquot was used to reamplify the fragments using the original primers for verification of target fragment recovery.

The DNA was ligated to the p-GEM-T vector (Promega) using the supplier's protocol. The vector was transformed into α-DHL-5 *E. coli* host cells and plated out onto LB media plates containing IPTG and X-Gal. Positive colonies were transferred to fresh LB plates and screened by stabbing each colony with a sterile pipet tip, mixing the cells with 10 µl of sterile H_2O, boiling for 10 minutes in a 1.5 ml microfuge tube, and amplifying a 2 µl aliquot using the same amplification profile that produced the RAPD marker. Plasmid DNA from clones selected for the appropriate insert size was isolated using Qiagen mini-prep spin columns. The plasmid DNA was digested with *Nco*I and *Sal*I enzymes and the products were electrophoresed on 1% agarose to confirm the origin of the inserts. Plasmid DNA from two selected clones was sequenced using flourescent dye-primer reactions run on an ABI 373A automated sequencer.

Sequence data was obtained from approximately 400 kb of the ends of each insert and used to design primers consisting of 19-24 bp corresponding to the terminal ends of the cloned fragments. These primer pairs (forward and reverse) were used to amplify DNA from the original seedling populations using 30 cycles of 1 min. at 94°C, and 2 min. at 68°C, 2 min. at 72°C, followed by a 15 min. soak at 72°C and held at 4°C until recovery. Amplification products were separated by electrophoresis on 1% agarose, stained, and photographed under UV illumination.

These primers were also used to test a panel of resistant and susceptible germplasm representing some of the experimental cultivars that are part of the hazelnut breeding population.

RESULTS

Inoculation assays

Among the 'Willamette' × 'VR 6-28' progeny tested, 42 were scored as resistant and 62 as susceptible phenotypes. In the 'OSU 23-17' × 'VR 17-19' population, 22 were scored as resistant and 28 were found to be susceptible. Although the observed numbers of resistant and susceptible phenotypes in both populations appear to deviate slightly from the expected ratio, chi-square analysis did not show a significant difference from the expected 1:1 segregation ratio (Table 1).

Primer screening

500 decamer primers (Operon Technologies kits A-J, UBC primers 101-250, 301-400, and odd # primers between 701 and 800) were screened using the bulked segregant strategy. Among these 458 primers successfully amplified fragments ranging from 300 to 3,000 base pairs in length. Typically, 5 to 10 fragments per primer were produced, although as many as 18 distinct fragments and even single amplification products were observed among the primers tested. Initially seven primers produced clear polymorphisms between the resistant and susceptible bulks. Five of these primers proved to be consistently amplifiable and produced the same polymorphic fragment in the resistant parent but not in the susceptible one (Figure 1).

Linkage analysis

The progeny from both populations were scored for the presence of the amplified marker fragments (Figures 2 and 3) and the data analyzed using MAPMAKER 3.0.

Table 1. Chi-square analysis for resistance locus and RAPD markers.

Population	Locus[a]	Expected ratio	Observed frequency	X^2	Probability
'Willamette' x 'VR 6-28'	Res/Susc	1:1	42:62	3.85	0.050
	$OPA\text{-}08_{700}$	1:1	43:61	3.12	0.078
	$OPH\text{-}17_{1250}$	1:1	42:62	3.85	0.050
	$OPH\text{-}19_{600}$	1:1	42:62	3.85	0.050
	$UBC\ 152_{800}$	1:1	42:62	3.85	0.050
	$UBC\ 173_{500}$	1:1	43:61	3.12	0.078
'OSU 23-17' x 'VR 17-19'	Res/Susc	1:1	22:28	0.72	0.396
	$OPA\text{-}08_{700}$	1:1	23:27	0.32	0.571
	$OPH\text{-}17_{1200}$	1:1	22:28	0.72	0.396
	$OPH\text{-}19_{600}$	1:1	21:29	1.28	0.258
	$UBC\ 152_{800}$	1:1	22:28	0.72	0.396
	$UBC\ 173_{500}$	1:1	36:14[b]	9.68	0.002

[a]Marker designation: subscript number is approximate size in base pairs of RAPD marker generated by each primer. OP = Operon Technologies, UBC = British Columbia.

[b]Appears to segregate 3:1 (X^2 = 0.223, P = 0.637).

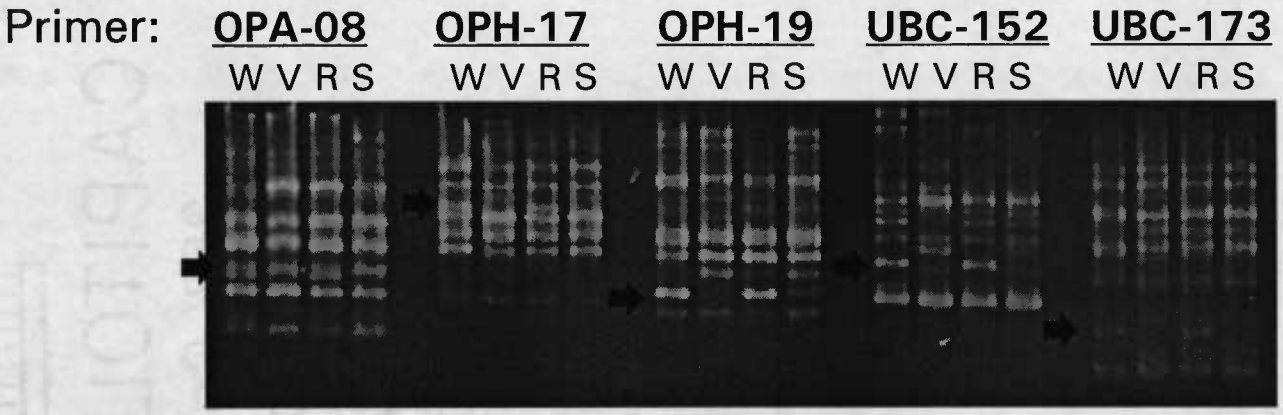

Figure 1. RAPD polymorphisms among bulked samples and parents. W ='Willamette', V ='VR 6-28', R = resistant bulk, S = susceptible bulk. Arrows indicate location of polymorphism.

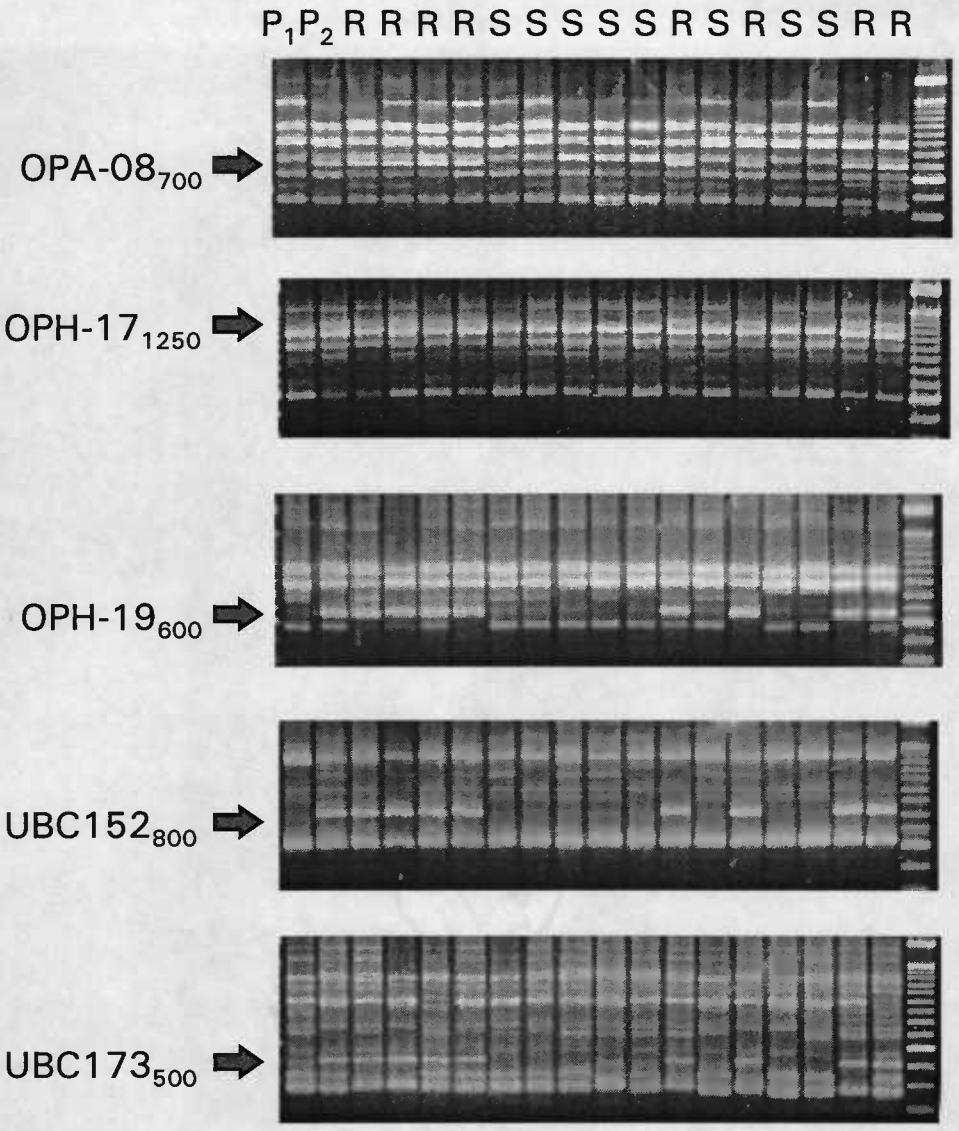

Figure 2. Segregating RAPD markers for 'Willamette' x 'VR 6-28' progeny. P_1 ='Willamette', P_2 = 'VR 6-28', R = resistant, S = susceptible.

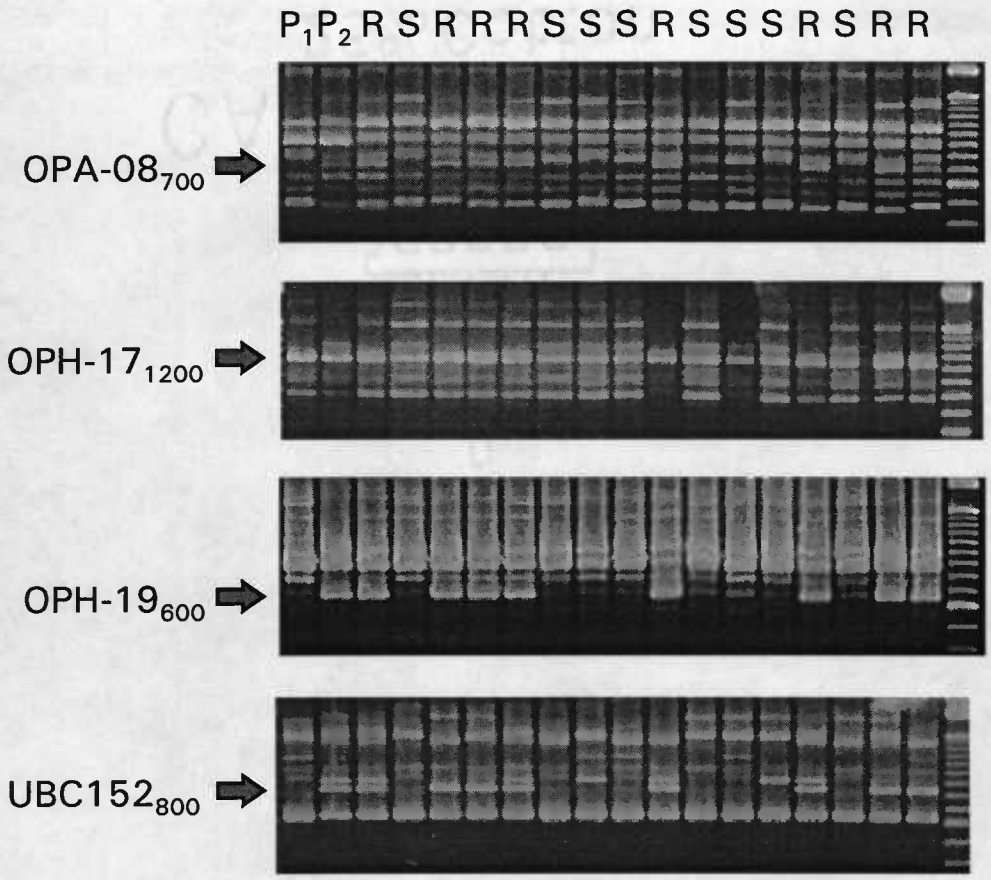

Figure 3. Segregating RAPD markers for 'OSU 23-17' x 'VR 17-19' progeny. P_1 ='OSU 23-17', P_2 = 'VR 17-19', R = resistant, S = susceptible.

These markers, designated OPA-08_{700}, OPH-17_{1250}, OPH-19_{600}, UBC152_{800}, and UBC173_{500}, were found to be closely linked in coupling to the resistance locus at distances of 6.7, 0.0, 2.0, 2.0, and 2.0 cM, respectively, for the 'Willamette' × 'VR 6-28' cross (Table 2). Three of these markers, OPA-08_{700}, OPH-19_{600}, and UBC152_{800}, were also found to be linked in coupling to the resistance locus in the 'OSU 23-17' × 'VR 17-19' cross at distances of 6.0, 6.0, and 4.0 cM, respectively (Table 2). In this second population primer OPH-17 amplified a fragment of the same size and relative intensity as that found in the 'Willamette' × 'VR 6-28' progeny, however the fragment cosegregated with the susceptible phenotye and was not found in any of the resistant progeny. Another polymorphic band approximately 1,200 base pairs in length (designated OPH-17_{1200}) was amplified by this primer and cosegregated with the resistance locus in the 'OSU 23-17' × 'VR 17-19' cross (Figure 3), with no recombinants observed. Primer UBC 173 appeared to segregate 3:1 in this population and was not included in the linkage analysis (Table 1).

Using the 'compare' command of MAPMAKER, the most likely map orders for both of the populations were generated (Figure 4). In the 'Willamette' × 'VR 6-28' cross the resistance locus appears to be flanked by markers on either side, with OPA-08_{700} on one side and OPH-19_{600}, UBC152_{800}, and UBC173_{500} on the opposite side. In the 'OSU 23-17' × 'VR 17-19' cross the resistance locus was also flanked by OPA-08_{700} on one side and OPH-19_{600} and UBC152_{800} on the opposite side. The markers OPH-17_{1250} and OPH-17_{1200} cosegregated with the resistance locus in their respective populations, with no recombinants observed.

Table 2. Recombination frequencies for resistance and marker loci.

Population	Locus	Observed Phenotypes[a] R/+	R/0	S/+	S/0	Recombination frequency[b]
'Willamette' × 'VR 6-28'	$OPA\text{-}08_{700}$	39	3	4	58	0.067
	$OPH\text{-}17_{1250}$	42	0	0	62	0.000
	$OPH\text{-}19_{600}$	41	1	1	61	0.019
	$UBC\ 152_{800}$	41	1	1	61	0.019
	$UBC\ 173_{500}$	41	1	1	61	0.019
	$SC152_{800}$	41	1	1	61	0.019
'OSU 23-17' × 'VR 17-19'	$OPA\text{-}08_{700}$	21	1	2	26	0.060
	$OPH\text{-}17_{1200}$	22	0	0	28	0.000
	$OPH\text{-}19_{600}$	20	2	1	27	0.060
	$UBC\ 152_{800}$	21	1	1	27	0.040
	$SC152_{800}$	21	1	1	27	0.040

[a]Phenotypes: R = resistant, S = susceptible, + = marker present, 0 = marker absent.
[b]Recombination frequency = (Sum of R/0 and S/+ seedlings)/ total # seedlings.

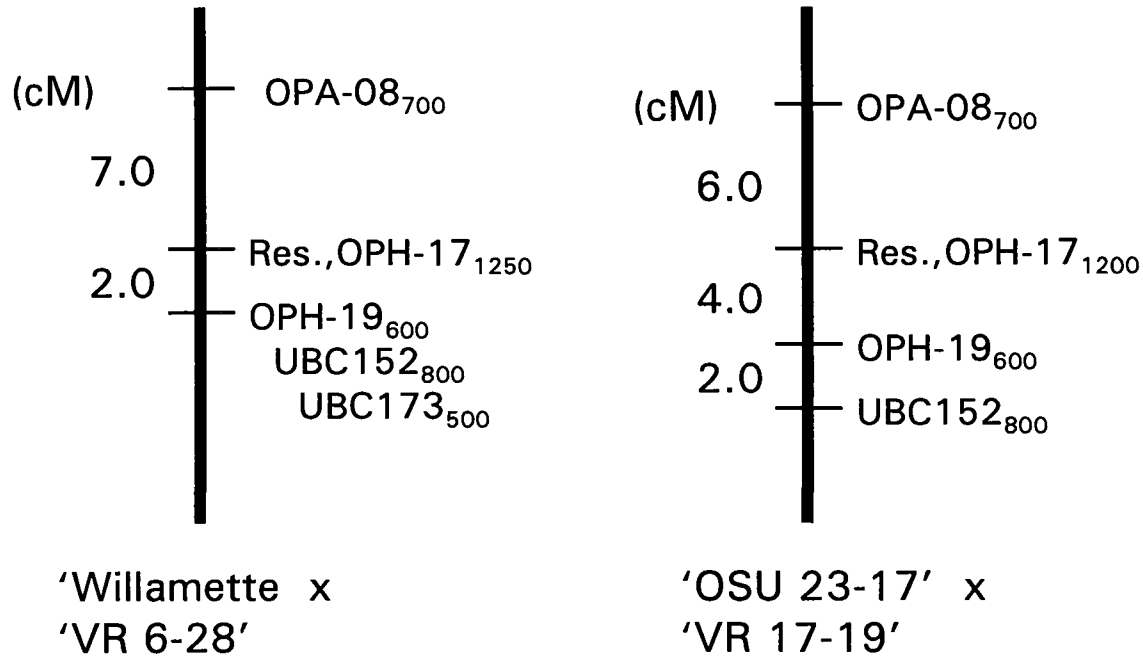

Figure 4. Most likely map orders of RAPD markers and resistanlocus for 'Willamette' x 'VR 6-28' and 'OSU 23-17' x 'VR 17-19' populations.

SCAR development

The polymorphic fragments $UBC152_{800}$ and $OPH\text{-}17_{1250}$ were cloned and the ends partially sequenced. From this data, forward and reverse primers were synthesized corresponding to the ends of each fragment. From the cloned product $UBC152_{800}$ a 24-mer primer (designated 152F) was synthesized using the sequence at one end of the fragment, and a 19-mer primer (152R) was derived from the sequence at the opposite end. From the cloned product $OPH\text{-}17_{1250}$, two 23-mer primers (17F, 17R) were synthesized, one for each end of the fragment. Each of these newly synthesized primers contained the original 10-mer sequences at their termini (Table 3).

The amplification product produced by the 152F and 152R primers (designated $SC152_{800}$) appeared to be the same size as the original RAPD marker, and the same segregation pattern was observed among the progeny of both crosses (Figure 5). The 17F and 17R primers also amplified a fragment corresponding to the size of the original RAPD marker, but was no longer polymorphic among the progeny of either cross.

The 152F and 152R primers were also used to amplify DNA extracted from 36 accessions that were used as parents in crosses performed in 1995 to generate progeny segregating for resistance (Figure 6). Twenty of these accessions had been previously scored as resistant phenotypes, and the rest were susceptible to infection by the fungus. Amplification of DNA from the all of the resistant parents produced the same size fragment corresponding to the cloned SCAR marker. Seven of the susceptible parents also generated amplification products. Three of these amplified a single product of the same size as the original marker, two parents amplified a single fragment approximately

Table 3. Sequence data for termini of cloned OPH-17_{1250} and $UBC152_{800}$ markers.

Marker	Primer designation	Sequence[a]	Polymorphism
OPH-17_{1250}	17F	CACTCTCCTCCTCCATCGGATTG	None
	17R	CACTCTCCTCCAGCAGCAACAAG	
$UBC152_{800}$	152F	CGCACCGCACATATTCCCCATTGG	Dominant
	152R	CGCACCGCACTCACACATC	

[a]Underlinded sequences are the original 10-mer primer sequences.

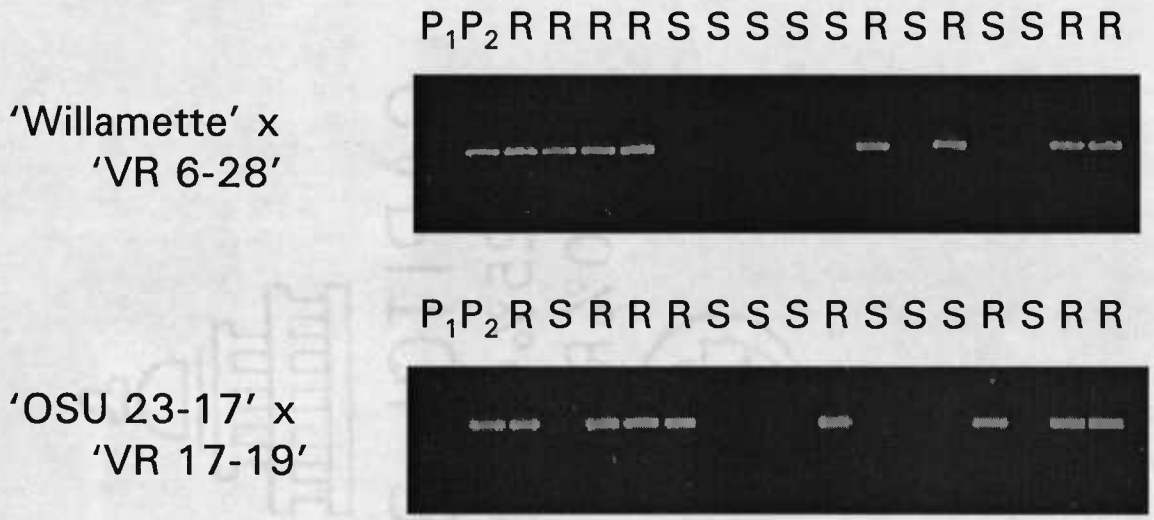

Figure 5. Segregation of $SC152_{800}$ marker among 'Willamette' x 'VR 6-28'and 'OSU 23-17' x 'VR 17-19' progeny. P_1 = susceptible parent, P_2 = resistant parent, R = resistant, S = susceptible.

Figure 6. Amplification of $SC152_{800}$ marker among germplasm accessions. R =resistant, S =susceptible.

100 bp smaller than the original marker, and two parents amplified both of these fragments .

DISCUSSION

Accurate genotyping of segregating populations is critical for the successful implementation of linkage analysis and MAS. The potential for infection 'escapes' in resistance scoring was a significant concern in our two populations. Therefore, all plants that were scored as resistant the first year were regrafted and tested again the following year. In addition, two assay procedures, microscopic examination and ELISA, were employed to verify results. Using this redundant screening approach, five escapes in the 'Willamette' × 'VR 6-28' cross and two in the 'OSU 23-17' × 'VR 17-19' cross were identified by ELISA testing after the second round of screening.

These two populations resemble a backcross configuration with respect to the resistance locus, therefore a 1:1 segregation ratio was expected among the progeny. This 1:1 ratio has been confirmed by previous findings using crosses between susceptible parents and selections having the 'Gasaway' gene (Osterbauer et al., 1997; Coyne et al., 1998). Although chi-square analysis supports a 1:1 segregation ratio, both of these populations appeared to be somewhat skewed towards susceptible genotypes. This small amount of bias may be attributed to loss of seedlings due to unsuccessful germination and poor seedling growth, discarding of non-dormant genotypes (25% in the 'Willamette' × 'VR 6-28' cross) before they are planted in the field, and the eventual death of some trees in the field after planting.

The bulked segregant analysis technique proved to be an efficient way to rapidly screen for markers linked to traits of interest without the benefit of a saturated molecular marker map. Bulk sizes typically range from five to fifteen individuals. Ten individuals

per bulk appears to be an optimal number for detection of markers that are tightly linked (<10 cM) to target loci. Using smaller bulk sizes would increase the likelihood of picking up markers that are weakly linked (>10 cM), while larger bulk sizes risk exceeding the ability to detect recombinants that may be present in the phenotypic pools.

Repeatability is an inherent problem with RAPD's (Kesseli et al., 1992; Paran and Michelmore, 1993; Kelly, 1995). The pattern of amplification products can vary slightly with different reaction conditions (template and primer concentration), annealing temperatures, or purity of DNA templates (Weeden et al., 1992). Therefore only primers that produced a consistent polymorphism under uniform conditions were considered for further evaluation. Several potential markers were discounted for this reason, even though they originally produced polymorphisms between the two bulks when first screened. We successfully found five primers that fit these criteria, each producing a polymorphic fragment that was repeatable and was tightly linked in coupling to the resistance locus in the 'Willamette' × 'VR 6-28' population. All of these markers were easily scored, although the primer OPH-17 produced a polymorphic fragment that was relatively faint compared to the other markers, and was adjacent to another fragment of slightly smaller size, which required more careful screening for linkage analysis.

Three of these markers were found to be tightly linked to the resistance locus in the 'OSU 23-17' × 'VR 17-19' population. Although the patterns of the amplification products varied slightly from the 'Willamette' × 'VR 6-28' progeny, the polymorphic marker fragments appeared to be the same size, as estimated by the adjacent molecular weight standards. The amplified marker fragment produced by OPH-17 exhibited a shift

from coupling to repulsion phase in the second population, and also amplified an additional fragment that was slightly larger in size and was linked in coupling to the resistance locus. The primer UBC 173 produced a product that is present in both the 'OSU 23-17' and 'VR 17-19' parents, and appears to segregate in a 3:1 ratio in this population.

Only coupling-phase markers were selected for linkage analysis. While repulsion-phased markers may provide greater efficiency in MAS for some breeding strategies (Haley et al., 1994b; Johnson et al., 1995), coupling-phased markers are well suited for selecting resistant individuals in backcross breeding programs. Our two populations are examples of modified backcrosses (Figure 7). When breeding highly heterozygous tree crops, a backcross strategy using a single recurrent parent would result in severe inbreeding depression (Mehlenbacher, 1995). To minimize inbreeding and avoid problems with self-incompatibility a different horticulturally desirable "recurrent parent" is used at each step of the backcrossing. This maintains the backcross configuration with respect to the target locus, thus allowing for the use of coupling-phased markers for selection of the disease resistance trait among the segregating progeny.

Some molecular marker maps may be useful for specific crosses only, especially when evaluating and selecting for quantitative traits. For the purpose of gene introgression using backcross breeding, however, maps containing markers tightly linked to resistance genes should be useful across a wide range of crosses containing the introgressed germplasm. The most likely map order for these five markers in the

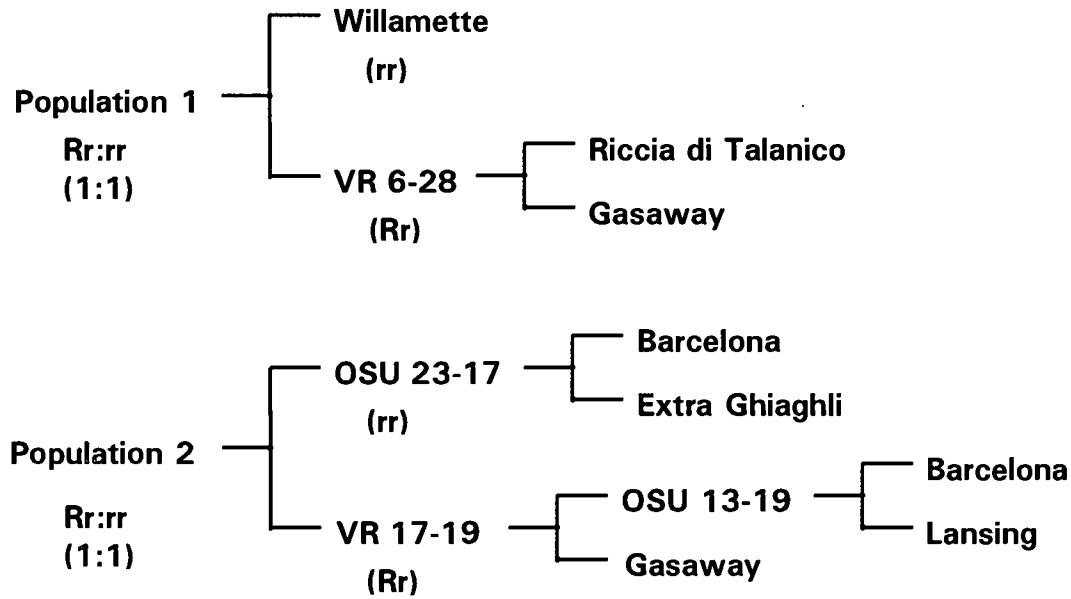

Figure 7. Pedigrees for 'Willamette' x 'VR 6-28' and 'OSU 23-17' x 'VR 17-19' segregating populations.

'Willamette' × 'VR 6-28' cross suggests that the resistance gene is flanked by the marker OPA-08_{700} on one side and the three markers OPH-19_{600}, UBC$152_{800,}$ and UBC173_{500} on the opposite side, with OPH-17_{1250} cosegregating with the resistance locus. In the 'OSU 23-17' × 'VR 17-19' cross the most likely map order places OPA-08_{700} on one side of the resistance locus and the markers OPH-19_{600} and UBC152_{800} on the opposite side, with OPH-17_{1200} also cosegregating with the resistance locus. These two maps are nearly identical with respect to the order of the markers around the resistance locus, and the recombination values are similar considering the 'OSU 23-17' × 'VR 17-19' population is half the size of the 'Willamette' × 'VR 6-28' cross. These markers (OPA-08_{700}, OPH-17_{1250}/OPH-17_{1200}, OPH-19_{600}, and UBC152_{800}) should have potential for applying MAS to the resistance breeding program in hazelnut.

For cloning, the two markers OPH-17_{1250} and UBC152_{800} were chosen for conversion to SCAR markers. OPH-17_{1250} was selected because it was most tightly linked to resistance in the first population, and the marker UBC152_{800} was chosen for its relatively large and distinct polymorphism which would be easier to isolate from the agarose gel. Excising and cloning RAPD bands from agarose gels often results in cloning more than one target fragment due to the carry over of neighboring bands in the agarose gel (Paran and Michelmore, 1993). When we reamplified the polymorphic bands from the agarose gels we often found one to two adjacent fragments that also reamplified. These extraneous cloned fragments were easily identified with the UBC152_{800} cloning, however the OPH-17_{1250} cloned products could not be easily distinguished from the adjacent fragment that is slightly smaller in size. The SCAR marker produced by this

cloning attempt amplified two different fragments, one a monomorphic band approximately 1,250 bp in length that was present in all of the progeny, and the other a polymorphic fragment slightly smaller in size that did not cosegregate with the resistance locus.

The OPH-17_{1250} clone was not confirmed as being from the original RAPD polymorphism, therefore it is unclear whether the loss of the original polymorphism was due to having cloned the wrong RAPD fragment or a result of the nature of the original polymorphism. Since the production of RAPD polymorphisms can occur if there is a mismatch in the primer site of the template DNA, a longer specific primer may be able to successfully anneal and amplify a fragment that was not produced by the shorter primer, giving a monomorphic banding pattern among the progeny. One possible approach to successfully isolating the OPH-17_{1250} fragment would be to run the RAPD amplification products on a polyacrylamide sequencing gel to further separate the bands and silver stain the DNA fragments (Bassam et al., 1991). The polymorphic band can then be directly reamplified by band-stabbing the gel (Wilton et al., 1997) and the newly amplified fragment run on another acrylamide gel alongside the original RAPD to verify the correct size of the isolated band. The marker would now be ready for cloning as previously described.

The marker UBC152_{800} initially produced two fragments (800 and 1,100 bp) using an annealing temperature of 60° C. The larger of these appeared as a monomorphic band across all of the progeny, while the 800 bp fragment cosegregated as expected with resistance. When the annealing temperature was increased to 68° C, only the 800 bp

fragment was amplified. This provided a more simplified pattern which makes scoring easier and may enable the use of ethidium bromide plate assay procedures in lieu of running agarose gels to detect the amplified marker.

The 800 bp SCAR marker successfully amplified in all of the seedlings of both crosses which showed the original RAPD marker. This suggests that the marker will be useful across different crosses and make it possible to use MAS in EFB resistance breeding. To test this further we examined a panel representing a wide range of germplasm currently being used in the resistance breeding program. The SCAR produced the expected fragment in all of the resistant germplasm in this panel, but in addition amplified products in eight of the 16 susceptible selections (Figure 6). In this case we cannot exclude the possibility that the SCAR primers are annealing to other homologous sequences found among other germplasm sources. The introgression of desirable genes into existing cultivars invariably results in the addition of extraneous unlinked germplasm along with the target genes. Even after several backcrosses large portions of the donor chromosome may still be retained in the recurrent parent (Young and Tanksley, 1989; Stam and Zeven, 1981). Even tightly linked markers do not necessarily represent portions of the target genes, and recombination occurring in previous generations can result in rearrangements of marker loci and other random sequences. In this situation it would be necessary to test all of the parents of crosses that are to be considered for MAS with the $UBC152_{800}$ marker.

Several SCAR markers have recently been developed as a potential means of selecting for disease resistance in such crops as wheat (Dedryver et al., 1996; Procunier et

al., 1997), common bean (Melotto et al., 1996; Adam-Blondon et al., 1994), pea (Timmerman et al., 1994: Gu et al., 1995), lettuce (Paran and Michelmore, 1993; Maisonneuve et al., 1994), tomato (Ohmori et al., 1996), and peanut (Garcia et al., 1996). Although there have been some instances of the use of RAPD's for screening tree crops (Virscek Marn et al., 1996), there are few published reports of the development and use of SCAR markers for MAS breeding. Gianfrancheschi et al. (1996) reported the development of markers for the scab resistance genes V_f and V_r in apple. Cheng et al. (1998) have developed a SCAR marker for the V_m gene conferring resistance to apple scab, and are evaluating its potential for MAS in apple breeding. Our research is among the few examples of the development of SCAR markers for disease resistance breeding in tree crops and the first work of its kind in hazelnuts.

The potential application of the cloned $UBC152_{800}$ marker in breeding for EFB resistance in hazelnuts is clearly demonstrated in this study. The marker has shown to be useful in identifying resistant phenotypes in two crosses with diverse parentage. By testing parents that are to be used for resistance breeding, it should be possible to determine which crosses will lend themselves to MAS using this marker. Successful cloning and sequencing of the remaining linked RAPD markers may yield additional SCAR markers that will adequately detect the presence of the introgressed resistance gene in a wider range of crosses. The availability of SCAR markers that flank the resistance locus would provide an even more powerful detection assay, as the probability of selecting a susceptible genotype becomes the product of the recombination frequencies of each marker.

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APPENDIX

APPENDIX - Data files for resistance and marker scoring

Willamette x VR 6-28

Sample	Date extr.	ug/ul	ul/1ug	EFB'94	EFB'95	Res/Susc	OPA-08	OPH-17	OPH-19	UBC152	UBC173	SC152
Willam.	7/30/94	0.32	3.13	NT	i	S	-	-	-	-	-	-
VR 6-28	7/30/94	0.43	2.33	NT	oo	R	+	+	+	+	+	+
Res. bulk	7/25/94	1.10	0.91	NT	NT	R	+	+	+	+	+	+
Sus. bulk	7/25/94	0.88	1.14	NT	NT	S	-	-	-	-	-	-
437.004	8/12/94	1.49	0.67	ooo	ooo	R	+	+	+	+	+	+
437.005	9/14/94	0.28	3.57	NA	NA	R	+	+	+	+	+	+
437.006	9/14/94	0.69	1.45	NA	NA	R	+	+	+	+	+	+
437.007	8/28/94	0.50	2.00	ooo	ooo	R	+	+	+	+	+	+
437.008	9/14/94	0.81	1.23	NA	NA	S	-	-	-	-	-	-
437.009	8/28/94	0.36	2.78	ooo	ooo	R	+	+	+	+	+	+
437.010	9/14/94	0.29	3.45	NA	NA	S	-	-	-	-	-	-
437.011	8/28/94	0.38	2.63	i	NT	S	-	-	-	-	-	-
437.012	9/14/94	1.17	0.85	i	NT	S	-	-	-	-	-	-
437.013	9/14/94	0.42	2.38	ooo	i	S	-	-	-	-	-	-
437.014	9/14/94	1.42	0.70	ooo	i	S	-	-	-	-	-	-
437.015	9/14/94	0.66	1.50	i	NT	S	+	-	-	-	-	-
437.016	9/14/94	0.78	1.28	oi	NT	S	-	-	-	-	-	-
437.017	8/12/94	1.26	0.79	i	NT	S	+	-	-	-	-	-
437.018	9/14/94	1.12	0.89	i	NT	S	-	-	-	-	-	-
437.019	9/14/94	0.99	1.01	i	NT	S	-	-	-	-	-	-
437.020	9/14/94	0.74	1.35	ooo	ooo	R	+	+	+	+	+	+
437.022	9/14/94	0.64	1.56	NA	NA	R	+	+	+	+	+	+
437.024	9/14/94	0.94	1.06	i	NT	S	-	-	-	-	-	-
437.027	9/14/94	0.49	2.04	i	NT	S	-	-	-	-	-	-
437.028	9/14/94	0.46	2.17	NA	NA	R	+	+	+	+	+	+
437.029	9/14/94	0.21	4.76	ooo	ooo	R	+	+	+	+	+	+
437.030	9/14/94	0.82	1.22	ooo	ooo	R	-	+	+	+	+	+
437.031	9/15/94	0.41	2.44	ooo	ooo	R	+	+	+	+	+	+
437.032	8/28/94	0.53	1.89	ooo	ooo	R	+	+	+	+	+	+

437.033	9/15/94	0.53	1.89	ooo	i	S	-	-	-	-	-	-
437.034	9/15/94	0.15	6.67	ii	NT	S	-	-	-	-	-	-
437.035	9/15/94	0.25	4	i	NT	S	-	-	-	-	-	-
437.036	9/15/94	0.51	1.96	i	NT	S	-	-	-	-	-	-
437.037	8/2/95	0.51	1.96	oi	NT	S	-	-	-	-	-	-
437.039	8/2/95	1.46	0.68	NA	NA	S	-	-	-	-	-	-
437.040	8/12/94	0.42	2.38	ooo	ooo	R	+	+	+	+	+	+
437.041	8/2/95	1.26	0.79	i	NT	S	-	-	-	-	-	-
437.042	9/15/94	0.54	1.85	ooo	ooo	R	+	+	+	+	+	+
437.044	9/15/94	0.26	3.85	ooo	ooo	R	-	+	+	+	+	+
437.045	8/12/94	0.37	2.7	ooo	ooo	R	+	+	+	+	+	+
437.046	8/27/94	0.57	1.75	i	NT	S	-	-	-	-	-	-
437.047	9/15/94	0.28	3.57	i	NT	S	-	-	-	-	-	-
437.048	8/12/94	0.47	2.13	i	NT	S	-	-	-	-	-	-
437.049	9/15/94	0.19	5.26	i	NT	S	-	-	-	-	-	-
437.050	8/2/95	1.13	0.88	ooo	ooo	R	+	+	+	+	+	+
437.051	8/2/95	1.44	0.69	oo	ooo	R	+	+	+	+	+	+
437.052	7/13/96	0.54	1,85	i	NT	S	-	-	-	-	-	-
437.053	8/2/95	1.14	0.88	ooo	ooo	R	+	+	+	+	+	+
437.054	8/2/95	1.30	0.77	ooo	ooo	R	+	+	+	+	+	+
437.055	8/2/95	0.85	1.18	i	NT	S	-	-	-	-	-	-
437.057	8/12/94	0.67	1.49	i	NT	S	-	-	-	-	-	-
437.058	9/17/94	0.63	1.59	oi	NT	S	-	-	-	-	-	-
437.059	8/12/94	1.24	0.81	i	NT	S	-	-	-	-	-	-
437.060	9/17/94	0.81	1.23	i	i	S	-	-	-	-	-	-
437.061	9/17/94	0.98	1.02	ooo	ooo	R	+	+	+	+	+	+
437.062	9/17/94	1.23	0.81	ooo	ooo	R	+	+	+	+	+	+
437.063	9/17/94	1.47	0.68	ii	NT	S	-	-	-	-	-	-
437.064	8/27/94	1.06	0.94	ooi	NT	S	-	-	-	-	-	-
437.066	8/12/94	0.40	2.50	ooo	ooo	R	+	+	+	+	+	+
437.068	9/17/94	1.12	0.89	i	NT	S	-	-	-	-	-	-
437.069	9/17/94	0.92	1.09	i	NT	S	-	-	-	-	-	-
437.071	9/17/94	0.90	1.11	ooo	i	S	-	-	-	-	-	-

437.072	8/12/94	1.14	0.88	ooo	o	R	+	+	+	+	+	+
437.073	9/17/94	1.18	0.85	ooo	oo	R	+	+	+	+	+	+
437.074	8/27/94	0.53	1.88	o	ooo	R	+	+	+	+	+	+
437.075	9/17/94	0.33	3.03	oi	i	S	+	-	-	-	-	-
437.076	8/12/94	0.73	1.37	i	NT	S	-	-	-	-	-	-
437.079	8/27/94	0.75	1.33	ooo	ooo	R	+	+	+	+	+	+
437.080	9/17/94	1.43	0.70	ooo	ooo	R	+	+	+	+	+	+
437.081	8/12/94	0.83	1.20	oi	NT	S	-	-	-	-	-	-
437.082	8/28/94	0.34	2.94	ooi	NT	S	-	-	-	-	-	-
437.083	8/12/94	0.34	2.94	ooo	i	S	-	-	-	-	-	-
437.084	8/28/94	0.44	2.27	oi	NT	S	-	-	-	-	-	-
437.085	9/17/94	0.71	1.41	ooo	ooo	R	+	+	+	+	+	+
437.086	9/17/94	0.29	3.45	ooo	ooo	R	+	+	+	+	+	+
437.088	9/17/94	0.84	1.19	i	NT	S	-	-	-	-	-	-
437.091	8/27/94	1.26	0.79	i	NT	S	-	-	-	-	-	-
437.092	8/27/94	0.43	2.33	ooo	ooo	R	+	+	+	+	+	+
437.093	9/17/94	0.42	2.38	ooo	ooo	R	+	+	+	+	+	+
437.094	9/17/94	0.31	3.23	ooo	ooo	R	+	+	+	+	+	+
437.096	9/17/94	0.88	1.14	i	NT	S	-	-	-	-	-	-
437.097	8/27/94	0.85	1.18	ooo	ooo	R	+	+	+	+	+	+
437.098	9/17/94	0.49	2.04	ii	NT	S	-	-	-	-	-	-
437.099	9/26/94	0.72	1.39	ooo	ooo	R	+	+	+	+	+	+
437.101	8/27/94	0.48	2.08	ooo	ooo	R	+	+	+	+	+	+
437.102	8/2/95	0.76	1.32	NA	NA	S	-	-	-	-	-	-
437.103	8/12/94	0.76	1.32	ii	NT	S	-	-	-	-	-	-
437.104	8/28/94	0.46	2.17	o?o	i	S	-	-	-	-	-	-
437.106	9/26/94	0.98	1.02	ooo	ooo	R	+	+	+	+	+	+
437.109	9/26/94	1.10	0.91	i	NT	S	-	-	-	-	-	-
437.110	9/26/94	0.97	1.03	i	NT	S	-	-	-	-	-	-
437.111	9/26/94	0.94	1.06	i	NT	S	-	-	-	-	-	-
437.112	9/26/94	0.66	1.51	i	NT	S	-	-	-	-	-	-
437.113	9/26/94	0.42	2.38	ooo	ooo	R	+	+	+	+	+	+
437.114	9/26/94	1.03	0.97	i	NT	S	-	-	-	-	-	-

437.115	8/28/94	2.01	0.49	ooo	ooo	R	+	+	+	+	+	+
437.116	8/12/94	0.72	1.39	ooo	ooo	R	+	+	+	+	+	+
437.118	8/28/94	0.54	1.85	oi	NT	S	-	-	-	-	-	-
437.120	9/26/94	0.78	1.28	i	NT	S	-	-	-	-	-	-
437.121	8/12/94	0.39	2.56	i	NT	S	-	-	-	-	-	-
437.122	9/26/94	0.96	1.04	i	NT	S	-	-	-	-	-	-
437.123	9/26/94	0.59	1.69	ooi	i	S	-	-	-	-	-	-
437.124	9/26/94	0.52	1.92	i	NT	S	-	-	-	-	-	-
437.125	8/2/95	0.67	1.49	NA	NA	S	-	-	-	-	-	-
437.126	8/27/94	0.30	3.33	ooo	ooo	R	+	+	-	-	-	-
437.128	9/26/94	0.80	1.25	ooo	ooo	R	-	+	+	+	+	+
437.131	8/2/95	0.51	1.96	NA	NA	S	-	-	+	+	+	+
437.133	9/26/94	0.27	3.7	NA	NA	S	+	-	-	-	-	-
Gasaway	8/27/94	1.86	0.54	NT	ooo	R	+	+	+	+	+	+

23-017 x VR 17-019

Seedling	Date extr.	ug/ul	ul/1ug	EFB'94	EFB'95	Res./Sus.	OPA-08	OPH-17	OPH-19	UBC152	SC152
23-017	7/22/94	0.24	4.17	NT	i	S	-	-	-	-	-
VR 17-19	7/22/94	0.15	6.67	NT	o?	R	+	+	+	+	+
498.001	6/95	1.15	0.87	ooo	ooo	R	+	+	+	+	+
498.003	6/95	2.34	0.43	i	NT	S	-	-	-	-	-
498.004	6/95	1.91	0.52	ooo	ooo	R	+	+	+	+	+
498.007	6/95	2.36	0.42	ooo	ooo	R	+	+	+	+	+
488.046	6/95	1.71	0.58	ooo	ooo	R	+	+	+	+	+
488.047	6/95	1.50	0.67	ooi	i	S	-	-	-	-	-
488.048	6/95	3.29	0.30	oi	NT	S	-	-	-	-	-
488.049	6/95	2.31	0.43	ooo	ooo	R	+	+	-	+	+
488.050	6/95	1.98	0.51	i	NT	S	-	-	-	-	-
488.051	6/95	0.72	1.39	ooo	ooo	R	+	+	+	+	+
488.052	6/95	1.56	0.64	i	NT	S	-	-	-	-	-
488.053	6/95	1.93	0.52	i	NT	S	-	-	-	-	-
488.054	6/95	2.66	0.38	i	NT	S	-	-	-	-	-
488.055	6/95	1.77	0.56	i	NT	S	-	-	-	-	-

488.056	6/95	1.95	0.51	i	NT	S	-	-	-	-	-
488.057	6/95	2.83	0.35	i	NT	S	+	-	-	-	-
488.058	6/95	2.72	0.37	i	NT	S	-	-	-	-	-
488.059	6/95	1.20	0.83	i	NT	S	-	-	-	-	-
488.060	6/95	0.98	1.02	oo	ooo	R	-	+	+	+	+
488.061	6/95	0.50	2.00	ooo	oi	S	-	-	-	-	-
488.062	6/95	0.50	2.00	ooo	o	R	+	+	+	+	+
488.063	6/95	1.42	0.70	oo	?oo	R	+	+	+	+	+
488.064	6/95	0.98	1.02	i	NT	S	-	-	-	-	-
488.065	6/95	1.32	0.76	ooi	NT	S	-	-	-	-	-
488.066	6/95	1.43	0.70	ooo	ooo	R	+	+	+	+	+
488.067	6/95	1.59	0.63	ooo	ooo	R	+	+	+	+	+
488.068	6/95	1.35	0.74	i	NT	S	-	-	-	-	-
488.069	6/95	2.56	0.39	ooo	ooo	R	+	+	+	+	+
488.070	6/95	0.82	1.22	ooo	ooo	R	+	+	+	+	+
488.071	6/95	1.28	0.78	i	NT	S	-	-	-	-	-
488.072	6/95	0.65	1.54	oo?	ooo	R	+	+	+	+	+
488.073	6/95	0.68	1.47	ooo	ooo	R	+	+	+	+	+
488.074	6/95	0.44	2.27	i	NT	S	-	-	-	-	-
488.075	6/95	1.44	0.69	i	NT	S	-	-	-	-	-
488.076	6/95	1.48	0.68	i	NT	S	-	-	-	-	-
488.077	6/95	1.22	0.82	ooo	ooo	R	+	+	+	+	+
488.078	6/95	1.45	0.69	ooo	?oo	R	+	+	+	+	+
488.079	6/95	0.64	1.56	i	NT	S	-	-	+	+	+
488.080	6/95	1.13	0.88	oi	NT	S	-	-	-	-	-
488.081	6/95	1.47	0.68	oii	i	S	-	-	-	-	-
488.082	6/95	0.67	1.49	ooo	ooo	R	+	+	+	+	+
488.083	6/95	0.42	2.38	i	NT	S	-	-	-	-	-
488.084	6/95	0.31	3.23	i	NT	S	+	-	-	-	-
488.085	6/95	0.41	2.44	ooo	ooo	R	+	+	+	+	+
488.086	6/95	1.31	0.76	ooo	ooo	R	+	+	+	+	+
488.087	6/95	0.86	1.16	ii	NT	S	-	-	-	-	-
488.088	6/95	0.27	3.70	oo	ooo	R	+	+	+	+	+

488.089	6/95	0.44	2.27	ooo	i	S	-	-	-	-	-
488.090	6/95	1.48	0.68	i	NT	S	-	-	-	-	-
488.091	6/95	0.92	1.09	ooo	ooo	R	+	+	-	-	-

Symbols:
o = no infection detected
i = infection detected
NA = not available, previously tested (1993) for resistance phenotype
NT = not tested
R = resistant phenotype
S = susceptible phenotype
+ = marker present
- = marker absent