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Title: New sources and linked AFLP markers for eastern filbert blight resistance in hazelnut

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Eastern filbert blight (EFB), caused by *Anisogramma anomala* (Peck) E. Müller, is a devastating disease to orchards of the European hazelnut (*Corylus avellana* L.) in the Willamette Valley, Oregon. Host resistance is the most desirable means of disease control. Fifty-eight hazelnut accessions, including European cultivars and interspecific hybrids were evaluated for their response to eastern filbert blight following greenhouse inoculation using an enzyme-linked immunosobent assay (ELISA) and visual inspection. Of the fifty-eight accessions, forty-four showed infections, twelve remained free of infection and two gave inconclusive results. The twelve accessions showing complete resistance to the disease are: European hazels 'Culpla' from Spain and 'COR 187' from Finland; *C. americana* × *C. avellana* hybrids 'COR 506', 'G081S' and Weschcke selections TP1, TP2 and TP3; *C. colurna* × *C. avellana* hybrids Chinese Trazel Gellatly #6 and #11, Turkish Trazel Gellatly #3 and a [(*C. colurna* × *C. avellana*) × *C. avellana*] backcross hybrid 'Lisa'; and a *C. heterophylla* var. *sutchuensis* × *C. avellana* hybrid 'Estrella #1'. These new sources of complete resistance can be

readily crossed to European cultivars and thus useful in the hazelnut breeding program.

OSU 408.040 showed complete resistance in a previous study and appears to be a new source of resistance apart from 'Gasaway'. Segregation analysis of three progenies from crosses with susceptible genotypes [97035 (OSU 245.098 × OSU 408.040), 97036 (OSU 474.013 × OSU 408.040) and 99035 (OSU 665.012 × OSU 408.040)] indicated that a single gene controls the resistance. AFLP methodology was employed to identify markers linked to EFB resistance in OSU 408.040. A total of 64 primer combinations were screened using progeny 97035. Five AFLP markers linked in coupling to resistance were identified, with recombination as shown: A4-265 (9.2 cM), A8-150 (0.0 cM), B2-125 (4.1 cM), C2-175 (5.9 cM) and D8-350 (2.5 cM). B2-125 was located on one side of the resistance locus and A4-265, C2-175 and D8-350 on the other side. Three of these markers (B2-125, C2-175 and D8-350) were also linked in coupling in a similar order in the second population 97036. The markers identified in this study are the first step toward marker-assisted selection for the OSU 408.040 source of resistance.

A Spanish cultivar 'Ratoli' showed complete resistance in a previous study and also appears to be a new source of resistance. Segregation analysis of two progenies [99035 (OSU 309.074 × Ratoli) and 99036 (OSU 665.012 × Ratoli)] indicated that 'Ratoli' transmitted resistance to 67% of its progenies. AFLP methodology was employed to identify markers linked to EFB resistance in

'Ratoli'. A total of 64 primer combinations were screened using progeny 99036. Two AFLP markers linked to Ratoli resistance were identified with recombination as shown: dA1-135 (13.7 cM) and C4-255 (4.2 cM). Marker dA1-135 is linked in repulsion to the resistance locus, whereas, C4-255 is linked in coupling. C4-255 and dA1-135 are on the same side of the resistance locus. One of the markers C4-255 was present in the second population 99035. Further effort is needed to find additional markers closely linked to the resistance locus in 'Ratoli'.

New sources and linked AFLP markers for eastern filbert blight resistance in hazelnut

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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.

Honglin Chen, Author

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Dr. Shawn Mehlenbacher was the main designer of the experiments (Chapters 2, 3, 4). Dr. Mehlenbacher also provided facilities and funding. David Smith grafted all the plant materials in the greenhouse for disease evaluation and was involved in field data collection.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF APPENDIX TABLES

NEW SOURCES AND LINKED AFLP MARKERS FOR EASTERN FILBERT BLIGHT RESISTANCE IN HAZELNUT

CHAPTER 1

INTRODUCTION

Eastern Filbert Blight of Hazelnut

The European hazelnut, *Corylus avellana* L., is produced commercially on 12,000 ha in Oregon's Willamette Valley, which represents 98% of the production in the United States and has a value of $15-42 million (FAO Production Yearbook, 2003). Oregon produces 3-5% of the world hazelnut crop (Hazelnut Marketing Board, 2003). However, the Oregon hazelnut industry is threatened by eastern filbert blight (EFB), a fungal disease caused by the pyrenomycete *Anisogramma anomala* (Peck) E. Müller.

A. anomala was first reported by Peck (1876) as a pathogen causing small cankers on the American hazel, *Corylus americana* Marsh., a common shrub in deciduous forests of the eastern United States. However, the fungus causes severe stem cankers on the commercially important European hazelnut. Perennial cankers often girdle branches and limbs, resulting in canopy dieback, yield loss and eventually tree death in 5 to 12 years if control measures are not practiced (Pinkerton et al., 1993). The disease prevented establishment of commercial production of the European hazelnut in the eastern United States (Fuller, 1910; Barss, 1930). The fungus was introduced from the eastern United States into southwestern Washington probably in the mid-1960s, and was first reported by

Davison and Davidson (1973). Eastern filbert blight (EFB) has moved southward into Oregon at an average rate of 2 to 3 km per year (Johnson et al., 1996) and it is now firmly established in the Willamette Valley of Oregon. Currently, 30% to 40% of Oregon's hazelnut orchards are affected or in proximity to diseased orchards (Pinkerton, 1992, 1996). Most of the plantings initially diseased have been destroyed, and the percentage of plantings infected with the pathogen continues to increase annually. EFB was recently discovered just north of Albany, OR (W. Chambers, pers. comm.) and near Eugene (R. Penhallegon, pers. comm.).

The infection cycle and developmental biology of the fungus have been well characterized. *A. anomala* is an obligate biotrophic fungus and has a two year life cycle (Johnson et al., 1994; Pinkerton et al., 1995). The pathogen infects immature hazelnut shoots in the spring at and following budbreak. Over the summer, *A. anomala* hyphae invade the phloem, cambium, and the outermost layer of the xylem, without obvious symptoms of disease (Stone et al., 1992; Johnson et al., 1994, 1996). The following spring, usually 13 to 16 months after initial infection, a perennial canker becomes visible with the formation of stromata by the pathogen. The maturation of two-celled ascospores begins in perithecia in August and spores are shot out from the perithecia forcibly during periods of prolonged branch wetness throughout the winter and spring (Gottwald and Cameron, 1980; Pinkerton et al., 1998a). Ascospores are dispersed to other branches and nearby trees by wind, wind-driven rain, and splashing raindrops. This dispersal can last

from autumn to mid-summer (Gottwald and Cameron, 1980; Pinkerton et al., 1992, 1995, 1998b, 2001). But infection occurs in a limited time in the spring.

In order to prevent the spread of eastern filbert blight to Oregon, a quarantine was established in 1922, prohibiting the importation of hazelnuts from east of the Rocky Mountains. This quarantine was further amended to prevent movement of hazelnut plant parts from the state of Washington and a portion of Columbia County, Oregon (Cameron, 1976). At present, the movement of plants from infected regions to those that are not yet infected is prohibited. Furthermore, retail sales of hazelnut tress have been prohibited by the Oregon Department of Agriculture (J. Hedberg, pers. comm.).

Current control practices include scouting, fungicide application, and therapeutic pruning (Johnson et al., 1993; Johnson et al., 1994). However, none of these methods is 100% effective. Extreme pruning of diseased branches cause a dramatic reduction in yield due to the loss of plant canopy. Commercial fungicides effective against EFB include protectants such as Bordeaux, chlorothalonil and copper hydroxide, as well as demethylation-inhibiting fungicides (DMI's) such as fenarimol, propaconazole and tebuconazole (Johnson and Pscheidt, 1993). A control level of 99% was provided when trees were treated with one or two sprays of chlorothalonil at budbreak followed by one or two sprays of a DMI product at two weeks intervals (Pscheidt, 2000). However, the cost of fungicide treatment is usually high relative to the benefits obtained.

Thus, the use of resistant varieties and pollenizers is the most desirable and economic means of disease control (Mehlenbacher, 1994).

Host Genetic Resistance to Eastern Filbert Blight

Within *C. avellena* L., the European hazelnut, the response of cultivars to eastern filbert blight varies widely from complete resistance (immunity) to high susceptibility. Cultivars such as 'Daviana', 'Ennis', 'DuChilly', 'Tonda Gentile delle Langhe', 'Tonda Romana', 'Casina', and 'Negret' are highly susceptible to eastern filbert blight. Meanwhile, cultivars like 'Hall's Giant', 'Willamette', and 'Barcelona', the latter of which is planted in 80% of the orchards in Oregon, show an intermediate response but the level of resistance is insufficient for sustained commercial production in the absence of fungicides (Pinkerton, 1993). Cultivars that show increased resistance to eastern filbert blight are 'Clark', 'Lewis', 'Gem', and 'Tonda di Giffoni'. In contrast, cultivars such as 'Gasaway', 'Zimmerman', 'Closca Molla', and 'Ratoli' exhibit complete resistance to the disease (Mehlenbacher and Thompson, 1991; Coyne, 1995; Lunde et al., 2000).

Complete resistance to eastern filbert blight was first discovered in 'Gasaway', an obsolete pollenizer that was found free of symptoms in a heavily infected 'DuChilly' orchard (Cameron, 1976). The resistance from 'Gasaway' is controlled by a single dominant gene (Mehlenbacher et al., 1991). This gene has been the major source of resistance utilized in the hazelnut breeding program at Oregon State University. Because of the many undesirable attributes of

'Gasaway', a modified backcross approach is being used to combine the resistance gene with desirable attributes in the creation of a commercially acceptable genotype (Mehlenbacher, 1994). This approach led to the release of four completely resistant pollinizers 'VR4-31', 'VR11-27', 'VR20-11', and 'VR23-18' to replace the highly susceptible 'Daviana' (Mehlenbacher, 1991) and more recently 'Gamma' and 'Delta' as pollinizers for 'Barcelona', 'Clark' and Lewis' (Mehlenbacher and Smith, 2003). Many promising selections are under evaluation at the OSU Smith Horticulture Research Farm. Hopefully, a completely resistant variety carrying the 'Gasaway' resistance gene will be released in the near future.

Segregation for resistance to eastern filbert blight was also studied in seedlings of 'Zimmerman', an uninfected seedling found by chance next to a severely infested orchard near Boring, Oregon. The 3:1 (resistant: susceptible) segregation ratio in progenies from crosses of three susceptible genotypes with 'Zimmerman' was a different inheritance pattern (Lunde, 1999). But the presence in these progenies of the $UBC152_{800}$ RAPD marker that co-segregates with the resistance gene in 'Gasaway' indicates the 'Zimmerman' source of resistance is the same or similar to that of 'Gasaway'. 'Epsilon' and 'Zeta', two pollinizers released recently from the OSU hazelnut breeding program, contain the 'Zimmerman' source of resistance.

The heritability of quantitative resistance to eastern filbert blight has also been investigated. Osterbauer et al. (1997) reported that high levels of partial resistance were transmitted by the pollen parents 'Gem' and 'Tonda di Giffoni',

and the seed parent 'Willamette'. Heritability estimated for disease incidence, number of cankers, and proportion of wood diseased was 0.21, 0.39 and 0.47, respectively (Osterbauer et al., 1997). This study suggested that quantitative resistance is exploitable in breeding for more durable resistance.

Other *Corylus* species possess significant resistance to eastern filbert blight as well. Resistant species include the American hazel (*C. americana*) (Ellis and Everhart, 1892), Turkish tree hazel (*C. colurna* L.) (Farris, 1969), Pacific hazel [*C. cornuta* var. *californica* (A.DC.) Sharp] (Barss, 1930; Davison and Davidson, 1973), beaked hazel (*C. cornuta* var. *cornuta* Marsh.) (Fuller, 1910; Barss, 1930), and two Asian shrub species, *C. heterophylla* Fisch. and *C. sieboldiana* Blume (Coyne et al., 1998). Greenhouse inoculation and field screening have confirmed that accessions of *C. cornuta* var. *cornuta*, *C. cornuta* var. *californica*, *C. heterophylla*, and *C. sieboldiana* are highly resistant (Coyne et al., 1998). Although *C. cornuta* var. *cornuta* and *C. sieboldiana* are hard to cross with *C. avellana* (Erdogan and Mehlenbacher, 2000), all others can be hybridized with the European hazel, if the latter is used as the pollen parent (Schuster, 1924; Reed, 1936; Gellatly, 1950, 1956a, 1956b, 1964, 1966; Weschcke, 1954; Farris 1969; Erdogan and Mehlenbacher, 2000). Thus, they offer additional resistant germplasm for use in breeding.

Early efforts in hazelnut breeding in North America attempted to combine the disease resistance of the *Corylus* species listed above, especially *C. americana*,

with the larger nut size and thinner shells of the European species. Interspecific hybridization at the New York State Agricultural Experiment Station in Geneva was made mainly between *C. americana* and *C. avellana*. In several cases, these interspecific hybrids showed resistance to eastern filbert blight. For example, the New York hybrid selections 'NYF-45', [Snyder × (Rush × DuChilly)], 'NY104' (Rush × DuChilly), 'NY110' (Rush × DuChilly), 'NY200' (Rush × Bollwyller), 'NY 616' (Rush × Barcelona), 'NY 1329' (Rush × Cosford), 'NY 1408' (Rush × Cosford), and 'NY 1464' (Rush × Cosford) were tested in the greenhouse and field and shown to be highly resistant to the disease (Coyne et al., 1998). 'Potomac', a *C. americana* × *C. avellana* hybrid, was also found to be resistant to eastern filbert blight (Lunde et al., 2000). One of four *C. cornuta* var. *californica* × *C. avellana* and two of three *C. heterophylla* × *C. avellana* hybrids also showed a high level of resistance in greenhouse inoculations and field screening (Coyne, 1998). 'Grand Traverse', a one-quarter *C. colurna* interspecific hybrid, showed no signs of the pathogen or symptoms of disease after greenhouse inoculation (Lunde et al., 2000).

Marker-Assisted Selection (MAS)

In recent years, several types of DNA markers have been developed, and a few have become important tools in applied plant breeding programs. DNA

markers, in conjunction with conventional breeding methods, have the potential to facilitate the incorporation of desirable traits. Four types of markers that have been used in plant genetics research are restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs) or microsatellites. Among them, RFLP is a hybridization-based technique, while RAPD, AFLP and SSR markers are generated by the polymerase chain reaction (PCR).

RFLPs involve the generation of a DNA probe, often from a cDNA library, digestion of genomic DNA, and Southern blotting to follow segregation in a population (Tanksley et al., 1989). Polymorphisms result from differences in the length of fragments obtained from digestion with restriction enzymes. RFLP markers are inherited in a co-dominant manner. However, the technique requires a large amount of high-quality DNA and usually the use of a radioactive isotope, but with only a few polymorphisms revealed per assay.

A RAPD marker is obtained through the amplification of genomic DNA using arbitrary primers, usually 8-12 mers (Welsh and McClelland, 1990; Williams et al. 1990). The PCR products are then subjected to electrophoresis and visualized by ethidium bromide (EtBr) staining. RAPD markers are usually dominant since the polymorphism is a result of the presence or absence of the priming sites in the genomic DNA. The assay is easy to perform and can reveal

moderate amounts of polymorphism. However, this technique is sensitive to amplification conditions such as primer concentration, $MgCl_2$ concentration, annealing temperature and other factors and hence less reproducible than other types of markers. As a result, some RAPD markers cannot be reliably generated and scored. This may be compensated by finding many markers and using the robust ones.

The AFLP technique combines the strengths of RFLPs and RAPDs, and it requires no knowledge of the sequence of the target region. AFLPs are the products of selective amplification of restriction fragments from digested genomic DNA (Vos et al., 1995). In an AFLP analysis, genomic DNA is digested by two restriction endonucleases, a rare cutter such as EcoR I and a frequent cutter such as Mse I. The resulting fragments are ligated to adapters, and then amplified by PCR in two steps using primers that contain the common sequences of the adapters, the restriction sites, and one to three arbitrary nucleotides as selective sequences. The polymorphisms are revealed by separating the amplified DNA fragments by electrophoresis on a sequencing gel, and visualized by silver staining, or radioactivity, or fluorescent dyes. With 50 or more bands amplified by each reaction, the AFLP procedure reveals a high level of polymorphism, and it is also reproducible.

An SSR locus is amplified using primers based on DNA sequences flanking the short tandem repeats, and polymorphism is due to differences in the

number of repeats (Morgante and Olivieri, 1993). SSR analysis often exhibits a high level of polymorphism and many alleles per locus. However, the development of SSRs requires a considerable investment and is time-consuming because an 'enriched' library has to be created, and DNA sequences obtained, in order to design primers for each SSR locus. On the other hand, once the markers are developed, they are cost-effective and convenient for use, especially when combined with capillary electrophoresis or fluorescent labeling methods that allow simultaneous genotyping using several (3-15) loci.

With the availability of these types of DNA markers and other newer user-friendly marker technologies that are on the horizon, marker assisted selection (MAS), originally proposed by Sax (1923) and Thoday (1961), has become a reality. MAS is a breeding strategy that takes advantage of the association, also called linkage disequilibrium, between agronomic traits with allelic variants of molecular markers, which are present in experimental populations such as backcross (BC), F_2, recombinant inbred line (RIL) or doubled haploid (DH) populations. It allows plant breeders to monitor the introgression of desirable alleles from each parent via molecular markers that are closely linked to the genes of interest.

Identification of DNA markers linked to genes of interest is an essential step toward MAS. Bulked segregant analysis (BSA) is an approach for rapid identification of markers linked to genes of interest (Michelmore et al., 1991). In

this approach, two bulked DNA samples are generated from seedlings in a segregating population from a controlled cross. Each bulk contains individuals that are identical for a particular trait (e.g. either resistant or susceptible to a particular disease). As a result, polymorphisms are revealed for the markers linked to the locus of interest, whereas all other unlinked markers appear monomorphic. When using the BSA approach, accurate phenotyping of the plants in the two bulks is critical for the successful identification of linked markers. Infection 'escapes' and recombination events are significant concerns that can lead to a failure to find markers linked to the target locus. An alternative approach is to use individuals (usually 3-5 for each phenotype) instead of bulks to overcome these problems (T. Kubisiak, pers. comm.).

The practical application of large-scale MAS in applied plant breeding programs requires high-throughput, cost-effective, reliable and easy to score marker assays. RFLP technology is not compatible with the need for high throughput, and is an expensive and tedious process. Though the start-up costs in the development of SSR markers are high, the technique should be useful in a MAS system because the markers are locus-specific, highly polymorphic, reliable and easy to use. The inherent problem of reproducibility of some RAPD markers gives rise to uncertainty as to their direct application in MAS. Although the AFLP technique is reliable, it is difficult to employ directly because it is complicated, technologically demanding, and costly. Conversion of RAPDs and AFLPs to

sequence-characterized amplified regions (SCARs) (Paran and Michelmore, 1993) or to cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel, 1993) provides alternatives for routine use of these markers.

Similar to RAPDs or AFLPs, SCARs are usually dominant markers. A SCAR is produced by isolating the polymorphic RAPD or AFLP band from the gel, sequencing the two ends of the fragment and designing two longer primers which are usually 17-24 bp in length. These long primers are annealed at higher temperatures (50-65 °C) and generally produce a single locus-specific amplified fragment corresponding to the original polymorphic band. SCAR markers can thereby be used in high-throughput screening processes. However, the original polymorphism of the RAPD or AFLP marker is frequently lost when converted to a SCAR marker, as a fragment is amplified in both resistant and susceptible seedlings.

A CAPS assay is usually performed if the polymorphism of the original markers is lost during the conversion to SCARs, or if co-dominant markers are needed. Two oligonucleotide primers are synthesized on the basis of known DNA sequences of the amplicon. Similar to SCARs, they specifically amplify single fragments. The polymorphism of CAPS is revealed by post-amplification digestion of template DNA, often with a 'frequent cutter' restriction enzyme that recognizes a 4 bp sequence in one allele but not in the other.

Though the efficiency of MAS for complex traits still needs validation, MAS has been shown to be extremely powerful and efficient for traits that are simply inherited, and either difficult or expensive to evaluate by conventional methods (Lande and Thompson, 1990; Dudley, 1993; Mehlenbacher, 1995; Young, 1999; Luby and Shaw, 2001; Dreher et al., 2002). Disease resistance, which is often simple and oligogenic in nature (Young, 1996) yet for which it is hard to establish reliable inoculation and scoring methods, are ideally suited to MAS analysis (Melchinger, 1990; Kelly, 1995). For example, unreliable inoculum has hampered the inoculation process in pepper breeding for potato virus Y (PVY) resistance, conferred by a single dominant gene *Pvr4*, because of the high observed rates of changes of PVY isolates from one pathotype to another. Thus, RAPD and SCAR markers linked to the *Pvr4* gene are useful for identifying PVY-resistant genotypes and facilitate the development of PVY-resistant lines (Arnedo-Andres et al., 2002). Screening for resistance to barley covered smut, caused by *Ustilago hordei* (Pers.) Lagerh, is time consuming and expensive because the disease phenotypes are not expressed until a late developmental stage. MAS accelerates the breeding program by screening the plants at a very early stage (Ardiel et al., 2002). MAS also facilitates breeding for rust [*Uromyces viciae fabae* (Pers.) J. Schrot.] resistance in faba bean where disease screening is difficult because of the race-specific nature of the resistance which requires the screening of plants simultaneously or even sequentially with several races (Avila et al., 2003). Young (2002) reported that MAS is efficient in predicting resistance to

soybean cyst nematode (*Heterodera glycines*), conferred by a single gene, *rhg1*, because it overcomes many of the deficiencies of greenhouse screening, which are time-consuming, labor intensive, and experience wide fluctuations in cyst counts. Roche et al. (1997) also suggest the use of a MAS strategy in the introgression of the *Sd1* gene in apple conferring resistance to biotypes 1 and 2 of rosy leaf curling aphid [*Dysaphis plantaginea* (Passerini)] to eliminate the problems in conventional screening experiments caused by the occurrence of different aphid biotypes, and the activities of aphid predators. In breeding for disease resistance in tree crops, disease screening can be complicated with juvenility and the large size of individual plants, which usually impose tremendous costs on the breeding programs. MAS has been demonstrated to be of considerable value in overcoming those problems in breeding for resistance to olive leaf spot caused by *Spilocaea oleagina* (Cast.) Hughes (Mekuria et al., 2001), to apple scab caused by *Venturia inaequalis* (Cooke) Winter (Gianfranceschi et al., 1996; King et al., 1998), to citrus tristeza virus (CTV) (Gmitter et al., 1996), and to root-knot nematode (*Meloidogyne* spp.) in peach (Lu et al., 1999).

Current methods for evaluation of eastern filbert blight resistance are slow and expensive. Field evaluation by observing canker development usually takes place 15-20 months after initial exposure. Quarantine regulations require isolation of disease tests from commercial orchards and breeding activities. An indirect enzyme-linked immunosorbent assay (ELISA) following greenhouse inoculation

shortens the detection time to 6 months (Coyne et al., 1996). However, this process is still time-consuming, and in some cases, yields ambiguous results even with repeated tests.

Therefore, MAS offers a way to improve selection efficiency in breeding for eastern filbert blight resistance. RAPD markers tightly linked to the 'Gasaway' gene for resistance have been identified (Davis et al., 1997, Mehlenbacher et al., 2003). Several of these markers have been sequenced and could be converted into SCARs. One of these RAPD markers, $UBC152_{800}$, is easy to score and robust to amplification conditions. It has been routinely used in MAS for several years in the hazelnut breeding program at Oregon State University. The availability of these tightly linked markers has provided a means of screening genotypes for eastern filbert blight resistance at an early stage. It is also useful for confirming the phenotyping results of promising selections. Only seedlings with the appropriate markers are planted in the field and used as parents for the next generation, leading to a greatly enriched gene pool for selection for other traits. The application of MAS improves selection efficiency and greatly facilitates the breeding of new resistant varieties.

Research Objectives

The major hazelnut breeding objective at Oregon State University is to develop new varieties that are suited to the blanched kernel market. Desirable horticultural traits include round nut shape, small to medium nut size, high percent kernel, easily blanched kernels, few defects, precocity, early nut maturity, free-falling nuts, and high yield (Mehlenbacher, 1991). New cultivars need to be at least moderately resistant to eastern filbert blight. To date, much effort has been placed on combining the 'Gasaway' resistance gene with the horticulturally desirable traits listed above, using a marker-assisted selection strategy to supplement conventional breeding methods. Due to the concern that a new race or isolate of *A. anomala* could potentially overcome the 'Gasaway' resistance gene (Johnson, 1996), investigation of additional sources of genetic resistance has been an emphasis in the breeding program.

In this research, the overall objective is to identify new sources of complete resistance to eastern filbert blight and facilitate their use in the breeding program, and is presented in three parts. In the first part, a large number of hazelnut accessions was evaluated as potential new sources of complete resistance to eastern filbert blight. In the second part, a study of the inheritance of resistance from OSU 408.040 was carried out and AFLP markers linked to resistance were then identified. In the third part, the same approaches were used to study segregation for resistance in seedlings of 'Ratoli' and to identify AFLP markers

linked to resistance. 'Ratoli' is superior in many horticultural respects to 'Gasaway', and its use should require fewer generations to combine resistance with other traits needed in a commercially acceptable variety. The markers identified in this study will allow MAS to be used for the introgression of resistance from these two additional sources.

References

Ardiel, G.S., T.S. Grewal, P. Deberdt, B.G. Rossnagel and G.J. Seoles. 2002. Inheritance of resistance to covered smut in barley and development of a tightly linked SCAR marker. Theor. Appl. Genet. 104:457-464.

Arnedo-Andres, M.S., R. Gil-Ortega, M. Luis-Arteaga and J.I. Hormaza. 2002. Development of RAPD and SCAR markers linked to the *Pvr4* locus for resistance to PVY in pepper (*Capsicum annuum* L.). Theor. Appl. Genet. 105:1067-1074.

Avila, C.M., J.C. Sillero, D. Rubiales, M.T. Moreno and A.M. Torres. 2003. Identification of RAPD markers linked to the *Uvf-1* gene conferring hypersensitive resistance against rust (*Uromyces viciae-fabae*) in *Vicia faba* L. Theor. Appl. Genet. (in press).

Barss, H.P. 1930. Eastern filbert blight. California Agr. Dept. Bull. 19:489-490.

Cameron, H.R. 1976. Eastern filbert blight established in the Pacific Northwest. Plant Dis. Rptr. 60:737-740.

Coyne, C.J. 1995. Genetic resistance to eastern filbert blight. MS thesis. Department of Horticulture, Oregon State University, Corvallis, Oregon.

Coyne, C.J., S.A. Mehlenbacher, R.O. Hampton, J.N. Pinkerton and K.B. Johnson. 1996. Use of ELISA to rapidly screen hazelnut for resistance to eastern filbert blight. Plant Disease 80:1327-1330.

Coyne, C.J., S.A. Mehlenbacher, K.B. Johnson, J.N. Pinkerton and D.C. Smith. 2000. Comparison of two methods to evaluate quantitative resistance to eastern filbert blight in European hazelnut. J. Amer. Soc. Hort. Sci. 125:603-608.

Coyne, C.J., S.A. Mehlenbacher, and D.C. Smith. 1998. Sources of resistance to eastern filbert blight in hazelnut. J. Amer. Soc. Hort. Sci. 123:253-257.

Davison, A.R. and R.M. Davidson, Jr. 1973. *Apioporthe* and *Monochaetia* cankers reported in western Washington. Plant Dis. Rep. 57:522-523.

Davis, J.W. and S.A. Mehlenbacher. 1997. Identification of RAPD markers linked to eastern filbert blight resistance in hazelnut. Acta Hort. 445:553-556.

Dreher, K., M. Morris, M. Khairallah, M. Ribaut, P. Shivaji, S. Ganesan, S. Pandey, G. Srinivasan, R.E. Evenson, V. Santaniello and D. Zilberman. 2002. Is marker-assisted selection cost-effective compared with conventional plant breeding methods? The case of quality protein maize. Economic and Social Issues in Agricultural Biotechnology 15:203-236.

Dudley, J.W. 1993. Molecular markers in plant improvement: manipulation of genes affecting quantitative traits. Crop Science 33:660-668.

Ellis, J.B. and B.M. Everhart. 1892. The North American Pyrenomycetes. Ellis and Everhart, Newfield, New Jersey. p. 531- 532.

Erdoğan, V. and S.A. Mehlenbacher. 2000. Interspecific hybridization in hazelnut (*Corylus*). J. Amer. Soc. Hort. Sci. 125:489-497.

FAO production yearbook. 2003. http://www.fao.org/DOCREP/003/X4484E/x4484e03.html.

Farris, C.W. 1969. Hybridization of filberts. p.299- P300. In: R. A. Jaynes (ed.). Handbook of North American nut trees. Northern Nut Growers Assn., Humphrey Press, Hamden, Conn.

Farris, C.W. 1970. Inheritance of parental charactheristics in filbert hybrids. Ann. Rept. Northern Nut Growers Soc. 61:54-58.

Fuller, A.S. 1910. Filbert or hazelnut. p. 118-146. In: The nut culturist. Orange Judd, New York.

Gellatly, J.U. 1950. Description of filazel varieties. Ann. Rept. Northern Nut Growers Assn. 41:116-117.

Gellatly, J.U. 1956a. Tree hazels-*Corylus colurna*. Ann. Rept. Northern Nut Growers Assn. 47:110-112.

Gellatly, J.U. 1956b. Filazels. Ann. Rept. Northern Nut Growers Assn. 47:112-114.

Gellatly, J.U. 1964. Filazels. Ann. Rept. Northern Nut Growers Assn. 55:153-155.

Gellatly, J.U. 1966. Tree hazels and their improved hybrids. Ann. Rept. Northern Nut Growers Assn. 57:98-106.

Gianfranceschi, L., B. Koller, N. Seglias, M. Kellerhals and C. Gessler. 1996. Molecular selection in apple for resistance to scab caused by *Venturia inaequalis*. Theor. Appl. Genet. 93:199-204.

Gmiter, F.G.Jr., S.Y. Xiao, S. Huang, X.L. Hu, S.M. Garnsey and Z. Deng. 1996. A localized linkage map of the citrus tristeza virus resistance gene region. Theor. Appl. Genet. 92:688-695.

Gottwald, T.R. and H.R. Cameron. 1980. Infection site, infection period, and latent period of canker caused by *Anisogramma anomala* in European filbert. Phytopathology 70:1083-1087.

Hazelnut Marketing Board. 2003. http://oregonhazelnuts.org/

Johnson, K.B. and J.W. Pscheidt. 1993. Evaluation of chlorothalonil, fenarimol, and flusilazole for control of eastern filbert blight. Plant Disease 77:831-837.

Johnson, K.B., J.N. Pinkerton, S.M. Gaudreault and J.K. Stone. 1994. Infection of European hazelnut by *Anisogramma anomala*: Site of infection and effect of host developmental stage. Phytopathology 84:1465-1470.

Johnson, K.B., S.A. Mehlenbacher, J.K. Stone and J.W. Pscheidt. 1996. Eastern filbert blight of European hazelnut – it's becoming a manageable disease. Plant Disease 80:1308-1316.

Kelly, J.D. 1995. Use of random amplified polymorphic DNA markers in breeding for major gene resistance to plant pathogens. HortScience 30:461-465.

King, G.J., F.H. Alston, L.M. Brown, E. Chevreau, K.M. Evans, F. Dunemann, J. Janse, F. Laurens, J.R. Lynn, C. Maliepaard, A.G. Manganaris, P. Roche, H. Schmidt, S. Tartarini, J. Verhaegh and R. Vrielink. 1998. Multiple field and glasshouse assessments increase the reliability of linkage mapping of the *Vf* source of scab resistance in apple. Theor. Appl. Genet. 96:699-708.

Konieczny, A. and F.M. Ausubel. 1993. A procedure for mapping Arabidopsis mutations using co-dominant ecotype-specific PCR-based markers. Plant J. 4:403-410.

Lande, R. and R. Thompson. 1990. Efficiency of marker-assisted selection in the improvement of quantitative traits. Genetics 124:743-756.

Lunde, C.F., S.A. Mehlenbacher and D.C. Smith. 2000. Survey of hazelnut cultivars for response to eastern filbert blight inoculation. HortScience 35:729-731.

Lu, Z.X., K.S. Alaoui, G.L. Reighard, W.V. Baird and A.G. Abbott. 1999. Development and characterization of a co-dominant marker linked to root-knot nematode resistance, and its application to peach rootstock breeding. Theor. Appl. Genet. 99:115-122.

Luby, J.J. and D.V. Shaw. 2001. Does marker-assisted selection make dollars and sense in a fruit breeding program? HortScience 36:872-879.

Mekuria, G.T., G. Collins and M. Sedgley. 2001. Identification of genetic markers in olive linked to olive leaf spot resistance and susceptibility. J. Amer. Soc. Hort. Sci. 126:305-308.

Melchinger, A.E. 1990. Use of molecular markers in breeding for oligogenic disease resistance. Plant Breeding 104:1-19.

Mehlenbacher, S. 1991. New developments in the Oregon hazelnut breeding program. Ann. Rept. Northern Nut Growers Assn. 82:103-110.

Mehlenbacher, S.A., M.M. Thompson and H.R. Cameron. 1991. Occurrence and inheritance of resistance to eastern filbert blight in 'Gasaway' hazelnut. HortScience 26:410-411.

Mehlenbacher, S. 1994. Genetic improvement of the hazelnut. Acta Hort. 351:23-38.

Mehlenbacher, S. 1995. Classical and molecular approaches to breeding furit and nut crops for disease resistance. HortScience 30:466-477.

Mehlenbacher, S.A. and D.C. Smith. 2003. Hazelnut pollenizers 'Gamma', 'Delta', 'Epsilon' and 'Zeta'. HortScience (in press).

Mehlenbacher, S.A., R.N. Brown, J.W. Davis, H. Chen, N. Bassil and D.C. Smith. 2003. RAPD markers for eastern filbert blight resistance in *Corylus avellana*. Theor. Appl. Genet (in press).

Michelmore, R. W., I. Paran and R. V. Kesseli. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Nat. Acad. Sci. USA. 88:9829-9832.

Morgante, M. and A.M. Olivieri. 1993. PCR-amplified microsatellites as markers in plant genetics. Plant J. 3:175-182.

Osterbauer, N. K., K. B. Johnson, S.A. Mehlenbacher and T.L. Sawyer. 1997. Analysis of resistance to eastern filbert blight in *Corylus avellana*. Plant Disease 81:388-394.

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

Pinkerton, J.N. 1992. Distribution and characteristics of the eastern filbert blight epidemic in western Oregon. Plant Disease 76:1179-1182.

Pinkerton, J.N. 1993. Susceptibility of European hazelnut clones to eastern filbert blight. Plant Disease 77:261-266.

Pinkerton, J.N., J.K. Stone, S.J. Nelson and K.B. Johnson. 1995. Infection of European hazelnut by *Anisogramma anomala*: ascospore adhesion, mode of penetration of immature shoots, and host response. Phytopathology 85:1260-1268.

Pinkerton, J.N., K.B. Johnson, J.K. Stone, K.L. Ivors. 1998a. Factors affecting the release of ascospores of *Anisogramma anomala*. Phytopathology 88:122-128.

Pinkerton, J.N., K.B. Johnson, J.K. Stone and K.L. Ivors. 1998b. Maturation and seasonal discharge pattern of ascospores of *Anisogramma anomala*. Phytopathology 88:1165-1173.

Pscheidt, J.W and S. Cluskey. 2001. Fungicide control of eastern filbert blight. Acta Hort. 556:411-418.

Reed, C.A. 1936. New filbert hybrids. J. Hered. 27:427-431.

Roche, P., F.H. Alston, C. Maliepaard, K.M. Evans, R. Vrielink, F. Dunemann, T. Markussen, S. Tartarini, L.M. Brown, C. Byder, G.J. King. 1997. RFLP and RAPD markers linked to the rosy leaf curling aphid resistance gene (Sd1) in apple. Theor. Appl. Genet. 94:3-4.

Sax, K. 1923. The association of size differences with seed-coat pattern and pigmentation in *Phaseolus vulgaris*. Genetics 8:552-560.

Schuster, C.E. 1924. Filberts: 2. Experimental data on filbert pollination. Oregon Agr. Expt. Sta. Bull. 208.

Stone, J.K., K.B. Johnson, J.N. Pinkerton J.N. and J.W. Pscheidt. 1992. Natural infection period and susceptibility of vegetative seedlings of European hazelnut to *Anisogramma anomala*. Plant Disease 76:348-352.

Tasias-Valls, J. 1975. EI avellano en la provincia de Tarragona (in Spanish) Escma. Diputacion provincial de Tarragona. Fundacion Servicio Agropecuario Provincial. Tarragona, Spain.

Tanksley, S.D., N.D. Young, A.H. Paterson and M.W. Bonierbale. 1989. RFLP mapping in plant breeding: new tools for an old science. Bio/Technol 7:257-263.

Thoday, J.M. 1961. Location of polygenes. Nature 191:368-370.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper and M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23:4407-4414.

Weschcke, C. 1954. Growing nuts in the north. Webb, St. Paul. p. 24-38.

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

Williams, J.G.K., A.R. Kubelic, K.J. Livak, J.A. Rafalsky and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531-6535.

Young, N.D. 1996. QTL mapping and quantitative disease resistance in plants. Annu. Rev. Phytopathol. 34:479-501.

Young, N.D. 1999. A cautiously optimistic vision for marker-assisted breeding. Molecular Breeding 5:505-510.

Young, N.D., J. Mudge, J.L. Starr, R. Cook and J. Bridge. 2002. Marker-assisted selection for soybean cyst nematode resistance. p. 241-252. In: Plant Resistance to Parasitic Nematodes. CABI Publishing, Wallingford, UK.

Chapter 2

HAZELNUT ACCESSIONS PROVIDE NEW SOURCES OF RESISTANCE TO EASTERN FILBERT BLIGHT

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Abstract

Fifty-eight hazelnut accessions, including *Corylus avellana* L. and interspecific hybrids, were evaluated for their response to eastern filbert blight caused by *Anisogramma anomala* (Peck) E. Müller following greenhouse inoculation using an enzyme-linked immunosobent assay (ELISA) and visual inspection. Of the fifty-eight accessions, forty-four showed infections, twelve remained free of infection and two gave inconclusive results. The twelve accessions showing complete resistance to the disease are: European hazels 'Culpla' from Spain and 'COR 187' from Finland; *C. americana* × *C. avellana* hybrids 'COR 506', 'G081S' and Weschcke TP1, TP2 and TP3; *C. colurna* × *C. avellana* hybrids Chinese Trazel Gellatly #6 and #11, Turkish Trazel Gellatly #3 and a [(*C. colurna* × *C. avellana*) × *C. avellana*] backcross hybrid 'Lisa'; and a *C. heterophylla* var. *sutchuensis* × *C. avellana* hybrid 'Estrella #1'. These new sources of complete resistance can be readily crossed to commercial European varieties and thus useful in the hazelnut breeding program.

Introduction

Production of the European hazelnut, *Corylus avellana* L. in Oregon's Willamette Valley represents 98% and 3-5% of the production in the United States and the world, respectively (FAO Production Yearbook, 2003). However, the Oregon hazelnut industry is threatened by eastern filbert blight, a disease caused

by the pyrenomycete *Anisogramma anomala* (Peck) E. Müller. The fungus causes severe cankers, rapid yield loss and eventually tree death in 5 to 12 years if control measures are not practiced (Pinkerton et al., 1993). Current control practices include fungicide applications and therapeutic pruning. However, due to the expense of fungicides and dramatic yield loss caused by severe pruning of cankers, genetic resistance is the most desirable and economic means of disease control (Mehlenbacher, 1994). Therefore, developing varieties resistant to eastern filbert blight is a major goal of the Oregon State University (OSU) hazelnut breeding program.

Complete resistance to eastern filbert blight was first discovered in the obsolete pollenizer 'Gasaway' (Cameron, 1976). Genetic studies showed that immunity is conferred by a single dominant gene (Mehlenbacher and Thompson, 1991). 'Gasaway' has been the major source of resistance utilized in the OSU breeding program. However, 'Gasaway' has extremely poor agronomic attributes, which demands more effort, especially in breeding for perennial woody plant, in combining the resistance gene with desirable attributes in the creation of a commercially acceptable variety. Furthermore, concern exists about the durability of a single resistance gene because a new race or isolate of *A. anomala* could potentially overcome it (Johnson, 1996). Therefore, the identification of additional sources of genetic resistance would be highly desirable.

Surveys of the response within the European hazelnut to eastern filbert blight have revealed additional sources of complete resistance. 'Zimmerman'

remained uninfected by the disease both in the field (Pinkerton, pers. comm.) and following greenhouse inoculations (Coyne, 1995). Though it is possible that 'Zimmerman' carries the same resistance gene as 'Gasaway', it is still valuable because it transmits resistance to a higher percentage (75%) of its offspring (Lunde, 1999). 'Closca Molla' and 'Ratoli', both superior in many horticultural respects to 'Gasaway', displayed no signs or symptoms of eastern filbert blight following greenhouse inoculations (Lunde et al., 2000).

Studies have shown that complete resistance also exits in other *Corylus* species. These species include the American hazel (*C. americana* Marsh.) (Ellis and Everhart, 1892), Turkish tree hazel (*C. colurna* L.) (Farris, 1969), Pacific hazel [*C. cornuta* var. *californica* (A.DC.) Sharp] (Davison and Davidson, 1973), beaked hazel (*C. cornuta* var. *cornuta* Marsh.) (Fuller, 1910; Barss, 1930), and two Asian shrub species, *C. heterophylla* Fisch., and *C. sieboldiana* Blume (Coyne et al., 1998). Greenhouse inoculation and field screening have confirmed that accessions of *C. cornuta* var. *cornuta*, *C. cornuta* var. *californica*, *C. heterophylla*, and *C. sieboldiana* are immune or near immune to eastern filbert blight (Coyne et al., 1998). Most of these species, except *C. cornuta* var. *cornuta* and *C. sieboldiana*, can be hybridized with the European hazel if the latter is used as the pollen parent (Schuster, 1924; Reed, 1936; Weschcke, 1954; Farris 1970; Erdogan and Mehlenbacher, 2000). Interspecific hybrid selections 'NYF-45', [Snyder × (Rush × DuChilly)], 'NY104' (Rush × DuChilly), 'NY110' (Rush × DuChilly), 'NY200' (Rush × Bollwyller), 'NY 616' (Rush × Barcelona), 'NY 1329' (Rush ×

Cosford), 'NY 1408' (Rush × Cosford), and 'NY 1464' (Rush × Cosford) were obtained from crosses of *C. americana* × *C. avellana* and showed complete resistance to eastern filbert blight when tested in the greenhouse and field (Coyne et al., 1998). Lunde et al. (2000) reported that 'Potomac', a *C. americana* × *C. avellana* hybrid, 'Yoder #5', a suspected *C. americana* × *C. avellana* hybrid, and 'Grand Traverse', a one-quarter *C. colurna* interspecific hybrid, were completely resistant to eastern filbert blight after greenhouse inoculations. They offer additional resistant germplasm for use in the breeding program.

The fungus *A. anomala* has a two-year life cycle and a long incubation period ranging from 12 to 14 months before symptoms are expressed (Gottwald et al., 1980; Johnson et al., 1994; Pinkerton et al., 1995). Thus, field evaluation by observing canker development is a slow process. An indirect enzyme linked immunosorbent assay (ELISA) test following greenhouse inoculation shortens the detection time to 6 months and offers a reliable method for evaluation of genotypes for complete resistance (Coyne et al., 1996).

In this study, the objective was to evaluate 58 hazelnut accessions from the collections of the OSU hazelnut breeding program and the U. S. Dept. of Agriculture, Agricultural Research Service (USDA, ARS) National Clonal Germplasm Repository (NCGR), Corvallis, Oregon potential new sources of complete resistance to eastern filbert blight.

Materials and Methods

Plant materials

Scions of fifty-eight clones were collected in Dec. 2000. They were stored at 0 °C until three scions per accession were grafted onto *C. avellana* rooted layers in spring, 2001. Grafted trees were planted in 5 liter pots containing a mix of equal volumes of peat, pumice, fine bark dust, and 9g of Sierra 3-4 month release fertilizer (18N-6P-12K) (Peters Professional, Allentown, PA). Three grafted trees of each genotype were kept in the greenhouse under optimal conditions (24°C day/18°C night) until they were ready for inoculation, usually 3-4 weeks later. 'Gasaway', the resistant control, and 'Ennis' or 'Daviana', the susceptible controls, were also included.

Greenhouse inoculations

Diseased twigs with mature stromata were collected from the North Willamette Research and Extension Center in Aurora, Oregon, in November, 2000 and 2001. They were stored at –20 °C in polyethylene bags until they were used as inoculum. Inoculation chambers were set up in the greenhouse, using polyvinyl chloride tubing (1.27 cm diameter) placed on top of benches (1.22 m× 0.44 m) and covered with white 4 mm polyethylene sheeting. A humidifier was

placed in each inoculation chamber and programmed to run from noon to 6 pm and from midnight to 4 am. Plants were inoculated when shoots had four to five nodes (Coyne et al., 1996). Perithecia were dissected from the stromata of diseased twigs and ground with a mortar and pestle to release ascospores. The ascospore suspensions were then diluted in distilled water to 1×10^6 spores/ml. The suspensions contained in a squeeze bottle were sprayed three times to the tip(s) of one or two actively growing shoots on each tree. The sites of inoculation were indicated by tape placed two to three nodes below the apical meristem. The inoculations were repeated three times at 3-day intervals. After inoculation, the trees remained in the greenhouse under optimal growing conditions for six months prior to the infection assay.

Disease evaluation

One replication of each greenhouse-inoculated accession was tested to score for the presence or absence of the fungus 6 months after inoculation using the ELISA method developed by Coyne (1996) as slightly modified by Lunde (2000). If the first tree showed infection, the other two trees of that accession were transported to the field at the North Willamette Station in fall 2001, planted in a nursery row and the development of cankers was monitored in winter 2003. If the first tree showed a negative or inconclusive result, the other two trees were also tested by ELISA. The accessions that were free of infection after the first year

were re-inoculated in spring, 2002, when new shoots had grown out, and were re-assayed six months later. A genotype was scored as susceptible if one or more of the three trees showed a postive ELISA score or symptoms or signs were observed, and was scored as completely resistant if all three trees showed a negative ELISA score and no symptoms or signs were observed for two years.

Results and Discussion

Using the ELISA method, the response of the hazelnut accessions to eastern filbert blight was separated into two distinct categories, i.e. complete resistance or susceptibility (Table 2.1 and 2.2), except that 'Estrella #2' and 'Chinese Trazel Gellatly #4' showed inconclusive results and 'G227S' grafts failed in both years (Table 2.3). For the trees moved to the field at the North Willamette Station, 88% of them developed cankers of various lengths after 16-18 months (Table 2.2). 'Gasaway', the negative control and 'Ennis' or 'Daviana', the positive controls, behaved as expected. A total of twelve accessions showed complete resistance to *A. anomala* following the greenhouse inoculations. They included *C. avellana* and different types of interspecific hybrids.

Corylus avellana accessions. Two accessions of *C. avellana*, 'Cupla' and 'COR 187', remained free of infection. 'Culpla', which originated in Spain, is similar in appearance to 'Closca Molla', and has round, medium sized but more

oblate nuts. It has medium-high productivity and is resistant to big bug mite (*Phytoptus avellanae* Nal.). Lunde et al. (2000) showed that two other Spanish cultivars, 'Closca Molla' and 'Ratoli' are completely resistant to EFB. Whether they carry the same resistance alleles warrants further study. COR 187 from Finland produces round, small sized nuts. Unfortunately, it has very few female flowers, low nut yield, and also shows male sterility (S. Mehlenbacher, pers. comm).

Corylus americana - *Corylus avellana* hybrids. The American hazel (*C. americana*) is the native host of the fungus *A. anomala*. Infection by the fungus results in small cankers on susceptible genotypes of the American hazel, but infected areas are walled off in resistant genotypes (Weschcke, 1954). Though the mode of inheritance remains unclear, early breeding work by J.F. Jones dating back to 1917 used the American hazelnut as a source of resistance to eastern filbert blight as well as cold-hardiness. Many completely resistant hybrids from crosses of *C. americana* with *C. avellana* have been reported (Ourecky and Slate, 1969; Rutter, 1991; Coyne et al., 1998; Lunde et al., 2000). 'COR 507', 'G081S', 'Weschcke TP1', 'Weschcke TP2' and 'Weschcke TP3' trace to the work of Carl Weschcke at his farm in Wisconsin. The hybrids are quite variable in nut size, shape and productivity. The TP selections were made by Tom Plocher of Hugo, MN at Carl Weschcke's farm in Wisconsin. 'Weschcke TP1' appears to have the largest nuts, but they also have the thickest shells. 'Weschcke TP2' produces a

heavy crop of medium sized nuts with a round-compressed shape and thin shells, which make it promising for use in breeding. 'COR507', 'G081S' and 'Weschcke

Table 2.1 Hazelnut accessions resistant to *A. anomala* following greenhouse inoculation.

Cultivar	Location	CCOR#*	Origin
***C. avellana* L.**			
Culpla	N07.08	255	Spain
COR 187	N05.28	187	Finland
C. americana* × *C. avellana			
COR 507	N06.35	507	MN, US
G081S	566.026	-----	MN, US
Weschcke TP1	567.031	-----	WI, US
Weschcke TP2	566.022, 567.033	-----	WI, US
Weschcke TP3	N04.46	561	WI, US
C. colurna* × *C. avellana			
Chinese Trazel Gellatly #6	N01.07	138	BC, Canada
Chinese Trazel Gellatly #11	N02.32	173	BC, Canada
Turkish Trazel Gellatly #3	N01.12	407	BC, Canada
(*C. colurna* × *C. avellana*) × *C. avellana*			
Lisa	567.021	-----	MI, US
***C. heterophylla* var. *sutchuensis* × *Holder* (*C. avellana*)**			
Estrella #1	N01.15	139	MI, US

* Corvallis *Corylus* (CCOR) accession number assigned by USDA, ARS National Clonal Germplasm Repository, Corvallis, OR.

Table 2.2 Hazelnut accessions infected by *Anisogramma anomala* following greenhouse inoculations.

Cultivar	Location	CCOR#	Origin	Canker Length (cm)*
C. avellana				
AL55 (COR 625)	N09.05	625	Albania	----
Arneson's Rootstock	N01.33	182	OR, US	14+0
Blumberger	212.053	205	Germany	0+0
C. avellana 76-1824	639.047	----	Arboretum	14+0
Camponica	N06.01	40	Italy	15+16
Carrello	N02.57	376	Italy	----
COR 626	N09.04	626	Albania	8+ 0
COR 627	N09.06	627	Sweden	24+2
Corabel	N09.09	482	France	15+12
Frango #4	N10.05	660	Poland	----
Frango #5	N10.06	661	Poland	43+10
G114S	566.025	----	MN, US	----
Goc	N10.07	662	Poland	0+0
Karol	N10.08	663	Poland	30+20
Kruse	N07.21	25	US	30+7
Locale di Piazza Armerina	N02.56	371	Italy	10+7
Maria	N10.13	668	Poland	6+0
Nonpareil	N07.05	37	OR, US	4+22
Nostrale	N05.50	335	Italy	0+3
not Tonda Gentile Romana	N06.36	----	Italy	18+25
Pinyolenc	W10a	339	Spain	0+30

Table 2.2 Continued

Cultivar	Location	CCOR#	Origin	Canker Length (cm)*
Romisondo G1	R01.14 or 22	----	Italy	3+3
Rosset de Valls	N01.16	379	Spain	0+0
Royal	N04.39	77	OR, US	7+5
Tonda di Giffoni	----	22	Italy	7+3
Volle Zellernuss	639.055	----	Germany	0+0
Warsaw Red	N08.08	181	Poland	----
Woodford	N05.09	12	OR, US	7+0
C. colurna* × *C. avellana				
Chinoka	N07.35	199	BC, Canada	17+33
Chinese Trazel Jemtegaard #1	N02.25	170	OR, US	2+0
Chinese Trazel Jemtegaard #2	N04.31	164	OR, US	10+6
Eastoka	N07.25	148	BC, Canada	35+27
Erioka	N07.39	201	BC, Canada	----
Faroka	N12.03	405	BC, Canada	0+0
Filcorn	N03.02	53	OR, US	----
Freeoka	N06.28	154	BC, Canada	5+ 60
Karloka	N07.58	406	BC, Canada	23+17
Laroka	N05.41	57	BC, Canada	----
Morrisoka	N03.05	33	BC, Canada	0+7
Turkish Trazel Gellatly #15	N07.57	408	BC, Canada	19+40
Turkish Trazel Gellatly #2	N07.37	200	BC, Canada	8+24
Turkish Trazel Gellatly #5	N02.23	169	BC, Canada	4+0
Zeroka	N01.20	409	BC, Canada	16+30

Table 2.2 Continued

Cultivar	Location	CCOR#	Origin	Canker Length (cm)*
		C. maxima		
Pell. Rouge	N02.07	38	France	3+0

* canker length of two trees per genotype is presented as x+x.

Table 2.3 Hazelnut accessions that showed inconclusive results following greenhouse inoculations with *Anisogramma anomala*.

Cultivar	Location	CCOR#	Origin
		C. avellana	
G227S*	566.027	-----	MN,US
		C. heterophylla* var *sutchuensis* × *Holder (C. avellana)	
Estrella #2	N01.04	140	MI,US
		C. colurna*× *C. avellana	
CTG4	N01.25	174	BC,Canada

* All grafts failed

TP3' produce moderate crops of round nuts. Similar to 'Yoder #5' (Lunde et al., 2000), 'Weschcke TP3' has a deficiency in that it is highly susceptible to big bud mite, which appears to derive from its *C. americana* parent as Ourecky and Slate (1969) found that 'Rush', the *C. americana* parent frequently used in breeding, transmitted its high susceptibility to big bud mite to most of its seedlings.

Tree hazel hybrids. The Turkish tree hazel, *Corylus colurna,* was used by J.U. Gellatly beginning in the early 1950s to combine the hardiness and non-suckering growth habit of the tree hazel with the nut size of the European hazel, and the hybrids were named 'trazels' (Gellatly, 1956, 1966). Complete resistance to eastern filbert blight in *C. colurna* has been reported in previous studies (Farris, 1969, 1970; Pinkerton et al., 1993; Coyne et al., 1998). But Coyne (1998) discovered that two interspecific hybrids of *C. colurna* × *C. avellana*, 'Newberg' and 'Dundee', were highly susceptible to the disease. In this study, though most of the trazels were infected by *A. anomala*, three selections ('Chinese Trazel Gellatly #6', 'Chinese Trazel Gellatly #11', and 'Turkish Trazel Gellatly #3') remained free of eastern filbert blight. Field observation also showed that they are highly resistant to big bud mite, which is consistent with reports that the tree hazel is highly resistant to *Phytopus avellanae* (Farris, 1970), and this resistance is transmitted to its offspring (Farris, 1988). These three hybrids closely resemble *C. colurna* in their nut shape and husk morphology. 'Chinese Trazel Gellatly #6' and 'Chinese Trazel Gellatly #11' have quite acceptable nuts and kernels and were used as parents in the breeding program in 1990. Among six samples of 'Chinese Trazel Gellatly #4' evaluated by ELISA, three test values were just above and the other three were just below the thresholds. The cultivar displayed no symptoms or signs of infection after 18 months in the field. The disease response of Chinese Trazel Gellatly #4 should be investigated further. 'Lisa', selected by Cecil Farris in 1989 from seedlings obtained through the open pollination of 'Grand Traverse'

(Lukasiewicz, 1992), phenotypically appears to be a hybrid of 'Grand Traverse' and *C. avellana*. Lunde et al. (2000) showed that 'Grand Traverse' is completely resistant to EFB and Farris (1990) reported resistance to big bud mite, which explains the resistance of 'Lisa'. 'Lisa' has attractive features such as good flavor, thin shells, smooth kernels and precocity (Farris, 1990). Unfortunately, it has long shaped nuts and long husks, and matures in late September.

C. heterophylla var. *sutchuensis* - *C. avellana* hybrids. The interspecific hybrids designated 'Estrella' were obtained by Farris in the 1980s from a cross of *C. heterophylla* var. *sutchuensis* with *C. avellana.* Estrella #1 yields well and the medium sized nuts have a slightly long shape. However, it is male-sterile (Farris, 1982). Estrella #2 is fully fertile, early maturing and has nut size and shape about equal to 'Holder', the pollen parent. In this study, four out of five Estrella #2 trees evaluated by ELISA showed no infection. The conflicting data may be the result of mistakenly inoculating a shoot from the rootstock, as it is harder to distinguish between scion and rootstock after two years' growth in the greenhouse. Its disease response should be investigated further.

According to Simmonds (1983), if a vertical resistance (VR) strategy is used in a disease resistance breeding program, cautions on the durability of the resistance should be taken. The single dominant resistance gene from 'Gasaway' conferring immunity to eastern filbert blight has been the major gene utilized in the OSU hazelnut breeding program. Thus, other sources of reliable VR genes are

highly desirable. In this study, the identification of additional sources of complete resistance to eastern filbert blight from *C. avellana* and several interspecific hybrids are of considerable value in enriching the resistant germplasm. Also, they will be useful in the OSU hazelnut breeding program because these resistant *C. avellana* cultivars and interspecific hybrids can be readily backcrossed to commercial European varieties.

References

Barss, H. P. 1930. Eastern filbert blight. California Agr. Dept. Bull. 19:489-490.

Cameron, H. R. 1976. Eastern filbert blight established in the Pacific Northwest. Plant Dis. Rptr. 60:737-740.

Coyne, C.J. 1995. Genetic resistance to Eastern filbert blight. MS thesis. Department of Horticulture, Oregon State University, Corvallis.

Coyne, C.J., S.A. Mehlenbacher, R.O. Hampton, J.N. Pinkerton and K.B. Johnson. 1996. Use of ELISA to rapidly screen hazelnut for resistance to eastern filbert blight. Plant Disease 80:1327-1330.

Coyne, C.J., S.A. Mehlenbacher, and D.C. Smith. 1998. Sources of resistance to eastern filbert blight in hazelnut. J. Amer. Soc. Hort. Sci. 123:253-257.

Davison, A.R. and R.M. Davidson, Jr. 1973. *Apioporthe* and *Monochaetia* cankers reported in western Washington. Plant Dis. Rep. 57:522-523.

Ellis, J.B. and B.M. Everhart. 1892. The North American Pyrenomycetes. Ellis and Everhart, Newfield, New Jersey. p. 531- 532.

FAO production yearbook. 2003. http://www.fao.org/DOCREP/003/X4484E/x4484e03.html.

Farris, C.W. 1969. Hybridization of filberts. p.299- P300. In: R.A. Jaynes (ed.). Handbook of North American nut trees. Northern Nut Growers Assn., Humphrey Press, Hamden, Conn.

Farris, C.W. 1970. Inheritance of parental charactheristics in filbert hybrids. Ann. Rept. Northern Nut Growers Assn. 61:54-58.

Farris, C.W. 1976. An introduction to the stars: A new family of filbert hybrids. Ann. Rept. Northern Nut Growers Assn. 67:80-82.

Farris, C.W. 1982. A progress report on the development of F2 hybrids of *Corylus colurna* × *C. avellana.* Ann. Rept. Northern Nut Growers Assn. 71:15-16

Farris, C.W. 1988. Potential for genetic improvement in hazelnuts. Ann. Rept. Northern Nut Growers Assn. 81:46-47

Farris, C.W. 1989. Grand Traverse hazelnut. Ann. Rept. Northern Nut Growers Assn. 80:97-99

Farris, C.W. 1990. Hazelnut cultivar development: A progress report. Ann. Rept. Northern Nut Growers Assn. 82:118-119.

Fuller, A.S. 1910. Filbert or hazelnut. p. 118-146. In: The nut culturist. Orange Judd, New York.

Gellatly, J.U. 1956. Tree hazels-*Corylus colurna*. Ann. Rept. Northern Nut Growers Assn. 47:110-112.

Gellatly, J.U. 1966. Tree hazels and their improved hybrids. Ann. Rept. Northern Nut Growers Assn. 57:98-106.

Gottwald, T.R. and H.R. Cameron. 1980. Infection site, infection period, and latent period of canker caused by *Anisogramma anomala* in European filbert. Phytopathology 70:1083-1087.

Johnson, K.B., J.N. Pinkerton, S.M. Gaudreault and J.K. Stone. 1994. Infection of European hazelnut by *Anisogramma anomala*: Site of infection and effect of host developmental stage. Phytopathology 84:1465-1470.

Johnson, K.B., S.A. Mehlenbacher, J.K. Stone and J.W. Pscheidt. 1996. Eastern filbert blight of European hazelnut – it's becoming a manageable disease. Plant Disease 80:1308-1316.

Lunde, C.F. 1999. Investigation of novel sources of genetic resistance to eastern filbert blight. MS thesis. Department of Horticulture, Oregon State University, Corvallis.

Lunde, C.F., S.A. Mehlenbacher and D.C. Smith. 2000. Survey of hazelnut cultivars for response to eastern filbert blight inoculation. HortScience 35:729-731.

Lukasiewicz, J. 1992. Experimental nut plantings at the southwest Michigan research and extension station. Ann. Rept. Northern Nut Growers Assn. 83:12-14.

Mehlenbacher, S.A., M.M. Thompson and H.R. Cameron. 1991. Occurrence and inheritance of resistance to eastern filbert blight in 'Gasaway' hazelnut. HortScience 26:410-411.

Mehlenbacher, S. 1994. Genetic improvement of the hazelnut. Acta Hort. 351:23-38.

Ourecky, D.K. and G.L. Slate. 1969. Susceptibility of filbert varieties and hybrids to the filbert bud mite, *Phytoptus avellane* Nal. Ann. Rept. Northern Nut Growers Assn. 60:89-91.

Pinkerton, J.N. 1993. Susceptibility of European hazelnut clones to eastern filbert blight. Plant Disease 77:261-266.

Pinkerton, J.N., J.K. Stone, S.J. Nelson and K.B. Johnson. 1995. Infection of European hazelnut by *Anisogramma anomala*: ascospore adhesion, mode of penetration of immature shoots, and host response. Phytopathology 85:1260-1268.

Reed, C.A. 1936. New filbert hybrids. J. Hered. 27:427-431.

Rutter, M. 1991. Variation in resistance to eastern filbert blight in hybrid hazels. Ann. Rept. Northern Nut Growers Assn. 82:159-162.

Schuster, C.E. 1924. Filberts: 2. Experimental data on filbert pollination. Oregon Agr. Expt. Sta. Bull. 208.

Simmonds, N.W. 1983. Strategy of disease resistance breeding. FAO Plant Prot. Bull. 31:2-10.

Tasias-Valls, J. 1975. El avellano en la provincia de Tarragona (in Spanish) Escma. Diputacion Provincial de Tarragona. Fundacion Servicio Agropecuario Provincial. Tarragona, Spain.

Weschcke, C. 1954. Growing nuts in the north. p. 24-38. Webb, St. Paul.

Chapter 3

AFLP MARKERS LINKED TO EASTERN FILBERT BLIGHT RESISTANCE IN OSU 408.040 HAZELNUT

Honglin Chen, Shawn A. Mehlenbacher and David C. Smith

Abstract

Eastern filbert blight (EFB), caused by *Anisogramma anomala* (Peck) E. Müller, is a devastating disease to European hazelnut orchards in the Willamette Valley, Oregon. Selection OSU 408.040 showed no symptoms or signs and tested negative for the fungus in greenhouse inoculations followed by ELISA. Segregation analysis of three progenies indicates that a single dominant gene controls the EFB resistance. AFLP methodology was employed to identify markers linked to EFB resistance in OSU 408.040. A total of 64 primer combinations were screened using three resistant and three susceptible individuals as well as the parents of progeny 97035. Primer combinations that showed no more than one recombination event in the initial six seedlings were investigated further in a group of 30 seedlings. Markers that showed less than 15% recombination with resistance were further investigated in the remaining samples of the population. Five AFLP markers linked in coupling to resistance were identified: A4-265 (9.2 cM), A8-150 (0.0 cM), B2-125 (4.1 cM), C2-175 (5.9 cM) and D8-350 (2.5 cM). B2-125 was located on one side of the resistance locus and A4-265, C2-175 and D8-350 on the other side. Three of these markers B2-125, C2-175 and D8-350 were also linked in coupling in a similar order in the second population 97036. The application of these markers in MAS for breeding of new hazelnut cultivars with eastern filbert blight resistance is discussed.

Introduction

Production of the European hazelnut, *Corylus avellana* L., in Oregon's Willamette Valley represents 98% and 3-5% of the production in the United States and the world, respectively (FAO Production Yearbook, 2003). However, the Oregon hazelnut industry is threatened by eastern filbert blight, a disease caused by the pyrenomycete *Anisogramma anomala* (Peck) E. Müller. The fungus causes severe cankers, rapid yield loss and eventually tree death in 5 to 12 years if control measures are not practiced (Pinkerton et al., 1993). Current control practices include fungicide applications and therapeutic pruning. However, due to the expense of fungicides and the dramatic yield loss caused by severe pruning of cankers, genetic resistance is the most desirable and economical means of disease control (Mehlenbacher, 1994). Therefore, developing varieties resistant to eastern filbert blight is a major goal of the Oregon State University (OSU) hazelnut breeding program.

Complete resistance to eastern filbert blight was first discovered in the obsolete pollenizer 'Gasaway' (Cameron, 1976). Immunity is conferred by a single dominant gene (Mehlenbacher et al., 1991). This gene has been the major source of resistance utilized in the OSU breeding program, while the search for new sources of complete resistance has continued. Selection OSU 408.040, grown from seeds labeled 'Weschcke hybrid' collected at the research farm of the University of Minnesota in 1987, showed no symptoms or signs and tested negative for the fungus in greenhouse inoculations followed by an enzyme-linked

immunosorbent assay (ELISA) (Mehlenbacher, pers. comm.). The selection phenotypically appears to be pure *C. avellana*, resembling wild populations in Europe, rather than *C. americana*. OSU 408.040 is not precocious, is highly susceptible to big bud mite (*Phytoptus avellanae* Nal.), and has nuts that are small and very long-shaped and kernels that do not blanch, but the nuts mature early. Some selected progeny of OSU 408.040 are a great improvement over their parent in the above traits.

Current methods for evaluation of eastern filbert blight resistance are slow and expensive. Field observation of canker development usually takes place 16-20 months after initial exposure. Quarantine regulations require isolation of disease tests from commercial orchards and breeding activities. ELISA following greenhouse inoculation shortens the detection time to 6 months (Coyne et al., 1996). However, this process is still time-consuming, and in some cases, yields ambiguous results even with repeated tests. Marker-assisted selection (MAS) provides a means of screening genotypes for eastern filbert blight resistance at an early stage. It is also useful for confirming the phenotypes of promising selections. Only seedlings with the appropriate markers are planted in the field and used as parents for the next generation, leading to a greatly enriched gene pool for selection for other traits. RAPD markers tightly linked to the 'Gasaway' gene for resistance have been identified (Davis et al., 1997; Mehlenbacher et al., 2003). Several of these markers have been sequenced and could be converted into SCARs

(Paran and Michelmore, 1993). One of these RAPD markers, $UBC152_{800}$, is easy to score and robust to amplification conditions. It has been routinely used in MAS for several years in the hazelnut breeding program at Oregon State University.

In this study, the objectives were to examine the inheritance of resistance from selection OSU 408.040 and to identify AFLP markers linked to resistance. The potential for use of these markers in a MAS program is also discussed.

Materials and Methods

Plant materials

In 1997 and 1999, three seedling populations were obtained from controlled crosses of the resistant selection OSU 408.040 as the pollen parent with the susceptible selections OSU 245.098, OSU 474.013, and OSU 665.012. The seedlings were assigned progeny numbers 97035, 97036, and 99035, respectively (Figure 3.1). For progenies 97035 and 97036, scions were collected from 75 and 64 seedlings, respectively, in Dec. 2000, from trees growing at the Oregon State University Smith Horticulture Research Farm. They were stored at 0 °C for two to three months until they were grafted onto *C. avellana* rooted layers in spring, 2001 (three grafted trees per genotype). For the 99035 population, seeds were collected in fall, 2000, stratified, sown in flats in the greenhouse when they sprouted, and grown to about 20 cm tall. The seedlings of progeny 99035 and grafted trees of

progenies 97035 and 97036 were planted in 5 liter pots containing a mixture of equal volumes of peat, pumice, fine bark dust, and 9g of Sierra 3-4 month release fertilizer (18N-6P-12K) (Peters Professional, Allentown, PA). They were kept in the greenhouse under optimal conditions (24°C day/18°C night) until they were ready for inoculation and DNA extraction. 'Gasaway', the resistant control, and 'Ennis', the susceptible control, were also included.

Greenhouse inoculation

Diseased twigs with mature stromata were collected from the North Willamette Research and Extension Center in Aurora, Oregon, in November 2000 and 2001. They were stored at –20 °C in polyethylene bags until they were used as inoculum. Perithecia were dissected from the stromata of infected twigs and ground with a mortar and pestle to release ascospores. The ascospore suspensions were then diluted in distilled water to 1×10^6 spores/ml. The suspensions contained in a squeeze bottle were sprayed three times to the tip(s) of one or two actively growing shoots on each tree. The sites of inoculation were indicated by tape placed two to three nodes below the apical meristem. Inoculation chambers were set up in the greenhouse, using polyvinyl chloride tubing (1.27 cm diameter) placed on top of benches (1.22m × 0.44m) and covered with white 4 mm polyethylene sheeting. A humidifier was placed in each inoculation chamber and programmed to run from noon to 6 pm and from midnight to 4 am. Plants were

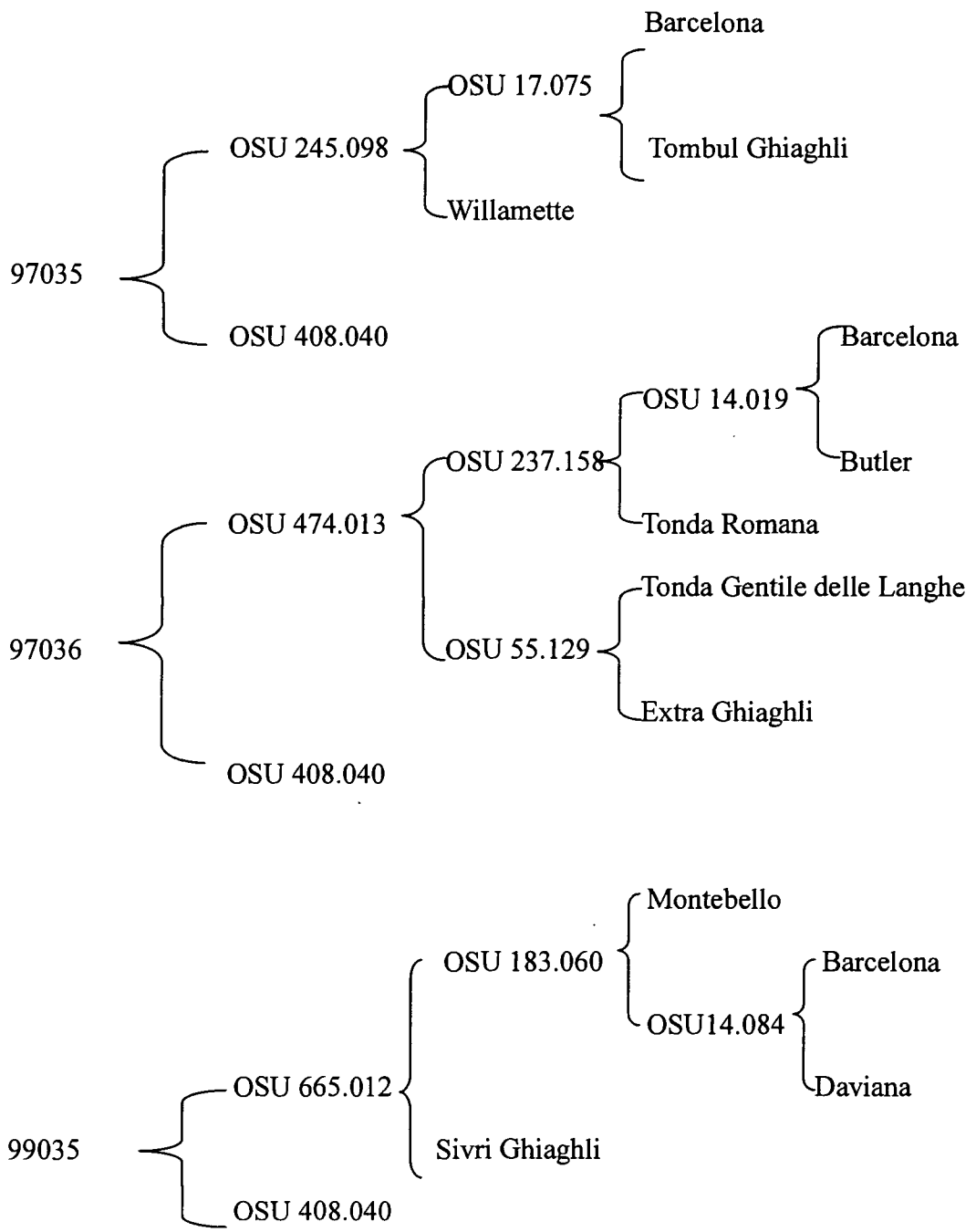

Figure 3.1 Pedigrees of progenies 97035, 97036 and 99035.

inoculated when shoots had four to five nodes (Coyne et al., 1996). The inoculations were repeated three times at 3-day intervals. After inoculation, the trees remained in the greenhouse under optimal growing conditions for six months prior to the infection assay.

EFB resistance evaluation

For progenies 97035 and 97036, one greenhouse-inoculated grafted tree was tested to score for the presence or absence of the fungus 6-8 months after inoculation using the ELISA method developed by Coyne (1996) as slightly modified by Lunde (2000). If the first tree showed infection, the other two trees of that genotype were transported to the North Willamette Station in fall 2001, planted in a nursery row and the development of cankers was noted the following winter. If the first tree showed a negative or inconclusive result, the other two trees were also tested using ELISA, and the tree remained in the greenhouse to be re-inoculated and re-assayed in 2002. A genotype was scored as susceptible if one or more of the three trees showed a positive ELISA score, whereas, it was scored as resistant if all three trees remained free of infection for two years. For progeny 99035, each seedling was inoculated and assayed once in 2001, and then they were transported to the North Willamette Station, planted in a nursery row and the development of cankers was visually inspected 18 months later. A genotype was scored as susceptible if the ELISA score was above the threshold and/or cankers

were observed, and as resistant if the ELISA test was negative and no cankers were observed.

DNA extraction

Fresh young leaves of field-planted trees of 74 seedlings of progeny 97035, 64 seedlings of progeny 97036 and the parents were collected in spring, 2002. Young leaves of field-planted trees of 48 additional seedlings of progeny 97035 were collected in spring, 2003. DNA was extracted from these leaves following the method of Lunde et al. (2000) with minor modifications, and RNA was removed by incubation at 37 °C in the presence of RNase A for one hour in a shaker, followed by 25 phenol: 24 chloroform: 1 isoamyl alcohol extraction. DNA was extracted four times from each seedling and stored at -4 °C until AFLP assays. Some DNA samples were further purified using QIAprep Spin Miniprep Kit (Qiagen, Chatsworth, Calif.) to ensure successful amplification. DNA was quantified using a Hoefer® DyNA Quant™ 2000 Fluorometer (Amersham Bioscience, San Francisco, Calif.).

AFLP analysis

Three EFB resistant seedlings, three EFB susceptible seedlings and the two parents of progeny 97035 were used in the pre-screening process in the search for potential linked AFLP markers. In this process, the AFLP assay was performed

using GIBCO BRL AFLP analysis system kit I (Cat. No 10544, Rockville, MD) following the manufacturer's protocol. A total of sixty-four primer combinations (*EcoRI* +AAC, AAG, ACA, ACT, ACC, ACG, AGC, AGG and *MseI* + CAA, CAC, CAG, CAT, CTA, CTC, CTG, CTT) were used. Primer combinations that generated a band that was present in the resistant parent and all 3 resistant seedlings but absent in the susceptible parent and all 3 susceptible seedlings were investigated further in a group of 30 additional seedlings. Primer pairs that generated a band that showed one recombinant in the 6 seedlings were also investigated further. Markers that showed less than 15% recombination with resistance in the 30 samples were further surveyed on the remaining seedlings in the population. In the post pre-screening process, AFLP marker analysis was conducted based on the protocol of Vos (1995) with some modifications (Appendix A). Briefly, 250ng of genomic DNA were digested with *EcoRI* and *MseI* by incubation at 37 °C overnight, and then held at 72 °C for 15 minutes to inactivate the enzymes. The DNA fragments were ligated with *EcoRI* and *MseI* adapters at 16 °C for two hours. For pre-selective amplification, 5µl of a 10-fold diluted ligation mixture was amplified for 20 cycles of 94 °C for 30s, 56 °C for 60s, and 72 °C for 60s using primers *EcoRI* + A and *MseI* + C. For selective amplification, 5µl of the combination of *EcoR I* +AXX and *Mse I* + CXX primers was mixed with 5µl of 50-fold diluted preamplified DNA and PCR buffer. The mixture was amplified for 1 cycle of 94 °C for 30s, 65 °C for 30s, 72 °C for 60s, then lowering annealing temperature 0.7 °C each cycle for 12 cycles, and then 23

cycles of 94 °C for 30s, 56 °C for 30s, and 72 °C for 60s. The PCR products were checked for evidence of amplification by electrophoresis on 1% agarose gels using 5µl of the aliquot with 3µl of loading dye (15% Ficoll® 400, 0.03% xylene cyanol FF, 0.4% orange G, 10mM Tris-HCL pH 7.5, and 50mM EDTA). The amplicons were mixed with an equal volume of loading dye and loaded on 6% polyacrylamide denaturing sequencing gels (Appendix B). Gels were run at 75W (1600V) constant power for 3.5 hours and stained with silver (Appendix C). The size of markers was estimated by comparison to a 100-1500bp ladder (Promega, Madison, WI).

Data analysis

Segregation analysis for resistance to eastern filbert blight in three progenies of OSU 408.040 was performed using the Chi-square test. A test of heterogeneity was also performed to decide whether the data from three progenies were sufficiently uniform to be pooled. AFLP markers potentially linked to the disease resistance gene were analyzed on 122 seedlings from the 97035 population and 64 seedlings from the 97036 population. Among 122 seedlings of the progeny 97035, 48 seedlings have no phenotypic data but were used in the AFLP assay to determine the linkage order of the promising markers. The potential AFLP markers were scored as 1 indicating the presence and 0 the absence of a band. Similarly, the phenotypic data was scored as 1 for resistance and 0 for susceptibility. The data was entered in a Microsoft Excel file, and then

saved as a tab-delimited file and imported into MAPMAKER EXP 3.0 (Lander et al., 1987) using the f2 backcross function and default linkage criteria of LOD=3.0, and a maximum recombination frequency of 0.25. The most likely map order was determined using the 'compare' command. The Kosambi mapping function (Kosambi, 1944) was used to convert the recombination frequency into map distances in centiMorgans (cM). The 'error detection on' command tells MapMaker to perform the analysis assuming incomplete penetrance and a mistyping error rate of 1%. The suspicious double recombinant data points were reexamined by selective amplification of new DNA template from the same tree. Mistakes potentially exist in genotyping and phenotyping processes and true double recombinant events may exist, so both maps are reported with the options of 'error detection on' and 'error detection off'. Maps were drawn using the 'draw map' command of MapMaker and the resulting PS file was visualized using Ghostview software.

Results

Segregation for resistance to EFB in progenies of OSU 408.040

Disease evaluation was carried out on the 64 seedlings of progeny 97036, 74 of progeny of 97035 and 54 of progeny 99035. All three progenies of OSU 408.040 showed good fit to an expected ratio of 1 resistant: 1 susceptible separately. The heterogeneity Chi-square test showed that the data from three progenies were indeed homogeneous. Thus, data were pooled, which also gave a

good fit to the expected ratio, indicating control by a single locus with a dominant allele for resistance (Table 3.1).

Table 3.1 Segregation for resistance to EFB in progenies of OSU 408.040

Population	Plant number		Expected ratio	χ^2	
	Resistant	Susceptible		Value	*P*
245.098×408.040 (97035)	32	42	1:1	1.35	0.25
474.013×408.040 (97036)	30	34	1:1	0.63	0.42
665.012×408.040 (99035)	29	27	1:1	0.07	0.79
Pooled data	91	103	1:1	0.74	0.39
Heterogeneity χ^2 (degrees of freedom = 2)				1.31	0.52

Identification of AFLP markers linked to eastern filbert blight resistance in OSU 408.040

Progeny 97035 was used in the search for potential AFLP markers. All 64 primer combinations successfully amplified fragments ranging from 1000 to less than 100 base pairs in length. Typically, 30 to 50 fragments per primer combination were produced and an average of 20% polymorphism between the resistant parent and the susceptible parent was observed.

Five markers, designated A4-265 (Figure 3.2), A8-150 (Figure 3.2), B2-125 (Figure 3.3), C2-175 (Figure 3.3) and D8-350 (Figure 3.4), were found to be closely linked in coupling to the resistance locus in OSU 408.040. All of the markers segregated in the expected 1:1 (present: absent) ratio (Table 3.2). The linkage analysis with a LOD score of 3.0 placed the five AFLP markers in the same linkage group as the resistance locus. A map spanning 13.3 cM was constructed with the function 'error detection on' with markers in the order A4-265, C2-175, D8-350, A8-150 and B2-125 (Figure 3.5a). With the 'error detection off' function, a map covering 19.8 cM was obtained with markers in the same order (Figure 3.5b). Four markers, A4-265, C2-175, D8-350 and A8-150, were located on one side of the resistance locus and marker B2-125 was located on the other side.

To further confirm the segregation and linkage of five linked markers to the resistance locus, 64 seedlings from the 97036 population were screened with the same AFLP primer combinations as in the 97035 population. The results showed that three of the markers, B2-125, C2-175 and D8-350, segregated in the expected ratio of 1 present: 1 absent (Table 3.2). They were also found linked in coupling to the resistance locus with the same linkage order, with the function 'error detection on' (Figure 3.6). Markers C2-175 and D8-350 co-segregated. Unfortunately, the polymorphism was lost for A8-150 and A4-265 as a fragment of the same size and relative intensity was amplified in the susceptible and resistant parents.

Table 3.2 AFLP markers and their segregation among 122 seedlings of progeny 97035 and 64 seedlings of progeny 97036.

Populations	Primer designation	Primer combinations	AFLP marker designation	Approximate size of markers (bp)	Observed frequency (present: absent)	Expected ratio	χ^2	
							Value	*P*
245.098 X 408.040	A4	EAAC/MCAT	A4-265	265	64:58	1:1	0.30	0.65
	A8	EAAC/MCTT	A8-150	150	61:61	1:1	0.00	1.00
	B2	EAAG/MCAC	B2-125	125	62:60	1:1	0.03	1.00
	C2	EACA/MCAC	C2-175	175	62:60	1:1	0.03	1.00
	D8	EACT/MCTT	D8-350	350	61:61	1:1	0.00	1.00
474.013 X 408.040	B2	EAAG/MCAC	B2-125	125	28:36	1:1	1.00	0.38
	C2	EACA/MCAC	C2-175	175	32:32	1:1	0.00	1.00
	D8	EACT/MCTT	D8-350	350	32:32	1:1	0.00	1.00

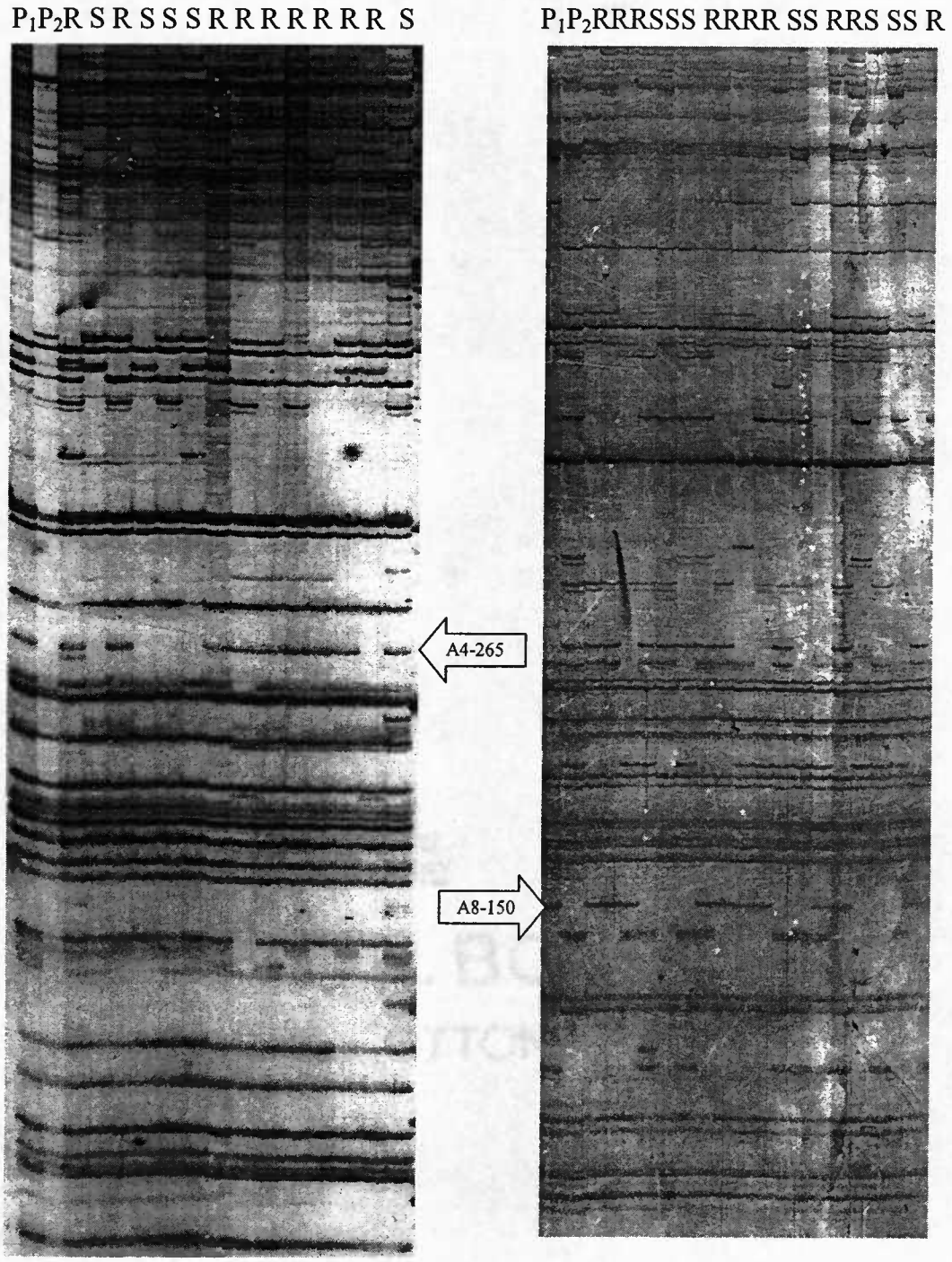

Figure 3.2 Segregating markers A4-265 and A8-150 for OSU 245.098 (P_2) × OSU 408.040 (P_1) progeny. R= resistant, S= susceptible. The arrow indicates the location of the polymorphism.

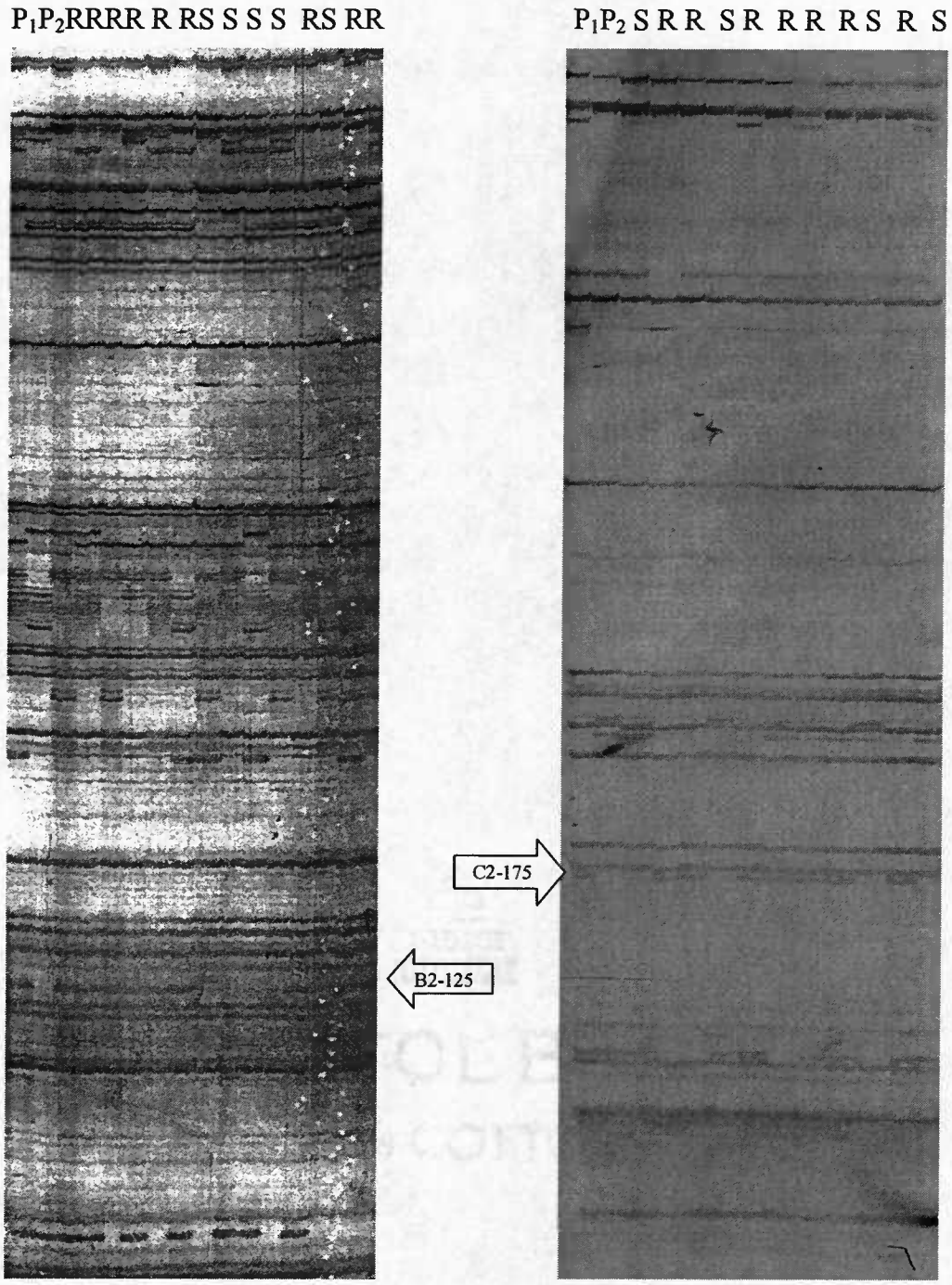

Figure 3.3 Segregating markers B2-125 and C2-175 for OSU 245.098 (P_2) × OSU 408.040 (P_1) progeny. R= resistant, S= susceptible. The arrow indicates the location of the polymorphism.

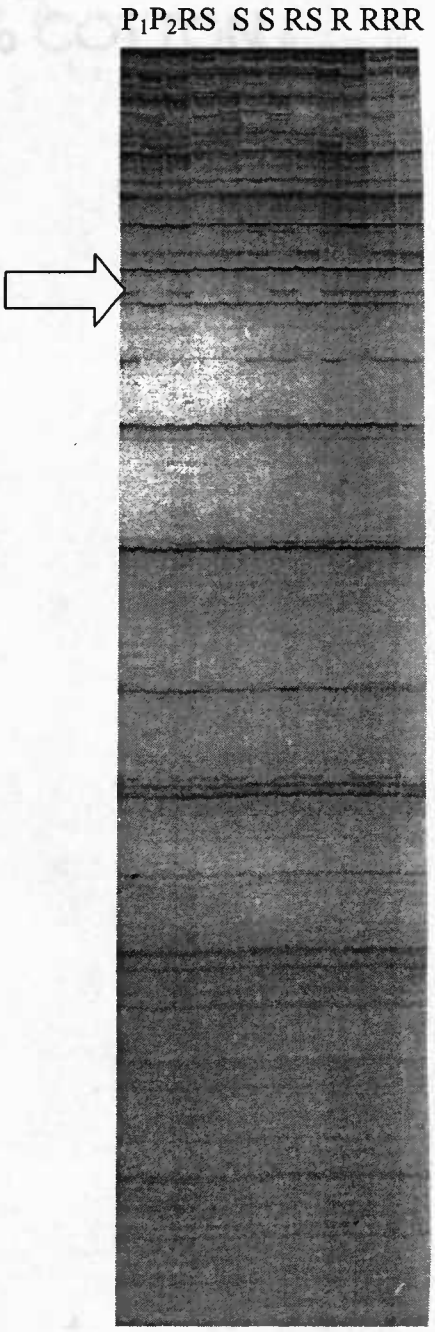

Figure 3.4 Segregating D8-350 marker for OSU 245.098 (P_2) × OSU 408.040 (P_1) progeny. R= resistant, S= susceptible. The arrow indicates the location of the polymorphism.

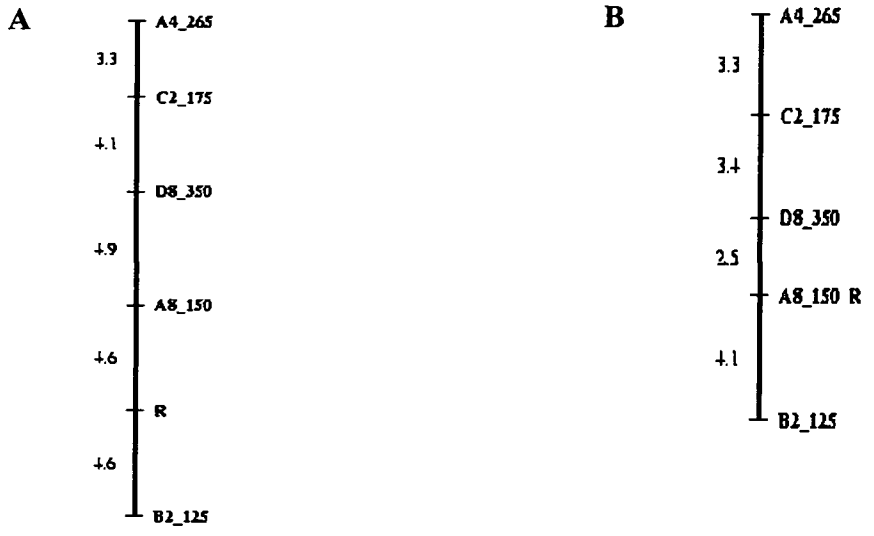

Figure 3.5 Most likely map orders of AFLP markers and the resistance locus for progeny 97035. (A) Error detection on. (B) Error detection off

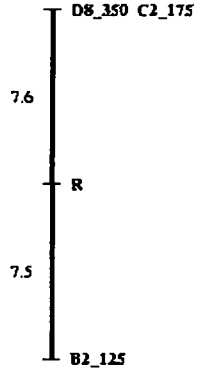

Figure 3.6 Most likely map order of AFLP markers and the resistance locus for 97036, with 'error detection on'.

Discussion

Disease resistance conferred by a single major gene is much easier than that conferred by multiple minor genes to incorporate into well-adapted, high-yielding cultivars using the modified backcross method, though it may be less durable (Mehlenbacher, 1995). Mehlenbacher et al. (1991) showed that resistance from 'Gasaway' is controlled by a single dominant gene. This gene has been the major source of resistance utilized in the hazelnut breeding program at Oregon State University. The RAPD marker, $UBC152_{800}$, which is tightly linked to the 'Gasaway' resistance allele and has been routinely used in marker-assisted selection, is absent in OSU 408.040. Thus, OSU 408.040 appears to be a novel source of genetic resistance to eastern filbert blight. The inheritance of resistance from selection OSU 408.040 indicates that a single dominant gene is involved, which means a similar approach can be used to incorporate the OSU 408.040 source of resistance into commercially acceptable varieties. Furthermore, the identification of this new source of resistance will be useful in gene pyramiding to create varieties with more durable resistance to EFB.

The strategy used in isolating AFLP markers linked to the resistance locus was based on screening a limited number of samples with a relatively large number of primer pairs to ensure that many loci can be screened with a limited effort. Candidate markers that were identified in this way were then screened on a larger number of phenotypically well-characterized samples to confirm their

linkage. In this study, three EFB resistant and three EFB susceptible individuals as well as the parents were used in the first screening process. Primer pairs that showed no more than one recombinant in the 6 seedlings were investigated in the secondary screen using 30 well-characterized seedlings. Any loci that exhibited less than 15% recombination were further surveyed on the remaining seedlings of the population. This level of stringency successfully ensured that no tightly linked markers were missed, while preventing pursuit of loosely linked markers for further mapping. The strategy used in this study can also overcome a problem in the bulked segregant analysis (BSA) approach (Michelmore et al., 1991) in which infection 'escapes' and recombination events can prevent the identification of linked markers.

MAS has been shown to be extremely powerful and efficient for traits that are simply inherited, and either difficult or expensive to evaluate by conventional methods, such as many types of disease resistance (Lande and Thompson, 1990; Melchinger, 1990; Dudley, 1993; Kelly, 1995; Mehlenbacher, 1995; Young, 1999; Luby and Shaw, 2001; Dreher et al., 2002). The identification of these AFLP markers, especially those that are tightly linked (<5cM) (Tanksley, 1983; Mohan et al., 1997) to the resistance locus in OSU 408.040, provides a step toward a marker-assisted selection program. AFLP markers will be more useful in a MAS program if they are applicable across a range of genetic backgrounds (Kelly, 1995). The marker linked closest to the resistance locus, A8-150, only exists in the

OSU 245.098 × OSU 408.040 cross and thus is only useful for selection in this progeny. The marker B2-125 appears to be a promising one as it exists in both 97035 and 97036 populations. The practical application of large-scale MAS in applied plant breeding program requires high-throughput, cost-effective, reliable and easy to score marker assays. Although the AFLP technique is reliable, it is difficult to employ directly because it is complicated, technologically demanding, and costly. Further studies should be carried out to convert the AFLP markers identified in this study to sequence-characterized amplified regions (SCARs) (Paran and Michelmore, 1993) or to cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel, 1993) for their direct application in a MAS program.

References

Cameron, H. R. 1976. Eastern filbert blight established in the Pacific Northwest. Plant Dis. Rptr. 60:737-740.

Coyne, C.J., S.A. Mehlenbacher, R.O. Hampton, J.N. Pinkerton and K.B. Johnson. 1996. Use of ELISA to rapidly screen hazelnut for resistance to eastern filbert blight. Plant Disease 80:1327-1330.

Davis, J.W. and S.A. Mehlenbacher. 1997. Identification of RAPD markers linked to eastern filbert blight resistance in hazelnut. Acta Hort. 445:553-556.

Dreher, K., M. Morris, M. Khairallah, M. Ribaut, P. Shivaji, S. Ganesan, S. Pandey, G. Srinivasan, R.E. Evenson, V. Santaniello and D. Zilberman. 2002. Is marker-assisted selection cost-effective compared with conventional plant breeding methods? The case of quality protein maize. Economic and Social Issues in Agricultural Biotechnology 15:203-236.

Dudley, J.W. 1993. Molecular markers in plant improvement: manipulation of genes affecting quantitative traits. Crop Science 33:660-668.

FAO Production Yearbook. 2003. http://www.fao.org/DOCREP/003/X4484E/x4484e03.html

Kelly, J.D. 1995. Use of random amplified polymorphic DNA markers in breeding for major gene resistance to plant pathogens. HortScience 30:461-465.

Konieczny, A. and F.M. Ausuble. 1993. A procedure for mapping Arabidopsis mutations using co-dominant ecotype-specific PCR-based markers. Plant J. 4:403-410.

Kosambi, D.D. 1944. The estimation of map distances from recombination values. Ann. Eugenics 12:172-175.

Lande, R. and R. Thompson. 1990. Efficiency of marker-assisted selection in the improvement of quantitative traits. Genetics 124:743-756.

Lander, E.S., P. Green, J. Abrahamson, A. Barlow, M.J. Daly, S.E. Lincoln and L. Newburg. 1987. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174-181

Luby, J.J. and D.V. Shaw. 2001. Does marker-assisted selection make dollars and sense in a fruit breeding program? HortScience 36:872-879.

Lunde, C.F., S.A. Mehlenbacher and D.C. Smith. 2000. Survey of hazelnut cultivars for response to eastern filbert blight inoculation. HortScience 35:729-731.

Melchinger, A.E. 1990. Use of molecular markers in breeding for oligogenic disease resistance. Plant Breeding 104:1-19.

Mehlenbacher, S.A., M.M. Thompson and H.R. Cameron. 1991. Occurrence and inheritance of resistance to eastern filbert blight in 'Gasaway' hazelnut. HortScience 26:410-411.

Mehlenbacher, S. 1994. Genetic improvement of the hazelnut. Acta Hort. 351:23-38.

Mehlenbacher, S. 1995. Classical and molecular approaches to breeding fruit and nut crops for disease resistance. HortScience 30:466-477.

Mehlenbacher, S.A. and D.C. Smith. 2003. Hazelnut Pollenizers 'Gamma', 'Delta', 'Epsilon' and 'Zeta'. HortScience (in press).

Michelmore, R. W., I. Paran and R. V. Kesseli. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Nat. Acad. Sci. USA. 88:9829-9832.

Mohan, M, S. Nair, A. Bhagwat, T.G. Krishna, M. Yano, C.R. Bhatia and T. Sasaki. 1997. Genome mapping, molecular markers and marker-assisted selection in crop plants. Mol. Breeding 3:87-103.

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

Pinkerton, J.N. 1993. Susceptibility of European hazelnut clones to eastern filbert blight. Plant Disease 77:261-266.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper and M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23:4407-4414.

Tanksley, S.D. 1983. Molecular markers in plant breeding. Plant Mol. Biol. Reporter 1:3-8.

Young, N.D. 1999. A cautiously optimistic vision for marker-assisted breeding. Molecular Breeding 5:505-510.

Chapter 4

AFLP MARKERS FOR EASTERN FILBERT BLIGHT RESISTANCE IN 'RATOLI' HAZELNUT

Honglin Chen and Shawn A. Mehlenbacher

Abstract

Eastern filbert blight (EFB), caused by *Anisogramma anomala* (Peck) E. Müller, is a devastating disease to European hazelnut orchards in the Willamette Valley, Oregon. The Spanish cultivar 'Ratoli' showed no symptoms or signs and tested negative for the fungus in greenhouse inoculations followed by ELISA. Segregation analysis of two backcross progenies 99035 (susceptible OSU 309.074 × Ratoli) and 99036 (susceptible OSU 665.012 × Ratoli) indicated that 'Ratoli' transmitted the resistance to 67% of its progenies. AFLP methodology was employed to identify markers linked to EFB resistance in 'Ratoli'. A total of 64 primer combinations were screened using three resistant and three susceptible individuals as well as the parents of progeny 99036. Primer combinations that showed no more than one recombination event in 6 seedlings were investigated further in a group of 16 seedlings. Markers that showed less than 25% recombination with resistance were further surveyed on the remaining seedlings of the population. Two AFLP markers linked to resistance were identified: dA1-135 (13.7 cM) and C4-255 (4.4 cM), both on the same side of the resistance loucs. Marker dA1-135 is linked in repulsion, whereas C4-255 is linked in coupling. Only marker C4-255 was present in the second population 99035. Further effort is needed to find markers more closely linked to the resistance locus in 'Ratoli'.

Introduction

Production of the European hazelnut, *Corylus avellana* L., in Oregon's Willamette Valley represents 98% and 3-5% of the production in the United States and the world, respectively (FAO Production Yearbook, 2003). However, the Oregon hazelnut industry is threatened by eastern filbert blight, a disease caused by the pyrenomycete *Anisogramma anomala* (Peck) E. Müller. The fungus causes severe cankers, rapid yield loss and eventually tree death in 5 to 12 years if control measures are not practiced (Pinkerton et al., 1993). Current control practices include fungicide applications and therapeutic pruning. However, due to the expense of fungicides and the dramatic yield loss caused by severe pruning of cankers, genetic resistance is the most desirable and economical means of disease control (Mehlenbacher, 1994). Therefore, developing varieties resistant to eastern filbert blight is a major goal of the Oregon State University (OSU) hazelnut breeding program.

Complete resistance to eastern filbert blight was first discovered in the obsolete pollenizer 'Gasaway' (Cameron, 1976). Immunity is conferred by a single dominant gene (Mehlenbacher et al., 1991). This gene has been the major source of resistance utilized in the OSU breeding program, although the search for new sources of complete resistance continues. 'Ratoli', a European hazelnut from Spain, showed no symptoms or signs and tested negative for the fungus in greenhouse inoculations followed by an enzyme-linked immunosorbent assay (ELISA) (Lunde et al., 2000). 'Ratoli' is superior to 'Gasaway' in many

horticultural aspects, including higher yield, high percent kernel (53%), and better blanching ability of kernels. 'Ratoli' has been used in the breeding program for the development of new resistant varieties that are suited to the blanched kernel market.

Current methods for evaluation of eastern filbert blight resistance are slow and expensive. Observation of cankers usually takes place 16 months after initial exposure to the causal fungus. Quarantine regulations require isolation of disease tests from commercial orchards and breeding activities. ELISA following greenhouse inoculation shortens the detection time to 6 months (Coyne et al., 1996). However, this process is still time-consuming, and in some cases, yields ambiguous results even with repeated tests. Marker-assisted selection (MAS) provides a means of screening genotypes for eastern filbert blight resistance at an early stage. It is also useful for confirming the phenotypes of promising selections. Only seedlings with DNA markers linked to the allele for resistance are planted in the field, and thus selection for other traits is in a population of resistant seedlings, leading to a greatly enriched gene pool. RAPD markers tightly linked to the 'Gasaway' gene for resistance have been identified (Davis et al., 1997, Mehlenbacher et al., 2003). Several of these markers have been sequenced and could be converted into SCARs (Paran and Michelmore, 1993). One of these RAPD markers, $UBC152_{800}$, is easy to score and robust to amplification

conditions. It has been routinely used in MAS for several years in the hazelnut breeding program at OSU.

In this study, the objectives were to examine segregation for resistance in seedlings of 'Ratoli' and to identify AFLP markers linked to resistance. The potential for use of these markers in a MAS program is also discussed.

Materials and Methods

Plant materials

In 1999, two seedling populations were obtained from controlled crosses of susceptible selections OSU 309.074 and OSU 665.012 with 'Ratoli', generating progenies 99038 and 99039, respectively (Figure 4.1). Seeds were collected in August, 2000, stratified, sown in flats in the greenhouse as they sprouted and grown in the flats to about 20cm tall. Seedlings were planted in 5 liter pots containing a mixture of equal volumes of peat, pumice, fine bark dust, and 9g of Sierra 3-4 month release fertilizer (18N-6P-12K) (Peters Professional, Allentown, PA). Seventy-two seedlings of progeny 99039 were transplanted to the field at the Oregon State University Smith Horticulture Research Farm in fall, 2001, and 51 others were kept in the greenhouse under optimal conditions (24°C day/18°C night) until they were ready for inoculation and DNA extraction. 'Gasaway', the

resistant control, and 'Ennis', the susceptible control, were also included in the greenhouse tests.

Greenhouse inoculation

Diseased twigs with mature stromata were collected from the North Willamette Research and Extension Centre in Aurora, Oregon, in November, 2000 and 2001. They were stored at –20 °C in polyethylene bags until they were used as inoculum. Perithecia were dissected from the stromata from diseased twigs and ground with a mortar and pestle to release ascospores. The ascospore suspensions were then diluted in distilled water to 1×10^6 spores/ml. The suspensions were placed in a squeeze bottle and sprayed three times to the tip(s) of one or two actively growing shoots on each tree. The sites of inoculation were indicated by tape placed two to three nodes below the apical meristem. Inoculation chambers were set up in the greenhouse, using polyvinyl chloride tubing (1.27 cm diameter) placed on top of benches (1.22 m× 0.44m) and covered with white 4 mm polyethylene sheeting. A humidifier was placed in each inoculation chamber and programmed to run from noon to 6 pm and from midnight to 4 am. Plants were inoculated when shoots had four to five nodes (Coyne et al., 1996). The inoculations were repeated three times at 3-day intervals. After inoculation, the trees remained in the greenhouse under optimal growing conditions for six months prior to the infection assay.

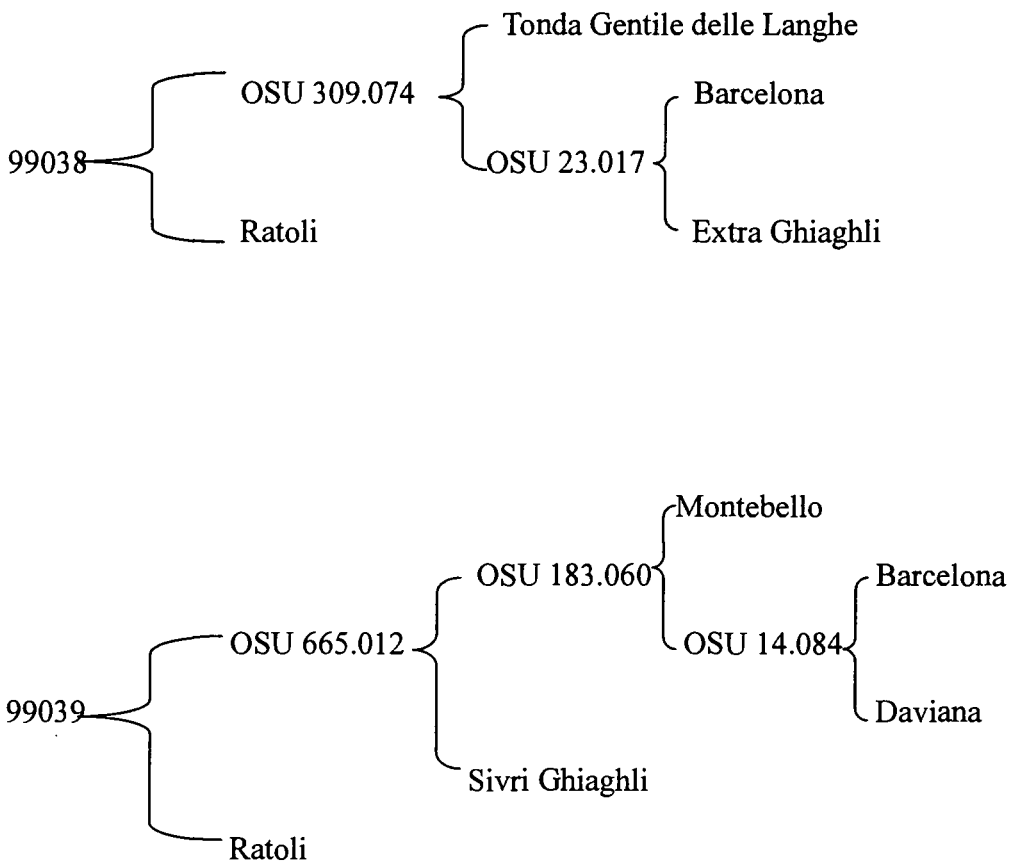

Figure 4.1 Pedigrees of hazelnut progenies 99038 and 99039.

EFB resistance evaluation

27 seedlings of progeny 99038 and 51 seedlings of progeny 99039 were tested for the presence or absence of the fungus 6-8 months after inoculation using the ELISA method developed by Coyne (1996) as slightly modified by Lunde (2000). The seedlings remained in the greenhouse to be re-inoculated and re-assayed in 2002. A genotype was scored as susceptible if the ELISA score was above the threshold or any symptoms or signs were seen when the plants were inspected 18 months later, and resistant if the ELISA test was negative and no disease symptoms or signs were observed.

DNA extraction

Fresh young leaves were collected from the greenhouse in spring, 2002 from 27 seedlings of progeny 99038, 49 seedlings of progeny 99039 (two died in the greenhouse) and the two parents, and from the field in spring, 2003 for an additional 72 seedlings of the 99039 population. DNA was extracted following the method of Lunde et al. (2000) with minor modifications, and RNA was removed by incubation at 37 °C in the presence of RNase A for one hour in a shaker, followed by 25 phenol: 24 chloroform: 1 isoamyl alcohol extraction. Some DNA samples were further purified using QIAprep Spin Miniprep Kit (Qiagen, Chatsworth, Calif.) to ensure successful amplification. DNA was extracted four times from each seedling and stored at -4 °C until used in AFLP assays. DNA

concentration was quantified using a Hoefer® DyNA Quant™ 2000 Fluorometer (Amersham Bioscience, San Francisco, Calif.).

AFLP analysis

Eight samples (three EFB resistant, three EFB susceptible seedlings, and two parents) were used with a GIBCO BRL AFLP analysis system kit I (Rockville, MD) following the manufacturer's protocol. Eight EcoRI (*EcoRI* +AAC, AAG, ACA, ACT, ACC, ACG, AGC, AGG) and eight MseI (*MseI* + CAA, CAC, CAG, CAT, CTA, CTC, CTG, CTT) primers were used for a total of 64 combination pairs. Primer combinations that generated a band associated in either coupling or repulsion with resistance were investigated further in a second group of 16 seedlings. Primer combinations that generated a band with one recombinant in the 6 seedlings were also investigated futher. Markers that showed less than 25% recombination with resistance were further surveyed in an additional 100 seedlings. In the post pre-screening process, the AFLP procedure of Vos (1995) was used with some modifications (Appendix A). Briefly, 250ng of genomic DNA were digested with *EcoRI* and *MseI* by incubation at 37 °C overnight, and then held at 72 °C for 15 minutes to inactivate the enzymes. The DNA fragments were ligated with *EcoRI* and *MseI* adapters at 16 °C for two hours. For pre-selective amplification, 5µl of a 10-fold diluted ligation mixture was amplified for 20 cycles of 94 °C for 30s, 56 °C for 60s, and 72 °C for 60s using primers *EcoRI* + A and *MseI* + C. For selective amplification, 5µl of the combination of *EcoR I* +AXX

and *Mse I* + CXX primers was mixed with 5µl of 50-fold diluted preamplified DNA and PCR buffer. The mixture was amplified for 1 cycle of 94 °C for 30s, 65 °C for 30s, 72 °C for 60s, then lowering annealing temperature 0.7 °C each cycle for 12 cycles, and then 23 cycles of 94 °C for 30s, 56 °C for 30s, and 72 °C for 60s. Amplification of PCR products was verified by electrophoresis on 1% agarose gels using 5µl of the aliquot and 3µl of loading dye (15% Ficoll® 400, 0.03% xylene cyanol FF, 0.4% orange G, 10mM Tris-HCl pH 7.5, and 50mM EDTA). The amplicons were mixed with an equal volume of loading dye and loaded on 6% polyacrylamide denaturing sequencing gels (Appendix B). Gels were run at 75W (1600V) constant power for 3.5 hours and then stained with silver (Appendix C). The size of markers was estimated by comparison to a 100-1500bp ladder (Promega, Madison, WI).

Data analysis

Goodness-of-fit to expected segregation ratios was performed using the Chi-square test. Heterogeneity Chi-square was also calculated to decide whether the data from the two progenies were sufficiently uniform to be pooled. Linkage of the AFLP markers and disease resistance was analyzed using 121 seedlings from progeny 99039 and 27 seedlings from progeny 99038. 59% of the seedlings of the 99039 population have no phenotypic data but were used to determine the linkage order of the markers. The AFLP markers were scored as 1 for the presence of a band and 0 for its absence. Similarly, the phenotypic data was

scored as 1 for resistance and 0 for susceptibility. The data were entered into a Microsoft Excel spreadsheet, and then saved as a tab-delimited text file and analyzed with MAPMAKER EXP 3.0 (Lander et al., 1987) using the f2 backcross function and default linkage criteria of LOD=3.0, and a maximum recombination frequency of 0.25. The most likely map order was determined using the 'compare' command. The Kosambi mapping function (Kosambi, 1944) was used to convert the recombination frequency into map distances in centiMorgans (cM). The 'error detection on' command tells MapMaker to perform the analysis assuming incomplete penetrance and a mistyping error rate of 1%. Suspicious double recombinant data points were reexamined by selective amplification of new DNA template from the same tree. Mistakes potentially were made in genotyping or phenotyping and true double recombinants may exist, so maps are reported with the options of 'error detection on' and 'error detection off'. Maps were drawn using the 'draw map' command of MapMaker and the resulting PS file was visualized using Ghostview software.

Results

Segregation for resistance to EFB in progenies of 'Ratoli'

In progeny 99038, 18 seedlings were scored as resistant and 9 as susceptible. In progeny 99039, 32 were scored as resistant and 19 as susceptible. The heterogeneity Chi-square test showed that the data from these two progenies were indeed homogeneous. Thus, the data were pooled. Although the Chi-square

test did not reject fit to a segregation ratio of 1:1 at a p-value of 0.05 for each separate population, the observed ratio in the pooled data was different from the 1:1 expected ratio (Table 3.1). The data indicated that 'Ratoli' transmitted resistance to a higher percentage (67%) of its seedlings.

Table 4.1 Segregation for resistance to EFB in seedlings of 'Ratoli'

Population	Plant number		Expected ratio	χ^2	
	Resistant	Susceptible		Value	*P*
309.074×Ratoli (99038)	18	9	1:1	3.00	0.08
665.012×Ratoli (99039)	32	19	1:1	3.31	0.06
Pooled data	50	28	1:1	6.21	0.01
Heterogeneity χ^2 (degree of freedom = 1)				0.10	0.75

Identification of AFLP markers linked to eastern filbert blight resistance in 'Ratoli'

Progeny 99039 was used in the search for potential AFLP markers. All 64 primer pairs successfully amplified fragments ranging from 1000 to less than 100 base pairs in length. Typically, 40 to 80 fragments were produced per primer

Table 4.2 AFLP markers and their segregation among 121 seedlings of progeny 99039 and 27 seedlings of progeny 99038.

Populations	Primer designation	Primer combinations	AFLP marker designation	Approximate size of markers (bp)	Observed frequency (present: absent)	Expected ratio	χ^2	
							Value	*P*
665.012 × Ratoli	A1	EAAC/MCAA	dA1-135	135	68:53	1:1	1.86	0.17
	C4	EACA/MCAT	C4-255	255	70:51	1:1	2.98	0.08
309.074 × Ratoli	C4	EACA/MCAT	C4-255	255	18:9	1:1	3.00	0.08

* Statistically significant at p=0.05 critical level.

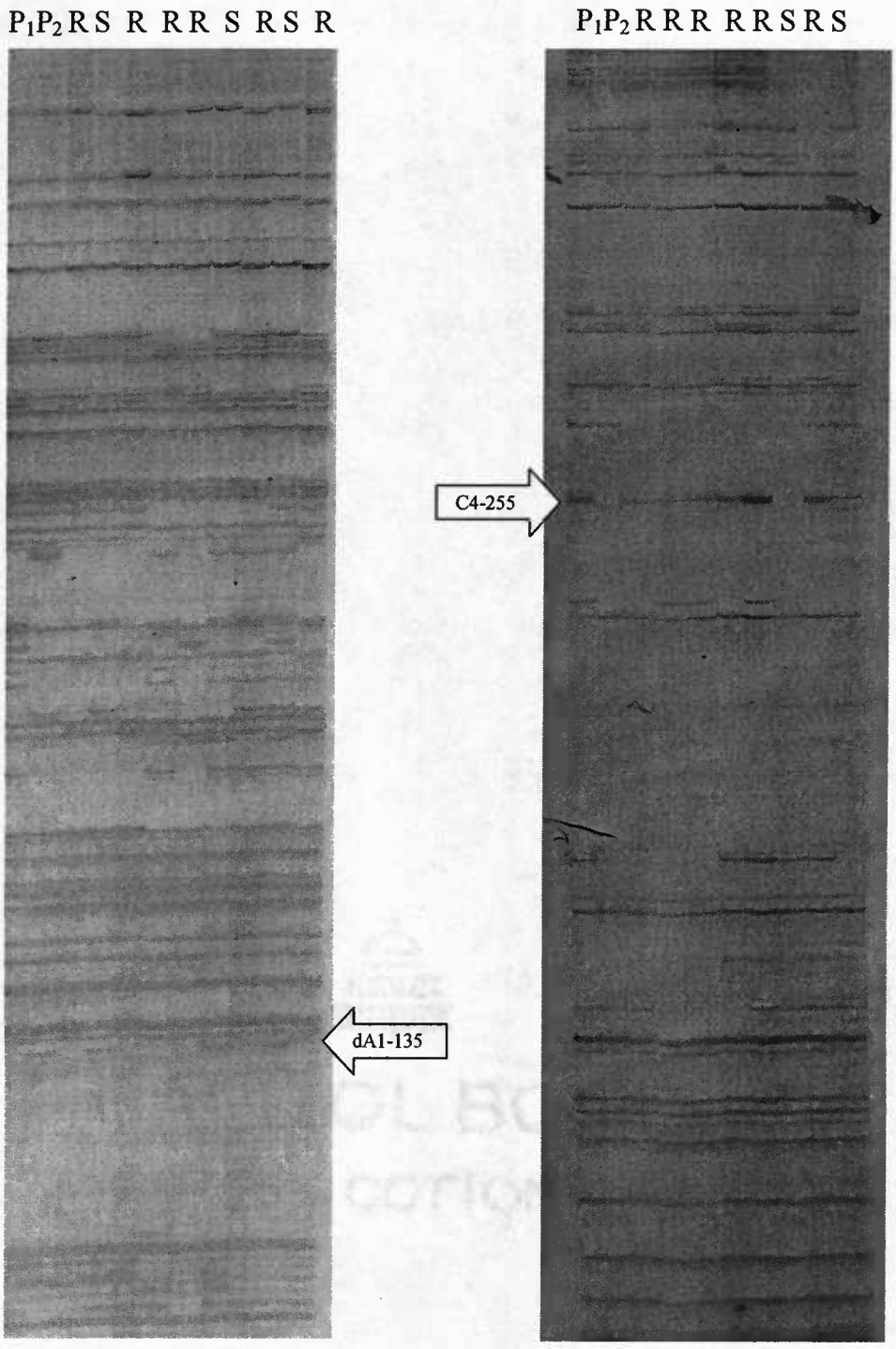

Figure 4.2 Segregating markers dA1-135 and C4-255 for OSU 665.012 (P_2) × Ratoli (P_1) progeny. R= resistant, S= susceptible. The arrow indicates the location of the polymorphism.

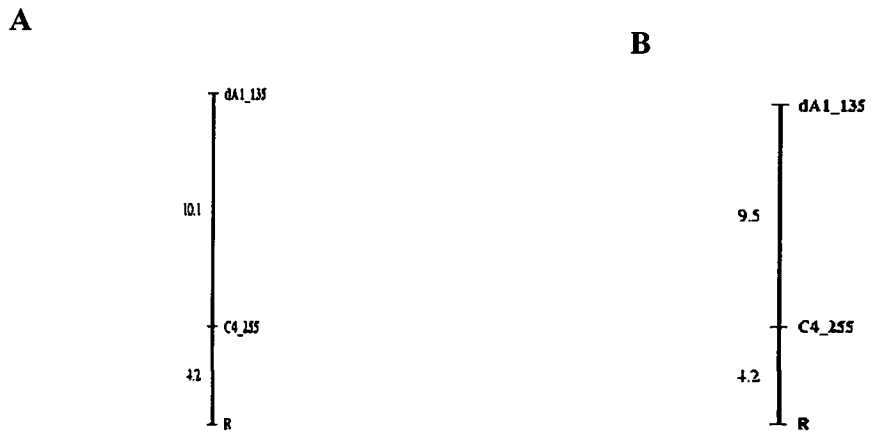

Figure 4.3 Most likely map orders of AFLP markers and the resistance locus for 99039. (A) Error detection off. (B) Error detection on.

combination and an average of 15% polymorphism between the resistant parent and the susceptible parent was observed.

A total of 15 markers was identified as putatively linked in either coupling or repulsion to resistance in 'Ratoli'. The linkage analysis using MapMaker placed seven AFLP markers in the same linkage group as the resistance locus. The remaining eight markers were placed in a second linkage group and thus they are not useful in MAS. Among the seven markers in the same linkage group as the resistance locus, only two showed recombination frequencies less than 25% and they were designated dA1-135 and C4-255 (Figure 4.2). The marker C4-255 was a polymorphic fragment adjacent to another fragment of slightly smaller size in a block of faint smear, and thus required careful examination during scoring. Marker dA1-135 is linked in repulsion, whereas C4-255 is linked in coupling. Both markers segregated in the expected 1:1 ratio of presence to absence (Table 4.2). With 'error detection off', a map spanning 14.3 cM was constructed for progeny 99039 (Figure 4.3a). When error detection was turned on, a map covering 13.7 cM was constructed (Figure 4.3b). Both markers were placed on the same side of the resistance locus.

To further confirm the segregation and linkage of these linked markers to the resistance locus, 27 seedlings from the 99038 population were screened with the same AFLP primer combinations used in the 99039 population. The results showed that only C4-255 was also present in this population and segregated in the expected 1:1 ratio (Table 4.2). However, the linkage analysis placed the marker

23.9 cM away from the resistance. This may due to the small number of seedlings in this progeny.

Discussion

Segregation studies for complete resistance to EFB have been carried out on 'Gasaway', 'Zimmerman' and selection OSU 408.040. They showed that a single dominant gene controls the resistance in 'Gasaway' (Mehlenbacher et al., 1991) and the selection OSU 408.040 (Chapter 2). Lunde (1999) reported that the segregation for resistance to EFB in 'Zimmerman' followed a 3:1 (resistant: susceptible) ratio. However, in this study, 'Ratoli' transmitted its resistance to 67% of its progenies, instead of 50% or 75%. This segregation distortion may be due to the insufficient sample size used and the uncertainty in the phenotyping data. 23 out of 51 seedlings of 99039 in the greenhouse were dead or nearly dead in the greenhouse at the end of the first year and thus their phenotype could not be confirmed in the second year. Thus, further study is needed to determine the inheritance pattern of 'Ratoli' source of resistance.

Disease resistance conferred by a single major gene is much easier to incorporate into well-adapted, high-yielding cultivars using the modified backcross method, though it may be less durable (Mehlenbacher, 1995). Resistance from 'Gasaway' is controlled by a single dominant gene (Mehlenbacher et al., 1991), and this gene has been the major source of resistance utilized in the hazelnut

breeding program at Oregon State University. The resistance of 'Ratoli' also appears to be conferred by a single gene, which indicates that a similar approach can be used to incorporate the 'Ratoli' resistance into commercially acceptable cultivars. The RAPD marker $UBC152_{800,}$ which is tightly linked to the 'Gasaway' resistance and has been routinely used in marker-assisted selection, is absent in 'Ratoli'. Thus, 'Ratoli' appears to be a novel source of genetic resistance to eastern filbert blight (Lunde et al., 2000). The identification of this new source of resistance will also be useful in the gene pyramiding to create varieties with more durable resistance to EFB.

The strategy used in isolating AFLP markers linked to the resistance locus was based on screening a limited number of samples with a relatively large number of primer pairs to ensure that many loci can be screened with a limited effort. Candidate markers that were identified in this way were then screened on a larger number of phenotypically well-characterized samples to confirm their linkage. In this study, three susceptible and three resistant seedlings were used in the pre-screening process. Primer pairs that generated a band associated in either coupling or repulsion to resistance or one of the six seedlings was a recombinant were investigated further in a second group of 16 seedlings. Any loci that exhibited less than 25% recombination were used to screen the remaining 100 seedlings, which gave 15 markers putatively linked to the resistance loci. However, 8 of them are not linked to the resistance locus at LOD=3.0 and they

remained unlinked until LOD was lowered to 1.0. Among the seven markers that were placed in the same linkage group as the resistance locus, only two showed less than 25% recombination frequency. Picking up these loosely linked markers may be because the criterion used in screening markers in the secondary screening process was not stringent enough. In addition, an appropriate amount of phenotypically well-characterized samples was not available for the secondary screen.

Marker-assisted selection has been shown to be extremely powerful for disease resistance breeding when it is difficult or expensive to evaluate the progeny by conventional methods (Lande and Thompson, 1990; Melchinger, 1990; Dudley, 1993; Kelly, 1995; Mehlenbacher, 1995; Young, 1999; Luby and Shaw, 2001; Dreher et al., 2002). The identification of markers tightly linked to the resistance locus (<5 cM) is an important step toward a marker-assisted selection program. In this study, only marker C4-255 was close enough to be useful in MAS. Thus, further effort is needed to narrow down the interval spanning the resistance locus or loci of 'Ratoli'.

References

Cameron, H. R. 1976. Eastern filbert blight established in the Pacific Northwest. Plant Dis. Rptr. 60:737-740.

Coyne, C.J., S.A. Mehlenbacher, R.O. Hampton, J.N. Pinkerton and K.B. Johnson. 1996. Use of ELISA to rapidly screen hazelnut for resistance to eastern filbert blight. Plant Disease 80:1327-1330.

Davis, J.W. and S.A. Mehlenbacher. 1997. Identification of RAPD markers linked to eastern filbert blight resistance in hazelnut. Acta Hort. 445:553-556.

Dreher, K., M. Morris, M. Khairallah, M. Ribaut, P. Shivaji, S. Ganesan, S. Pandey, G. Srinivasan, R.E. Evenson, V. Santaniello and D. Zilberman. 2002. Is marker-assisted selection cost-effective compared with conventional plant breeding methods? The case of quality protein maize. Economic and Social Issues in Agricultural Biotechnology 15:203-236.

Dudley, J.W. 1993. Molecular markers in plant improvement: manipulation of genes affecting quantitative traits. Crop Science 33:660-668.

FAO Production Yearbook. 2003. http://www.fao.org/DOCREP/003/X4484E/x4484e03.html

Kelly, J.D. 1995. Use of random amplified polymorphic DNA markers in breeding for major gene resistance to plant pathogens. HortScience 30:461-465.

Konieczny, A. and F.M. Ausuble. 1993. A procedure for mapping Arabidopsis mutations using co-dominant ecotype-specific PCR-based markers. Plant J. 4:403-410.

Kosambi, D.D. 1944. The estimation of map distances from recombination values. Ann. Eugenics 12:172-175.

Lande, R. and R. Thompson. 1990. Efficiency of marker-assisted selection in the improvement of quantitative traits. Genetics 124:743-756.

Lander, E.S., P. Green, J. Abrahamson, A. Barlow, M.J. Daly, S.E. Lincoln and L. Newburg. 1987. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural

populations. Genomics 1:174-181

Luby, J.J. and D.V. Shaw. 2001. Does marker-assisted selection make dollars and sense in a fruit breeding program? HortScience 36:872-879.

Lunde, C.F., S.A. Mehlenbacher and D.C. Smith. 2000. Survey of hazelnut cultivars for response to eastern filbert blight inoculation. HortScience 35:729-731.

Melchinger, A.E. 1990. Use of molecular markers in breeding for oligogenic disease resistance. Plant Breeding 104:1-19.

Mehlenbacher, S.A., M.M. Thompson and H.R. Cameron. 1991. Occurrence and inheritance of resistance to eastern filbert blight in 'Gasaway' hazelnut. HortScience 26:410-411.

Mehlenbacher, S. 1994. Genetic improvement of the hazelnut. Acta Hort. 351:23-38.

Mehlenbacher, S. 1995. Classical and molecular approaches to breeding fruit and nut crops for disease resistance. HortScience 30:466-477.

Mehlenbacher, S.A. and D.C. Smith. 2003. Hazelnut Pollenizers 'Gamma', 'Delta', 'Epsilon' and 'Zeta'. HortScience. (in press)

Michelmore, R. W., I. Paran and R. V. Kesseli. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Nat. Acad. Sci. USA. 88:9829-9832.

Mohan, M, S. Nair, A. Bhagwat, T.G. Krishna, M. Yano, C.R. Bhatia and T. Sasaki. 1997. Genome mapping, molecular markers and marker-assisted selection in crop plants. Mol. Breeding 3:87-103.

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

Pinkerton, J.N. 1993. Susceptibility of European hazelnut clones to eastern filbert blight. Plant Disease 77:261-266.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper and M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23:4407-4414.

Tanksley, S.D. 1983. Molecular markers in plant breeding. Plant Mol. Biol. Reporter 1:3-8.

Young, N.D. 1999. A cautiously optimistic vision for marker-assisted breeding. Molecular Breeding 5:505-510.

CHAPTER 5

SUMMARY

Fifty-eight hazelnut accessions, including *Corylus avellana* L. and interspecific hybrids were evaluated for their response to *Anisogramma anomala* (Peck) E. Müller, the causal fungus of eastern filbert blight, by greenhouse inoculation using the methods of enzyme-linked immunosorbent assay (ELISA) and visual inspection of cankers. Twelve accessions remained free of infection: European hazels 'Culpla' from Spain and 'COR 187' from Finland; *C. americana* × *C. avellana* hybrids 'COR 506', 'G081S' and Weschcke TP1, TP2 and TP3; *C. colurna* × *C. avellana* hybrids 'Chinese Trazel Gellatly #6 and #11, Turkish Trazel Gellatly #3 and a (*C. colurna* × *C. avellana*) × *C. avellana* backcross hybrid 'Lisa'; and a *C. heterophylla* var. *sutchuensis* × *C. avellana* hybrid 'Estrella #1'. These new sources of complete resistance can be readily crossed to commercial European varieties and thus will facilitate the development of new hazelnut varieties with resistance to eastern filbert blight.

Segregation for resistance to eastern filbert blight in three progenies obtained from the controlled crosses of three different susceptible parents with OSU 408.040, a new source of complete resistance to EFB, showed that a single gene controls the EFB resistance. AFLP methodology was employed to identify markers linked to EFB resistance in OSU 408.040. A total of 64 primer

combinations were screened and resulted in the discovery of five AFLP markers linked in coupling to resistance. They are A4-265 (9.2 cM), A8-150 (0.0 cM), B2-125 (4.1 cM), C2-175 (5.9 cM) and D8-350 (2.5 cM), with the latter one located on the other side of the resistance locus. Three of these markers B2-125, C2-175 and D8-350 were also linked in coupling in a similar order in a second population. The markers identified in this study are a first step toward marker-assisted selection for the OSU 408.040 source of resistance.

Likewise, investigation of the inheritance of eastern filbert blight resistance from the Spanish cultivar 'Ratoli', which showed complete resistance in a previous study and appears to be a new source of resistance, indicated that resistance is tranmitted to 67% of its progenies. A total of 64 primer combinations were screened to find AFLP markers linked to the 'Ratoli' source of resistance. Two AFLP markers were identified: dA1-135 (13.7 cM) and C4-255 (4.4 cM), both on the same side of the resistance loucs. Marker dA1-135 is linked in repulsion, whereas C4-255 is linked in coupling. Only C4-255 was present in a second population. Further effort is needed to narrow down the interval spanning the resistance locus in 'Ratoli' prior to marker-assisted selection.

BIBLIOGRAPHY

Ardiel, G.S., T.S. Grewal, P. Deberdt, B.G. Rossnagel and G.J. Seoles. 2002. Inheritance of resistance to covered smut in barley and development of a tightly linked SCAR marker. Theor. Appl. Genet. 104:457-464.

Arnedo-Andres, M.S., R. Gil-Ortega, M. Luis-Arteaga and J.I. Hormaza. 2002. Development of RAPD and SCAR markers linked to the *Pvr4* locus for resistance to PVY in pepper (*Capsicum annuum* L.). Theor. Appl. Genet. 105:1067-1074.

Avila, C.M., J.C. Sillero, D. Rubiales, M.T. Moreno and A.M. Torres. 2003. Identification of RAPD markers linked to the *Uvf-1* gene conferring hypersensitive resistance against rust (*Uromyces viciae-fabae*) in *Vicia faba* L. Theor. Appl. Genet. (in press).

Barss, H.P. 1930. Eastern filbert blight. California Agr. Dept. Bull. 19:489-490.

Cameron, H.R. 1976. Eastern filbert blight established in the Pacific Northwest. Plant Dis. Rptr. 60:737-740.

Chen, H.L. and S.A.Mehlenbacher. 2003. New sources and linked AFLP markers for eastern filbert blight resistance in hazelnut. MS thesis. Department of Horticulture, Oregon State Univeristy, Corvallis.

Coyne, C.J. 1995. Genetic resistance to Eastern filbert blight. MS thesis. Department of Horticulture, Oregon State University, Corvallis.

Coyne, C.J., S.A. Mehlenbacher, R.O. Hampton, J.N. Pinkerton and K.B. Johnson. 1996. Use of ELISA to rapidly screen hazelnut for resistance to eastern filbert blight. Plant Disease 80:1327-1330.

Coyne, C.J., S.A. Mehlenbacher, and D.C. Smith. 1998. Sources of resistance to eastern filbert blight in hazelnut. J. Amer. Soc. Hort. Sci. 123:253-257.

Coyne, C.J., S.A. Mehlenbacher, K.B. Johnson, J.N. Pinkerton and D.C. Smith. 2000. Comparison of two methods to evaluate quantitative resistance to eastern filbert blight in European hazelnut. J. Amer. Soc. Hort. Sci. 125:603-608.

Davison, A.R. and R.M. Davidson, Jr. 1973. *Apioporthe* and *Monochaetia* cankers reported in western Washington. Plant Dis. Rep. 57:522-523.

Davis, J.W. and S.A. Mehlenbacher. 1997. Identification of RAPD markers linked to eastern filbert blight resistance in hazelnut. Acta Hort. 445:553-556.

Dreher, K., M. Morris, M. Khairallah, M. Ribaut, P. Shivaji, S. Ganesan, S. Pandey, G. Srinivasan, R.E. Evenson, V. Santaniello and D. Zilberman. 2002. Is marker-assisted selection cost-effective compared with conventional plant breeding methods? The case of quality protein maize. Economic and Social Issues in Agricultural Biotechnology 15:203-236.

Dudley, J.W. 1993. Molecular markers in plant improvement: manipulation of genes affecting quantitative traits. Crop Science 33:660-668.

Ellis, J.B. and B.M. Everhart. 1892. The North American Pyrenomycetes. Ellis and Everhart, Newfield, New Jersey. p. 531- 532.

Erdoğan, V. and S. A. Mehlenbacher. 2000. Interspecific hybridization in hazelnut (*Corylus*). J. Amer. Soc. Hort. Sci. 125:489-497.

FAO Production Yearbook. 2003. http://www.fao.org/DOCREP/003/X4484E/x4484e03.html.

Farris, C.W. 1969. Hybridization of filberts. p. 299- P300. In: R. A. Jaynes (ed.). Handbook of North American nut trees. Northern Nut Growers Assn., Humphrey Press, Hamden, Conn.

Farris, C.W. 1970. Inheritance of parental charactheristics in filbert hybrids. Ann. Rept. Northern Nut Growers Assn. 61:54-58.

Farris, C.W. 1976. An introduction to the stars: A new family of filbert hybrids. Ann. Rept. Northern Nut Growers Assn. 67:80-82.

Farris, C.W. 1982. A progress report on the development of F2 hybrids of *Corylus colurna* × *C. avellana*. Ann. Rept. Northern Nut Growers Assn. 71:15-16

Farris, C.W. 1988. Potential for genetic improvement in hazelnuts. Ann. Rept. Northern Nut Growers Assn. 81:46-47

Farris, C.W. 1989. Grand Traverse hazelnuts. Ann. Rept. Northern Nut Growers Assn. 80:97-99

Farris, C.W. 1990. Hazelnut cultivar development: A progress report. Ann. Rept. Northern Nut Growers Assn. 82:118-119.

Fuller, A.S. 1910. Filbert or hazelnut. p. 118-146. In: The nut culturist. Orange Judd, New York.

Gellatly, J.U. 1950. Description of filazel varieties. Ann. Rept. Northern Nut Growers Assn. 41:116-117.

Gellatly, J.U. 1956a. Tree hazels-*Corylus colurna*. Ann. Rept. Northern Nut Growers Assn. 47:110-112.

Gellatly, J.U. 1956b. Filazels. Ann. Rept. Northern Nut Growers Assn. 47:112-114.

Gellatly, J.U. 1964. Filazels. Ann. Rept. Northern Nut Growers Assn. 55:153-155.

Gellatly, J.U. 1966. Tree hazels and their improved hybrids. Ann. Rept. Northern Nut Growers Assn. 57:98-106.

Gianfranceschi, L., B. Koller, N. Seglias, M. Kellerhals and C. Gessler. 1996. Molecular selection in apple for resistance to scab caused by *Venturia inaequalis*. Theor. Appl. Genet. 93:199-204.

Gmiter, F.G. Jr., S.Y. Xiao, S. Huang, X.L. Hu, S.M. Garnsey and Z. Deng. 1996. A localized linkage map of the citrus tristeza virus resistance gene region. Theor. Appl. Genet. 92:688-695.

Gottwald, T.R. and H.R. Cameron. 1980. Infection site, infection period, and latent period of canker caused by *Anisogramma anomala* in European filbert. Phytopathology 70:1083-1087.

Hazelnut Marketing Board. 2003. http://oregonhazelnuts.org/

Johnson, K.B. and J.W. Pscheidt. 1993. Evaluation of chlorothalonil, fenarimol, and flusilazole for control of eastern filbert blight. Plant Disease 77:831-837.

Johnson, K.B., J.N. Pinkerton, S.M. Gaudreault and J.K. Stone. 1994. Infection of European hazelnut by *Anisogramma anomala*: Site of infection and effect of host developmental stage. Phytopathology 84:1465-1470.

Johnson, K.B., S.A. Mehlenbacher, J.K. Stone and J.W. Pscheidt. 1996. Eastern filbert blight of European hazelnut – it's becoming a manageable disease. Plant Disease 80:1308-1316.

Kelly, J.D. 1995. Use of random amplified polymorphic DNA markers in breeding for major gene resistance to plant pathogens. HortScience 30:461-465.

King, G.J., F.H. Alston, L.M. Brown, E. Chevreau, K.M. Evans, F. Dunemann, J. Janse, F. Laurens, J.R. Lynn, C. Maliepaard, A.G. Manganaris, P. Roche, H. Schmidt, S. Tartarini, J. Verhaegh and R. Vrielink. 1998. Multiple field and glasshouse assessments increase the reliability of linkage mapping of the *Vf* source of scab resistance in apple. Theor. Appl. Genet. 96:699-708.

Konieczny, A. and F.M. Ausuble. 1993. A procedure for mapping Arabidopsis mutations using co-dominant ecotype-specific PCR-based markers. Plant J. 4:403-410.

Kosambi, D.D. 1944. The estimation of map distances from recombination values. Ann. Eugenics 12:172-175.

Lander, E.S., P. Green, J. Abrahamson, A. Barlow, M.J. Daly, S.E. Lincoln and L. Newburg. 1987. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174-181

Lande, R. and R. Thompson. 1990. Efficiency of marker-assisted selection in the improvement of quantitative traits. Genetics 124:743-756.

Lu, Z.X., K.S. Alaoui, G.L. Reighard, W.V. Baird and A.G. Abbott. 1999. Development and characterization of a co-dominant marker linked to root-knot nematode resistance, and its application to peach rootstock breeding. Theor. Appl. Genet. 99:115-122.

Luby, J.J. and D.V. Shaw. 2001. Does marker-assisted selection make dollars and sense in a fruit breeding program? HortScience 36:872-879.

Lukasiewicz, J. 1992. Experimental nut plantings at the southwest Michigan research and extension station. Ann. Rept. Northern Nut Growers Assn. 83:12-14.

Lunde, C.F. 1999. Investigation of novel sources of genetic resistance to eastern filbert blight. MS thesis. Department of Horticulture, Oregon State University, Corvallis.

Lunde, C.F., S.A. Mehlenbacher and D.C. Smith. 2000. Survey of hazelnut cultivars for response to eastern filbert blight inoculation. HortScience 35:729-731.

Mekuria, G.T., G. Collins and M. Sedgley. 2001. Identification of genetic markers in olive linked to olive leaf spot resistance and susceptibility. J. Amer. Soc. Hort. Sci. 126:305-308.

Melchinger, A.E. 1990. Use of molecular markers in breeding for oligogenic disease resistance. Plant Breeding 104:1-19.

Mehlenbacher, S. 1991. New developments in the Oregon hazelnut breeding program. Ann. Rept. Northern Nut Growers Assn. 82:103-110.

Mehlenbacher, S.A., M.M. Thompson and H.R. Cameron. 1991. Occurrence and inheritance of resistance to eastern filbert blight in 'Gasaway' hazelnut. HortScience 26:410-411.

Mehlenbacher, S. 1994. Genetic improvement of the hazelnut. Acta Hort. 351:23-38.

Mehlenbacher, S. 1995. Classical and molecular approaches to breeding fruit and nut crops for disease resistance. HortScience 30:466-477.

Mehlenbacher, S.A. and D.C. Smith. 2003. Hazelnut Pollenizers 'Gamma', 'Delta', 'Epsilon' and 'Zeta'. HortScience. (in press)

Mehlenbacher, S.A., R.N. Brown, J.W. Davis, H. Chen, N. Bassil and D.C. Smith. 2003. RAPD markers for eastern filbert blight resistance in *Corylus avellana*. Theor. Appl. Genet. (in press)

Michelmore, R. W., I. Paran and R. V. Kesseli. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Nat. Acad. Sci. USA. 88:9829-9832.

Mohan, M, S. Nair, A. Bhagwat, T.G. Krishna, M. Yano, C.R. Bhatia and T. Sasaki. 1997 Genome mapping, molecular markers and marker-assisted selection in crop plants. Mol. Breeding 3:87-103.

Morgante, M. and A.M. Olivieri. 1993. PCR-amplified microsatellites as markers in plant genetics. Plant J. 3:175-182.

Osterbauer, N. K., K. B. Johnson, S.A. Mehlenbacher and T.L. Sawyer. 1997. Analysis of resistance to eastern filbert blight in *Corylus avellana*. Plant Disease 81:388-394.

Ourecky, D.K. and G.L. Slate. 1969. Susceptibility of filbert varieties and hybrids to the filbert bud mite, *Phytoptus avellane* Nal. Ann. Rept. Northern Nut Growers Assn. 60:89-91.

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

Pinkerton, J.N. 1992. Distribution and characteristics of the eastern filbert blight epidemic in western Oregon. Plant Disease 76:1179-1182.

Pinkerton, J.N. 1993. Susceptibility of European hazelnut clones to eastern filbert blight. Plant Disease 77:261-266.

Pinkerton, J.N., J.K. Stone, S.J. Nelson and K.B. Johnson. 1995. Infection of European hazelnut by *Anisogramma anomala*: ascospore adhesion, mode of penetration of immature shoots, and host response. Phytopathology 85:1260-1268.

Pinkerton, J.N., K.B. Johnson, J.K. Stone, K.L. Ivors. 1998a. Factors affecting the release of ascospores of *Anisogramma anomala*. Phytopathology 88:122-128.

Pinkerton, J.N., K.B. Johnson, J.K. Stone and K.L. Ivors. 1998b. Maturation and seasonal discharge pattern of ascospores of *Anisogramma anomala*. Phytopathology 88:1165-1173.

Pscheidt, J.W and S. Cluskey. 2001. Fungicide control of eastern filbert blight. Acta Hort. 556:411-418.

Reed, C.A. 1936. New filbert hybrids. J. Hered. 27:427-431.

Roche, P., F.H. Alston, C. Maliepaard, K.M. Evans, R. Vrielink, F. Dunemann, T. Markussen, S. Tartarini, L.M. Brown, C. Byder, G.J. King. 1997. RFLP and RAPD markers linked to the rosy leaf curling aphid resistance gene (Sd1) in apple. Theor. Appl. Genet. 94:3-4.

Rutter, M. 1991. Variation in resistance to eastern filbert blight in hybrid hazels. Ann. Rept. Northern Nut Growers Assn. 82:159-162.

Sax, K. 1923. The association of size differences with seed-coat pattern and pigmentation in *Phaseolus vulgaris*. Genetics 8:552-560.

Schuster, C.E. 1924. Filberts: 2. Experimental data on filbert pollination. Oregon Agr. Expt. Sta. Bull. 208.

Simmonds, N.W. 1983. Strategy of disease resistance breeding. FAO Plant Prot. Bull. 31:2-10.

Stone, J.K., K.B. Johnson, J.N. Pinkerton J.N. and J.W. Pscheidt. 1992. Natural infection period and susceptibility of vegetative seedlings of European hazelnut to *Anisogramma anomala*. Plant Disease 76:348-352.

Tasias-Valls, J. 1975. EI avellano en la provincia de Tarragona (in Spanish) Escma. Diputacion provincial de Tarragona. Fundacion Servicio Agropecuario Provincial. Tarragona, Spain.

Tanksley, S.D. 1983. Molecular markers in plant breeding. Plant Mol. Biol. Reporter 1:3-8.

Tanksley, S.D., N.D. Young, A.H. Paterson and M.W. Bonierbale. 1989. RFLP mapping in plant breeding: new tools for an old science. Bio/Technol 7:257-263.

Thoday, J.M. 1961. Location of polygenes. Nature 191:368-370.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper and M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23:4407-4414.

Weschcke, C. 1954. Growing nuts in the north. p. 24-38. Webb. St. Paul.

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

Williams, J.G.K., A.R. Kubelic, K.J. Livak, J.A. Rafalsky and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531-6535.

Young, N.D. 1996. QTL mapping and quantitative disease resistance in plants. Annu. Rev. Phytopathol. 34:479-501.

Young, N.D. 1999. A cautiously optimistic vision for marker-assisted breeding. Molecular Breeding 5:505-510.

Young, N.D., J. Mudge, J.L. Starr, R. Cook and J. Bridge. 2002.. Marker-assisted selection for soybean cyst nematode resistance. p. 241-252. In: Plant Resistance to Parasitic Nematodes. CABI publishing, Wallingford, UK.

APPENDICES

Appendix A AFLP Protocol for Hazelnut

Modification based on Vos's protocol (per reaction)

1. Digestion

250.0 ng	DNA template
0.25ul	EcoRI (20U/ul) BioLab
0.5 ul	MseI (10U/ul)
2.5 ul	EcoRI 10× buffer
0.25ul	BSA
ul	H_2O
25.0 ul	volume

Double digest overnight, then 72 °C for 15min to inactivate enzymes.

2. Adapter preparation

Add STE (10 mM Tris pH 8.0, 50 mM NaCl, 1mM EDTA) to each oligo (Integrated DNA Company, Coralville, IA) to make 100uM stock. Mix 100ul of sense oligo and antisense oligo, respectively, into a tube. Put it in a water bath heated up to 95°C for 15 min. Turn the power off and leave it until the temperature drops to room temperature (6 hours; overnight is fine). The concentration of the adapter is 100 pmole.

3. Ligation

0.5 ul	EcoRI adapter (100pmole/ul)
5.0 ul	MseI adapter (100pmole/ul)
0.05ul	T4 ligase (100,000U/ul) BioLab
2.75ul	T4 10× buffer (include ATP)
8.9 ul	H_2O
12.5 ul	DNA template
27.5 ul	volume

Ligation at 16°C for two hours. Perform 1:10 dilution of the ligation product.

4. Pre-amplification

1.0 ul	EcoRI pre-amplification primer (50ng/ul) (Qiagen, Chatsworth, Calif.)
1.0 ul	MseI pre-amplification primer (50ng/ul) (Qiagen, Chatsworth, Calif.)
4.0 ul	2mM dNTP
0.2 ul	Biolase (5U/ul) (Bioline, Canton, MA)
4.0 ul	10× buffer
1.6 ul	50mM $MgCl_2$
5.0 ul	DNA template
23.2ul	H_2O
40.0 ul	volume

Run at the program based on manufacturer's manual in GibcoBRL/Invitrogen

AFLP analysis system kit I.

Perform 1:50 dilution of the PCR product.

5. Selective amplification

Mix I	0.5ul	EcoRI selective primer (10ng/ul) (Qiagen)
	3.0ul	MseI selective primer (10ng/ul) (Qiagen)
	1.5ul	2mM dNTP
	5.0ul	
Mix II	7.1ul	H_2O
	2.0ul	10× buffer
	0.8ul	50mM $MgCl_2$
	0.1ul	Biolase (5U/ul)
	10.0ul	
	5.0ul	Mix I
	10.0ul	Mix II
	5.0ul	DNA template
	20.0ul	volume

Run at the program based on manufacturer's manual in GibcoBRL/Invitrogen

AFLP analysis system kit I.

Appendix B. Protocol for PAGE for AFLP Markers in Hazelnut

IPC (Biolab) modification of instructions from Biolab

6% PAGE	**10% APS**
12ml 19% acrylamide/bis acrylamide	.025g ammonium persulfate (make fresh)
25.2g urea	100ul nanopure H_2O
6ml 10X TBE	
42ml nanopure H_2O	
60ml	
See the instructions	

Preparing IPC and plate

1. Wash the IPC and the glass plate with distilled water and detergent. Rinse well.
2. Soak the glass plate overnight in 1N NaOH (2L) in plastic boxes. (Solutions are reused numerous times).
3. Rinse the glass plate with tap water repeatedly and scrub with Sequesoap, then rinse with DI water repeatedly. Dry with Kimwipe or allow to air dry away from dust.
4. Choose best side of the glass plate (free of chips and scratches). Clean the glass plate and IPC with 95% ethanol by wiping with a Kimwipe.
5. Before assembly, wipe the glass plate with methacryloxypropyltrimethoxysilane (Bind Silane) solution in 2 perpendicular directions with a Kimwipe, make sure plate is completely covered. Dry it in the fume hood for at least one hour.

6. Wipe the IPC with Rainx (just enough to cover entire side of plate, e.g. enough to dampen a Kimwipe) using a Kimwipe in perpendicular directions. Dry it in the fume hood for ten minutes.

Casting the gel

1. Place the 0.4mm spacers on the sides of the IPC.
2. Place the glass plate on top of the IPC (treated side down). Be careful to ensure that the spacers are aligned on either side of the gel plates. Adjust plates so that they are even on all sides.
3. Be sure the combs are clean and ready to use.
4. Prepare the polyacrylamide gel solution (see recipes above). Mix well. Degas for ~10 minutes using the vacuum apparatus in the fume hood. Attach rubber hose to side arm of flask. Cover the tip with a rubber bung.

Sequencing gel apparatus (Fisher) modification of protocol from barley group

6% PAGE

21ml
44.1g urea
10.5ml 10X TBE
69.5ml nanopure H_2O
100ml

10% APS

.025g ammonium persulfate (make fresh)
100ul nanopure H_2O

Preparing plates

1. Wash the long and notched plates with distilled water and detergent. Rinse well.

2. Soak the plates overnight in 1N NaOH (2L) in the respective plastic boxes. (Solutions are reused numerous times). Be sure not to mix up the long plates and notched plates.
3. Rinse the plates with tap water repeatedly and scrub with Sequesoap, then rinse with DI water repeatedly. Dry with Kimwipe or allow to air dry away from dust.
4. Choose best side of the glass plate (free of chips and scratches). Clean plates with 95% ethanol by wiping with a Kimwipe.
5. Before assembly, wipe the long plate with methacryloxypropyltrimethoxysilane (Bind Silane) solution in 2 perpendicular directions with a Kimwipe, make sure plate is completely covered. Dry it in the fume hood for at least one hour.
6. Wipe the notched plate with Rainx (just enough to cover entire side of plate, e.g. enough to dampen a Kimwipe) using a Kimwipe in perpendicular directions. Dry it in the fume hood for at least ten minutes.

Casting the gel

1. Place the long plate (treated side up) on the casing stand and place the 0.4mm spacers on the sides of the long plate.
2. Place the notched plate on the top of the long plate (treated side down). Be careful to ensure that the spacers are aligned on either side of the gel plates. Adjust plates so that they are even on all sides.

3. Place 3 clamps on each side of the gel, in the slots on the casing stand. They should clamp on top of the spacers. One more clamp on each side of the plate will be placed at the first slot after pouring the gel.
4. Prepare the polyacrylamide gel solution (see recipes above). Mix well. Degas for ~10 minutes using the vacuum apparatus in the fume hood. Attach rubber hose to side arm of flask. Cover the tip with a rubber bung.
5. Add APS and TEMED and mix by gently swirling (avoid aerating the mix)
6. Immediately after adding APS and TEMED, slowly dispense the polyacrylamide solution into the space between the two plates at the upper edge. It can be poured directly from the flask, or dispensed with a pipette.
7. Be sure the comb is clean and free of debris and dust. On the top of the gel, insert the comb, flat side first. Make sure the comb is even and only ½'' in the gel (the holes in the comb should line up with the edge of the glass. If the comb is too deep it will be very difficult to load the gel. Place four clamps over the ends of the comb and one clamp on each side of the plate at the first slot of the casting stand.
8. Let the gel sit for at least two hours, or leave it overnight. If leaving overnight, extra care has to been carried out. Wet paper towers should be used to cover the top opening of the gel to prevent its dehydration, which causes shrinking at the top or bottom of the gel. Use plastic to cover the bottom of the gel too (wet paper towers are not recommended here, since

they will cause shrinkage at the top of the gel). Or if it is possible, pour the gel in a cold room to prevent shrinkage.

9. Carefully check the edges for signs of polymerization. After polymerization, take off the clamps. Loosen the comb with a clean razor blade or spatula. Avoid damaging the teeth of the comb with the razor blade or spatula. Carefully and smoothly pull out the comb. Avoid twisting the comb or tugging on only one side. Clean the comb well with DI water. Rinse out the top of the gel (where the comb was inserted) with DI water.

Gel loading buffer/dye:

98% formamide	1.0 ml
xylene cyanol FF	1.0 mg
bromophenol blue	1.0 mg
0.5 M EDTA pH 8.0	20 ul

Store at -20 °C.

Electrophoresis

Be sure the upper buffer drain is closed.

When using Fisher sequencing apparatus

1. Make up running buffer: 1L of 1 X TBE
2. Moisten the rubber band with running buffer solution to get a better seal Place gel sandwich in the sequencing apparatus with the short plate facing inward. Rest the bottom edge of the plates on the ribbed gel support blocks in the lower buffer tray. Secure the gel sandwich with integral clamps along the sides of the apparatus. Tighten knobs securely, but not too tight.

Over-tightening can lead to leaks, gel sticking to both plates and deformation of the gel.

3. Fill the upper buffer tray with approximately 450ml of the running buffer and the lower buffer tray to the fill line.
4. Cover the lids of the upper buffer tray and lower buffer tray. Connect the leads to the power pack in the correct orientation [Black (-) to Red (+)]. Check settings on the power source.
5. Pre-run the gel at ~70 Watts (~1500-1600 Volts) for one hour.
6. Turn off the power. Use a syringe filled with 1X TBE and carefully squirt buffer into the top of the gel (where the comb was inserted) to remove urea and air bubbles. Make sure to remove all small bits of polyacrylamide from the top of the gel. Do not contact the bottom of the well or you will damage it.
7. Insert the comb with the teeth pointing in. Insert the teeth until they go into the acrylamide about 1-2mm. This step is critical to the success of the gel. If the insertion is not deep enough, leaking may occur between wells. If the insertion is too deep, it may be hard to load the samples. Do not pull the comb out once the teeth have entered the gel.
8. Add an equal volume of loading dye to DNA samples. Add 6ul of dye to 0.5ul of DNA ladder (Promega, Madison, WI). Load 6 ul of the mixture and the DNA ladder into a plate and denature DNA samples for three

minutes at 95°C using a thermocycler. Place the sample on ice immediately after denaturing to prevent re-annealing.

9. Load the samples using loading tips (Fisher Scientific, Tustin, CA.). Try to load them as fast as possible (within 20 minutes), or the samples will diffuse into the gel, or leak to adjacent wells.
10. Turn the power back on at ~70 Watts (~1500 Volts) and run for 3.5 hours.
11. After electrophoresis, open the upper buffer drain and empty the upper chamber. Remove the gel sandwich from the apparatus and carefully separate the plates using a wedge or spatula. The gel should stick to the long plate. Rinse the upper and lower buffer tray with DI water.

<u>When using Biolab IPC apparatus</u>

Follow manufacturer's protocol.

Appendix C Protocol for Silver Staining for AFLP Markers in Hazelnut

Solution preparation

Fix/stop solution: 10% glacial acetic acid in 1 L of DI water. (900 ml DI water and 100 ml acetic acid)

Silver stain solution: dissolve 1.3g of silver nitrate ($AgNO_3$) in 1 L of DI water. Immediately before use, add 1.5 ml of 37% formaldehyde.

Developing solution: dissolve 30g of sodium carbonate (Na_2CO_3) in 1L of DI water. Chill to 4-10 °C. Immediately before use, add 1.5 ml of 37% formaldehyde and 0.04g sodium thiosulfate.

Silver staining and developing

1. Fix the gel by covering it with the fix/stop solution and agitating gently for 30-60 minutes. After fixing save the fix/stop solution. It will be used again to terminate the developing reaction.
2. Rinse the gel 3 times (for 3 minutes each) with DI water using gentle agitation. Lift the gel out of the wash and let it drain 10-20 seconds before transferring to the next wash.
3. Stain the gel by transferring it to the silver stain solution and agitating for 30-60 minutes. Be careful, as the stain solution will stain you too.
4. Develop the gel. Remove the gel from the staining solution and rinse in a tray containing DI water for 5-10 seconds (the timing of the rinse step is very important; longer rinses could result in weak or no signal) and then

remove. Drain the plate for 3 minutes to reduce the background. If rinse goes too long, the stain can be repeated.

5. Develop the gel by transferring it to the developing solution and agitate gently until solution begins to turn a brownish color or until the bands begin to appear. It usually takes 4 to 5 minutes. Do not wait too long, which will result in dark background and thus low resolution.
6. Lift the gel and place it into fix/stop solution immediately to terminate the developing reaction. Agitate for 5 minutes. The gel will turn light yellow.
7. Rinse the gel 2 times (2 minutes each) in DI water.
8. Dry the gel at room temperature overnight away from sources of dust.

Appendix D ELISA data file for resistance to EFB

I* (Threshold, average absorbent value of the sample): just above threshold
NT: moved to the field, not tested
- : no data
I: infected
O: clean

Table D.1 ELISA data file for EFB in hazelnut accessions

Accessions	Location	EFB'02	EFB'03	Canker length	Res/Susc
AL55	N09.05	OII	I	Pustules	S
Arneson's Rootstock	N01.33	I	NT	14+0	S
Blumberger	212.053	I	NT	0	S
C. avellana 76-1824	639.047	I	NT	14+0	S
Camponica	N06.01	I	NT	15+16	S
Carello	N02.57	IO	I	Pustules	S
Chinoka	N07.35	I	NT	17+33	S
COR 187	N05.28	OOO	OO	-	R
COR 507	N06.35	OOO	OOO	-	R
COR 626	N09.04	I	NT	8+0	S
COR 627	N09.06	I	NT	24+2	S
Corabel	N09.09	I	NT	15+12	S
Chinese Trazel Gellatly #11	N02.32	OOO	OOO	-	R
Chinese Trazel Gellatly #4	N01.25	I*I*O	I*OO	-	inconclusive
Chinese Trazel Gellatly #6	N01.07	OOO	OOO	-	R
Chinese Trazel Jemtegaard #1	N02.25	I	NT	2+0	S
Chinese Trazel Jemtegaard #2	N04.31	I	NT	10+6	S
Culpla	N07.08	OOO	OOO	-	R
Eastoka	N07.25	I	NT	35+27	S
Erioka	N07.39	OO	I	Pustules	S
Estrella #1	N01.15	OOO	OOO	-	R
Estrella #2	N01.04	OO	IOO	-	inconclusive
Filcorn	N03.02	NT	I	-	S
Frango #4	N10.05	NT	II	-	S
Frango #5	N10.06	I	NT	43+10	S
Freeoka	N06.28	I	NT	5+60	S
G081S	566.026	OOO	OOO	-	R
G114S	566.025	I	I	-	S
G227S	566.027	NT(dead)	NT(dead)	-	-
Goc	N10.07	I	NT	0	S
Karloka	N07.58	I	NT	23+17	S

Table D.1 Continued.

Accessions	Location	EFB'02	EFB'03	Canker length	Res/Susc
Karol	N10.08	I	NT	30+20	S
Kruse	N07.21	I	NT	30+7	S
Laroka	N05.41	OII	I	Pustules	S
Lisa	567.021	OOO	OOO	-	R
Locale di Piazza Armerina	N02.56	I	NT	10+7	S
Maria	N10.13	I	NT	6+0	S
Morrisoka	N03.05	I	NT	0+7	S
Nonpareil	N07.05	I	NT	4+22	S
Nostrale	N05.50	I	NT	0+3	S
not TGR	N06.36	I	NT	18+25	S
Pell. Rouge	N02.07	I	NT	3+0	S
Pinyolenc	W10a	I	NT	0+30	S
RG 1	R01.14 or 22	I	NT	3+3	S
Rosset de Valls	N01.16	I	NT	0	S
Royal	N04.39	I	NT	7+5	S
Tonda di Giffoni	----	I	NT	7+3	S
Turkish Trazel Gellatly #15	N07.57	I	NT	19+40	S
Turkish Trazel Gellatly #2	N07.37	I	NT	8+24	S
Turkish Trazel Gellatly #3	N01.12	OOO	OOO	-	R
Turkish Trazel Gellatly 5	N02.23	I	NT	5	S
Volle Zeller	639.055	I	NT	0	S
Warsaw Red	N08.08	I	I	-	S
Weschcke TP1	567.031	OOO	OOO	-	R
Weschcke TP2	566.022, 567.033	OOO	OOO	-	R
Weschcke TP3	N04.46	OOO	OOO	-	R
Woodford	N05.09	I	NT	7+0	S
Zeroka	N01.20	I	NT	16+30	S

Table D.2 ELISA data file for seedlings of OSU 408.040

245.098 X 408.040				
Sample	EFB'02	EFB'03	Canker length	Res/susc
859.009	OOO	OOI*(.055,.067)	-	R
859.012	OII	I	pustules	S
859.014	OOO	OOO	-	R
859.018	OOO	OI*(.053,.065)O	-	R
859.019	IO	OOI*(.060,.062)	-	R
859.022	OOO	OO	-	R
859.023	OOO	OI*(.053,.055)O	-	R
859.025	I	NT	10	S
859.026	I	NT	1	S
859.027	OOO	I*(.049,.058)I*(.062,.080)O	-	R
859.028	I	NT	15	S
859.034	I(.086,.093)	NT	8	S
859.037	I	NT	6	S
859.039	I	NT	0	S
859.040	I	NT	24+12	S
867.001	OI*(.054,.071)	I*(.053,.055)OO	-	R
867.008	OOO	OOO	-	R
867.009	I	NT	22	S
867.012	II	II	pustules	S
867.014	I	NT	2+7	S
867.016	OOO	OOO	-	R
867.020	OOO	OOI*(.051,.053)	-	R
867.024	I	NT	24	S
867.027	I	NT	45	S
867.028	I	NT	11+1	S
867.029	OOO	OI*(.048,.046)I*(.055,.058)	-	R
867.033	OOO	OOO	-	R
867.034	I	NT	0	S
867.038	I	NT	15+3	S
867.044	I	NT	5+11	S
889.002	I	NT	1	S
889.003	OOO	OOI*(.028,.030)	-	R
889.004	I	NT	6+8	S
889.005	OOO	OI*(.053,.064)O	-	R
889.006	OOO	II	-	S
889.007	OOO	OI*(.049,.056)I	-	R
889.008	OOO	OOO	-	R
889.009	I	NT	5+1	S
889.010	OOO	OOO	-	R

Table D.2 Continued.

245.098 X 408.040				
Sample	EFB'02	EFB'03	Canker length	Res/susc
889.011	OOO	II	15	S
889.012	I	NT	31	S
889.013	OOO	OOO	-	R
889.014	I	NT	27+19	S
889.015	IO	I*(.049,.075)I*(.052,.054)	-	S
889.016	OOO	OOO	-	R
889.017	OOO	OOO	-	R
889.018	I	NT	6	S
889.019	I	NT	0	S
889.020	I	NT	14+16	S
889.021	I	NT	14+9	S
889.022	I	NT	27	S
889.023	I	NT	30+10	S
889.024	I	NT	20	S
889.025	OII	OII	-	S
889.026	I	NT	1	S
889.027	OOO	OOO	-	R
889.028	OOO	OOO	-	R
889.029	OOO	OO	-	R
889.030	OII	I	-	S
889.031	OOO	I*(.050,.061)OI	-	R
889.032	I	NT	19	S
889.033	I(.099,.145)	OOO	-	R
889.034	OOO	OI*(.051,.061)O	-	R
889.035	I*(.077,.087)	NT	0	R
889.036	OOO	OOO	-	R
889.037	I	NT	sunken	S
889.038	I	NT	29	S
889.039	OOO	OI*(.053,.069)O	-	R
889.040	OOO	OOO	-	R
889.041	I	NT	32	S
889.042	I	NT	5+1	S
889.043	I	NT	21	S
889.044	OOO	OOO	-	R
889.045	I	NT	0	S

Table D.2 Continued

474.013 X 408.040				
Sample	EFB'02	EFB'03	Canker length	Res/susc
851.052	OOO	OOO	-	R
851.053	OO	OOI	-	R
851.054	I	NT	0	S
851.055	I*(.056, .069)	NT	0	R
851.056	I	NT	0	S
851.057	I	NT	29+12	S
851.058	I	NT	30+7	S
851.059	O	OO	-	R
851.060	OOO	OOO	-	R
851.061	OO	I*(.060, .075)OO	-	R
851.062	I	NT	5	S
851.063	OOO	OOO	-	R
859.044	I	NT	1	S
859.045	OOO	OOO	-	R
859.046	I	NT	2	S
859.047	I	NT	0	S
859.048	OOO	III	-	S
859.050	OO	I*(.050, .078)O	-	R
859.051	OO	I*(.040, .095)OI	-	R
859.052	OO	OI*(.053, .060)O	-	R
859.053	I	NT	0	S
859.054	OO	OOO	-	R
859.055	I	NT	1	S
859.056	I	NT	22	S
859.057	I	NT	2	S
859.058	OO	OI*(.050,.061)O	-	R
859.059	OOO	OI*(.060, .064)	-	R
859.060	O	II	-	S
859.061	OOO	OOO	-	R
859.062	OI	I	-	S
859.063	I	NT	0	S
859.064	O	I*(.055, .059)O	-	R
859.065	I	NT	5	S
859.066	I	I	-	S
859.067	I	NT	18	S
859.068	I	NT	0	S
859.069	OIO	I	Pustules	S
859.070	OOI	IIO	-	S
859.071	OOO	OOO	-	R

Table D.2 Continued

474.013 X 408.040				
Sample	EFB'02	EFB'03	Canker length	Res/susc
859.072	OOO	OOO	-	R
859.073	OO	OI*(.048, .055)O	-	R
859.074	I	NT	33	S
859.075	OOO	OOO	-	R
859.076	OOO	OI*(.048,.090)O	-	R
859.077	OOO	I*(.062, .078)OO	-	R
859.078	I	NT	16	S
859.079	O	OOO	-	R
859.080	I	NT	2+5	S
859.081	I	I	-	S
859.082	OOO	II	-	S
859.083	O	II	-	S
859.084	I	NT	3	S
859.085	OOI	II	Pustules	S
859.086	OO	OOO	-	R
859.087	I*(.057, .064)	NT	0	R
859.088	I	NT	12	S
859.089	OO	OOO	-	R
859.090	OOO	OOO	-	R
859.091	O	OOO	-	R
910.071	I	NT	10+10	S
910.072	I*(.066,.078)	NT	0	R
910.073	I	NT	0	S
910.074	I	NT	3	S
910.075	O	OO	-	R
665.012 × 408.040				
99035-01	I	NT	0	S
99035-02	I	NT	0	S
99035-03	O	NT	0	R
99035-04	O	NT	0	R
99035-05	I	NT	0	S
99035-06	O	NT	0	R
99035-07	I	NT	0	S
99035-08	O	NT	0	R
99035-09	O	NT	0	R
99035-10	O	NT	0	R

Table D.2 Continued

665.012 × 408.040				
Sample	EFB'02	EFB'03	Canker length	Res/Susc
99035-11	I	NT	0	S
99035-12	O	NT	0	R
99035-13	O	NT	0	R
99035-15	I	NT	14+11	S
99035-16	O	NT	0	R
99035-17	O	NT	0	R
99035-18	I	NT	0	S
99035-19	O	NT	0	R
99035-20	O	NT	0	R
99035-21	O	NT	0	R
99035-22	I	NT	1	S
99035-23	I	NT	0	S
99035-24	O	NT	0	R
99035-25	O	NT	0	R
99035-26	I	NT	4	S
99035-27	O	NT	0	R
99035-28	I	NT	9	S
99035-29	O	NT	0	R
99035-30	I	NT	4	S
99035-31	O	NT	0	R
99035-32	I	NT	10+4	S
99035-33	I	NT	7	S
99035-34	I	NT	0	S
99035-35	I	NT	0	S
99035-36	O	NT	0	R
99035-37	I	NT	0	S
99035-38	I	NT	3+6	S
99035-39	I	NT	1	S
99035-40	I	NT	10+4	S
99035-41	I	NT	20+7	S
99035-42	O	NT	0	R
99035-43	O	NT	0	R
99035-44	O	NT	0	R
99035-45	O	NT	0	R
99035-46	I	NT	20	S
99035-47	O	NT	0	R
99035-48	I	NT	0	S
99035-49	O	NT	0	R

Table D.2 Continued

665.012 × 408.040				
Sample	EFB'02	EFB'03	Canker length	Res/Susc
99035-50	O	NT	0	R
99035-51	O	NT	0	R
99035-52	I	NT	0	S
99035-54	O	NT	0	R
99035-55	O	NT	0	R
99035-56	I	NT	20+7	S
99035-57	I	NT	0	S
99035-58	I	NT	0	S

Table D.3 ELISA data file for seedlings of 'Ratoli'

665.012 X Ratoli				
Sample	EFB'02	EFB'03	Canker length	Res/susc
99039-01	O	NT	Pustules	S
99039-02	O	I*(.036,.042)	-	R
99039-03	I	NT	-	S
99039-04	I	NT	-	S
99039-05	O	NT	-	R
99039-06	O	O	-	R
99039-07	O	O	-	R
99039-08	I*(.034, .048)	NT	-	R
99039-09	O	NT	-	R
99039-10	O	NT	-	R
99039-11	I	NT	-	S
99039-12	I	I	-	S
99039-13	O	O	-	R
99039-14	I	NT	-	S
99039-15	I*(.036,.040)	NT	-	R
99039-16	I	I	-	S
99039-17	O	NT	-	R
99039-18	O	NT	-	R
99039-20	O	NT	-	R
99039-21	O	O	-	R
99039-22	I*(.054,.074)	O	-	S
99039-23	I	NT	-	S

Table D.3 Continued

665.012 X Ratoli				
Sample	EFB'02	EFB'03	Canker length	Res/susc
99039-24	O	O	-	R
99039-25	O	O	-	R
99039-26	I	I	-	S
99039-27	I	NT	-	S
99039-28	O	I*(.036,.054)	-	R
99039-29	I*(.033,.080)	O	-	R
99039-30	I	NT	-	S
99039-32	I	NT	-	S
99039-33	O	O	-	R
99039-34	O	O	-	R
99039-35	I	I*(.051,.066)	-	S
99039-36	I	I	-	S
99039-37	O	I*(.045,.056)	-	R
99039-38	I	NT	Pustules	S
99039-39	O	O	-	R
99039-40	I	NT	-	S
99039-42	O	I*(.035,.038)	-	R
99039-43	I	NT	-	S
ND99039-01	I	O	-	S
ND99039-02	I	NT	-	S
ND99039-03	O	O	-	R
ND99039-04	O	NT	-	R
ND99039-06	O	I*(.045,.078)	-	R
ND99039-07	I*(.053,.075)	NT	-	S
ND99039-08	O	O	-	R
ND99039-09	O	O	-	R
ND99039-10	O	O	-	R
ND99039-11	I	O	-	R
ND99039-12	O	O	-	R
309.074 X Ratoli				
99038-01	O	O	-	R
99038-02	O	I*(.036,.038)	-	R
99038-03	I	I	-	S
99038-04	O	I*(.037,.047)	-	R
99038-05	O	O	-	R
99038-06	O	O	-	R
99038-07	I	NT	-	S
99038-08	O	O	-	R

Table D.3 Continued

309.074 X Ratoli				
Sample	EFB'02	EFB'03	Canker length	Res/susc
99038-09	I*(.034,.035)	I	-	S
99038-10	O	O	-	R
99038-11	I	I	-	S
99038-12	O	O	-	R
99038-13	O	O	-	R
99038-14	I	I	-	S
99038-15	O	O	-	R
99038-16	O	O	-	R
99038-17	O	O	-	R
99038-18	O	O	-	R
99038-19	I	I	-	S
99038-20	I	I	Pustules	S
99038-21	O	O	-	R
99038-22	O	O	-	R
99038-23	I	I	-	S
99038-24	O	O	-	R
99038-25	O	O	-	R
99038-26	O	O	-	R
99038-27	I	I	-	S

Appendix E AFLP Marker Scoring for Seedlings of OSU 408.040 and 'Ratoli'

Table E.1 AFLP marker scoring for seedlings of OSU 408.040

245.098 X 408.040							
Sample	Code	Res/susc	A4-265	C2-175	D8-350	A8-150	B2-125
859.009	MX61	R	1	1	1	1	1
859.012	MX62	S	0	0	0	0	0
859.014	MY63	R	0	0	0	1	1
859.018	MX64	R	0	1	1	1	1
859.019	MX65	R ?	1	1	1	1	1
859.022	MX66	R	1	1	1	1	1
859.023	MX67	R	1	1	1	1	1
859.025	MX68	S	0	0	0	0	0
859.026	MX69	S	0	0	0	0	1
859.027	MX70,MY70	R	1	1	1	1	1
859.028	MX71	S	0	0	0	0	0
859.034	MX72	S	1	1	1	1	1
859.037	MX73	S	0	0	0	0	0
859.039	MX74	S	0	0	0	0	1
859.040	MX75	S	0	0	0	0	0
867.001	MX46	S (R)	1	1	1	1	1
867.008	MX47	R	1	1	1	1	1
867.009	MX48,MY48	S	0	0	0	0	0
867.012	MX49,MY49	S	0	0	0	0	0
867.014	MX50,MY50	S	0	0	0	0	0
867.016	MX51	R	1	1	1	1	1
867.020	MX52	R	1	1	1	1	1
867.024	MX53,MY53	S	1	0	0	0	0
867.027	MX54	S	0	0	0	0	0
867.028	MX55	S	0	0	0	0	0
867.029	MX56	R	1	1	1	1	1
867.033	MX57	R	1	1	1	1	1
867.034	MX58,MY58	S	0	0	1	0	0
867.038	MX59	S	0	0	0	0	0
867.044	MX60	S	0	0	0	0	0
889.002	MX2	S	0	0	0	0	0
889.003	MX3,MY3	R	1	1	1	1	1
889.004	MX4	S	0	0	0	0	0
889.005	MX5	R	1	1	1	1	1

889.001 S

Table E.1 Continued

245.098 X 408.040							
Sample	Code	Res/susc	A4-265	C2-175	D8-350	A8-150	B2-125
889.006	MX6	S	0	0	0	0	0
889.007	MX7	(R)S	0	0	1	1	1
889.008	MX8,MY8	R	1	1	1	1	1
889.009	MY9	S	0	0	0	0	0
889.010	MX10	R	1	1	1	1	1
889.011	MX11	S	0	0	0	0	0
889.012	MX12	S	1	0	0	0	0
889.013	MX13	R	1	1	1	1	1
889.014	MX14,MY14	S	0	0	0	1	0
889.015	MX15	S	0	0	0	0	0
889.016	MY16	R	1	1	1	1	1
889.017	MX17	R	1	1	1	1	1
889.018	MX18	S	0	0	0	0	0
889.019	MX19	S	0	0	0	0	0
889.020	MX20	S	0	0	0	0	0
889.021	MX21	S	0	0	0	0	0
889.022	MX22	S	0	0	0	0	0
889.023	MX23	S	0	0	0	0	0
889.024	MX24	S	0	0	0	0	0
889.025	MX25,MY25	S	0	0	0	1	0
889.026	MX26	S	0	0	0	0	0
889.027	MY27	R	1	1	1	1	1
889.028	MX28	R	1	1	1	1	1
889.029	MX29	R	1	1	1	1	1
889.030	MX30	S	1	1	1	0	0
889.031	MX31,MY31	(R)S	0	0	0	0	0
889.032	MX32	S	0	0	0	0	0
889.033	MX33	(R)S	1	1	1	1	1
889.034	MX34	R	1	1	1	1	1
889.035	MX35	(R)S	1	1	1	1	1
889.036	MX36,MY36,ND36	R	1	1	1	1	1
889.037	MX37,MY37	S	0	0	0	0	0
889.038	MX38	S	1	0	0	0	0
889.039	MX39	R	1	1	1	1	1
889.040	MX40	R	1	1	1	1	1
889.041	MY41	S	0	0	0	0	0
889.042	MX42	S	0	0	0	0	0
889.043	MY43	S	0	0	0	0	0

889.054 S

Table E.1 Continued

245.098 X 408.040							
Sample	Code	Res/susc	A4-265	C2-175	D8-350	A8-150	B2-125
889.044	MX44	R	1	1	1	1	1
889.045	MX45	S	0	0	0	0	0
859.010	PD1	-	0	0	0	0	0
859.011	PD2	-	1	1	1	1	1
859.013	PD3	-	1	1	1	1	1
859.015	PD4	-	1	1	1	1	1
859.016	PD5	-	0	0	0	0	0
859.017	PD6	-	0	0	0	0	0
859.020	PD7	-	1	1	1	0	0
859.021	PD8	-	1	1	1	1	1
859.024	PD9	-	0	0	0	0	0
859.029	PD10	-	1	1	1	1	1
859.030	PD11	-	1	1	1	1	1
859.031	PD12	-	1	1	1	1	1
859.032	PD13	-	1	1	1	1	1
859.033	PD14	-	1	1	0	0	0
859.035	PD15	-	0	0	0	0	0
859.036	PD16	-	1	1	1	1	1
859.038	PD17	-	1	1	1	1	0
859.041	PD18	-	1	1	1	1	1
859.042	PD19	-	0	0	0	0	0
859.043	PD20	-	1	1	1	1	1
867.002	PD21	-	0	0	0	0	0
867.003	PD22	-	1	1	0	0	0
867.004	PD23	-	0	0	0	0	0
867.005	PD24	-	1	1	1	1	1
867.006	PD25	-	0	0	0	0	0
867.007	PD26	-	1	1	1	1	1
867.010	PD27	-	1	1	1	1	1
867.011	PD28	-	1	1	1	1	1
867.013	PD29	-	1	1	1	1	1
867.015	PD30	-	1	1	1	1	1
867.017	PD31	-	1	1	1	1	1
867.018	PD32	-	0	0	0	0	0
867.019	PD33	-	1	1	1	1	1
867.021	PD34	-	1	1	1	1	1
867.022	PD35	-	0	0	0	0	1
867.023	PD36	-	1	1	1	1	1

Table E.1 Continued

245.098 X 408.040							
Sample	Code	Res/susc	A4-265	C2-175	D8-350	A8-150	B2-125
867.025	PD37	-	1	1	1	1	1
867.026	PD38	-	1	1	1	1	1
867.030	PD39	-	0	0	0	0	0
867.031	PD40	-	0	0	0	0	0
867.032	PD41	-	0	0	0	0	0
867.035	PD42	-	0	0	0	0	0
867.036	PD43	-	0	0	0	0	1
867.037	PD44	- S	0	0	0	0	0
867.039	PD45	-	1	1	1	1	1
867.040	PD46	-	1	1	1	1	1
867.041	PD47	-	1	1	1	1	1
867.042	PD48	-	1	1	0	0	0

474.013 X 408.040					
Sample	Code	Res/susc	C2-175	D8-350	B2-125
851.052	MX76	R	1	1	1
851.053	MX77	R	1	1	1
851.054	MX78	S	0	0	0
851.055	MX79	R	1	1	1
851.056	MX80	S	1	1	0
851.057	MX81	S	0	0	0
851.058	MX82	S	0	0	0
851.059	MX83	R	1	1	1
851.060	MX84	R	1	1	0
851.061	MX85	R	0	0	1
851.062	MX86	S	0	0	0
851.063	MX87	R	1	1	1
859.044	MX88	S	0	0	0
859.045	MX89	R	1	1	1
859.046	MX90	S	0	0	0
859.047	MX91	S	0	0	0
859.048	MX92	S	0	0	0
859.050	MX94	R	1	1	1
859.051	MX95	R	1	1	1
859.052	MX96	R	1	1	1
859.053	MZ1	S	0	0	0
859.054	MZ2	R	1	1	1
859.055	MZ3	S	0	0	0
859.056	MZ4	S	0	0	0

859 ou 9 - S

Table E.1 Continued

474.013 X 408.040					
Sample	Code	Res/susc	C2-175	D8-350	B2-125
859.057	MZ5	S	0	0	0
859.058	MZ6	R	1	1	1
859.059	MZ7	R	1	1	0
859.060	MZ8	S	0	0	0
859.061	MZ9	R	0	0	0
859.062	MZ10	S	0	0	1
859.063	MZ11	S	0	0	0
859.064	MZ12	R	1	1	1
859.065	MZ13	S	0	0	0
859.066	MZ14	S	0	0	0
859.067	MZ15	S	0	0	0
859.068	MZ16	S	0	0	0
859.069	MZ17	S	0	0	0
859.070	MZ18	S	0	0	0
859.071	MZ19	R	1	1	1
859.072	MZ20	R	1	1	1
859.073	MZ21	R	1	1	1
859.074	MZ22	S	0	0	0
859.075	MZ23	R	1	1	1
859.076	MZ24	R	1	1	1
859.077	MZ25	R	1	1	1
859.078	MZ26	S	1	1	0
859.079	MZ27	R	1	1	1
859.080	MZ28	S	0	0	0
859.081	MZ29	S	0	0	0
859.082	MZ30	S	0	0	0
859.083	MZ31	S	0	0	0
859.084	MZ32	S	0	0	0
859.085	MZ33	S	0	0	0
859.086	MZ34,NF34	R	1	1	1
859.087	MZ35	R	1	1	1
859.088	MZ36	S	0	0	0
859.089	MZ38	R	1	1	1
859.090	NF38	R	1	1	1
859.091	MZ39	R S	1	1	0
910.071	MZ40,NF40	S	0	0	0
910.072	MZ41	R S	1	1	1
910.073	MZ42	S	1	1	0

Table E.1 Continued

474.013 X 408.040					
Sample	Code	Res/susc	C2-175	D8-350	B2-125
910.074	MZ43,NF43	S	1	1	1
910.075	MZ44	R	1	1	1

Table E.2 AFLP marker scoring for seedlings of 'Ratoli'

665.012 X Ratoli				
Sample	Code	Res/susc	dA1-135	C4-255
99039-01	MW40	S	0	0
99039-02	MV76	R	1	1
99039-03	MW41	S	0	0
99039-04	LY42,MV42	S	0	0
99039-05	MV43	R	1	1
99039-06	MV44	R	1	1
99039-07	MV74,MW74	R	1	1
99039-08	MV45,MW45	R	1	1
99039-09	MV46,MW46	R	1	1
99039-10	MV47,MW47	R	1	1
99039-11	no DNA	S	-	-
99039-12	LY48	S	0	0
99039-13	LY49,MW49	R	1	1
99039-14	MW50	S	1	0
99039-15	MV73	R	1	1
99039-16	LY51,MW51	S	0	0
99039-17	MV52	R	1	1
99039-18	MW53,LZ53	R	1	1
99039-20	MV54	R	1	1
99039-21	LY55,MW55	R	1	1
99039-22	no code	S	0	0
99039-23	MW56	S	0	0
99039-24	LZ57	R	1	1
99039-25	MW58,MV58	R	1	1
99039-26	MV59	S	0	0
99039-27	MW60,MV60	S	0	0
99039-28	MV61	R	1	1
99039-29	MV62	R S	0	0
99039-30	no DNA	S	-	-

Table E.2 Continued

665.012 X Ratoli				
Sample	Code	Res/susc	dA1-135	C4-255
99039-32	MW63	S	0	0
99039-33	MW64,MV64	R	0	1
99039-34	MW65	R	1	1
99039-35	LZ66	S	0	0
99039-36	MW67,MV67	S	1	0
99039-37	MV68,MW68	R	1	1
99039-38	MV75,MW75	S	0	0
99039-39	MV69	R	1	1
99039-40	MV70,MW70	S	0	0
99039-42	MV71,MW71	R	1	1
99039-43	MW72,MV72	S	0	0
ND99039-01	MV29	S	1	0
ND99039-02	MV30	S	0	0
ND99039-03	MW31	R	1	1
ND99039-04	MV32	R	0	0
ND99039-06	MV33,MW33	R	1	1
ND99039-07	MV34	S	0	0
ND99039-08	MW35	R	1	1
ND99039-09	MW36	R	1	1
ND99039-10	MW37	R	1	1
ND99039-11	MV38	R	1	1
ND99039-12	MV39	R	1	1
960.046	PO01,PP01	-	0	0
960.047	PO02,PP02	-	1	0
960.049	PO03,PP03	-	1	1
960.050	PO04	-	1	1
960.053	PO05	-	0	0
960.057	PO06,PP06	-	1	1
960.058	PO07,PP07	-	1	1
960.059	PO08	-	1	1
960.060	PO09,PP09	-	0	1
960.061	PO10	-	1	1
960.062	PO11	-	1	1
960.064	PO12,PP12	-	0	1
960.065	PO13,PP13	-	1	1
960.066	PO14	-	1	1
960.067	PO15	-	0	0
960.069	PO16	-	1	1
960.070	PO17	-	1	1

Table E.2 Continued

665.012 X Ratoli				
Sample	Code	Res/susc	dA1-135	C4-255
960.071	PO18	-	1	1
960.073	PO19	-	1	1
960.074	PO20	-	1	1
960.075	PO21	-	1	1
960.077	PO22,PP22	-	1	1
960.079	PO23	-	0	0
960.080	PO24,PP24	-	1	1
960.081	PO25	-	0	0
960.082	PO26	-	1	1
960.083	PO27	-	1	1
960.084	PO28,PP28	-	1	1
960.085	PO29,PP29	-	0	1
960.086	PO30	-	0	0
960.087	PO31	-	1	1
960.088	PO32,PP32	-	1	1
960.091	PO33,PP33	-	0	0
993.001	PO34	-	0	0
993.002	PO35	-	1	1
993.003	PO36,PP36	-	1	1
993.004	PO37	-	1	1
993.005	PO38	-	0	0
993.006	PO39	-	0	0
993.007	PO40	-	1	1
993.008	PO41,PP41	-	1	1
993.009	PO42	-	0	0
993.010	PO43	-	0	0
993.011	PO44	-	1	1
993.012	PO45	-	0	0
993.042	PO46	-	0	0
993.014	PO47,PP47	-	1	1
993.044	PO48	-	0	0
993.016	PO49,PP49	-	0	1
993.017	PO50,PP50	-	0	0
993.018	PO51	-	1	1
993.019	PO52,PP52	-	1	1
993.020	PO53	-	0	0
993.021	PO54,PP54	-	1	0
993.022	PO55	-	0	0
993.023	PO56,PP56	-	1	1

Table E.2 Continued

665.012 X Ratoli				
Sample	Code	Res/susc	dA1-135	C4-255
993.024	PO57	-	0	0
993.025	PO58	-	0	1
993.026	PO59	-	0	1
993.027	PO60	-	0	0
993.028	PO61,PP61	-	0	0
993.029	PO62,PP62	-	0	0
993.030	PO63,PP63	-	0	0
993.045	PO64,PP64	-	1	1
993.032	PO65	-	0	0
993.046	PO66	-	1	1
993.034	PO67,PP67	-	1	1
993.035	PO68,PP68	-	0	0
993.047	PO69	-	1	1
993.037	PO70	-	0	0
993.038	PO71	-	0	0
999.001	PO72	-	0	0
309.074 X Ratoli				
sample	code	Res/susc	C4-255	
99038-01	MV1	R	1	
99038-02	MV2	R	1	
99038-03	MV3	S	0	
99038-04	MV4	R	1	
99038-05	MV5	R	1	
99038-06	LY6	R	1	
99038-07	MV7	S	1	
99038-08	MV8	R	0	
99038-09	MV9	S	1	
99038-10	MV10	R	1	
99038-11	MV11	S	0	
99038-12	MV12	R	1	
99038-13	MV13	R	1	
99038-14	MV14	S	0	
99038-15	MV15	R	1	
99038-16	MV16	R	1	
99038-17	MV17	R	1	
99038-18	MV18	R	1	
99038-19	MV19	S	1	
99038-20	MV20	S	0	
99038-21	MV21	R	0	

Table E.2 Continued

309.074 X Ratoli			
sample	code	Res/susc	C4-255
99038-22	MV22	R	1
99038-23	MV23	S	0
99038-24	MV24	R	1
99038-25	MV25	R	1
99038-26	MV26	R	0
99038-27	MV27	S	0