AN ABSTRACT OF THE THESIS OF

China F. Lunde for the degree of Master of Science in Horticulture presented on April 1, 1999. Title: Investigation of Novel Sources of Genetic Resistance to Eastern Filbert Blight

Abstract approved: _____
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To identify novel host genetic resistance to eastern filbert blight, caused by the fungus *Anisogramma anomala* (Peck) E. Müller, ninety European hazelnut (*Corylus avellana* L.) varieties and interspecific hybrids (with *C. americana* Marsh. or *C. colurna* L.) were screened for response to the eastern filbert blight pathogen after inoculation in a severe greenhouse test. Six varieties were discovered which did not display signs of the pathogen or symptoms of eastern filbert blight: 'Closca Molla', 'Ratoli', 'Yoder #5', 'Potomac', 'Medium Long', and 'Grand Traverse'. Two of these, 'Ratoli' and 'Closca Molla', are minor varieties from Spain and are superior agronomic types to 'Gasaway' which is the principle source of resistance currently being used in the Oregon State University (OSU) breeding program. Further studies are required to ascertain if the genetics of resistance in these newly evaluated varieties is unique. None of the six varieties has the UBC 152_{800} RAPD marker that segregates with the resistance gene found in 'Gasaway'.

Inheritance of resistance to eastern filbert blight also was investigated in 'Zimmerman', a volunteer seedling that was found in 1989 displaying no disease symptoms despite its proximity to a orchard heavily infested with eastern filbert blight. Three progenies were created using 'Zimmerman' as the pollen parent in controlled crosses with three susceptible advanced selections from the OSU breeding program. The 'Zimmerman' progenies were grown from seed, grafted and subjected to the same severe inoculation as for the germplasm survey.

The progenies displayed significant deviation from the 1:1 ratio that would be expected if 'Zimmerman' was heterozygous for the 'Gasaway' gene. Resistant phenotypes make up about 83% of the populations which indicates that the genetics of resistance in 'Zimmerman' differs at least partially, from that found in 'Gasaway'. The data were congruent with a 3 resistant :1 susceptible ratio which suggests 'Zimmerman' may possess a second, independent resistance gene. Yet, a well-characterized RAPD marker, linked to the resistance gene in 'Gasaway', segregates with the resistant phenotype in all three progeny populations with very little recombination (2 %, 4 %, and 6%). These newly found sources of resistance to eastern filbert blight will aid in the release of resistant cultivars and may increase the stability of genetic resistance to *A. anomala.*

Investigation of Novel Sources of Genetic Resistance to Eastern Filbert Blight

By

China F. Lunde

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed April 1, 1999
Commencement June, 1999

Master of Science thesis of China F. Lunde presented on April 1, 1999

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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.

China F. Lunde, Author

ACKNOWLEDGEMENTS

My ability to complete this work was greatly aided my many people. In particular, I would like to thank Dr. Shawn Mehlenbacher for his interest and wonderful mentoring. Exceptional technical advice was given by: Joel Davis, Dr. Nahla Bassil, Dr. Ruth Martin, Dr. Robert Martin, and Dr. David Martin. David Smith applied his fine growing and grafting skills on the trees for my project. My committee, including Drs. Anita Azarenko, Kenneth Johnson, and Patrick Hayes, also provided much helpful advice.

The Horticulture Department as a whole was very friendly and I enjoyed my time at OSU very much. I have a deep appreciation for all my friends especially, Nahla Bassil, Ruth Martin, Elizabeth Harrison, Rebecca Brown, and Veli Erdogan. I also was greatly pleased by the camaraderie I experienced during planting and harvesting. The field crew is comprised of many people and I thank every one.

The Oregon Hazelnut Commission provided funding for my project and the USDA-ARS National Clonal Germplasm Repository provided plant materials. Dr. Tony Chen's laboratory provided much of the needed equipment.

My family deserves recognition as well. I was lucky enough to escape to McKenzie Bridge for visits with my Grandmother (Alice Lunde), my Aunt Jan and Uncle Milt. They gave me much encouragement, many fine meals, and great companionship. My parents were extremely supportive as well.

CONTRIBUTION OF AUTHORS

Dr. Shawn Mehlenbacher planned much of this work and performed much of its execution. He was also instrumental to the editing of the manuscripts. David Smith grew the 'Zimmerman' seedlings, collected scionwood, grafted and maintained all of the trees for these projects.

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CHAPTER 1

INTRODUCTION

Host Genetic Resistance

Host genetic resistance is an important tool plant breeders use against plant pathogens. There are a number of types of resistance. Complete resistance is that which completely prevents all growth of the pathogen population. Incomplete resistance is that which is not complete (Parlevliet, 1989); the host allows limited growth of the pathogen. Durability is the critical factor when breeding for genetic resistance. Roy Johnson (1984) views durable resistance as "resistance that remains effective during its prolonged and widespread use in an environment favorable to the disease." Its discovery depends on testing both over a long period of time and over a large area where the disease is prevalent and favored by the environment. Durable resistance may be conferred by various genetic mechanisms, and controlled by one or multiple genes.

Eastern Filbert Blight of Hazelnut

Genetic resistance is desired as a control measure for eastern filbert blight, a serious canker disease of European hazelnut *(Corylus avellana* L.), that has been

ruinous to the Oregon hazelnut industry since the causal fungus was introduced into Washington state from the Eastern United States in the mid-1960's. This fungus, *Anisogramma anomala* (Peck) E. Müller (Diaporthales, Gnomoniaceae) was reported in 1876 as a pathogen of *C. americana* Marsh. in New York (Cameron, 1976). *A. anomala* is a pyrenomycete whose hyphae directly penetrate young hazelnut shoots, permeate and destroy the cambial layer, and eventually form perithecia and ascospores within ascostromata. Direct penetration of hazelnut shoots occurs most effectively at the first internode. Older tissues have undergone cell wall and cuticular thickening, vacuolation, and phenolic production rendering them impenetrable (Pinkerton et al., 1995). A latent period of 12-15 months is required for the formation of stromata which erupt in lines and turn black at maturity. These stromata bear perithecia that lie within perennial cankers, actively discharging ascospores during rainy periods. In the Willamette Valley, this disease moves southward about 2-3 km each year (Johnson et al., 1996) and the annual pattern is dictated mostly by the timing and amount of precipitation (Pinkerton et al., 1998b).

Since 1937, there has been a quarantine against the importation of *Corylus* plant material from east of the Rocky Mountains into Oregon. With the establishment of eastern filbert blight in the Pacific Northwest, the quarantine became an important control measure. The quarantine area was extended in 1974 following its discovery in Washington, and in 1987 it was extended again to include at least parts of Multnomah, Clackamas, Washington, and Yamhill counties

due to discovery of the disease in Clackamas County near Damascus and Boring, OR. Planning for the control area was continued and in 1996 a quarantine proposal was put in place and will be reviewed later this year. Retail sales of *Corylus* nursery stock is prohibited throughout the state. Plants from the generally infested areas are not allowed into those areas that are as yet uninfected. Now, the infested areas include the counties previously mentioned and parts of Marion county as well (Johnson, 1996). Movement of materials from infested areas to those that are not (including Linn and Benton Counties, where the OSU hazelnut breeding program is housed) is prohibited.

Current control practices are diverse and time-consuming. Active scouting of orchards, especially in the path of the pathogen (south of Salem, OR) is vital. Only newly emergent shoots are susceptible to pathogen ingress but, since shoots develop gradually the canopy is vulnerable throughout elongation. Once an infestation is found, trees are aggressively pruned especially if branches or trunks displaying long cankers are discovered. Current recommendations are to cut about 1 meter below a visible canker. The resulting loss of canopy can dramatically decrease yield. Suckers emerging from the bases of older trees provide continual emergent tissue and must repeatedly be removed. Bravo® 720 (chlorothalonil) and copper-containing fungicides such as Kocide® (copper hydroxide) are the most popular and economical means of chemical control (Pscheidt and Wallace, 1997). Another class of fungicides, demethylation inhibitors such as Rubigan EC (fenarimol) (Johnson et al., 1993) and Orbit (propiconazole) (Pscheidt, pers.

comm.), also are effective but they are more expensive. Fungicides are expensive in general and growers are looking to resistant varieties as an alternative. Resistance to eastern filbert blight is an important objective of the OSU hazelnut breeding project (Mehlenbacher, 1995b; Thompson et al., 1996).

Genetic Resistance to Eastern Filbert Blight

Within the genus *Corylus* the following species possess genetic resistance to eastern filbert blight: *C. americana*, *C. colurna* L., *C. heterophylla* Fisch., *C. seiboldiana* Blume, *C. cornuta* var. *californica* (A.DC.) Sharp and *C. cornuta* var. *cornuta* Marsh. (Barss, 1930). The mechanisms and inheritance of resistance in these species have not yet been elucidated (Coyne et al., 1998; Johnson et al.,1996) but attempts are underway to transfer resistance from these sources to *C. avellana*. The number of unacceptable traits in these species will prolong the development of suitable varieties. Thus, additional sources of resistance, within *C. avellana* or hybrids with this species, would benefit the breeding program.

Some early interspecific hybridizations were performed to develop varieties suited to the harsh winters and high disease pressure from *A. anomala* in the eastern U. S. Often, *C. americana* has been used for this purpose. J. F. Jones was the first breeder to use the *C. americana* selection 'Rush' in crosses with *C. avellana*, from 1917-1919. He discovered that the cross only produced seed when 'Rush' was used as the pistillate parent (Reed, 1948). The New York State Agricultural Research Experiment Station in Geneva performed some of this work after receiving about

500 hybrids between 'Rush' and *C. avellana* as trees from W.G. Bixby of Rochester, N.Y. The cultivars, 'Potomac' ('Rush' x 'DuChilly') and 'Reed'('Rush' x 'Hall's Giant') were developed in Beltsville, MD by J.W. McKay, H.L. Crane, and C.A. Reed (ASHS, 1997).

Some of these NY hybrids have been tested for disease response at the OSU hazelnut breeding program. Inoculation by growing grafted, potted trees of the clones under structures overlain with sporulating branches was unable to cause disease in any of 4 'Rush' x *C. avellana* clones tested. Two of these had 'DuChilly' as a pollen parent whereas 'Hall's Giant' and 'Barcelona' were the pollen parents for the other two. Greenhouse inoculation of 18 clones of pure *C. americana* resulted in infection in two of the genotypes tested (COR 59-59 and COR 59-61). Included in this test was one genotype of *C. colurna* (N13) which had a small sunken canker the first year it was observed but the canker had healed eight months later (Coyne et al., 1998).

Hybridizations of European hazelnut with *C. colurna* were performed by J.U. Gellatly starting in the mid-1930s. He did not make controlled pollinations so it is difficult to know the pedigrees of his selections. However, he collected seed from the *C. colurna* parent and the phenotypes of the resulting seedlings suggested that the pollen source had been *C. avellana*. He named these hybrids "trazels" (Gellatly, 1966). A private breeder in Michigan, Cecil Farris, released 'Grand Traverse' from a cross between 'Faroka' (a Gellatly trazel) and 'Royal' in 1989 (Farris, 1989).

Corylus avellana L., the European hazelnut, displays wide variation in its response to the eastern filbert blight pathogen, from complete resistance to extreme susceptibility. In some cases, multiple genes appear to condition host response to this fungus (Osterbauer et al., 1997). The cultivars 'Gem', 'Giresun 54-21' and 'Giresun 54-56' showed no symptoms after being grown under structures overlaid with sporulating branches while a few varieties, 'Hall's Giant', 'Willamette' and 'Tonda di Giffoni' possess reasonably high levels of incomplete resistance (Pinkerton, 1993). 'Barcelona', the most widely planted cultivar in Oregon, is moderately susceptible and becomes unproductive eight to twelve years after infection (Johnson et al., 1996). Seedlings of 'Gem' display incomplete resistance (Osterbauer et al., 1997).

Dominant, single-gene resistance has been found in the outdated pollinizer, 'Gasaway' (Mehlenbacher et al., 1991) and is expressed as an ability to resist initial infection and/or an ability to restrict canker expansion after ingress by the fungus (Pinkerton, et al. 1995). Unfortunately, this cultivar produces very few, very small, long nuts that are late to mature, and the non-blanching kernels have poor flavor (Mehlenbacher et al., 1991). Nevertheless, the majority of the completely resistant advanced selections currently in the breeding program are derived from 'Gasaway'. It would be beneficial to have resistant material of higher agronomic quality. As hazelnut is highly heterozygous and is susceptible to inbreeding depression, a modified backcross approach is used to incorporate resistance from 'Gasaway' which is heterozygous for its resistance gene. In each generation, the best plants

carrying the resistance gene from 'Gasaway' are selected and then crossed to the best susceptible seed parents.

In addition, new resistance sources are desirable because of concern over the potential instability of the resistance mechanism found in 'Gasaway' (Johnson et al., 1996) due to the nature of the pathogen. *A. anomala* is an obligate biotroph producing copious airborne ascospores. Similar fungi, such as rusts and smuts, often evolve new virulence when presented with populations of resistant plants carrying single dominant resistance genes (Wolfe and Barrett, 1980) and a shift in virulence is a concern (Osterbauer et al., 1997). In apple (*Malus* x*domestica* Borkh.), *Venturia inaequalis* (Cooke) Aderh., the causal agent of apple scab, was able to overcome the V_m resistance gene (Williams and Kuc, 1969). It is hoped that a completely resistant variety will be released in 2004 (Mehlenbacher, pers. comm.). Natural selection pressure on *A. anomala* is likely to increase after widespread release of completely resistant cultivars in Oregon (Pinkerton et al., 1998).

Evaluation of Host Genetic Resistance

Following inoculation, susceptibility is usually indicated following observation of cankers in the field and microscope detection of stained hyphae in infected tissue, or by indirect ELISA (Coyne et al., 1996). The quarantine regulations require that field observation take place within areas infested by the pathogen. One method of exposing trees to *A. anomala* is to grow them under a

wooden frame topped with infected, sporulating branches. This technique is necessary for the detection of incomplete resistance but the method will not give reliable results for at least 16 months and our field plots are in Vancouver, WA. Disease incidence, number of cankers per tree, canker length, proportion of cankered wood and proportion of dead wood are used as measures of susceptibility to eastern filbert blight(Pinkerton et al., 1993). Microscopic observation of hyphae is not reliable and often leads to failures in detection of the fungus. The ELISA technique is much reliable (Coyne et al., 1996) however it depends on repeated inoculation in a glasshouse (including a six month waiting period before branches can be sampled) and a time-consuming analysis. Nonetheless, it is much faster than the field evaluations. Lower levels of disease resistance cannot be detected in glasshouse tests (Coyne et al., 1998).

ELISA has been routinely used to detect fungi for many applications. The protocol developed by Coyne, uses polyclonal antibodies specific to mycelia of *A. anomala*. Polyclonal antibodies to cell walls of *Thielaviopsis basicola* (Berk. and Broome) were developed for the detection of cotton plants having black root rot (Holtz et al., 1994). Spoilage fungi such as *Penicillium aurantiogriseum* Dierckx and *Aspergillus ochraceus* Wilhelm, that can produce aflatoxins have been detected using polyclonal antibodies specific to their extracellular exudates (Lu et al., 1994; Lu et al., 1995). Another plant pathogen, *Macrophomina phaseolina* (Tassi) Goid., is causal to charcoal/dry rot of many crops and is detected in soils using polyclonal antibodies specific to its mycelial proteins (Srivastava and Arora, 1997).

Challenges of Hazelnut Breeding

Tree crops present a number of challenges to breeders. As with apple (Gianfranceschi et al., 1996), the juvenile period reduces the rate at which new cultivars are released. There is an eight year breeding cycle for European hazelnut (Mehlenbacher, 1995b) with the most precocious trees bearing nuts in the third year. The genetic portion of variability for this trait is largely additive (Mehlenbacher and Smith, 1992). Objectives of the OSU hazelnut breeding program are: big bud mite (*Phytoptus avellanae* Nal.) resistance, round nut shape, high percent kernel, precocity, good yield, blanching of kernels, absence of defects (such as twins, moldy kernels, etc.), early maturity and nuts that fall to the ground for ease of mechanical harvest. In addition, varieties that are not completely resistant or do not have a high level of incomplete resistance to eastern filbert blight cannot be grown in areas infested by the eastern filbert blight pathogen.

The typical breeding cycle at the OSU program lasts eight years from the harvest of hybrid seed to the creation of new hybrid seed. In winter, controlled crosses of parents (usually those combining desirable attributes or those used for genetic studies) are made, the seed is collected in August and kept in a cool room until November when the kernels are put into stratification. Stratification consists of storage in damp vermiculite for 3-5 months at 4°C. In spring, the seeds are warmed for five days and the ones that have germinated are planted in the greenhouse where they remain for the summer (Thompson et al., 1996). By fall,

they are ready and about 5000 are transplanted to the field. In the second and third years non-dormant seedlings die. By year four, precocious trees have begun to set nuts and those with long nuts or very thick shells are discarded. In winter, they are evaluated for big bud mite susceptibility and those with many blasted buds are eliminated. In the fifth year, evaluations are for the same traits as in the fourth year as well as yield, percent kernel, blanching ability, and percent defects. Winter evaluation is for number of catkins and female flowers because low flower numbers lead to low seed set. Trees with few catkins, indicating they will set very few nuts in the sixth year, are discarded. In year six, evaluations are repeated as in the previous year, reducing the number of trees to 100. In year seven, yield and kernel defects become more important criteria. The remaining 25 selections (0.5 %) are multiplied by layering for placement in replicated yield trials. In year eight, the most promising trees are used as parents and the cycle begins again.

Breeding European hazelnut entails overcoming a number of additional obstacles. One of these is that the crop has a sporophytic self-incompatibility system (Thompson, 1979). Phenotypic differences in compatibility are due to pollen-stigma interactions that are determined by alleles at a single locus. At the present time, twenty-nine alleles have been identified (S. A. Mehlenbacher, pers. comm.). These alleles are always codominant in the style and dominant or codominant in the pollen (Thompson, 1979; Mehlenbacher and Thompson, 1988; Mehlenbacher, 1997). Incompatibility alleles have been used for cultivar identification in pineapple (Coppens d'Eeckenbrugge et al., 1997) and pyrethrum

clones (Brewer and Parlevliet, 1969). Hazelnut cultivars differ in the time of pollen shed and the time at which their stigmas are receptive—some being protandrous and some protogynous (Mehlenbacher, 1991). It is necessary that varieties be planted with suitable pollinizers, or seed set will be extremely poor. There is great need for late-shedding pollinizers having complete resistance to eastern filbert blight (S. A. Mehlenbacher, pers. comm.) and four have been released (Mehlenbacher and Thompson, 1991).

Molecular Tools for Breeding

Other tools available to the breeder include molecular markers of which there are myriad types. Some commonly used in breeding are: RFLPs (Restriction Fragment Length Polymorphisms), RAPDs (Random Amplified Polymorphic DNAs), SCARs (Sequence Characterized Amplified Regions), SSRs (Simple Sequence Repeats or microsatellites), and AFLPs (Amplified Fragment Length Polymorphisms). The suitability of these analyses depends on the purpose for which they will be used. RAPD markers were reported nearly simultaneously in two laboratories (Williams et al., 1990; Welsh and McClelland, 1990). These markers rely on the polymerase chain reaction (PCR) (Saiki et al., 1988) which is relatively inexpensive and requires limited experience by the investigator. Short primers (usually 10 bp) bind to short inverted repeats and amplify random sequences. These amplified DNA fragments are subjected to electrophoresis and later visualized by ethidium bromide staining. Once a RAPD marker linked to a

trait of interest is found, it is often converted to a SCAR by creating longer primers (18-24 bp as compared to 10 bp for a RAPD marker) based on the DNA sequence of the polymorphic band. With longer primers the PCR is generally more robust as higher annealing temperatures are used and gels are easier to score. A disadvantage is when PCR does fail those samples are indistinguishable from those that lack the polymorphic band. It is also possible that a long primer will anneal with a slight mismatch and the first product will become a template for succeeding cycles of amplification. This leads to amplification of DNA samples from genotypes that do not actually carry the trait of interest (Davis, 1998). DNA concentration often has a strong impact on the amplification reaction and is often variable when rough DNA extractions are performed causing false-negatives to be of concern.

RFLPs depend on restriction enzymes cutting divergent sequences of DNA and forming fragments of many lengths. These fragments are separated by electrophoresis on a gel, blotted to nitrocellulose (Southern hybridization), probed with a radioactively-labeled DNA probe, and visualized by autoradiography. An advantage is that these markers are codominant but polymorphisms are less common than for most other marker types. Construction of a genomic library is necessary for the use of SSRs (Staub et al., 1996) as is the use of polyacrylamide gels (Liu et al., 1995). The library is probed to find repetitive tandem sequences of 2 to 5 bases, the patterns of which are diagnostic of particular individuals. Their main uses are fingerprinting and mapping. AFLPs generate many bands for tasks such as fingerprinting, taxonomic studies and fine genetic mapping but are very

expensive (Staub et al., 1996). They require restriction with two different enzymes, and a ligation step as well as two PCR steps.

Therefore, RAPDs more easily lend themselves to the high sample throughput (Williams et al., 1990) that is nearly mandatory in breeding programs. RAPD markers are well-suited to marker-assisted selection in woody fruit crops (Weeden et al., 1994) and they are especially fitting for the selection of a dominant resistance gene in the absence of the pathogen (Mehlenbacher, 1995a). Although RAPD markers are dominant, this is not problematic in our situation because the gene from 'Gasaway' is dominant and heterozygotes display immunity to eastern filbert blight. One limitation is that in some crosses, the expected segregation is not found. For example, in the cross in which the linkage was established, the marker may co-segregate 1 present : 1 absent with a phenotype. With the use of a different parent, the polymorphism may be lost and all progeny will amplify a band of the same size as that which was polymorphic in the original cross. The informative band will be indistinguishable from the artifact band because it is of the same size. Unexplained ratios of 3 present : 1 absent have also been seen (Davis, 1998). By cloning the sequence of the RAPD fragment and comparing it to the sequence of the polymorphic band one can identify restriction enzymes that will differentiate the two cases. Digestion with a restriction enzyme that cuts at a unique site within the artifact band will resolve it into two smaller bands and cause its disappearance (Caetano-Annollés, 1993). A similar approach has been described for distinguishing alleles by Janssens et al. (1995) in apple where allele-specific

primers were synthesized based on cDNA sequences and the resulting PCR product was subjected to restriction enzyme digestion. This allowed for distinction between different alleles of a self-incompatibility gene. Sakurai et al. (1997) performed similar work for the same purpose.

In tree crops, the bulked segregant analysis approach has also been used to find markers linked to many diseases caused by fungi. Examples include markers for black leaf spot resistance in elm (Benet et al., 1995), for powdery mildew resistance in apple (Markussen et al., 1995) and in peach (Dirlewanger et al., 1994), for scab resistance in apple (Yang and Krüger, 1994; Hemmat et al., 1998), and for white pine blister rust in sugar pine (Devey et al., 1995). In 1995, bulked segregant analysis (Michelmore et al., 1991) was used and 5 RAPD markers linked to the resistance gene from 'Gasaway' were found (Davis and Mehlenbacher, 1997). Two of these markers (UBC 152_{800} and OPH 19_{600}) are easy to score and robust to amplification conditions and are routinely used to select resistant seedlings in populations segregating for this gene. In hazelnut, bulked segregant analysis was also used to find markers linked to the S_2 incompatibility allele (Pomper et al., 1998). Molecular markers allow early selection thereby avoiding the costs involved with planting and caring for large numbers of trees susceptible to eastern filbert blight. Moreover, because *A. anomala* is not present in Corvallis (Johnson, 1996) field studies are precluded at this location. This is another factor which makes marker-assisted selection attractive (Melchinger, 1990).

Segregation Distortion

Segregation distortion as a phenomenon is widespread in plants and affects annuals, perennials, outcrossing and selfing plants and may act as a cause of aberrant segregation of molecular markers as well as of phenotypic traits. As one might expect there are many mechanisms for it. Some causes of distortion are: directed chromosome loss, competition among gametes, incompatibility factors, and lethal alleles that can cause inbreeding depression via genetic load (Bradshaw and Stettler, 1994; Perfectti and Pascual, 1996). Both gametic and zygotic selection operates to cause segregation distortion in cherimoya (Perfectti and Pascual, 1996). In hazelnut, 10 of 46 (22%) of isozyme alleles were found to exhibit distortion (Rovira et al., 1993). Distortion due to differential pollen fertility is common in crops where production of huge numbers of gametes provides a buffer against poor fecundity (Lyttle, 1991). Pollen competition was cited as a cause of marker distortion in apple (Conner et al., 1997). In addition *Corylus* displays sympodial branching which increases the likelihood of somatic mutation by allowing possibly mutant stem sectors to become apical meristems. In turn, these subterminal buds undergo increased cellular differentiation in order to outgrow the terminal bud, thus increasing intercellular competition and mutation rate (Klekowski, 1988).

Research Objectives

The objectives of this work are to identify additional sources of complete resistance to eastern filbert blight and thereby diversify the genetic pool available

for breeding resistance to eastern filbert blight and to improve the marker-assisted selection of seedlings carrying the 'Gasaway' gene. Two approaches were taken toward the former objective. The first approach was to survey a large number of European hazelnut clones for complete resistance to the eastern filbert blight pathogen. Secondly, we evaluated if a resistant chance-seedling, designated 'Zimmerman', found near Troutdale, OR in winter of 1989 (J. Pinkerton, pers. comm.) carries the same resistance gene as that found in 'Gasaway'. Both trees share the combination of an eastern filbert blight resistant phenotype and the S_3 incompatibility allele. 'Gasaway' is (S_3S_{26}) and 'Zimmerman' is (S_1S_3). 'Gasaway' is heterozygous for a resistance gene to eastern filbert blight and progenies created by crossing with susceptible genotypes segregate in a 1:1 ratio for this trait (Mehlenbacher et al., 1991). Therefore if 'Zimmerman' were a seedling of 'Gasaway', we would expect a similar segregation ratio in crosses between 'Zimmerman' and susceptible genotypes. The overall aim of this research is to expedite the release of eastern filbert resistant hazelnut varieties.

CHAPTER 2

SURVEY OF HAZELNUT CLONES FOR RESPONSE TO THE EASTERN FILBERT BLIGHT PATHOGEN

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To be submitted to *HortScience*
American Society for Horticultural Science,
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Abstract

Ninety European hazelnut (*Corylus avellana* L.) genotypes including some interspecific hybrids with *C. americana* Marsh., and *C. colurna* L. were surveyed for response to the eastern filbert blight pathogen [*Anisogramma anomala* (Peck) E. Müller] following greenhouse inoculation. Six varieties were discovered which did not display signs of the pathogen or symptoms of disease: 'Closca Molla', 'Ratoli', 'Yoder #5', 'Potomac', 'Medium Long', and 'Grand Traverse'. Of these, 'Ratoli' and 'Closca Molla', both minor *C. avellana* varieties from Spain, are superior agronomic types to the resistant cultivar 'Gasaway' which has been an important resistance source. 'Potomac' and 'Yoder #5' have *C. americana* relatives and 'Grand Traverse' is one-quarter *C. colurna*. The origin of 'Medium Long' is uncertain. None of these varieties possess the UBC 152_{800} RAPD marker linked to the single resistance gene in 'Gasaway' and its progeny. These varieties provide breeders with novel sources of genetic resistance to this devastating disease.

Introduction

Oregon is the top producer of hazelnuts (*Corylus avellana* L.) in the United States, accounting for 99% of the U.S. crop and 3-5 % of the world hazelnut crop (Mehlenbacher and Olsen, 1997). Eastern filbert blight (EFB), caused by the pyrenomycete *Anisogramma anomala*, is a serious and widespread disease in Oregon. This disease can destroy the productivity of a moderately susceptible cultivar in 8-12 years (Johnson et al., 1996) so combating it is an important

industry goal. Due to the expense of fungicides and detrimental effects on yield caused by severe pruning of cankers, genetic resistance is an especially appealing approach to fighting this disease (Mehlenbacher, 1995).

Most of the resistant material in the Oregon State University (OSU) hazelnut breeding program has been derived from the single dominant resistance gene found in the outdated pollenizer variety 'Gasaway' (Mehlenbacher et al., 1991). This variety has extremely poor agronomic quality and developing cultivars for the kernel market that are completely resistant to eastern filbert blight is an important goal of the OSU breeding program (Mehlenbacher, 1995). Hazelnuts for the kernel market must fit strict standards for size, shape, shell thickness, ease of pellicle removal and flavor (Mehlenbacher, 1994). 'Gasaway' has poor quality in nearly all these areas. Therefore, it would be beneficial to find resistance sources that also have good nut quality.

One tool that breeders can use for early selection is marker-assisted selection. Of the many types of markers, RAPD markers are one of the least expensive and they are well-suited to the high throughput of breeding programs. RAPD markers were reported nearly simultaneously in two laboratories (Williams et al., 1990; Welsh and McClelland, 1990). These markers rely on the polymerase chain reaction (PCR) (Saiki et al., 1988) which requires limited experience by the investigator. Short primers (usually 10 bp) bind to short inverted repeats that amplify random sequences. These amplified DNA fragments are subjected to electrophoresis and later visualized by ethidium bromide staining. In 1995, bulked

segregant analysis (Michelmore et al., 1991) was used and 5 RAPD markers linked to the resistance gene from 'Gasaway' were found (Davis and Mehlenbacher, 1997). Two of these markers (UBC 152_{800} and OPH 19_{600}) are easy to score and robust to amplification conditions and are routinely used to select resistant seedlings in populations segregating for this gene (Mehlenbacher and Lunde, unpublished). Primer names are followed by subscripts denoting the size of the polymorphic band which each amplifies.

There is concern about the durability of this source of resistance as well (Pinkerton et al., 1998). The 'Gasaway' gene should be combined with others, in the same or separate new varieties and this is an important objective of the OSU hazelnut breeding program (Mehlenbacher, 1994). Thus, it would be highly beneficial to find new sources of genetic resistance to eastern filbert blight. The purpose of this study was to conduct a broad survey of germplasm from the collections of the OSU breeding program and the USDA-ARS (United States Department of Agriculture-Agricultural Research Station) National Clonal Germplasm Repository, Corvallis, Oregon for response to inoculation by the EFB pathogen.

Materials and Methods

Plant Materials

Three scions of each of 90 European hazelnut selections from the USDA-ARS National Clonal Germplasm Repository, Corvallis, OR and the collection of the OSU hazelnut breeding program as well as 'Gasaway' as a resistant control and 'Ennis' and 'Daviana' as susceptible controls (Table 2.1) were gathered in December 1996, and stored at 0° C until they were grafted onto *C. avellana* rootstocks in the spring of 1997. Grafted trees were placed in 3.7 liter or 5 liter pots in a mix containing equal volumes of peat, pumice and fine bark dust. In addition, 9 g of Sierra 3-4 month release fertilizer (17N-6P-12K with micronutrients) was added to each pot. Supplemental fertilizer treatments with Peter's (20N-8.7P-16.6K) formula were made as needed. Plants were kept in a glasshouse under optimal conditions (24° C day/18° C night) until they were ready for inoculation. Scionwood was collected from genotypes scored as resistant in the 1996 inoculations and grafted, inoculated, and retested in 1998.

Inoculations

Inoculations were started about 3-5 weeks after grafting, once shoots had 4-5 nodes, and followed the protocol outlined in Coyne et al. (1996). All actively growing branches (usually two or three) of the grafted seedlings were marked with tape three or four nodes from the apical meristem to indicate where inoculation

would occur. Shoots were inoculated with a 1 x 10^6 spores/mL in distilled water as described by Davis (1998). Chamber frames were constructed of polyvinyl chloride tubing (1.27 cm diameter) placed on top of a bench (1.22 m x 0.44 m) and covered with white 4 mm polyethylene sheeting. About 75 trees were placed in each of two inoculation chambers. A humidifier was placed in each chamber and the plastic was fastened with clothespins. The humidifiers were programmed to run from noon to 6pm and from midnight to 4 am. The chambers were opened five days after inoculation and left open for two days. Inoculations were then repeated. All trees received a total of three inoculations. They remained in the glasshouse (24° C day/18° C night) with optimal watering for six months prior to assaying for infection.

Infection Assays

Three assays were used to detect the presence of *A. anomala* in inoculated shoots using several assays. An ELISA assay was performed on inoculated shoots of two grafted trees from each original seedling as described in Coyne et al. (1996) except that Nunc Maxisorp (439454) microtiter plates were used instead of Corning (25860) plates. A second assay was used on one grafted tree of each genotype grafted in 1996. They were inoculated as above and transported to the Southwest Washington Experiment Station, Vancouver, WA where they were planted in February, 1997 and evaluated for canker incidence in June, 1998. Trees which allowed any infection by fungal hyphae were scored as susceptible. In winter of

Table 2.1 Locations and names of cultivars tested for reaction to *A. anomala*

Cultivar	Location	CCOR#	Origin
A Pellicola Bianca	N04.26	454.001	Italy
Alcover	N05.54	375.001	Italy
Amandi	W15a	566.001	Spain
Apolda	N05.49	360.001	Italy
Ata Baba	N06.02	480.001	Azerbaijan
Atlas	N03.28	389.001	Denmark
Aveline d'Angleterre	N02.58	387.001	England
Bandnuss	N03.52	382.001	England
Barcelloner Zeller[3]	N03.58	331.001	England
Bard	N05.20	514.003	England
Barr's Zeller	N03.57	333.001	England
Bearn (Du Bearn)	N04.35	461.001	France
Blumberger Zeller[3]	212.053	---------	Germany
Brixley's New	N07.49	288.001	USA-Oregon
Burchardt's Zeller	N03.32	334.001	Germany
Buttner's Zeller	CC05.63	329.001	Germany
Ceret	R20.19	508.001	Spain
Cherkesskii II	V13	544.001	Russia
Closca Molla	212.054	257.002	Spain
Comen	N05.06	362.002	Italy
Comun	R20.16	486.001	Portugal
Culpla[3]	212.055	255.002	Spain
da Viega	R20.17	487.001	Portugal
Daviana[2]	R02.32/LB	42.001	England
Des Anglais	N05.04	481.001	England
Ennis[2]	LB	11.001	USA-Washington
Espinaredo	R20.20	509.001	Spain
Faroka[3]	R21.33	---------	British Columbia
Frizzled Filbert	N01.21	218.001	England
Fructo Albo	N07.24	511.001	Italy
Garibaldi	N04.56	338.001	England
Garrofi	W8a	341.002	Spain
Gasaway[1]	N06.14/LB	54.001	USA-Washington
Gironell (Grossal)	212.079	---------	Spain
Grand Traverse	R20.10	559.001	USA-Michigan
Grifoll (Queixal de Llop)	212.059	443.002	Spain
Gunslebert (Guslebener Zeller)	N01.57	382.001	Germany
Gustav's Zeller	N02.38	206.001	Germany
Heynick's Zeller	CC05.42	390.001	Germany
Istarski Duguljasti	N03.14	272.001	Slovenia
Jean's	N03.18	264.001	Italy
Jeeve's Samling	N03.51	352.001	England
Kadetten Zeller	N04.49	323.001	Germany
Kunzemuller's Zeller	N03.56	353.001	Germany
Lange Landsberger	N03.55	325.001	Germany
Liegel's Zeller	N04.48	316.001	Germany
Louisen's Zeller	N05.39	207.001	Germany

Table 2.1 Continued

Cultivar	Location	CCOR#	Origin
Ludolph's Zeller	N04.50	330.001	Germany
Martorella	N03.40	---------	Spain
Medium Long	R18.32	---------	USA-New York
Molar	R20.18	488.001	Portugal
Mogulnuss	N03.49	324.001	England
Morell	N06.23	6.001	Spain
Multiflora	N04.54	322.001	England
Napoletana	N05.48	374.001	Italy
Napoletanedda	212.045	463.002	Italy
Noce Lungha	N07.52	296.001	Italy
Nociara	N02.50	385.001	Italy
Nottingham	N07.53	297.001	England
Palaz	N05.14	29.002	Turkey
Pallagrossa	N02.55	372.001	Italy
Pearson's Prolific	N04.57	335.001	England
Pere Mas	N04.02	---------	Spain
Pinyolenc	R19.09	339.002	Spain
Pioneer	V15a	548.001	Russia
Planeta	212.049	445.002	Spain
Potomac	CC05.29	377.001	USA-Maryland
Princess Royal	N04.53	327.001	England
Prolific Closehead	N03.47	326.001	England
Purple Aveline	R05.01	---------	England
Quiros	W16	279.001	Spain
Ratllada	R20.13	442.002	Spain
Ratoli	N05.57	344.001	Spain
Red Filbert	N03.30	317.001	England
Reed	CC05.38	383.001	USA-Maryland
Restiello	N07.10	280.002	Spain
Riekchen's Zeller	N04.04	393.001	Germany
Ros de la Selva	W09	260.002	Spain
Rossetta	N01.55	379.001	Spain
Sant Joan[3]	212.076	271.004	Spain
Sant Pere	R19.18	270.005	Spain
Sickler's Zeller[3]	N03.48	321.001	Germany
Sodlinger	566.086	---------	Italy
The Shah	N06.34	319.001	England
Tomasina	212.057	441.002	Spain
Tonda Bianca	R20.11	21.003	Italy
Tonda Rossa	R19.06	267.003	Italy
Truchsess' Zeller	N04.51	328.001	Germany
Ugbrooke	N02.45	245.001	New Zealand
Volle Zeller	CC05.45	---------	Germany
Webb's Prize Cob	N04.55	336.001	England
Witpit Lambertsnoot	CC05.02/V07	573.001	New Zealand
Yoder #5	639.031	---------	USA—Ohio

N=National Clonal Germplasm Repository, Smith Farm, Corvallis, OR
CCOR#=*Corylus* catalogue accession number
LB*= layer beds

[1] Negative control
[2] Positive controls
[3] Graft fails, 1996

1997, scionwood was collected from trees scored as resistant in the 1996 inoculations and new trees were grafted in spring 1998 and tested again. Microscope sectioning and visualization were used for trees inoculated in winter 1998 to search for hyphae in samples that gave conflicting results. Hyphae were stained with 0.05% trypan blue in lactophenol and observed with a lightmicroscope as described in Stone et al. (1992). If grafts of a genotype failed in 1996, they were grafted, inoculated and assayed again in 1998.

DNA Extraction and RAPD Screening

A very young apical meristem and first leaf were sampled from from field-planted trees and DNA samples were extracted. When samples were needed later in the season, catkins were used, as Cheng et al. (1997) had successfully extracted DNA from catkins. The samples were processed in the laboratory within an hour of field collection. DNA extractions were executed as described by Davis et al. (1998) with minor modifications.

PCR Assay

PCR reactions were done in a volume of 15μl containing 10 mM of Tris-HCl (pH 9.0), 50 mM KCl, 0.15% Triton X-100, 1.5 mM $MgCl_2$, 120 μM each of dATP, dCTP, dGTP, and dTTP, 0.1 μM of primer, 3-25 ng of DNA template, and

0.75 units of Taq polymerase (Promega, Madison, Wis.) (Davis, 1998). Ninety-six reactions were run simultaneously using a Geneamp® PCR System 9700 thermal cycler (Perkin-Elmer Corporation, Foster City, CA). Each genotype which had been scored as resistant and a control containing no DNA were also tested. The thermal cycler program consisted of denaturation for 1 min at 94° C, followed by 5 cycles of: 1 min at 94° C, 1 min 30 sec at 37° C, 30 sec at 54° C and 2 min at 72° C. Then 35 cycles followed: 15 sec at 94° C, 45 sec at 37° C, 30 sec at 54° C, and 1 min 30 sec at 72° C. Primer extension occurred for 7 min at 72° C and then samples were held at 4° C until they were retrieved. DNA from each tree was amplified using UBC 152 (University of British Columbia, Vancouver, B.C.) and OPH 19 (Operon Technologies, Alameda, CA). Amplification products were separated on 1.5% agarose gels, and stained with ethidium bromide. They were visualized with a transilluminator and photographed.

Results and Discussion

Of the ninety cultivars (Table 2.1), six were found to resist infection by *Anisogramma anomala* (Table 2.2). The six cultivars vary widely in agronomic quality (Table 2.2). Desired traits for the kernel market are: medium size, round shape, 50% percent kernel or better, about 2 nuts per cluster, very little fiber on the kernels or pellicles, good blanching ability, and few defects (less than 35%). It is important that nuts fall free from the husk because, in Oregon, nuts are

Table 2.2 Nut traits of cultivars found to be resistant to eastern filbert blight

Variety	Size[Z]	Shape[Y]	% K[W]	Husk[W, P]	N/Cl[V, P]	Fiber[U, P]	Blan[T, P]	%De[S, P]	%Fr[R, P]
Closca Molla	M	R	57[Q]	4	3	3	4	44	67
Grand Traverse	L	R	46	4	2	1	7	40	60
Medium Long	M	L	43	5	3	2	7	54	62
Potomac	M	Rpt	--	6	2	--	--	--	--
Ratoli	M	Rpt	53[Q]	4	3	--	--	--	--
Yoder # 5	L	Rpt	43	7	2	1	7	28	25
Gasaway	VS	Lcp	20	2	2	1	5	12	90

[Z]Approximate size categories in mm (diam.): VS < 17 < S < 18 < M < 19.5 < L < 22
[Y]Shape codes: R (round); Rpt (round with point); Lcp (long compressed)
[W]Approximate husk length categories in cm: category 1< 2.5 < category 7 < 10
[V]Approximate number of nuts per cluster
[U]Amount of fiber on kernel: 1 (none) < 4 (completely covered)
[T]Blanching ability: 1 (complete removal of pellicle) < 7 (no removal of pellicle)
[S]Percent defects out of 50 nut sample
[R]Percent nuts free from husk at harvest
[Q]Tasias-Valls (1975)
[P]Mehlenbacher (unpublished data, 1994-1998)

mechanically harvested off manicured orchard floors. These varieties seem to exhibit complete resistance to eastern filbert blight but the inheritance of this resistance requires further study.

It is likely that these six cultivars will be useful as parents in the development of new resistant varieties. 'Closca Molla' and 'Ratoli' have nicely shaped kernels and blanch better than the others. The former has nice thin shells but unfortunately low yield (Tasias-Valls, 1975). 'Closca Molla' has been noted for its high percent kernel and 'Ratoli' has been recommended as a potential variety due to the quality of its kernel traits (Tasias Valls, 1975). Both of these cultivars have potential for immediate use as cultivars. Release of completely resistant, late-shedding pollinizers is an objective of the breeding program (Mehlenbacher, pers. comm.). These six varieties have diverse alleles: 'Closca Molla' (S_2 S_5), 'Grand Traverse' (S_{11} S_{25}), 'Medium Long' (S_{11} S_{12}), 'Potomac' (S_5 S_{12}), 'Ratoli' (S_2 S_{10}), 'Yoder # 5' (S_{10} S_{23}). Incompatibility should not be a barrier to using these cultivars in breeding.

At least three of the other four clones are related to species other than *C. avellana*. 'Grand Traverse' was selected from a cross between 'Faroka' (*C. colurna* x *C. avellana*) and 'Royal' (*C. avellana*) by Cecil Farris (Farris, 1989). 'Faroka' was created by J. U. Gellatly in the 1950's by collecting seed from a *C. colurna* plant grown nearby a European hazel. Thus, 'Grand Traverse' ('Faroka' x 'Royal') is one-quarter *C. colurna* which explains its slightly bitter flavor and poor

precocity. Resistance in this variety was previously reported by Cecil Farris (Farris, 1989).

'Potomac' is half *C. americana* and the appearance of 'Yoder #5' clearly indicates that it too contains some *C. americana*. Neither of these varieties blanches well. 'Yoder #5' has the added deficiencies of being quite sensitive to big bud mite and being low in yield. Segregation of resistance to eastern filbert blight has been previously described in hybrids of European hazelnut with *C. americana* (Coyne et al., 1998) but its pattern is undiscernible. 'Potomac' is the result of a cross between 'Rush' (a *C. americana* selection) and 'DuChilly', a European hazelnut variety. However, there are conflicting reports about whether 'Rush' itself is susceptible to eastern filbert blight (Slate, 1947; Thompson et al., 1996). Likewise, the cultivar, 'Reed' ('Rush' x 'Bolwyller') shares its *C. americana* parent but was susceptible in the greenhouse inoculation. Other plants resulting from this cross and plants sharing the parentage of 'Potomac' have been resistant (Coyne et al., 1998). It is unknown whether 'Medium Long' is of purely *C. avellana* heritage. It may have been imported from Europe originally but, its resistance to EFB hints that our clone may be an interspecific hybrid. Its origin is the New York Agricultural Experiment Station where the European hazel was routinely crossed with *C. americana* (Slate, 1947).

The imminent release of varieties carrying the 'Gasaway' gene for resistance to eastern filbert blight and the nature of the causal fungus lead to the conclusion that multiple sources of resistance to this disease are needed for

durability. These six newly found sources of resistance should provide insurance against new virulence in the pathogen. These cultivars do not amplify the UBC 152_{800} marker which is linked to eastern filbert blight resistance in 'Gasaway' and its progeny. By deploying varied sources of genetic resistance to eastern filbert blight, there will be less selection pressure on the 'Gasaway' resistance gene.

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CHAPTER 3

SEGREGATION FOR RESISTANCE TO EASTERN FILBERT BLIGHT IN PROGENY OF 'ZIMMERMAN' HAZELNUT

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To be submitted to
Journal of the American Society for Horticultural Science
American Society for Horticultural Science,
Alexandria, Virginia

Abstract

Eastern filbert blight, caused by the fungus *Anisogramma anomala* (Peck) E. Müller, is an important disease of European hazelnut (*Corylus avellana* L.) in the Pacific Northwest. In 1989, a chance seedling free of eastern filbert blight was discovered adjacent to a severely diseased orchard near Troutdale, Oregon. This seedling, subsequently named 'Zimmerman', was crossed with three susceptible selections. These progeny populations were inoculated three times with spores of the pathogen in a greenhouse test and assayed by indirect ELISA and by observation of canker incidence. The pathogen was not detected in 86%, 80%, and 86% of the three progeny populations in contrast to the typical 50% incidence of infection found in progeny of 'Gasaway' when it is crossed to susceptible genotypes. A RAPD marker linked to the resistance gene in 'Gasaway' cosegregates with the resistant phenotype in all three populations (2 cM, 4 cM, 6 cM). 'Zimmerman' appears to differ at least partially from that found in 'Gasaway'.

Introduction

Oregon is the top producer of hazelnuts in the United States (Mehlenbacher, and Olsen, 1997) and the industry is under threat by the fungus *A. anomala* which causes the perennial canker disease, eastern filbert blight. This disease is detrimental to production (Johnson et al., 1996) and continues to move into uninfested areas (Johnson, 1996). Host genetic resistance is a desirable way to

avoid the expense and time commitment that is involved in scouting, pruning, and spraying to control this disease (Mehlenbacher, 1995).

As most of the resistant material in the OSU breeding program is derived from a single source, the obsolete pollinizer 'Gasaway', it would be beneficial to find new sources of genetic resistance to eastern filbert blight. Resistance from 'Gasaway' is conferred by a single dominant gene inherited in simple Mendelian fashion (Mehlenbacher et al., 1991; Coyne et al., 1998). There is concern that the imminent release (in 2004) of resistant varieties carrying the 'Gasaway' gene (S. A. Mehlenbacher, pers. comm.) will increase selection pressure on the pathogen, causing it to shift virulence (Pinkerton et al., 1998b). A similar scenario occurred in apple, in which the scab pathogen, *Venturia inaequalis* (Cooke) Aderh., overcame resistance conferred by the V_m gene (Williams and Brown, 1968) and the V_f gene (Parisi et al. 1993, Fischer, 1994).

In 1995, bulked segregant analysis was used to find five RAPD markers linked to the resistance gene in populations segregating for the 'Gasaway' gene (Davis and Mehlenbacher, 1997). Two of these have proven easy to score and useful for practicing early selection on seedlings segregating for resistance (Mehlenbacher and Lunde, unpublished).

In 1989, Jack Pinkerton, a USDA scientist found an uninfected chance seedling growing next to a severely infested orchard near Troutdale, Oregon. The orchard was owned by the Zimmerman family so the seedling was named 'Zimmerman' (J. Pinkerton, pers.comm.). Scions were collected, grafted,

inoculated with the pathogen several times in a severe greenhouse inoculation procedure and produced neither signs of the pathogen nor symptoms of eastern filbert blight. The reaction to inoculation is identical to that seen in 'Gasaway' and since they share a common incompatibility allele (S_3), it seemed likely that this chance-seedling derived its resistance from 'Gasaway'. In this paper we report the disease response observed in seedlings from crosses of 'Zimmerman' with three susceptible genotypes.

Materials and Methods

Plant Materials

In February 1992, three populations were created using three different advanced selections from the breeding program as seed parents with 'Zimmerman' as the pollen parent in controlled hybridizations. All of the chosen seed parents are susceptible to eastern filbert blight and have diverse pedigrees (Figure 3.1). Since progeny of 'Gasaway' segregate 1 resistant : 1 susceptible (Mehlenbacher et al., 1991) a similar ratio was expected in the three 'Zimmerman' populations.

Seeds were harvested in August of 1992 from pollinated branches, held in a cool room for two months, soaked in water for four days, and then stratified in moist vermiculite at 4°C for about four months. Those which failed to germinate were partially cracked by hand and treated with 50 ppm gibberellic acid. After germination, they were planted in 64 cell flats (44.13 cm x 44.13 cm) and later

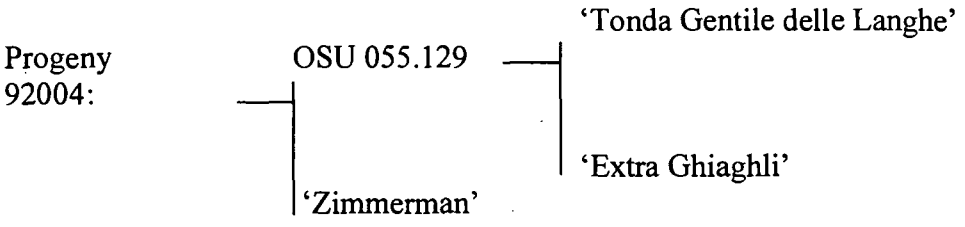
potted in 3.7 liter or 5 liter pots in a mix containing equal volumes of peat, pumice and fine bark dust. Later, 9 g of Sierra 3-4 month release fertilizer (17N-6P-12K with micronutrients) was added to each pot. Supplemental fertilizer treatments with Peter's (20N-8.7P-16.6K) formula were made as needed. Plants were grown in a glasshouse under optimal conditions (24° C day/18° C night), hardened in September, and planted in the field in October 1993 at a spacing of 0.92 m in rows spaced 3.05 m apart.

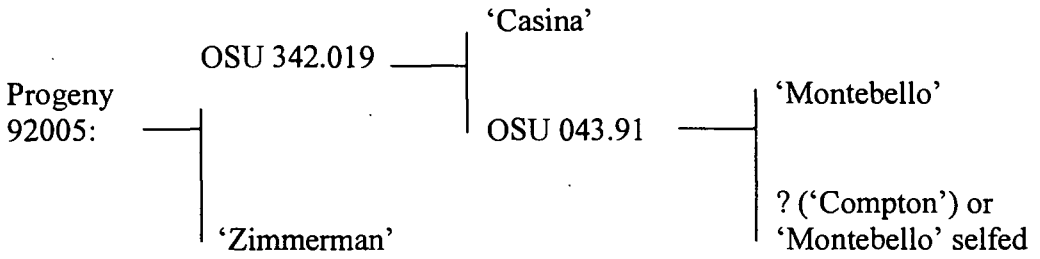
In December 1995, scions were collected. Scionwood was stored at 0°C until it was grafted onto *C. avellana* rootstocks in Spring of 1996. The grafted trees, three of each seedling, were potted as described above and kept in a glasshouse (24° C day/18° C night) with optimal watering until ready for inoculation. Scionwood was collected in December 1997 from trees that were scored as resistant in the first test and grafted, inoculated and assayed in 1998 in order to confirm their phenotype.

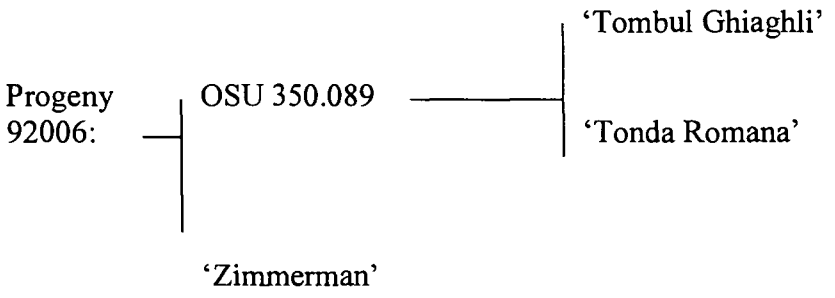
Inoculations

Inoculations began once 4-5 nodes had emerged (3-5 weeks), and followed the protocol outlined in Coyne et al. (1996). Actively growing branches (usually two or three) of the grafted seedlings were marked with tape three or four nodes from the apical meristem to indicate where the shoots would be inoculated. Spores

Figure 3.1 – Pedigrees of seed parents of populations 92004, 92005, 92006







of the pathogen were dissected from mature perithecia and inoculum consisted of 1 x 10^6 spores/mL suspended in distilled water as in Davis (1998). Chamber frames were constructed of polyvinyl chloride tubing (1.27 cm diameter) placed on top of a bench (1.22 m x 0.44 m) and covered with white 4 mm polyethylene sheeting. About seventy-five trees were placed in each of two inoculation chambers. A humidifier programmed to run from 12 pm to 6pm and from 12 am to 4 am was placed in each chamber and the plastic was fastened with clothespins. Five days later, the chambers were opened for two days. Inoculations were then repeated: all trees received a total of three inoculations. They remained in the glasshouse (24° C day/18° C night) with optimal watering for six months prior to beginning infection assays.

Infection Assays

A. anomala was detected in susceptible genotypes using three assays. The ELISA assay was performed on inoculated shoots of each of two grafted trees from the original seedling as described in Coyne et al. (1996) with the exception that Nunc Maxisorp (439454) microtiter plates were used instead of Corning (25860) plates. The third grafted tree of each genotype was inoculated as above and transported to the Southwest Washington Experiment Station, Vancouver, WA where they were planted in a nursery row in February, 1997. Evaluations for canker incidence were made in February, 1998 and in June, 1998. For trees inoculated in

winter 1998, microscope sections were stained with 0.05% trypan blue in lactophenol and observed with a light microscope as described in Stone et al. (1992). Sections were searched for hyphae in samples that gave conflicting results in other assays. Trees that showed any infection by fungal hyphae were scored as susceptible.

DNA Extraction and RAPD Screening

DNA samples were collected from the original, field-planted trees. A very young apical meristem and first leaf were sampled from each tree. When samples were needed from trees later in the season, catkins were used as in Cheng et al. (1997). The samples were rushed back to the laboratory and quickly ground. DNA extractions were executed as specified in Davis et al. (1998) with minor modifications (Appendix A).

PCR Assay

PCR reactions were done in a volume of 15µl containing 10 mM of Tris-HCl (pH 9.0), 50 mM KCl, 0.15% Triton X-100, 1.5 mM $MgCl_2$, 120 µM each of dATP, dCTP, dGTP, and dTTP, 0.1 µM of primer, 3-25 ng of DNA template, and 0.75 units of Taq polymerase (Promega, Madison, Wis.) (Davis, 1998). Ninety-six reactions were run simultaneously using the Geneamp® PCR System 9700 thermal

cycler (Perkin-Elmer Corporation, Foster City, CA). The parents of each progeny and a control containing no DNA were also included. The thermal cycler program consisted of denaturation for 1 min at 94° C, followed by 5 cycles of: 1 min at 94° C, 1 min 30 sec at 37° C, 30 sec at 54° C and 2 min at 72° C, and then 35 cycles of: 15 sec at 94° C, 45 sec at 37° C, 30 sec at 54° C, and 1 min 30 sec at 72° C. Primer extension occurred for 7 min at 72° C, and then samples were held at 4° C until they were retrieved. DNA from each tree was amplified using two 10-mer primers: UBC 152 (University of British Columbia, Vancouver, B.C.) and OPH 19 (Operon Technologies, Alameda, CA). Amplification products were separated on 1.5% agarose gels, stained with ethidium bromide, visualized with a transilluminator, and photographed. RAPD markers are denoted by the primer name followed by subscripts denoting the size of the polymorphic band that each amplifies ($UBC152_{800}$ or $OPH19_{600}$).

Marker Cloning

DNA samples from 'Zimmerman', 'Gasaway', and OSU 350.089 were used in 24, 15 µl PCR reactions with either primer UBC 152 or primer OPH 19 (Table 3.2). PCR samples were compiled and dried down or ethanol precipitated. Ten µl of the concentrated UBC 152_{800} fragment or the OPH 19_{600} fragment dissolved in autoclaved nano filtered water were run on 2% low-melting point agarose (NuSieve, FMC, Rockland, ME) gels. The fragments were excised from the gels

with a sterile blade and purified with the QIAquick™ kit (Qiagen, Inc., Chatsworth, Calif.) according to the manufacturer's instructions.

Table 3.1 Origin of cloned RAPD fragments

Selection	Phenotype	Primer
Zimmerman	Resistant	OPH 19_{600} $UBC152_{800}$
Gasaway	Resistant	OPH 19_{600} $UBC152_{800}$*
OSU 350.089	Susceptible	OPH 19_{600}

* Cloning and sequencing of $UBC152_{800}$ was previously performed and reported (Davis, 1998.)

This purified DNA fragment was poly-A tailed to facilitate ligation. Poly-A tailing was performed by combining 30 mM Tris-HCl (pH 7.3), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 0.2 mM dATP, 5 units of Taq polymerase, 6 µl of purified fragment (about 70 ng) , and 2.5 mM $MgCl_2$ in a volume of 10 µl and incubating for 30 min at 70 °C. Ligation reactions contained 30 mM Tris-HCl (pH 7.3), 10 mM $MgCl_2$, mM DTT, 1 mM ATP, 3 units ligase, about 40 ng purified insert, and 50 ng pGEM®-T Easy vector. The reactions were stored overnight at 4° C. JM109 competent *lacI*^{q}ZΔM15 *Escherichia coli* cells were transformed with the vector which contained an ampicillin resistance gene and the *lac* operon as selectable markers. Putative transformants were grown on LB + ampicillin media with X-Gal and IPTG for blue/white screening.

The presence of the desired insert was confirmed with PCR and by digestion with *EcoR*I (0.5 U of enzyme per 50 ng DNA at 37° C for one hour) to cut the plasmid at sites flanking the position where the insert should be. After electrophoresis, the fragment size was measured by comparison with a 100 bp ladder. Only colonies with an insert of the correct size were multiplied by overnight incubation in LB broth. Plasmid DNA was then isolated from these cultures with the QIAprep Spin Plasmid Miniprep kit (Qiagen) as described by the supplier. The concentration of the purified plasmid DNA was measured with a Hoefer DyNAQuant 200 fluorometer (Hoefer Pharmacia Biotech Inc.) and sequenced on an ABI 373A automated sequencer (Applied Biosystems, Perkin-Elmer). Sequences were edited using GDE (Smith et al., 1994). Alignment, sequence homology, and restriction enzyme sites were analyzed using GCG version 9 (University of Wisconsin Genetics Computer Group, Madison).

Pollen Germination Test

Mature elongated catkins were collected at the end of January, 1999. They were laid out on paper overnight. The anthers dehisced and the pollen was poured into glass vials, plugged with cotton and frozen until the germination test was performed. Germination media was prepared as in Kim et al. (1985). Pollen was sprinkled over petri dishes with quadrant dividers. Three plates were made for each genotype and plates were randomized on the bench top and left overnight (12-15

hours) at about 21°C. Pollen grains were scored as having germinated if pollen tubes were longer than the diameter of the grain. One reading of approximately 100 grains was made per quadrant. Pollen from 'Daviana' was included as a control. Data were recorded as percent germination.

Results

Resistant phenotypes represented about 83% of the total in all three progenies, which is far more than would be expected if 'Zimmerman' were heterozygous for the 'Gasaway' resistance gene. The observed ratio does approximate a 3:1 ratio (Table 3.2). A total of nine trees, including at least one in each progeny were found that displayed an intermediate phenotype. These had non-sporulating cankers in the field, but hyphae could not be detected with the ELISA technique or with microscopic examination of stained tissue. The UBC 152_{800} marker was present in all nine plants. When intermediate phenotypes were excluded, the ratio still was not 1 : 1 but the fit to a 3:1 was better (Table 3.2).

Categorization by phenotype was congruent using the ELISA technique and by scoring in the field (Table 3.3) indicating that the aberrant ratio is not due to a large number of disease escapes or error in technique. Furthermore, a second test was performed on all seedlings that were scored as being resistant in the first test. The RAPD marker UBC 152_{800} which is linked to the dominant resistance gene in 'Gasaway' segregates in almost exactly the same proportion as the resistant

Table 3.2 Segregation for eastern filbert blight resistance in 'Zimmerman' populations fits 3:1 ratio

Progeny code	Parents	No. plants		Expected	X^2	
		Resistant	Susceptible	ratio	Value	1-*P*
92004	OSU 055.129 X Zimmerman	43[Z]	7	3:1	3.23	0.071
92005	OSU 342.019 X Zimmerman	39[Y]	10	3:1	0.55	0.458
92006	OSU 350.089 X Zimmerman	43[W]	7	3:1	3.23	0.071
Pooled data		125	24		6.46	0.011
Heterogeneity						
92004	OSU 055.129 X Zimmerman	42[V]	7	3:1	3.00	0.773
92005	OSU 342.019 X Zimmerman	34[U]	10	3:1	0.62	0.511
92006	OSU 350.089 X Zimmerman	39[T]	7	3:1	2.39	0.723
Pooled data		115	25		3.81	0.821
Heterogeneity					2.20	0.333

[Z, Y, W] Includes selections with non-sporulating cankers in the field, but no hyphae detected by ELISA or microscopic examination

[V, U, T] Selections with non-sporulating cankers in the field, no hyphae detected by ELISA or microscopic examination were excluded

'Gasaway' was included as negative control. 'Daviana' and 'Ennis' included as susceptible controls.

Table 3.3 Resistance response to eastern filbert blight congruent between field observation and detection by ELISA*

Progeny code	Parents		No. plants		X^{2Z}	
			Resistant	Susceptible	Value	1-*P*
92004	OSU 055.129 X Zimmerman	ELISA	43	5	0.00	1.00
		Field	43	5		
92005	OSU 342.019 X Zimmerman	ELISA	35	9	0.00	1.00
		Field	35	9		
92006	OSU 350.089 X Zimmerman	ELISA	42	5	0.00	1.00
		Field	41	6		

*Plants showing intermediate phenotype (non-sporulating cankers) were excluded

phenotype, exhibiting only 2 %, 4 %, and 6% recombination between the marker and the resistance phenotype in the populations, respectively (Table 3.4). Inheritance of an eastern filbert blight resistant phenotype is not independent of ability to amplify the UBC 152_{800} marker. The RAPD marker OPH 19_{600} was monomorphic in all three populations. Cloning and sequencing of the OPH 19_{600} marker indicated that bands of nearly identical sequence are amplified from the susceptible seed parent, OSU 350.089 and from the resistant genotypes, 'Gasaway' and 'Zimmerman'. Cloning and sequencing of the UBC 152_{800} RAPD marker (Table 3.1) showed that the sequences amplified by 'Zimmerman' and 'Gasaway' were nearly identical (Figure 3.2).

Pollen of 'Zimmerman' germinated approximately 29% of the time. 'Barcelona' germinated 18% of the time. In contrast, the pollen germination rate for 'Gasaway' was 71%.

Discussion

Segregation distortion is common and affects diverse types of plants. The phenomenon is seen in annual as well as perennial plants and it is likely that there are many mechanisms behind it. It has been reported in perennial crops including poplar (Bradshaw et al., 1994), Douglas fir (Jermstad et al., 1994), eucalyptus (Grattapaglia and Sederoff, 1994), cherimoya (Perfectti and Pascual, 1996), and avocado (Torres et al., 1986). In hazelnut, distortion is common in isozyme

Table 3.4 Recombination frequencies for resistance to eastern filbert blight and UBC 152_{800}[Z].

Progeny code	Parents	Observed Phenotypes[Y]				Recombination Frequency[X]
		R/+	R/0	S/+	S/0	
92004	OSU 055.129 X Zimmerman	43	0	1	6	0.02
92005	OSU 342.019 X Zimmerman	39	0	2	8	0.04
92006	OSU 350.089 X Zimmerman	42	0	3	5	0.06

[Z] Intermediate phenotypes included with resistant phenotypes.
[Y] Phenotypes: R =resistant, S = susceptible, + = amplifies marker, 0 = does not amplify marker
[X] Recombination frequency = (Σ R/0 and S/+)/total number of plants

Figure 3.2 – Sequence homology between UBC 152_{800} band from 'Gasaway' vs. UBC 152_{800} band from 'Zimmerman' showing 803/807 identical basepairs.

```
Gap Weight:    50    Average Match: 10.000
Length Weight:  3    Average Mismatch: -9.000

Quality: 7738          Length:  808
Ratio: 9.636           Gaps:    3
Percent Similarity: 99.252  Percent Identity: 99.252

Match display thresholds for the alignment(s):
        | = IDENTITY
        : = 5
        . = 1

                .         .         .         .         .
  1 TCACACATCTAATAGATATGCATATGTATTTGTGTGTTATAGCTTGATTA 50
    |||||||||||||||||||||||||||||||||||||||||||||||||||
  1 TCACACATCTAATAGATATGCATATGTATTTGTGTGTTATAGCTTGATTA 50
                .         .         .         .         .
 51 GCACGTGTCGCCATTTGATTTGAGTAATTTATTTGGCAAACTGATGTGAT 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 51 GCACGTGTCGCCATTTGATTTGAGTAATTTATTTGGCAAACTGATGTGAT 100
                .         .         .         .         .
101 TTAGATGCTCCACAAAATTTAGCAGAGAAACTCTAGTGAAAGCCAAACAT 150
    ||||||||||||||||||||||||||||||||||||||||||||||||||
101 TTAGATGCTCCACAAAATTTAGCAGAGAAACTCTAGTGAAAGCCAAACAT 150
                .         .         .         .         .
151 ACTGGAGCCTTCTTCATCCCCAAATTCTTGTGCGTAGTTTAAGATTTTCA 200
    ||||||||||||||||||||||||||||||||||||||||||||||||||
151 ACTGGAGCCTTCTTCATCCCCAAATTCTTGTGCGTAGTTTAAGATTTTCA 200
                .         .         .         .         .
201 AAAATGATTAAGGAGAAAATTTCTTTAACTTCCAAGATCCATGCTTCATT 250
    ||||||||||||||||||||||||||||||||||||||||||||||||||
201 AAAATGATTAAGGAGAAAATTTCTTTAACTTCCAAGATCCATGCTTCATT 250
                .         .         .         .         .
251 CTGATTTCACATTT....TTTTTTAAAAATTCGCACTACACTTTTAGGCT 296
    ||||||||||||||    || || |||| ||||||||||||||||||||
251 CTGATTTCACATTTTGTGTTGTTAAAAATTTCGCACTACACTTTTAGGCT 300
                .         .         .         .         .
297 CCTTATTTGTGTTGATCAGCAAAAAGAGTGTAGAAGCAGAAAAGTAACTT 346
    |||||||||||||||||||||||||||||||| ||||| ||||||||||
301 CCTTATTTGTGTTGATCAGCAAAAAGAGTGTAGGAGCAG.AAAGTAACTT 349
                .         .         .         .         .
347 CTGGTTTTCCTTTGAAATTCTTCAACATAATTTGCT.CTTTCCATTTATG 395
    |||||||||||||||||||||||||||||||| ||||||||||||
350 CTGGTTTTCCTTTGAAATTCTTCAACATAATTTGCTCCTTTCCATTTATG 399
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Figure 3.2 Continued

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396 TAAAACGATTGTCTAGTATAACTCTTGCTATGCGCTAGGTAGCTGCAGCT 445
    |||||||||||||||||||||||||||||||||||||||||||||||||||
400 TAAAACGATTGTCTAGTATAACTCTTGCTATGCGCTAGGTAGCTGCAGCT 449

446 AGCAGCATCAATTGTTAGGGTGGATAATATTAGGGGTGAAACCCGGTCCC 495
    |||||||||||||||||||||||||||||||||||||||||||||||||||
450 AGCAGCATCAATTGTTAGGGTGGATAATATTAGGGGTGAAACCCGGTCCC 499

496 GATTCTCGGTTATTGACCCAAACTTTCATTTCCTCATTGTTAAGTTGAAA 545
    |||||||||||||||||||||||||||||||||||||||||||||||||||
500 GATTCTCGGTTATTGACCCAAACTTTCATTTCCTCATTGTTAAGTTGAAA 549

546 AAGACTGACAGAAGAGGAGTACATGTGCATGATACAGATGAAGCAGCTGA 595
    |||||||||||||||||||||||||||||||||||||||||||||||||||
550 AAGACTGACAGAAGAGGAGTACATGTGCATGATACAGATGAAGCAGCTGA 599

596 GGCCACAGCAGGAGAAGAAGACGATCACGATGTTGCCTCCCACGCCAATA 645
    |||||||||||||||||||||||||||||||||||||||||||||||||||
600 GGCCACAGCAGGAGAAGAAGACGATCACGATGTTGCCTCCCACGCCAATA 649

646 GGGAAGCAAGGTTTACGATCATAGCCAGATGTGAAGTAGCCTCGATCGGT 695
    |||||||||||||||||||||||||||||||||||  ||||||||
650 GGGAAGCAAGGTTTACGATCATAGCCAGATGTGAAGTAGAATCGATCGGT 699

696 GAGTTTAATTCTCATATATAAACTTGTTTTATGAGATCAACTATCGCAAG 745
    |||||||||||||||||||||||||||||||||||||||||||||||||||
700 GAGTTTAATTCTCATATATAAACTTGTTTTATGAGATCAACTATCGCAAG 749

746 ATTCTAATTTAATCAAAAGTTCTGCTATAGATGAACGATGGATGCCAATG 795
    |||||||||||||||||||||||||||||||||||||||||||||||||||
750 ATTCTAATTTAATCAAAAGTTCTGCTATAGATGAACGATGGATGCCAATG 799

796 GGGAATAT 803
    ||||||||
800 GGGAATAT 807
```

analyses (Cheng, 1992; Rovira et al., 1993). Clonally propagated crops seem especially prone to distortion and within this group, distortion has been attributed to genetic load (Bradshaw and Stettler, 1994) or accumulation of somatic mutations (Klekowski, 1988).

There are two possible explanations for the unexpected abundance of resistant genotypes in these populations. One is that 'Zimmerman' has indeed inherited its resistance gene from 'Gasaway' but that some mechanism of segregation distortion is acting in favor of resistant genotypes. Selection pressure on male or female germline cells or postzygotic selection pressure before the trait of interest is evaluated will affect segregation. These can be genes important during sporogenesis, for proper functioning of germ cells, for seed development, for germination, or for plant growth (Zamir and Tadmor, 1986; Xu et al., 1997). Similarly, artifical selection for traits linked to these pre- or post-zygotic factors, will distort segregation.

One cause of pre-zygotic selection is meiotic drive. This is the preferential retention of one member of a pair of heterozygous alleles or one heteromorphic chromosome such that it is transmitted to more than half of the resulting meiocytes (Lyttle, 1991). Chromosome loss can also function during meiosis but this is most common in interspecific crosses (Bradshaw and Stettler, 1994). Maternal abortion or competition between megaspores can cause distorted segregation, however, three unrelated seed parents were chosen decreasing the likelihood that this mechanism functions in the 'Zimmerman' progenies.

Another cause of pre-zygotic selection is certation, or abnormal pollen function. Competition due to differential pollen vigor acts from pollen germination through fertilization. In *Lycopersicon hirsutum* Humb. & Bonpl., genes expressed in pollen give advantage in low temperatures, exemplifying that even the haploid genome is vulnerable to selection (Zamir et al., 1982). In European hazelnut, fertilization requires five months. The pollen tube grows toward the ovule and forms a callosed resting stage, eventually fertilization occurs once the ovule has formed (Thompson et al., 1979). It is conceivable that pollen genotypes having lesser ability to complete this process would be selected against. If 'Zimmerman' is heterozygous for its resistance gene and the pollen carrying the susceptible allele is linked to gene(s) causing poor pollen fitness, susceptible genotypes would be compromised in the offspring resulting from its use as a pollen parent. The low rate of germination of 'Zimmerman' pollen may indicate that this is the cause of distortion in these populations. Pollination germination in 'Zimmerman' was similar to that in 'Barcelona', and only 40% of its pollen is viable (Salesses, 1973). This mechanism has been cited as a possible cause of distortion in the inheritance of isozymes in hazelnut (Rovira et al., 1993). Chromosome translocation has been cited as a cause of pollen sterility (Salesses, 1973).

Some post-zygotic mechanisms were discounted. Although non-dormant mutants are rogued or suffer early natural elimination, this phenotype was only present in two 'Zimmerman' populations. Population 92004 does not segregate for non-dormancy and the proportion of resistant plants in the populations is similar to

that observed in the other two (Table 3.2, Table 3.3). Likewise, no trend could be seen in the records of moldy or rotted kernels, stunted seedlings, seedlings with roots but no shoots, or twins indicating that selection during stratification or transplanting is not likely responsible for the high percent of resistant phenotypes. The stratification procedure is fairly long yet, the similarities in the percentage of resistant genotypes makes it unlikely that one of these is the cause of the observed distortion.

Chances that experimental error has caused misclassification of plant phenotypes are slight because field data and greenhouse data were consistent (Table 3.3) and inoculations of resistant seedlings were repeated and confirmed. A similar percentage (83%) of resistant genotypes was previously reported in other plants from population 92004 (Coyne, 1995). The UBC 152_{800} marker is well-characterized and its segregation with the resistance phenotype (Table 3.8, Table 3.9) is not due to improper scoring.

An alternative hypothesis is that 'Zimmerman' is heterozygous (AaBb) for two independent resistance genes and that only (aabb) genotypes are susceptible. This would explain why the data fit a 3 resistant : 1 susceptible ratio (Table 3.6). Following this reasoning, crosses of resistant 'Zimmerman' progeny from these crosses with susceptible plants should segregate: 3 resistant : 1 susceptible or 1 resistant : 1 susceptible. In 1993, a cross was made between VR 6-28 which carries the 'Gasaway' gene and 'Zimmerman'. Progeny from this cross should segregate 7

resistant : 1 susceptible following the two gene assumption. One of 18 plants was susceptible and this data fits a the expected ratio despite the small sample size.

However, if the UBC 152_{800} marker is only linked to the resistance gene from 'Gasaway', it should segregate 1 present : 1 absent which it does not (Table 3.8). In order to explain these results it is necessary to hypothesize that the second gene arose from duplication of the first and that the flanking region where the UBC 152 primer amplifies was also duplicated. It is interesting to note that the only recombinants seen were susceptible plants with the marker.

In summation, it is hoped that increasing knowledge about novel sources of resistance to eastern filbert blight will aid in the development of immune cultivars. Investigation of inheritance of eastern filbert blight resistance in progenies of the chance-seedling, 'Zimmerman', has provided us with very useful information. Although it seems likely that 'Zimmerman' carries the same resistance gene as 'Gasaway' and therefore will not provide us with a new resistance gene, we now know that we can use our RAPD marker in selecting resistant seedlings that inherit this trait from 'Zimmerman'. In addition, the proportion of resistant progeny in crosses of 'Zimmerman' is much higher than when 'Gasaway' is used as the resistance source.

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CHAPTER 4

SUMMARY

A survey of ninety hazelnut varieties, mostly *Corylus avellana* L. (but including a few hybrids of *C. americana* Marsh. or *C. colurna* L.), led to the discovery of six varieties displaying no disease symptoms after a severe inoculation with *Anisogramma anomala* (Peck) E. Müller, the causal agent of eastern filbert blight. These six varieties: 'Closca Molla', 'Ratoli', 'Yoder #5', 'Potomac', 'Medium Long', and 'Grand Traverse' will greatly aid the development of new hazelnut varieties completely resistant to this devastating disease. Moreover, two of these varieties, 'Closca Molla' and 'Ratoli' are minor Spainish cultivars that appear to have high agronomic quality. They are well suited to the kernel market and may have direct potential for use in Oregon.

The nut characteristics of the other cultivars are not ideal however, they are no worse than those of the obsolete pollinizer, 'Gasaway' from which the majority of the resistant material in the Oregon State University hazelnut breeding program is derived. None of these six varieties has the UBC 152_{800} RAPD marker that is linked to the 'Gasaway' resistance gene. These varieties potentially carry new genes that will diversify the resistance mechanisms that can be deployed against the eastern filbert blight pathogen.

Likewise, investigation into the inheritance of eastern filbert blight resistance in the volunteer seedling, 'Zimmerman', led to the conclusion that this too will be a useful source of resistance in breeding new varieties. In three populations, each having different unrelated susceptible seed parents, 'Zimmerman' transmitted complete resistance to 77%

of its progeny. Another 6% showed sunken, non-sporulating cankers in the field but hyphae was not detectable with the indirect ELISA technique or with microscopic examination. The UBC 152_{800} RAPD marker is linked to the resistance phenotype in 'Zimmerman' and its seedlings with little recombination. This indicates that 'Zimmerman' probably carries the resistance gene from 'Gasaway'. However, since 'Gasaway' is heterozygous for this single dominant gene, it transmits resistance to only 50% of its progeny, making 'Zimmerman' a more efficient parent for the development of new completely resistant varieties.

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APPENDICES

Appendix A – Modification of DNA Extraction Protocol for *C. avellana* from Davis et al. (1998)

Grinding buffer
750 ml ddH_2O
119.8 g sucrose
12.1 g Tris base
3.7 g KCl
9.3 g EDTA
50 g PVP (MW=40,000)

Lysing buffer
200 mL 0.5M EDTA pH 8.0
50 mL 1.0M Tris-HCl pH 8.0
750 mL ddH_2O
Volumize to 1 liter.

Lysing solution
787 mL lysing buffer
106.5 mL 20% sarkosyl
106.5 mL 25% Triton X-100
Add 585 µl Proteinase K (20mg/mL) to 124 mL lysing solution just prior to use

All centrifugation steps are at 13,000 rpm. All vortexing is done at maximum speed.

1. Harvest youngest leaf of hazelnut trees or suckers and transport on ice. Grind in cold grinding buffer using a juice press (MEKU, Wennigen, Germany). Collect about 500 µl of homogenate into a 1.7 mL microcentrifuge tube. Keep on ice.
2. Spin for 5 minutes. Discard supernatant. Aspirate to remove remaining liquid. Add 640 µl of lysing solution and resuspend pellet by vortexing. Incubate at 37° C for one hour on a rotary shaker.
3. Spin 5 minutes. Transfer 500 µl of supernatant to new 1.7 mL tube. Add 500 µl isopropanol. Cap, invert several times and store at -20° C at least 30 minutes. Spin 5 minutes. Discard supernatant. Aspirate to remove remaining liquid. Add 220 µl TE and resuspend pellet. Store at 4° C at least overnight.
4. Add 220 µl phenol: chloroform: IAA (25:24:1) and vortex 10 seconds. Spin 10 minutes. Transfer 150 µl of the supernatant (aqueous phase) to a new tube.

5. Add 300 µl 95% ethanol + .05% sodium acetate (3M, pH 5.5). Cap, invert several times and store at -20° C at least 30 minutes.
6. Spin 5 minutes. Pour off supernatant. Add 1 mL 70% ethanol, spin 5 minutes. Decant. Dry pellet under vacuum or in fume hood. Resuspend pellet in 50 µl TE or ddH_2O. This should yield about 125 ng/µl of DNA.

Appendix B Sequence homologies

B.1 – Sequence homology between OPH 19_{600} band from 'Gasaway' (top) and OPH 19_{600} band from 350.089 (bottom) showing that they are identical.

Gap Weight: 8 Average Match: 2.912
Length Weight: 2 Average Mismatch: -2.003

Quality: 3569 Length: 629
Ratio: 5.674 Gaps: 0
Percent Similarity: 100.000 Percent Identity: 100.000

Match display thresholds for the alignment(s):
| = IDENTITY
: = 2
. = 1

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          .         .         .         .         .
  1 CATGGAGTGTTGAAATACCTGCGCACAAGTAGTGCTCGCCATCTAGGCCA 50
    ||||||||||||||||||||||||||||||||||||||||||||||||||
  1 CATGGAGTGTTGAAATACCTGCGCACAAGTAGTGCTCGCCATCTAGGCCA 50
          .         .         .         .         .
 51 TCTCTTTTGACAATGTTTATTTAAGTCCCTGCAAAGATCTAAGTAATGGA 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 51 TCTCTTTTGACAATGTTTATTTAAGTCCCTGCAAAGATCTAAGTAATGGA 100
          .         .         .         .         .
101 AGCTCTTAACACAAGTGGTGTGGTAAAGCTTGTTGAAGAATTGGACCGCA 150
    ||||||||||||||||||||||||||||||||||||||||||||||||||
101 AGCTCTTAACACAAGTGGTGTGGTAAAGCTTGTTGAAGAATTGGACCGCA 150
          .         .         .         .         .
151 TCCTCTGGATTCAAGCAGATAGAAAGGATATTTCTTTCACGAAGTATCTC 200
    ||||||||||||||||||||||||||||||||||||||||||||||||||
151 TCCTCTGGATTCAAGCAGATAGAAAGGATATTTCTTTCACGAAGTATCTC 200
          .         .         .         .         .
201 CACGTCCTTAGTAGTGTCAATGAGGTTGTCGAGGAGTATGGCATAGGAAG 250
    ||||||||||||||||||||||||||||||||||||||||||||||||||
201 CACGTCCTTAGTAGTGTCAATGAGGTTGTCGAGGAGTATGGCATAGGAAG 250
          .         .         .         .         .
251 TGATCGTATCATCGCCTTTGGGGTAGCATTGCTCAAAGCTGATGAGATTC 300
    ||||||||||||||||||||||||||||||||||||||||||||||||||
251 TGATCGTATCATCGCCTTTGGGGTAGCATTGCTCAAAGCTGATGAGATTC 300
          .         .         .         .         .
301 TTGATTAAATGTTTCTGTGATCTCATGAATTCTTATTTGAGGAATTTCGA 350
    ||||||||||||||||||||||||||||||||||||||||||||||||||
301 TTGATTAAATGTTTCTGTGATCTCATGAATTCTTATTTGAGGAATTTCGA 350
          .         .         .         .         .
351 GGATGCCATTTCTAAATTTTATGTCAAACATATTTGCAGACTTACTCTGT 400
    ||||||||||||||||||||||||||||||||||||||||||||||||||
351 GGATGCCATTTCTAAATTTTATGTCAAACATATTTGCAGACTTACTCTGT 400
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B.1 Continued

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401 CTGAATTTGATTCCAGCTTCTAGGAGACGCGTAGCAGAAGGTACGGGTTC 450
    ||||||||||||||||||||||||||||||||||||||||||||||||||
401 CTGAATTTGATTCCAGCTTCTAGGAGACGCGTAGCAGAAGGTACGGGTTC 450
          .         .         .         .         .
451 CCATCTGTATGGCATATTCGATCCAAATTCTTCTACTCCCATTGATGAAA 500
    ||||||||||||||||||||||||||||||||||||||||||||||||||
451 CCATCTGTATGGCATATTCGATCCAAATTCTTCTACTCCCATTGATGAAA 500
          .         .         .         .         .
501 CCATCAATTTTTTAAACAAGTCAGGAATATGTTTGATGGCTTGAGGTGGA 550
    ||||||||||||||||||||||||||||||||||||||||||||||||||
501 CCATCAATTTTTTAAACAAGTCAGGAATATGTTTGATGGCTTGAGGTGGA 550
          .         .         .         .         .
551 TCAGATATCGTGGACGGGAATACGTTACGAAAGAATTCAATGGCAAGTTG 600
    ||||||||||||||||||||||||||||||||||||||||||||||||||
551 TCAGATATCGTGGACGGGAATACGTTACGAAAGAATTCAATGGCAAGTTG 600
          .         .
601 AATTAGGGTCTCCCCGTGGCATCTATTGT 629
    |||||||||||||||||||||||||||||
601 AATTAGGGTCTCCCCGTGGCATCTATTGT 629
```

B.2 – Sequence homology between OPH 19_{600} band from 'Gasaway' (top) and OPH 19_{600} band from 'Zimmerman' (bottom) showing that they are identical.

Gap Weight: 8 Average Match: 2.912
Length Weight: 2 Average Mismatch: -2.003

Quality: 3569 Length: 629
Ratio: 5.674 Gaps: 0
Percent Similarity: 100.000 Percent Identity: 100.000

Match display thresholds for the alignment(s):
| = IDENTITY
: = 2
. = 1

```
  1 CATGGAGTGTTGAAATACCTGCGCACAAGTAGTGCTCGCCATCTAGGCCA 50
    ||||||||||||||||||||||||||||||||||||||||||||||||||
  1 CATGGAGTGTTGAAATACCTGCGCACAAGTAGTGCTCGCCATCTAGGCCA 50
            .         .         .         .         .
 51 TCTCTTTTGACAATGTTTATTTAAGTCCCTGCAAAGATCTAAGTAATGGA 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 51 TCTCTTTTGACAATGTTTATTTAAGTCCCTGCAAAGATCTAAGTAATGGA 100
            .         .         .         .         .
101 AGCTCTTAACACAAGTGGTGTGGTAAAGCTTGTTGAAGAATTGGACCGCA 150
    ||||||||||||||||||||||||||||||||||||||||||||||||||
101 AGCTCTTAACACAAGTGGTGTGGTAAAGCTTGTTGAAGAATTGGACCGCA 150
            .         .         .         .         .
151 TCCTCTGGATTCAAGCAGATAGAAAGGATATTTCTTTCACGAAGTATCTC 200
    ||||||||||||||||||||||||||||||||||||||||||||||||||
151 TCCTCTGGATTCAAGCAGATAGAAAGGATATTTCTTTCACGAAGTATCTC 200
            .         .         .         .         .
201 CACGTCCTTAGTAGTGTCAATGAGGTTGTCGAGGAGTATGGCATAGGAAG 250
    ||||||||||||||||||||||||||||||||||||||||||||||||||
201 CACGTCCTTAGTAGTGTCAATGAGGTTGTCGAGGAGTATGGCATAGGAAG 250
            .         .         .         .         .
251 TGATCGTATCATCGCCTTTGGGGTAGCATTGCTCAAAGCTGATGAGATTC 300
    ||||||||||||||||||||||||||||||||||||||||||||||||||
251 TGATCGTATCATCGCCTTTGGGGTAGCATTGCTCAAAGCTGATGAGATTC 300
            .         .         .         .         .
301 TTGATTAAATGTTTCTGTGATCTCATGAATTCTTATTTGAGGAATTTCGA 350
    ||||||||||||||||||||||||||||||||||||||||||||||||||
301 TTGATTAAATGTTTCTGTGATCTCATGAATTCTTATTTGAGGAATTTCGA 350
            .         .         .         .         .
351 GGATGCCATTTCTAAATTTTATGTCAAACATATTTGCAGACTTACTCTGT 400
    ||||||||||||||||||||||||||||||||||||||||||||||||||
351 GGATGCCATTTCTAAATTTTATGTCAAACATATTTGCAGACTTACTCTGT 400
            .         .         .         .         .
401 CTGAATTTGATTCCAGCTTCTAGGAGACGCGTAGCAGAAGGTACGGGTTC 450
    ||||||||||||||||||||||||||||||||||||||||||||||||||
401 CTGAATTTGATTCCAGCTTCTAGGAGACGCGTAGCAGAAGGTACGGGTTC 450
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B.2 Continued

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451 CCATCTGTATGGCATATTCGATCCAAATTCTTCTACTCCCATTGATGAAA 500
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451 CCATCTGTATGGCATATTCGATCCAAATTCTTCTACTCCCATTGATGAAA 500
              .         .         .         .         .
501 CCATCAATTTTTTAAACAAGTCAGGAATATGTTTGATGGCTTGAGGTGGA 550
    ||||||||||||||||||||||||||||||||||||||||||||||||||
501 CCATCAATTTTTTAAACAAGTCAGGAATATGTTTGATGGCTTGAGGTGGA 550
              .         .         .         .         .
551 TCAGATATCGTGGACGGGAATACGTTACGAAAGAATTCAATGGCAAGTTG 600
    ||||||||||||||||||||||||||||||||||||||||||||||||||
551 TCAGATATCGTGGACGGGAATACGTTACGAAAGAATTCAATGGCAAGTTG 600
              .         .
601 AATTAGGGTCTCCCCGTGGCATCTATTGT 629
    |||||||||||||||||||||||||||||
601 AATTAGGGTCTCCCCGTGGCATCTATTGT 629
```

B.3 – Sequence homology between OPH 19_{600} band from 'Zimmerman' (top) and OPH 19_{600} band from 350.089 (bottom) showing that they are identical.

Gap Weight: 8 Average Match: 2.912
Length Weight: 2 Average Mismatch: -2.003

Quality: 3569 Length: 629
Ratio: 5.674 Gaps: 0
Percent Similarity: 100.000 Percent Identity: 100.000

Match display thresholds for the alignment(s):
| = IDENTITY
: = 2
. = 1

```
  1 CATGGAGTGTTGAAATACCTGCGCACAAGTAGTGCTCGCCATCTAGGCCA 50
    ||||||||||||||||||||||||||||||||||||||||||||||||||
  1 CATGGAGTGTTGAAATACCTGCGCACAAGTAGTGCTCGCCATCTAGGCCA 50
             .         .         .         .         .
 51 TCTCTTTTGACAATGTTTATTTAAGTCCCTGCAAAGATCTAAGTAATGGA 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 51 TCTCTTTTGACAATGTTTATTTAAGTCCCTGCAAAGATCTAAGTAATGGA 100
             .         .         .         .         .
101 AGCTCTTAACACAAGTGGTGTGGTAAAGCTTGTTGAAGAATTGGACCGCA 150
    ||||||||||||||||||||||||||||||||||||||||||||||||||
101 AGCTCTTAACACAAGTGGTGTGGTAAAGCTTGTTGAAGAATTGGACCGCA 150
             .         .         .         .         .
151 TCCTCTGGATTCAAGCAGATAGAAAGGATATTTCTTTCACGAAGTATCTC 200
    ||||||||||||||||||||||||||||||||||||||||||||||||||
151 TCCTCTGGATTCAAGCAGATAGAAAGGATATTTCTTTCACGAAGTATCTC 200
             .         .         .         .         .
201 CACGTCCTTAGTAGTGTCAATGAGGTTGTCGAGGAGTATGGCATAGGAAG 250
    ||||||||||||||||||||||||||||||||||||||||||||||||||
201 CACGTCCTTAGTAGTGTCAATGAGGTTGTCGAGGAGTATGGCATAGGAAG 250
             .         .         .         .         .
251 TGATCGTATCATCGCCTTTGGGGTAGCATTGCTCAAAGCTGATGAGATTC 300
    ||||||||||||||||||||||||||||||||||||||||||||||||||
251 TGATCGTATCATCGCCTTTGGGGTAGCATTGCTCAAAGCTGATGAGATTC 300
             .         .         .         .         .
301 TTGATTAAATGTTTCTGTGATCTCATGAATTCTTATTTGAGGAATTTCGA 350
    ||||||||||||||||||||||||||||||||||||||||||||||||||
301 TTGATTAAATGTTTCTGTGATCTCATGAATTCTTATTTGAGGAATTTCGA 350
             .         .         .         .         .
351 GGATGCCATTTCTAAATTTTATGTCAAACATATTTGCAGACTTACTCTGT 400
    ||||||||||||||||||||||||||||||||||||||||||||||||||
351 GGATGCCATTTCTAAATTTTATGTCAAACATATTTGCAGACTTACTCTGT 400
             .         .         .         .         .
```

B.3 Continued

```
401 CTGAATTTGATTCCAGCTTCTAGGAGACGCGTAGCAGAAGGTACGGGTTC 450
    ||||||||||||||||||||||||||||||||||||||||||||||||||
401 CTGAATTTGATTCCAGCTTCTAGGAGACGCGTAGCAGAAGGTACGGGTTC 450
          .         .         .         .         .
451 CCATCTGTATGGCATATTCGATCCAAATTCTTCTACTCCCATTGATGAAA 500
    ||||||||||||||||||||||||||||||||||||||||||||||||||
451 CCATCTGTATGGCATATTCGATCCAAATTCTTCTACTCCCATTGATGAAA 500
          .         .         .         .         .
501 CCATCAATTTTTTAAACAAGTCAGGAATATGTTTGATGGCTTGAGGTGGA 550
    ||||||||||||||||||||||||||||||||||||||||||||||||||
501 CCATCAATTTTTTAAACAAGTCAGGAATATGTTTGATGGCTTGAGGTGGA 550
          .         .         .         .         .
551 TCAGATATCGTGGACGGGAATACGTTACGAAAGAATTCAATGGCAAGTTG 600
    ||||||||||||||||||||||||||||||||||||||||||||||||||
551 TCAGATATCGTGGACGGGAATACGTTACGAAAGAATTCAATGGCAAGTTG 600
          .         .
601 AATTAGGGTCTCCCCGTGGCATCTATTGT 629
    |||||||||||||||||||||||||||||
601 AATTAGGGTCTCCCCGTGGCATCTATTGT 629
```

Appendix C - Pollen germination test

Cultivar	Percent pollen germination
Gasaway	71
Zimmerman	29
Barcelona[Z]	18
Daviana[Y]	64

[Z]Barcelona produces pollen that is half empty (Salesses, 1973).
[Y]Daviana was included as a control, usual germination rate is 78±10% (Craddock, 1987)

Appendix D Segregation for resistance to in 'Zimmerman' progenies

D.1 Segregation of 'Zimmerman' progeniess does not fit a 1:1 ratio

Progeny code	Parents	No. plants		Expected	X^2	
		Resistant	Susceptible	ratio	Value	1-*P*
92004	OSU 055.129 X Zimmerman	43[Z]	7	1:1	25.92	<0.001
92005	OSU 342.019 X Zimmerman	39[Y]	10	1:1	17.16	<0.001
92006	OSU 350.089 X Zimmerman	43[X]	7	1:1	25.92	<0.001
Pooled data		125	24		68.48	<0.001
Heterogeneity					0.52	0.471
92004	OSU 055.129 X Zimmerman	42[w]	7	1:1	25.00	<0.001
92005	OSU 342.019 X Zimmerman	34[v]	10	1:1	7.36	0.007
92006	OSU 350.089 X Zimmerman	39[u]	7	1:1	17.16	<0.001
Pooled data		115	25		49.52	<0.001
Heterogeneity					8.32	0.004

[Z, Y, X] Includes selections with non-sporulating cankers in the field, but no hyphae detected by ELISA or microscopic examination

[W,V, U,] Selections with non-sporulating cankers in the field, no hyphae detected by ELISA or microscopic examination were excluded

Appendix D.2 RAPD marker UBC 152_{800} is linked to eastern filbert blight resistance (intermediate phenotypes removed)*

Progeny code	Parents	Marker	No. plants		X^2	
			Resistant	Susceptible	Value	1-*P*
92004	OSU 055.129 X Zimmerman	Present	42	1	33.44	<0.001
		Absent	0	6		
92005	OSU 342.019 X Zimmerman	Present	34	2	28.21	<0.001
		Absent	0	8		
92006	OSU 350.089 X Zimmerman	Present	39	3	21.10	<0.001
		Absent	0	5		

* Chi-square contingency test for independence of two events

Appendix E Short report on improvement of marker-assisted selection

Abstract

Marker-assisted selection using a RAPD marker linked to the 'Gasaway' resistance gene has been very useful for the early selection of eastern filbert blight resistant plants. Unfortunately, one commonly used pollen parent, OSU 509.064, from the OSU breeding program transmits a band, indistinguishable from one that we use to select resistant genotypes, to all of its progeny. Early selection against susceptible genotypes is important in the breeding program to decrease expenditures of vital resources such as land and labor. Thus, optimization of the marker-assisted selection procedure is a worthwhile endeavor.

Introduction

RAPDs lend themselves to the high sample throughput (Williams et al., 1990) that is nearly mandatory in breeding programs. RAPD markers are well-suited to marker-assisted selection in woody fruit crops (Weeden et al., 1994) and they are especially fitting for the selection of a dominant resistance gene in the absence of the pathogen (Mehlenbacher, 1995a). Although RAPD markers are dominant, this is not problematic in our situation because the gene from 'Gasaway' is dominant and heterozygotes display immunity to eastern filbert blight. One limitation is that in some crosses, the expected segregation is not found. For example, in the cross in which the linkage was established, the marker may co-

segregate 1:1 with a phenotype. With the use of OSU 509.064, the polymorphism may be lost and all progeny will amplify a band of the same size as that which was polymorphic in the original cross. The informative band will be indistinguishable from the artifact band because it is of the same size.

By cloning the sequence of the band amplified by genotypes that cause aberrant patterns, and comparing them to the sequence of the polymorphic band one can identify restriction enzymes that will differentiate the two cases. Digestion with a restriction enzyme that cuts at a unique site within the artifact band will resolve it into two smaller bands and cause its disappearance (Caetano-Annollés, 1993). A similar approach has been described by Janssens et al. (1995) for distinguishing alleles of a self-incompatibility gene in apple (*Malus* x*domestica* Borkh.) where allele-specific primers were synthesized based on cDNA sequences and the resulting PCR product was subjected to restriction enzyme digestion. This allowed for distinction between different alleles. Sakurai et al. (1997) performed similar work for the same purpose.

Materials and Methods

DNA was extracted from an apical meristem and first leaf from each tree. The samples were processed within one hour from their collection DNA extractions were executed as specified in Davis et al. (1998) with minor modifications (Appendix A). PCR, marker cloning, and sequence analysis were performed as in Chapter 3.

Results

The cloned fragment amplified by UBC 152_{800} from OSU 509.064, showed almost no homology to 'Gasaway' or 'Zimmerman' (Figure E.1 and Figure E.2, respectively.) The following six restriction enzymes cut the UBC 152_{800} RAPD fragment from OSU 509.064 but not the band amplified by the cultivars 'Gasaway' or 'Zimmerman' (Table E.1).

Table E.1 Restriction enzymes that cut UBC 152 RAPD marker from OSU 509.064 but not marker in 'Gasaway' or 'Zimmerman'

Restriction enzyme	Cut site
AceIII	CAGCTCnnnnnnn'nnnn[Z,Y]
BmgI	GKGCCC[X]
Bsu36I	CC'TnA_G [W]
DdeI	C'TnA_G
BanI	G'GyrC_C
BciVI	GTATCCnnnnn_n'

[Z]Apostrophe marks cut site
[Y]n = Means any nucleotide
[X] K = Thymidine or Guanosine
[W]_ = Indicates that opposite strand is cut

Figure E.1 Sequence homology between UBC 152_{800} band from 'Gasaway' (top) and UBC 152_{800} band from 509.064 (bottom). Best homology only 54 of ~800 base pairs.

```
    Gap Weight:    50     Average Match: 10.000
 Length Weight:     3  Average Mismatch: -9.000

      Quality:   132          Length:    55
        Ratio: 2.400           Gaps:     0
Percent Similarity: 60.000  Percent Identity: 60.000

  Match display thresholds for the alignment(s):
        | = IDENTITY
        : =  5
        . =  1

490 ACCCGGTCCCGATTCTCGGTTATTGACCCAAACTTTCATTTCCTCATTGT 539
    ||||    ||  |||||| | ||   ||||| | ||   | ||  | | |
378 ACCCTCATCCCTTTCTCGCTCATGTGCCCAAGCCTTAGGTGCCATAATTT 427

540 TAAGT 544
    | |||
428 TGAGT 432
```

Figure 3.2 Lack of sequence homology between UBC 152_{800} band from 'Zimmerman' (top) and UBC 152_{800} band from 509.064 (bottom). Best homology only 26 of ~800 base pairs.

```
    Gap Weight:    50     Average Match: 10.000
 Length Weight:     3  Average Mismatch: -9.000

      Quality:   137          Length:    27
        Ratio: 5.074           Gaps:     0
Percent Similarity: 74.074  Percent Identity: 74.074

  Match display thresholds for the alignment(s):
        | = IDENTITY
        : =  5
        . =  1

246 TCATTCTGATTTCACATTTTTTTTTAA 272
    || | ||||  |||  || ||||||||
589 TCCTACTGAAATCAGGTTCTTTTTTAA 615
```

Figure E.3 Sequence map of UBC 152 RAPD marker from OSU 509.064 showing restriction enzymes that only cut once

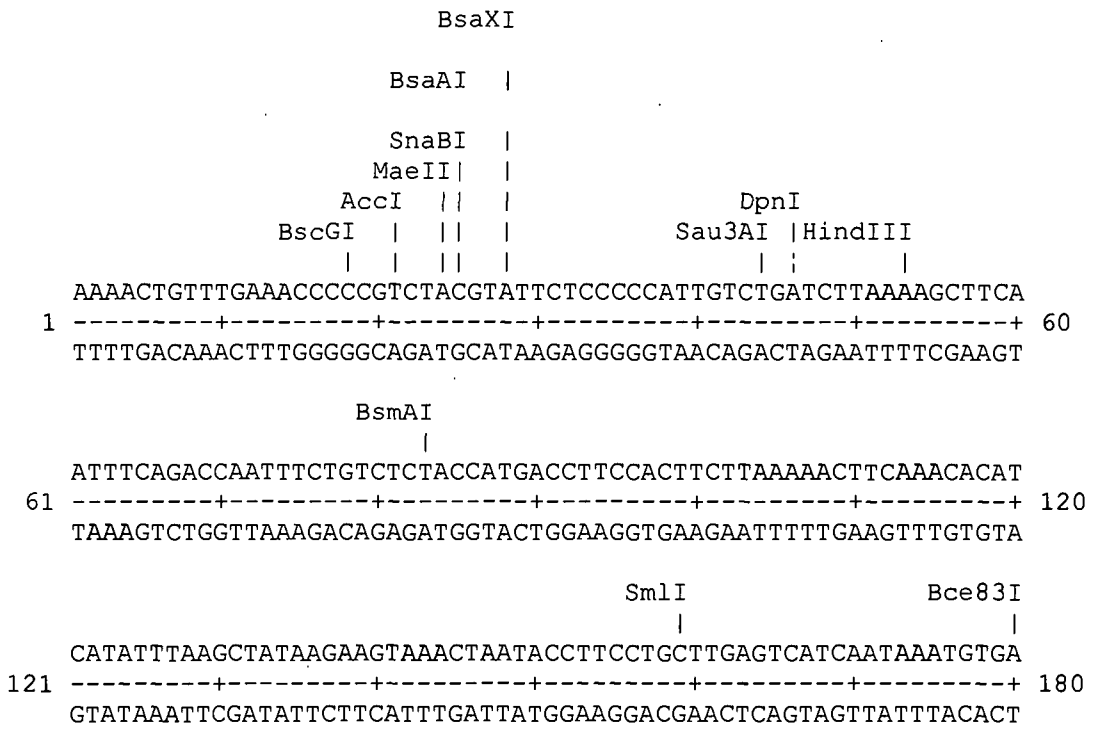

Figure E.3 Continued

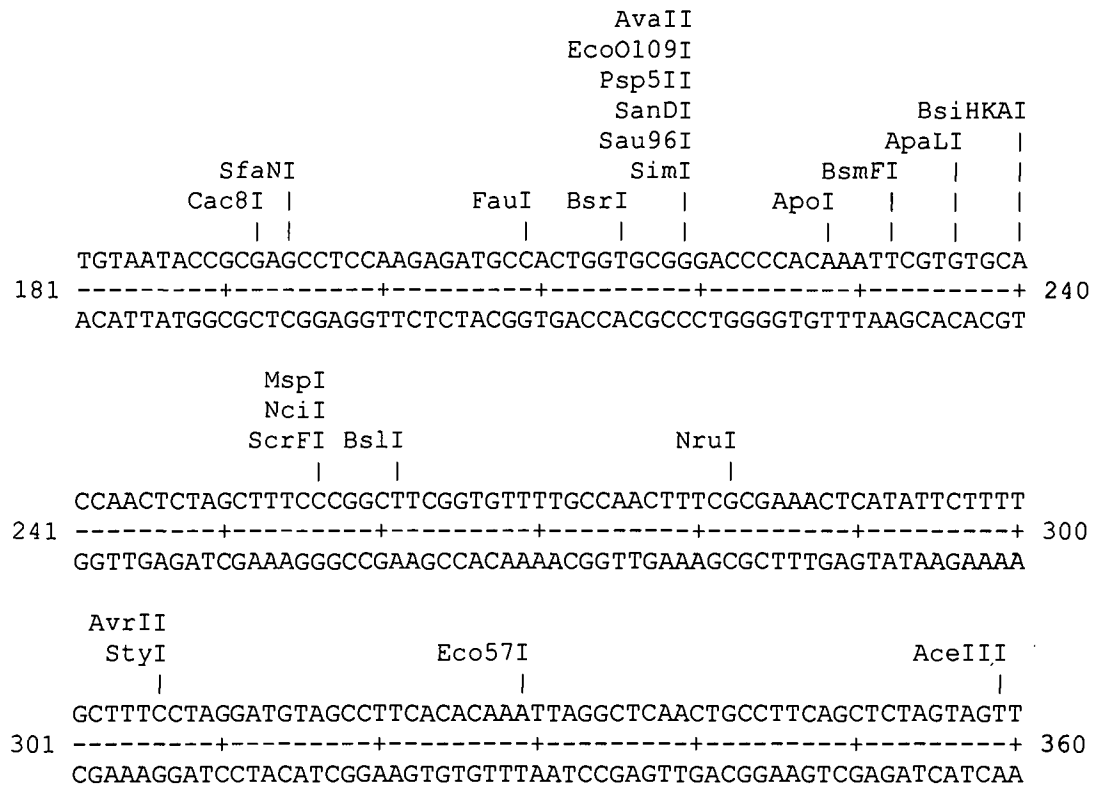

Figure E.3 Continued

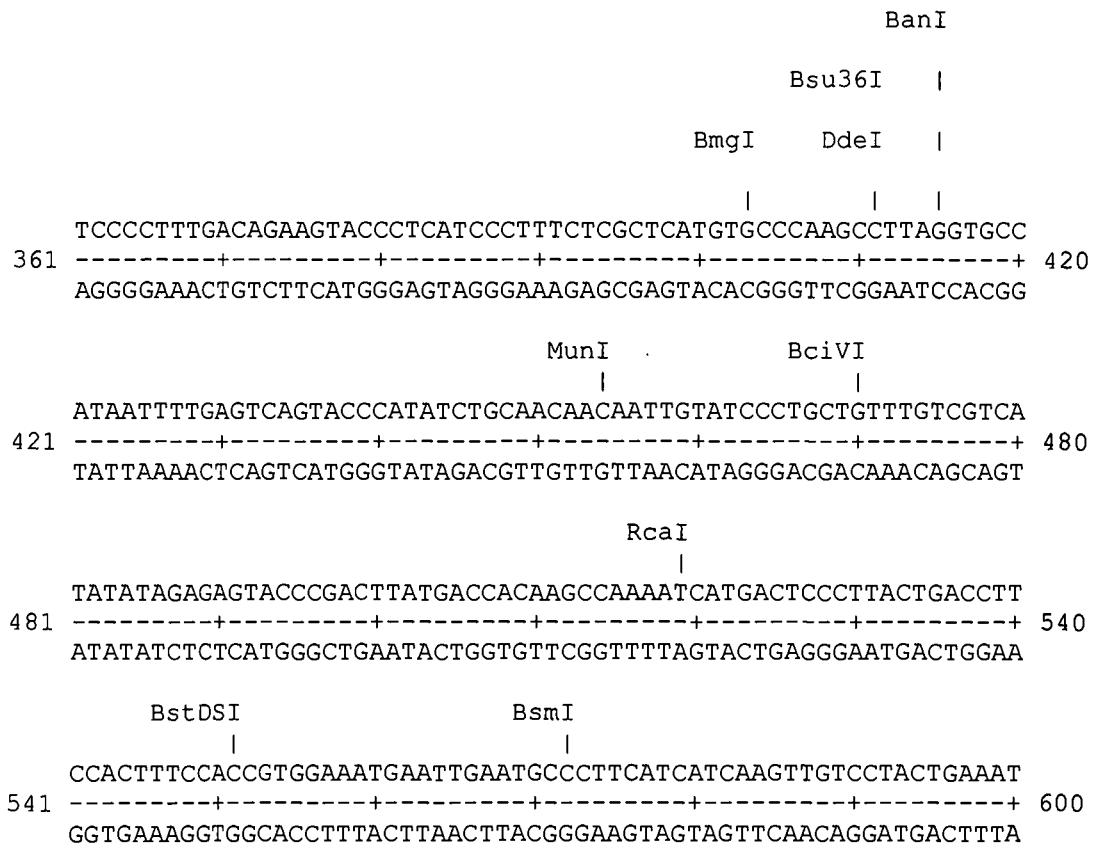

Figure E.3 Continued

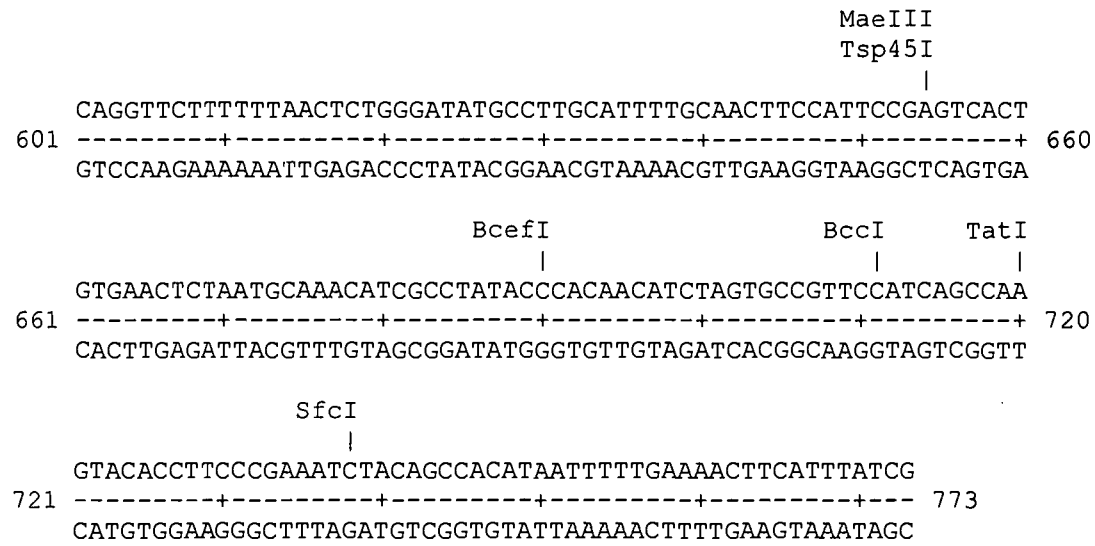

Figure E.3 Continued

Enzymes that do cut and were not excluded:

AccI	AceIII	ApaLI	ApoI	AvaII	AvrII	BanI	BccI
Bce83I	BcefI	BciVI	BmgI	BsaAI	BsaXI	BscGI	BsiHKAI
BslI	BsmI	BsmAI	BsmFI	BsrI	BstDSI	Bsu36I	Cac8I
DdeI	DpnI	Eco57I	EcoO109I	FauI	HindIII	MaeII	MaeIII
MspI	MunI	NciI	NruI	Psp5II	RcaI	SanDI	Sau96I
Sau3AI	ScrFI	SfaNI	SfcI	SimI	SmlI	SnaBI	StyI
TatI	Tsp45I						

Enzymes that do not cut:

AarI	AatII	AclI	AflII	AflIII	AhdI	AloI	AlwI
AlwNI	ApaI	AscI	AvaI	BamHI	BanII	BbsI	BbvI
BbvCI	BcgI	BclI	BglI	BglII	BmrI	BplI	BpmI
Bpu10I	Bpu1102I	BsaI	BsaBI	BsaHI	BsaWI	BsbI	BseMII
BseRI	BsgI	BsiEI	BsmBI	BspEI	BspGI	BspLU11I	BspMI
BsrBI	BsrDI	BsrFI	BsrGI	BssHII	BssSI	BstAPI	BstEII
BstXI	BstYI	BstZ17I	BtrI	BtsI	ClaI	DraI	DraIII
DrdI	DrdII	EaeI	EagI	EarI	EciI	Eco47III	EcoNI
EcoRI	EcoRII	EcoRV	Fnu4HI	FseI	FspI	GdiII	HaeI
HaeII	HaeIII	HaeIV	HgaI	HgiEII	HhaI	Hin4I	HincII
HpaI	HphI	KpnI	MboII	MluI	MmeI	MscI	MslI
MspA1I	NarI	NcoI	NdeI	NgoAIV	NheI	NotI	NsiI
NspI	NspV	PacI	Pfl1108I	PflMI	PinAI	PmeI	PmlI
PpiI	PshAI	PsiI	PstI	PvuI	PvuII	RsrII	SacI
SacII	SalI	SapI	SbfI	ScaI	SexAI	SfiI	SgfI
SgrAI	SmaI	SpeI	SphI	SrfI	Sse8647I	SspI	StuI
SunI	SwaI	TaqI	TaqII	TauI	TfiI	TseI	Tth111I
VspI	XbaI	XcmI	XhoI	XmnI			

Discussion

In crosses between seed parents heterozygous for the 'Gasaway' resistance gene and OSU 509.064 a RAPD band (UBC 153_{800}), that is usually linked to the resistance gene shows up in all progeny. Sequence differences should allow distinction between ~800 bp bands which are not linked to eastern filbert blight resistance (resulting from an artifact of the OSU 509.064 parent) and those which allow identification of plants that have the 'Gasaway' gene. This can be achieved by using restriction enzymes that cut at regions unique to one of the sequences (Figure E.3, Table 3.10). All of the enzymes listed (Table 3.10) cut about 400 bp into the fragment and so they should resolve the band into two much smaller bands that can easily seen by agarose electrophoresis. Although only 4 of ~800 base pairs differ between the (UBC 153_{800}) in 'Gasaway' and that in 'Zimmerman', this accounts for the difference in figures E.1 and E.2. The computer program used (the "Bestfit" command of GCG version 9), picks the region of greatest homology which was affected by this 4 base pair difference.