Characterizing the host response and genetic control of resistance in 'Honeycrisp' to apple scab (*Venturia inaequalis*)

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Dedication

I dedicate this dissertation to those who showed me how plants were important, interesting, and such a joy to explore. The list of those farmers and green thumbs is much too long to list here. So I dedicate this work to those who taught me to plant seeds, to graft trees, to divide rhizomes, to recognize and name different species, to put-up the bounty, and to enjoy cooking and eating the food that I produced.

Abstract

Two novel apple scab resistance loci have been identified in the apple cultivar Honeycrisp, an emerging cultivar in North America that is utilized in apple breeding programs worldwide. Greenhouse inoculation experiments with the apple scab fungal pathogen, *Venturia inaequalis,* identified a resistance defense response in 'Honeycrisp' and its ancestors 'Keepsake', 'Frostbite', and 'Northern Spy' seven days after inoculation. The defense response ranged from necrotic and chlorotic lesions to stellate necrosis and class 3b lesions (with sporulation). A hallmark of the resistance defense response is autofluorescence at the infection site in cleared leaf tissue. Several 'Honeycrisp' progeny populations were screened with monoconidial isolates of *V. inaequalis* and segregated 3:1 for resistance, suggesting two resistance genes inherited from 'Honeycrisp'. A consensus 'Honeycrisp' linkage map with 1091 SNP markers was constructed for use in mapping. Two resistance loci were mapped using linkage mapping and quantitative trait loci mapping approaches. Marker haplotypes were constructed to trace the inheritance of resistance loci. *Rvi19* mapped on linkage group 1 at ~50 cM. In the *Rvi19* haplotype, the 138 bp allele for the Ch-Vf1 marker cosegregates with resistance, and is identical by state (IBS) with the *Rvi17* resistance in 'Antonovka'. *Rvi19* is transmitted from 'Frostbite' to 'Keepsake' to 'Honeycrisp', and into the resistant progeny of 'Honeycrisp'. The other locus, *Rvi20*, mapped onto linkage group 15, and is IBS with a marker haplotype found in the susceptible cultivar Golden Delicious. *Rvi20* is transmitted to 'Honeycrisp' from an unknown parent. Molecular marker haplotypes were used to identify advanced selections in the University of Minnesota breeding program

with pyramided scab resistance. Candidate genes were identified at each haplotype that can serve as starting points for identifying the functional genes conferring resistance. A collection of *V. inaequalis* isolates was assembled and curated from six locations in Minnesota for screening scab resistance. These 80+ isolates were examined for genetic diversity and population structure and provide a snapshot of the diversity of the pathogen present at this time.

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Chapter 1

Introduction

Apple scab, caused by the fungal pathogen *Venturia inaequalis* (Cke.) Wint. (anamorph *Spilocaea pomi* Fr.), is the major disease affecting apples grown in temperate climates around the world. This disease can have a significant economic impact as diseased fruit are not marketable. Complete crop loss is possible if steps are not taken in the orchard to reduce infection. Regular fungicide application of up to 10-15 per season is the most effective ways to limit disease (Tartarini et al., 1999). Fungicides increase production costs and also lead to concerns over environmental pollution, health concerns for growers, and pesticide residues in foods for consumers (Ellis et al., 1998; Broggini et al., 2010). Organic management approaches are aimed to control the disease and include sanitation practices which include shredding leaves on the orchard floor in the fall or early spring to limit the disease pressure and thus the number of fungicide applications (Sutton et al., 2000). The deployment of durable scab resistant cultivars also offers growers sustainable options for reducing infection in an orchard.

Apple scab and apple have existed in concert through the domestication and spread of apple around the globe where the pathogen was able to infect other species such as *Malus sylvestris* in Europe (Gladieux et al, 2010) and N. American species such as *M. ioensis*. Paintings that depict apple fruit with scab lesions are known from Europe and American artists. A Renaissance painting called 'The Fall' (Hugo van der Goes c. 1468) shows Eve selecting an apple for Adam from a tree with obvious scab lesions on the fruit (Ameson, 1998). The still-life images 'Basket of Fruit' (c. 1596 by Michaelangelo Mersis Caravaggio) and 'Fruit still life with Chinese export basket' (c. 1824 by James

Peale) show infected apples as part of exquisite displays of grapes and other fruits.

The tolerance of blemishes, the lack of understanding of germ theory of disease, and the lack of effective controls would have allowed the disease to proliferate and move with humans throughout the domestication of apple. Prior to 1850, apples were not grown on large orchards as they are today due to economies of scale (Hancock et al., 2008). Europeans had brought apple seeds with them to North America, which created a wide diversity of apple germplasm in the United States due to its highly heterozygous nature. Locally adapted cultivars were grown in mixed orchards. This increased diversity may have reduced disease pressures due to host specificity. The planting of mixed cultivar orchards would impart differential resistance, thus influencing the adaptation of local *V. inaequalis* strains, slowing down the spread of highly virulent strains (Sierotzki et al., 1994). In America, apple seedlings were planted as part of the requirements for homesteading, further diversifying apple across the landscape, and leading to unique, chance seedlings which became cultivars that are still important today.

Apples were generally grown for fresh eating, baking, cider, and hog feed. The latter two for which scab blemishes would have been tolerated without concern for long-term storage. Apples that were packed and shipped had to be free of disease, as we all know that ancient idiom, "a rotten apple spoils the whole barrel." MacHardy (1996) points out that the first reports of apple scab most likely coincided with apple becoming a more important horticultural crop, with orchards of a limited number of susceptible cultivars being planted on the landscape. As Americans moved off farms and into cities, in conjunction with the development of rail transport and refrigeration, the need for large

scale production with high quality, uniform apples required interventions to reduce disease (Goland and Bauer, 2000). Consumers, no longer connected with growing their own food, were requiring high standards that needed to be met through the development of new cultivars, management practices, and disease control. These market pressures led to a great reduction in the number of cultivars grown, many of which had no scab resistance.

The Morrill Acts of 1862 and 1890 established land-grand colleges in the United States which provided public education in the field of agricultural research. The Hatch Act of 1887 further supported this mission through the establishment of experiment stations to study farming practices to meet State needs and educate farmers. These universities were charged with developing cultivars adapted for local conditions and in studying their management practices which included disease control. The coincidental timing of disease pressure on the landscape and a mechanism to study the disease has provided over 100 years of research in the apple-apple scab pathosystem. This includes cultural practices for disease management that remove inoculum (sanitation, leaf litter removal) and alter the growing environment (pruning). A number of fungicides and biocontrols have been utilized since the early 1900s to prevent infection and to reduce inoculum pressures. Additionally a number of tools have been developed for disease monitoring with models for forecasting infection as a way to effectively time scab control treatments (Mills, 1944). For a comprehensive exploration of these topics see MacHardy (1996).

The Host: Apple

Considered as the most important temperate fruit crop (Celton et al., 2008), cultivated apple (*Malus x domestica* Borkh.) production exceeded 75.48 million metric tonnes in 2011 worldwide (FAOSTAT, 2013). Losses from apple scab generally come from the reduction in fruit quality (Figure 1); however, infection can also effectively reduce overall fruit set. Apple scab can adversely affect the growth of young plants resulting in long-term structural damage. Severe infections can result in defoliation, a reduction in fruit bud formation, and a reduction in overall plant health and vigor. Severe infections with very visible symptoms can readily be identified in ornamental crabapples in the landscape that are generally not treated with fungicides.

New growth on apples is the most susceptible to infection, with timing of bud break coinciding with or just after ascospore release in the early spring, especially in warmer climates (Alves and Beresford, 2013). New vegetative (shoot) growth in apples persists well into the growing season which provides a supply of highly susceptible tissues for new infection sites for several months when environmental conditions favor the disease. Older leaf tissues demonstrate ontogenic resistance, but this can break down in the fall as senescing leaves again become susceptible and these lesions are thought to provide the most inoculum for the next season (MacHardy, 1996).

The development of disease resistant apple cultivars is intended to reduce the number of inputs required in disease management and is one of the major goals of most breeding programs. Very few apple scab resistance (R) genes have been identified in the cultivated apple. At the end of the 19th Century, variation for resistance in apple cultivars

was first observed and the hypothesis was formed that physiological races could be characterized based on the type of resistance reaction observed in the field (Aderhold, 1899). In the mid-20th Century, apple breeders in the United States began examining collections of related *Malus* spp. (crab apples) for field resistance to apple scab following major disease out breaks in 1943 (Janick, 2006). In North America, apple breeders from Purdue, Rutgers, and Illinois (PRI) have cooperatively developed a number of commercial cultivar releases with scab resistance (Crosby et al., 1992; Janick, 2006). Other scab resistance cultivars have come out of Cornell University (e.g. 'Freedom' and 'Liberty'), Nova Scotia (e.g. 'Nova Easygro', 'Novamac'), and Ontario (e.g. 'Murray') breeding programs.

Early research conducted at PRI on scab resistance identified a number of selections of *Malus* species and hybrids that displayed field resistance at research stations throughout the United States (Shay and Hough, 1952). These presumed resistant accessions were evaluated in the field and in greenhouse experiments to explore the host-pathogen interaction, and all accessions showed some level of resistance. These *Malus* species, including *M. baccata*, *M. floribunda M. hupenhensis, M. micromalus, M. prunifolia*, were implicated for use in fruit cultivar development, and several were utilized for introducing the resistance factors (i.e. the R genes) into commercial breeding programs for the development of interspecific hybrid cultivars (Shay and Hough, 1952). These resistant cultivars have not been widely accepted because of their lower fruit quality, but they remain important in breeding programs (Crosby et al., 1992). Related *Malus* species, especially those collected from the center of diversity, are important

resources which provide breeders with the unique genetic material for bringing in important R genes to scab (Luby et al., 2001; Bus et al., 2002) and fire blight (*Erwinia amylovora*) (Luby et al., 2002) not found currently in breeding programs.

The Pathogen: *V. inaequalis*

The *Malus*-*Venturia inaequalis* pathosystem is one of the earliest examples for which the gene-for-gene interaction was first described (Williams and Shay, 1957; Boone, 1971). Flor (1956) previously described the classic gene-for-gene model in the flax (*Linum usitatissimum*)-flax rust (*Melampsora lini*) pathosystem. The model defines the incompatible interaction in which the *R* locus is present in the host and the cognate *Avr* locus is present in the pathogen, resulting in a host phenotype disease resistance response (Dangl and Jones, 2001). When a spore lands and initiates an infection site on the plant, the presence of the Avr protein is recognized by the corresponding R protein which then triggers a defense cascade. Pathogen *Avr* genes can become virulent against the host through mutational selection pressures that result in avoiding recognition by the R proteins (Ellis et al., 1999; Abramovitch et al., 2006). The deployment of cultivars with single R genes has led to identification of new apple scab races (Races 6 and 7) which can overcome the cognate R gene (Parisi et al., 1993; Bénaouf and Parisi, 2000).

A number of excellent reviews (Gessler et al., 2006; Jha et al., 2009; Bowen et al., 2011) and an extensive monograph (MacHardy, 1996) describe the *Malus* - *V. inaequalis* pathosystem. An abbreviated diagram depicts the life cycle of the pathogen (Figure 2). *Venturia inaequalis* is a hemi-biotrophic ascomycete that produces both sexual spores (ascospores) and asexual spores (conidia) which are capable of causing infection on apple

trees referred to as scab or black spot. The infection begins each spring with the release of ascospores from pseudothecia (fruiting bodies) which have over wintered in leaf tissue on the ground from the previous season (MacHardy, 1996). The ascospores are the result of sexual reproduction between compatible mating types that had colonized the same host leaf, and overwintered in the dead leaf on the orchard floor. This sexual reproductive strategy with inherent host specificity can lead to ecological divergence, especially in the presence of resistance genes in the population (Gladieux et al., 2011; Leroy et al. 2013).

Ascospores are released from the asci into air currents that move the ascospores relatively short distances to the host plant where the primary infection occurs on young, emerging leaves and sepals. The scab lesions that develop from the primary inoculum produce conidia which are able to cause secondary infections. The conidia are dispersed in wet weather by wind and splashing rain leading to infection that can spread throughout the orchard on leaves, stems, and fruit. The polycyclic, secondary infection can persist throughout the growing season and into leaf senescence. Leaf senescence may be premature in heavily infected trees and leaves. In some cases, conidia are able to over winter on the tree on bud scales or on shoots which serve as the source of primary inoculum in the following spring without undergoing sexual recombination.

An infection site results from the penetration of a germinating spore (ascospore or conidia) into the cuticle of the leaf or immature fruit. In order to cause an infection, the spore adheres to the wet surface of the cuticle, germinates, and develops an appressorium where the fungus produces enzymes (cutinases) which hydrolyze the cuticle. The spore may also penetrate the cuticle directly without an appressorium (Smereka et al., 1987).

The fungus grows and develops into a network of hyphae through the subcuticular stroma from which it is able to derive nutrients from the host plant. In about 10-14 days, conidiophores develop from these hyphae. The conidiophores push through the epidermis and release the asexual spores, the conidia. Lesions also form on young fruit which can cause deformation, fruit drop, or blemishes that result in unsalable fruit. The young, infected fruit may drop because the infection distorts the fruit and prevents normal development (Wilcox, 2001).

The symptoms on leaves vary depending on the host-pathogen interaction. There are several known races (8) of the pathogen and numerous resistance factors (R genes, and polygenic resistance) found in the host. The classic symptoms in the compatible reaction include circular lesions that are green, necrotic, or chlorotic on the leaf surface. The margin may or may not be defined, and the lesion may change color as the older portion in the middle of the lesion and supporting leaf tissue dies. Sepals and pedicels may also be infected with similar symptoms. Symptoms may also include the presence of dark mycelial growth on either adaxial or abaxial leaf surface or fungal growth along the lateral and mid veins. Several phenotyping protocols have been utilized to describe the infections classes. The most widely utilized scale is based on a 0-4 rating that describes infection on spray-inoculated seedling leaves in greenhouse studies (Chevalier et al., 1991). Other scales attempt to quantify the proportion of leaves and fruits infected (Croxall et al., 1952a,b; Lateur and Populer, 1994). New high throughput phenotyping techniques like quantitative real-time PCR may offer improved quantification of infection to increase the precision in apple resistance QTL mapping (Gusberti et al., 2012). On

fruit, the disease symptoms are dark colored lesions with sharp, brown borders, or corky lesions. Mature fruit may have black spots called pin-point lesions which may develop in storage (Tomerlin et al., 1983).

Collection and Curation of Fungal Isolates

Single spore isolates are the preferred source of inoculum in screening seedling apple progenies for disease resistance in order to assess the gene-for-gene interaction with a range of pathogen races (and cognate *Avr* genes) when available. Although some breeders and pathologist have collections of diverse, typed isolates, these were not available for the current project due to regulatory limitations in shipping and transporting the pathogen.

Fruit and leaf lesions provide the conidia necessary for the initiation of spore culture. The requirements for culturing and studying the pathogen have been well described by Boone (1971). Briefly, air dried lesions are suspended in a small volume of water to release spores. These are diluted and then plated on 3% agarose plates for 8-24 h. The conidia germinate during this time and can be identified from other fungal spores under a microscope. The conidia are individually replated to a nutrient media such as potato dextrose agar and allowed to colonize and grow to ~1 cm (Barbara et al., 2008). Colonies can be subcultured, frozen, or washed to release spores for inoculation of leaves (see below) or placed on sterile filter paper for storage. Filter papers can be colonized on top of the nutrient media, air dried, and stored at -20º C (Bus, pers. communication, 2010). Similarly, cellophane sheets placed on nutrient media can be inoculated with a monoconidial suspension and used for increasing conidia for use in leaf inoculations or

long-term storage (Parker et al., 1995).

Inoculation and Disease Screening

Apple seedlings can be screened for resistance as early as the 2-leaf stage (Lamb and Hamilton 1969). Larger plants (6 leaves) are preferred as they are less likely to be permanently damaged or killed by the infection. For genetic studies, leaf material is needed for DNA extraction and sufficient leaf material may not be available at early stages. Greenhouse screening allows the researcher to elucidate resistance classes, whereas field screening typically can only detect differences between resistant or susceptible genotypes (Gardiner et al., 1996).

Field resistance can be screened using both natural and applied inoculum (Liebhard et al., 2003c). Assessment tools have been developed to quantify the amount of disease on a leaves and fruit in field experiments (Croxall et al., 1952a,b). These and other scales have been modified to describe infection on whole trees, but give no indication of a specific resistance reaction (Lateur and Populer, 1994; Lefrancq et al., 2003). The pathogen population in the field may be diverse and the specific gene-for-gene interaction cannot be resolved in field experiments.

Spray inoculation with *V. inaequalis* isolates or mixtures of isolates is useful to assess resistance and segregation patterns. Spray inoculations require large amounts of inoculum and often a second application to test escapes. Seedlings are inoculated in the greenhouse at a concentration of 1×10^5 conidia ml^{-1}. Plants are incubated in the greenhouse for 2 weeks at 20º C daytime temperatures and 16º C nighttime, with 95% relative humidity (Gardiner et al., 1996). After the incubation period, plants are scored

for the resistance reaction. Escape plants, i.e. those showing no symptoms of disease or resistance reaction, are then re-inoculated. Susceptible leaves with sporulating, monoconidial lesions can be collected, dried, and stored at -20 °C for future inoculation experiments, or recultured on nutrient media.

An additional downside to spray inoculation is that susceptible seedling plants may not be able to recover from some infections, thus only one isolate may be tested per progeny. Furthermore there may be bias between field and greenhouse studies (Lefrancq et al., 2003). Larger progeny populations (of the same cross) and more space are required to assess different isolates.

Bus and colleagues (2005a) have developed a method of droplet inoculation in which multiple races/isolates can be screened for pathogenicity on the same leaf. The method can then be repeated on emerging leaves for numerous replications, testing different isolates, or to re-test escapes. The droplet technique consists of a small inoculation chamber constructed from a microcentrifuge tube cap. The cap has an 8mm diameter, and a 2 mm hole bored into it. Two chambers are mounted to an alligator hairclip. The chambers are clipped to the adaxial leaf surface of the newly expanding leaf and 100 µl inoculum suspension (concentration ~ 4×10^4 conidia ml^{-1}) is pipetted into each chamber. The plants are incubated for 48 h in high humidity (100% when possible) to ensure that the inoculation suspension in the chambers does not evaporate. After 48 h, the chambers are removed and the plants are grown in 80% rh for 2-3 weeks. After several days/weeks, the newest leaves on the same plant can be utilized for droplet testing without concerns of contamination or adversely affecting previous symptom

development. At inoculation, each leaf can be tagged with identifying information for organizational purposes.

In addition to whole plant sprays and single leaf droplet assays, researchers have utilized other techniques to evaluate greenhouse, field, and in vitro grown plants. These include detached-leaf assay tissue culture leaves (Yepes and Aldwinckle, 1993), leaves from whole plants (Nicholson et al., 1973; Schenato et al., 2008), or the use of leaf discs (Martinez-Bilbao and Murillo, 2005).

Defense Response in Apple

Ontogenic resistance to apple scab is shown in most apple cultivars and species. Immature leaves are the most susceptible to infection. The young leaves are succulent and create a suitable microclimate for infection. Matured leaves demonstrate resistance due to strengthened cell walls and membranes, and changes in subcuticular pH (MacHardy, 1996). The rate of spore germination and appressoria development is the same between young and old leaves, but stroma development is progressively slower in older leaves (Gessler and Stumm, 1984).

In resistant and susceptible hosts, the initial stages of infection are similar, including spore germination, appressoria development, penetration of the cuticle, and hyphal development (Nicholson, 1977; Komjanc et al., 1999). Following the development of an infection site, the resistance response will fall on a continuum from complete resistance to complete susceptibility which depends on the pathogen race, resistance genes in the host, and incubation conditions (Shay and Hough, 1952).

Complete resistance conditioned by the hypersensitive response (HR) in apple is

known to be conditioned by several previously described R genes including *Rvi4*, *Rvi5* *Rvi7*, and *Rvi15* (Win et al., 2003; Galli et al., 2010; Bus et al., 2011). This "pin-point" reaction results from the rapid death and subsequent collapse of cells immediately surrounding the penetration site of the spore (Goodman and Novacky, 1994). The HR is generally visible as small depressions on the leaf surface (Nicholson et al., 1973). The HR observed in *Malus-Venturia* pathosystem has varied rates of reaction (2 to 11 days) depending on the R gene present (Shay and Hough, 1952; Galli et al., 2010; Bowen et al., 2011). The HR prevents colonization through programmed cell death (PCD) and may involve cell to cell signaling mediated in part by reactive oxygen species. Not all components of the PCD are known but various phytoalexins and the accumulation and oxidation of other phenolic compounds (phloridzin and phloretin) have been implicated in the HR (Nicholson, 1977). These compounds fluoresce under ultraviolet light which make them hallmarks in characterizing genetic resistance responses in cleared leaf tissue in apple and other genera (Vanderplank, 1982). In addition to PCD, apple plants produce phenolics and other compounds in response to the pathogen and can reduce germination and growth of *V. inaequalis* (Jha et al., 2009). Pathogenicity proteins (PR) have also been implicated in the resistant cultivars with chemical defenses like β-1,3-glucanase, chitinase, and cystein protease which attack the fungus itself (Gau et al., 2004).

In addition to HR, the range of macroscopic resistance reactions in apple to inoculation with *V. inaequalis* have been characterized by Chevalier et al. (1991) and are described as no lesions (0), HR (1), chlorotic lesions (2), necrotic/chlorotic lesions with

slight sporulation (3a-weak resistance), necrotic/chlorotic lesions with sporulation (3b-weak susceptibility), stellate necrosis (3-SN), and lesions with high sporulation (4).

The effectiveness of a plant defense system requires that the plant be able to detect the invasion of a pathogen. The development and evolution of avirulence genes in the pathogen is in direct response to selection pressure to avoid detection by the plant R protein. Many of the cloned R genes in plants are in a gene family which encodes for leucine rich repeats (LRR) and a nucleotide binding site (NBS; Baldi et al., 2004). It is proposed that the NBS-LRR genes act as receptors in the signal transduction pathway in response to the pathogen (Michelmore, 1995; Hammond-Kosack and Jones, 1997). By using an NBS-profiling technique, which preferentially uses NBS specific polymerase chain reaction (PCR) primers to develop markers polymorphic in a population, Calenge et al. (2004) identified R gene analogues for apple scab and mildew. Many of the R gene analogs mapped close to known major genes and QTL. At the *Rvi6* locus, the four resistance gene paralogs lack the NBS domain, but have unique LRR and transmembrane domains (Xu and Korban, 2002).

R genes are known to exist in clusters or R gene complexes throughout plant genomes (Young, 2000). Recombination (along with gene conversion) is proposed as one way in which new genetic variation is developed. The sequence similarity within a gene family, especially within a gene cluster, results in unequal crossing over. The misalignment during meiosis results in new haplotypes and alleles that may have a new specificity for monitoring pathogen effectors. Although it is technically possible for allelic diversity to be generated in this way, it appears that this occurs at a low rate and

varies among gene families (Hulbert et al., 2001). Allelic variation has been shown in maize due to recombination in existing *Rp1* paralogs resulting in new race specificity (Smith and Hulbert, 2005). This new variation can be observed in the first generation and is due to meiotic instability at these loci. The LRR domain appears to be under the most diversifying selection and also has the most changes in residues that are thought to interact directly or indirectly with pathogen effector molecules. Duplication events within the LRR domain can lead to mispairing and cycles of shorter and longer domains with their own specificity to effector molecules (Ellis et al., 2000).

Mutations, especially in the LRR region, may result in allelic variation for specificity in effector recognition (Ellis et al., 2000). Although no specific example is given, the sequence comparisons show that point mutations can result in new variants (Hulbert et al., 2001). Transposons have been proposed as a source of novel variation in resistance genes. Transposons may result in ectopic duplication events that allow for novel functions to evolve or whose function is changed by its new location in the genome when influenced by 'new' enhancers and promoters (Michelmore and Meyers, 1998). Insertions of transposons into a resistance gene may result in its gain or loss of function. The splicing of a transposon from a genic region may restore function in that gene. Somatic recombination may be a source of novel variation, especially in long-lived crops (McDowell and Simon, 2006). A portion of a tree may have an increased fitness advantage with these somatic variations (Michelmore and Meyers, 1998). These anomalies can only be transmitted if the tissue type leads to offspring. In apples, somaclonal variation is known to occur for a number of traits and can be induced in tissue

culture. Since apples are clonally propagated, these traits can be perpetuated through grafting. Somatic variation has been suggested as one way in which the *Vf* locus has diversified following duplication events as there is no support for the aforementioned exchange of sequences and mispairing (Xu and Korban, 2004).

Genetics and Mapping Approaches

The genetics involved in the inheritance of a single dominant major R gene is straightforward, following Mendelian segregation. Scab susceptible individuals are homozygous recessive for major gene resistance. The expected segregation ratio in the F_1 generation between Rr × rr is 1:1 (resistant:susceptible) and 3:1 in $R_1r_1r_2r_2 \times r_1r_1R_2r_2$ or $R_1r_1R_2r_2 \times r_1r_1r_2r_2$ (resistant:susceptible) (Crosby et al., 1992; MacHardy, 1996; Figure 3). Further, two resistant siblings can be crossed to determine if they are heterozygous (Rr) or homozygous (RR) for resistance. Resistant plants from the sibling cross may be backcrossed to the susceptible parent to determine if the resistant parent is hetero/homozygous. Crosses can be made to determine if multiple non-allelic major genes are involved in resistance, or in determining if different sources of major resistance correspond to the same locus (MacHardy, 1996).

Determining that resistance is conferred by a single gene (or a cluster of genes inherited as a single Mendelian factor) is also straightforward and is done by making the cross of a resistant parent × susceptible parent and screening seedling material with *V. inaequalis* isolates and evaluating phenotypic segregation. Mapping the R gene, detecting quantitative trait loci (QTL), and determining marker-trait associations can have utility for plant breeders for marker assisted breeding (MAB) (Liebhard et al.,

2003a).

Due to its highly heterozygous genome, high levels of inbreeding depression, and self-incompatibility, genetic studies in apple can be quite limited (Lawson et al., 1995). Pseudo test-cross populations are one method for linkage map construction (Maliepaard et al., 1997). This method takes advantage of the breeding approaches utilized in cultivar development instead of developing large and complex mapping populations. Association and pedigree-based approaches also offer apple breeders different tools in studying the genetics of complex traits (Rosyara et al., 2013).

Genetic mapping using bi-parental mapping populations is common in apple genetics, especially in developing molecular markers for monogenic traits like disease resistance (Tartarini and Sansavini, 2003; Schenato et al., 2008). A number of linkage maps have been developed to detect quantitative loci (QTLs) and genes for a range of important traits including resistance to apple scab and other diseases, acidity (*Ma*), and growth habit and developmental traits (Lawson et al., 1995; Maliepaard et al., 1998). The *Vf* gene has been more finely mapped using increased marker density (Xu & Korban, 2000; Gardiner et al., 1996) and a bacterial artificial chromosome (BAC) library utilized for positional cloning of the gene (Xu et al., 2001). More recently the apple genome was sequenced, providing researchers with additional genomics and genetics tools for studying apple (Velasco et al., 2010).

Bulked segregant analysis (BSA) is a useful tool in identifying markers from a bi-parental segregating population for a single dominant gene (Michelmore et al., 1991). DNA from resistant genotypes is pooled as well as a second pool of DNA from

susceptible genotypes. Polymorphisms that are observed between the pools are assumed to be linked to the loci for resistance. The pools are assumed to be equivalent for all other genes. Instead of using a bi-parental mapping population, Yang et al. (1997) used pools of susceptible and resistant cultivars in a BSA approach to identify the R gene *Vf*. A genome scanning approach with evenly spaced markers was used to screen resistant plants in a population to detect linked simple sequence repeat (SSR) DNA markers (Patocchi et al., 2005). Hyten et al (2009) utilized a high-throughput, single nucleotide polymorphism (SNP) array to map soybean rust resistance in soybean using a BSA approach. BSA can reduce the number of samples submitted for genotyping services such as arrays by pooling the DNA.

Known Resistance Genes

To date, twenty major R genes imparting scab resistance have been identified, many of which have been mapped (Table 1; Boone, 1971; Gessler et al., 2006; Jha et al., 2009; Bus et al., 2011). The first gene implicated in resistance to apple scab was identified among several *Malus* species [*M. floribunda* 821, *M. prunifolia* 19651, *M. atrosanguinea* 804, *M. baccata* (Hansen's #1), *M. microcarpa*, and *M.* sp. MA 1255] that were heterozygous for the same resistance allele, *Vf* (referring to *Venturia* resistance in *M. floribunda*) (MacHardy, 1996). The first attempt at introgressing this gene was in a cross between *M. floribunda* 821 and 'Rome Beauty' initially made in 1914 (Crandall, 1926). This cross serves as the basis for most of the modified backcross populations in scab resistance breeding. Known as *Vf* (*Rvi6*) this allele has been utilized in apple breeding programs worldwide with over 70 cultivars released, although none are planted

extensively (Janick et al., 1999). The *Vf* allele offers strong resistance to races 1-5 of *V. inaequalis* (Lespinasse et al., 2002). However, the use of *Vf* resistance in cultivars quickly resulted in emergence of a race (6) that can overcome this gene (Parisi et al., 1993). The new race is not able to overcome the resistance in *M. floribunda* 821 which suggests that *Vf* is a single gene and that another gene *Vfh* (*Rvi7*) in the original source has since been lost in cultivar development and segregates independently (Parisi et al., 1993; Bénaouf and Parisi, 2000).

Vf has been mapped by several research groups using different genetic marker platforms and populations. Gardiner et al. (1996) constructed a linkage map of ~28 cM consisting of 8 markers flanking *Vf*. King et al. (1998) used 24 molecular markers [isozyme, random amplified polymorphic DNA (RAPD), and restriction fragment length polymorphism (RFLP)] linked to the R gene to develop a linkage map that covers 54 cM containing the *Vf* gene in the cultivar Prima. Maliepaard (1998) mapped *Vf* with markers spanning the genome and confirmed that *Vf* mapped to linkage group 1. Additional, reliable sequence characterized amplified regions (SCAR) markers were developed which were closely linked to the *Vf* gene (Tartarini et al., 1999) which are useful in selecting genotypes containing multiple scab resistance genes. Xu and Korban (2000) saturated the *Vf* region to identify closely linked amplified fragment length polymorphism (AFLP) markers. Closely linked markers can be utilized to identify just the *Vf* gene and select against closely linked, deleterious genes responsible for the genetic drag which limits cultivar development (Tartarini, 1996). Other markers have been developed to improve marker order and examine introgression of *Vf* in *Malus* accessions

and cultivars (King et al., 1999).

In addition to mapping *Vf* and developing tightly linked markers, a map based positional cloning approach was suggested for *Vf* (Patocchi et al., 1999). Using a BAC library from *M. floribunda* 821, contigs spanning the *Vf* region were constructed (Xu et al., 2001). From the BAC library a cluster of 4 R gene paralogs was identified encoding the LRR domain, a receptor motif common in disease resistance genes (Xu and Korban, 2002).

Derived from *M. atrosanguinea* 804 (as well as *M. micromalus*), *Vm* (*Rvi5)* confers resistance to *V. inaequalis* races 1-4. A closely linked sequenced-tagged site (STS) marker was developed from a mapping population and was then used to identify *Vm* in other *Malus* spp. (Cheng et al., 1998). Using a genome scanning approach, the *Vm* gene was mapped to linkage group 17 and a tightly linked SSR marker was identified (Patocchi et al., 2005). Markers were developed and mapped for R genes *Va* (*Rvi17* from 'Antonovka') and *Vb* (*Rvi12* from *M. baccata* Hansen's #2) which are useful in distinguishing these from other markers that map closely to *Vf* (Hemmat et al., 2003). A genome scanning approach was later used to map *Vb* onto a different linkage group (12) thus segregating separately from *Vf* (Erdin et al., 2006).

The cultivar Antonovka, identified as scab resistant and used in breeding due to its large fruit size and eating quality which set it apart from the resistant crabapple species used (Schmidt, 1938). Two major genes, *Va1* (*Rvi17*) and *Va/Va2* (*Rvi10*), have been mapped on linkage group 1 (Bus et al., 2012). *Va1* (Rvi17) maps closely to *Vf* and the resistance phenotype (chlorosis) co-segregates with the Ch-Vf1 SSR marker (138 bp;

Dunemann and Egerer, 2010). The *Rvi10* locus maps above *Vf* and exhibits a resistance reaction unique from *Rvi17* (Dayton and Williams, 1968; Hemmat et al., 2003).

The crab apple *M. baccata jackii* also contains genetic resistance to scab, and one of these loci, *Vbj* (*Rvi11*), has been mapped to linkage group 2 (Dayton and Williams, 1968; Gygax et al., 2004). The resistance found in the Russian apple R12740-7A was originally considered as non-race specific (*Vr*). However, this apple appears to have at least two different resistant genes: *Vh2* (previously *Vr*-A; *Rvi2*) and *Vh4* (same as *Vx*; *Rvi4*) in addition to the race nonspecific *Vr*-DW (Bus et al., 2005b). The *Vh8* (*Rvi8*) gene, from *M. sieversii* is closely linked (if not allelic to) the *Vh2* (*Rvi2*) gene (Bus et al., 2005a). *Vr2* (*Rvi15*) is unique from *Vr* and is found in GMAL 2473 (Patocchi et al., 2004).

Venturia inaequalis race 6 was utilized in identifying *Vd* (*Rvi13*) on linkage group 10 in the cultivar 'Durello di Forli' and is characterized by the stellate necrosis resistance reaction (Tartarini et al., 2004). *Vd3* is a narrow spectrum gene with resistance to race 7 that was identified in genotype 1980-015-25, and mapped ~1 cm below *Vf* (Soriano et al., 2009). An R gene called *Vdr1* (*Rvi14*) was identified on linkage group 6 in the cultivar 'Dülmener Rosenapfel' and may be homeologous to *Vf* on linkage group 1. The resistance reaction is described as a chlorotic reaction type similar to that observed in 'Florina' which carries the *Vf* allele (Soufflet-Freslon et al., 2008).

The only R gene that has been identified in a widely cultivated apple was found in 'Golden Delicious' (*Vg*; *Rvi1*) and its progeny ('Prima' and 'Florina'--also containing *Vf*) when exposed to race 7 isolates (Bénaouf and Parisi, 2000). The pedigree of 'Golden

Delicious' is not known, so it is unclear from where this resistance is derived. However it is unique from (nonallelic to) other known R genes. 'Golden Delicious' has been used extensively in cultivar development, so this R gene may have been carried fortuitously into other cultivars and breeding lines that have not been screened with the race 7 isolate.

Quantitative and Polygenic Resistance

Early descriptions of polygenic resistance to known races were described by Williams and Kuc (1969). Polygenic resistance with multiple partial resistances is more durable. Bus et al. (2002) showed moderate levels of heritability ($h^2 = 0.30$) in scab resistant populations which would be useful in developing durable cultivars as part of a recurrent selection breeding program in addition to the introgression of known major R genes. Polygenic resistance is observed in apple and results in the variation observed in sporulation on individual apple genotypes (MacHardy, 1996). Partial, polygenic resistance has been shown in some apple cultivars (Liebhard et al., 2003b; Calenge et al., 2004). Polygenic resistance is more challenging to breed for and has increased sensitivity to the environment (Ignatov and Bodishevskaya, 2011).

The mapping of quantitative trait loci (QTL) for disease resistance is also useful in marker-assisted breeding (MAB) and for describing inheritance of resistance when no major R gene is detected (Young, 1996). QTL mapping requires genotyping all progeny in the resistant by susceptible cross. Examining the field resistance in a 'Fiesta' x 'Discovery' population for leaf and fruit scab, QTL were detected over eight genomic regions (Liebhard et al., 2003c). Selective genotyping of a sub-population of progeny of 'Gala' x 'Dülmener Rosenapfel' detected *Vdr1*, and three QTL (Soufflet-Freslon et al.

2008). One of these QTL mapped to *Vdr1* and the other 2 QTL were detected on LG11 and LG17. These QTL were further confirmed in a second subpopulation of the same cross (Soufflet-Freslon et al., 2008). QTL have been shown to have both specificity to isolates and broad spectrum resistance (Calenge et al., 2004).

Fruit Quality and the Cultivar Honeycrisp

Fruit quality traits are the most important characteristics evaluated and the most crucial component of a breeding project as the fruit are the saleable product driven by consumer demands. These quality traits include texture (King et al., 2000) and its components firmness (Pre-Aymard et al., 2005), juiciness, and crispness. Recently a QTL and functional SNP haplotypes for "fresh sensation" which combines components of crispness, juiciness, and acidity was detected in apple (Verma et al., 2013). Quality can also be defined by the experience of eating the fruit, nutritional value, the price, and other factors leading to consumer satisfaction (Harker et al., 2003).

The sensory and quality traits of some scab-resistant cultivars have been described (Kuhn and Thybo, 2001). Depending on which scab resistant cultivars were evaluated, the preference for the traditional cultivar 'Jonagold' varied (Cmelik et al., 2007; Kelley et al., 2010). The development of scab resistant cultivars faces genetic challenges (linkage drag) and marketing challenges. Any new cultivar (resistant or otherwise) must be an outstanding alternative or replacement to an existing, consumer-recognized cultivar.

Consumers have indicated a preference for reduced pesticide usage but have not shown a willingness to pay for such produce in the marketplace. This inconsistency may

be the result of poor advertising, product placement, or inappropriate pricing that inadequately taps into the consumer mindset. Consumers may not be aware of the availability of such products and the potential direct and indirect benefits over traditionally-produced apples (Baker, 1999). Consumer familiarity with a cultivar, and previous purchase of a particular apple cultivar, rank as top characteristics in selecting fruit to purchase (Kelley et al., 2010).

The apple cultivar Honeycrisp is reported to have outstanding flavor and textural traits that make it a superior cultivar (Luby and Bedford, 1992; Tong et al., 1999; Hoover et al., 2000). Although it does suffer from some storage disorders, 'Honeycrisp' can maintain crispness for 6-9 months in storage (Luby and Bedford, 1992; Tong et al., 1999). 'Honeycrisp' has also been shown to exhibit field resistance to foliar apple scab infection when grown under organic disease management practices (Berkett et al., 2008). This characteristic is important for 'Honeycrisp' growers who may be able to reduce fungicide inputs in their orchards. For the apple breeder, using 'Honeycrisp' as a parent offers the genetic background for superb fruit quality traits including the components of fresh sensation, long-term storage quality, and disease resistance traits that should be exploited. Identifying the R gene(s) in 'Honeycrisp' will give plant breeders additional tools for MAB, sometimes referred to as marker-assisted selection (MAS), in developing resistant cultivars.

Marker-Assisted Selection

The selection of desired genotypes for advancement in an apple breeding program is often an extremely long process. The development of a single cultivar can take as long

as 20-25 years. Cultivar development is hindered by constraints of a long juvenility and self-incompatibility (Maliepaard et al., 1998). Orchard space is limited, and the maintenance of individual trees from juvenility to fruit-bearing age is expensive and requires a large amount of space. The development of genetic markers to screen for important traits at the seedling stage (or even as seeds!) could greatly reduce the number of trees that need to be grown to maturity for phenotypic evaluation. Genetic marker development and MAB rely on being able to accurately phenotype the traits of interest (Luby and Shaw, 2001). To have the greatest impact, the traits must be well defined and also measurable.

Apples are asexually propagated and are grown on rootstocks to allow multiple growers to have an infinite number of trees of the same genotype. However, in the apple breeding program, it is not practical to have multiple replications of the same genotype, especially if it has not been proven in preliminary taste and sensory evaluations. For a breeding program in a woody species like apple, orchard resources are better suited for screening large numbers of families (~20 crosses each year, ~150-200 progeny per family) from high quality parents, than for large populations of a single cross for genetic mapping. Large mapping populations in apple are generally limited, however pedigree based QTL mapping techniques are being employed to capitalize on the smaller, related populations more common in breeding programs (Evans et al., 2012). Another restriction for genetics studies for many traits is that apple trees are often not randomized in the field but are planted as contiguous rows of full-sibs where space is available.

Traditional screening of seedlings with scab inoculum is equally effective in identifying resistant progeny with *Vf* (or other R gene). MAS can be useful as a substitute for a disease screen, to reduce linkage drag, and to select for resistance QTL in a population (Michelmore, 1995). MAS can be efficient for identifying multiple R genes (in gene pyramiding) and for selecting for other traits simultaneously (Maliepaard et al., 1998). Using markers tightly linked to an R gene, one can identify genotypes with the R gene but select against traits in the less desirable parent (Gianfranceschi et al., 1996).

Traditional glasshouse screening will remain a valuable tool to confirm that the R gene is still effective against the pathogen (Michelmore, 1995). In order to decrease the amount of time between generations and to determine scab resistance in a test cross progeny, Aldwinckle et al. (1976) developed a method for forcing the flowering of apple seedlings 16-20 months after germination. This unique tool could be used for rapid cultivar development and gene pyramiding while exploiting the tools of MAS (Aldwinckle et al., 1976).

Gene pyramiding or the stacking of R genes (with MAS) into a single cultivar is an approach to create durable resistance. Gene pyramiding reduces overall chemical applications for a variety of diseases or pests. Durable resistance and multiple resistances in an orchard can limit natural selection for virulent races (Ognjanov et al., 1999). The planting of a single resistant variety in an orchard increases the selection pressures on the pathogen, which could result in the development of a new race (Fischer, 2005). Deployment of scab resistant cultivars can also cause pathogen population segmentation (Leroy, 2013). The gene pyramiding approach fundamentally requires that genes being

stacked be functionally different in their modes of action (Patocchi et al., 2005). Pyramiding can include both major genes and minor genes, including quantitative and partial resistance (Baldi et al. 2004). Resistance alleles linked in repulsion at two loci will be passed to a smaller number of progeny than those in coupling or at unlinked loci (Patocchi et al., 2005).

Molecular Tools

Until recently, microsatellite markers (simple sequence repeat; SSR) were the tool of choice for gene and QTL mapping in apple. SSRs are highly repeatable across labs, affordable, and have been identified and mapped in apple. Many of these markers, including linkage map(s) position are available online (www.hidras.unimi.it). Construction of SSR linkage maps have allowed resistance loci to be linked to molecular markers for MAS. The advent of a high throughput SNP array in apple (Chagné et al., 2012) should be useful in more precise QTL detection, map development, and MAS. The International RosBREED SNP Consortium (IRSC) apple 8K SNP array is a highly repeatable assay with SNPs clustered in ~1 cM intervals across the genome, including within putatively expressed genes (Chagné et al., 2012). SSRs remain important in MAS and can be utilized in conjunction with the SNP assay. SSRs or other PCR based molecular markers can be developed from sequence information and tools available at Genome Database for Rosaceae [GDR; www.rosaceae.org (Jung et al. 2008)] based on relative positions of the physical and genetic map placement of the resistance locus.

In addition to genotyping apple samples for MAS, SSRs are useful in genotyping *V. inaequalis* isolates for population genetics studies. A number of neutral SSRs are

available for characterizing the pathogen (Tenzer et al., 1999; Guérin et al., 2004).

Candidate Genes

Resistance genes in plants encode proteins that function in recognizing the presence of the pathogen. These receptor proteins can detect the pathogenic avirulence (*Avr*) genes directly or indirectly, leading to a race-specific interaction. The most common class of R genes includes those encoding both NBS-LRR motifs. The NBS and LRR domains function in signal transduction and recognition, respectively (Young, 2000). Other protein classes include receptor like kinases and the Toll/interleukin receptor (TIR) domain which is often the N-terminus of the NBS-LRR (Dangl and Jones, 2001). The NBS domain has been used for identifying resistance gene analogs in apple (Calenge, 2004) but was not successful at identifying candidate genes at *Rvi6* (Xu and Korban, 2002).

The sequencing of the apple genome allowed for the identification of a consensus gene set that is annotated and available online at GDR (Jung et al. 2008, Velasco et al., 2010). Of the roughly 57,000 genes identified, 4,531 were classified as resistance genes with 992 containing variants of the NBS-LRR domains (Veleasco et al., 2010). One approach for identifying candidate genes associated with the resistance is to narrow the search interval from genome wide to a reduced interval flanking the resistance locus following gene/QTL mapping. Sequenced-based molecular markers (SSR, SNP) can be anchored to the physical map flanking the resistance locus, and contig sequences and/or consensus gene sequences can be downloaded and evaluated from within a genome browser. It is expected that the region will contain a number of unrelated genes, so

software that annotate the protein sequences into families (Pfam) and gene ontologies are used to further delimit the potential candidate genes (Punta et al., 2012). Because R genes often reside in clusters and mega-clusters a number of candidate genes will likely be identified (Young, 2000).

The candidate gene mining approach using the genome browser is an efficient alternative to other map-based positional cloning strategies such as the construction of bacterial artificial libraries. Expression profiling of candidate genes between parents, and among segregating offspring should further narrow the candidate gene pool (Wayne and McIntyre, 2002). This could be especially useful in a crop like apple, where the development of very large seedling populations required for fine-mapping is limited by the number of seeds in each fruit and the number of parental genotypes available for crossing. Furthermore, sequence information is available for ~25 founder genotypes in apple, including 'Honeycrisp' and its ancestors. Identifying the functional differences (insertion-deletions, SNPs, etc. within the open reading frame) between 'Honeycrisp' and susceptible genotypes will also reduce the number of candidate R genes. The most likely candidate genes can then be cloned and evaluated for function by transformation into susceptible genotypes (Joshi et al, 2011).

Transgenic Apple

A number of researchers have transformed apple with trans- and cisgenic approaches as a way to test the function of candidate genes, and to examine the effects of pathogen related proteins in disease resistance. *HcrVf2* has been cloned from *M. floridbunda*-821 into 'Gala' and was shown to provide resistance (Belfanti et al., 2004).

A cisgenic approach was taken to produce 'Gala' plants containing a single copy of the *HcVf2* gene under its own promoter without other foreign DNA (Vanblaere et al., 2011). The *Vfa1, Vfa2* and *Vfa4* paralogs at *Rvi6* were transformed into the susceptible 'McIntosh' and 'Galaxy' with their native promoter and 3'-UTR region, but only *Vfa1* and *Vfa2* showed increased resistance (Malnoy et al, 2008). It was later demonstrated that only *Vfa2* is functional (Joshi et al, 2010). Transgenic approaches have also been taken in apple to control the other critical disease in apple, such as fireblight (*Erwinia amylovora*), harmful insect pests, tree architecture, and herbicide tolerance. Aldwinckle and Malnoy (2009) produced a comprehensive review of transgenic approaches and objectives in Rosaceous crops including apple. A susceptible apple clone of 'McIntosh' was transformed with a fungal endochitinase which proved to reduce apple scab, but also resulted in low vigor plants (Bolar et al., 2000).

Venturia inaequalis Population Structure

Eight physiological races of *V. inaequalis* have been identified based on symptom differences observed within a differential set of apple genotypes. For race determination, an inoculum suspension derived from a single spore is applied to each of the genotypes and the observed resistance reactions are associated with the Avr genes in the pathogen. The differential set has been expanded and modified over time to contain apple genotypes with only one known R gene in an international effort to monitor apple scab (Bus et al., 2011).

Natural gene flow of the pathogen can be very slow, as splash dispersal is the main mechanism of movement of spores. Infected leaf litter is spatially restricted within

an orchard and the development and/or movement of races to other orchards may be slow or non-existent. Local adaptation of strains and cultivars develops because of limited movement of the pathogen within and among orchards. Knowing the races present in an orchard or growing region through continual monitoring can give insight as to how R genes are defeated or as to how resistant cultivars and management practices should be implemented (www.vinquest.ch). How the presence of 'Honeycrisp' which demonstrates disease resistance, and is planted around globe, is influencing the evolution of the pathogen and thus population genetic structure is unclear.

The movement of pathotypes (races 6 and 7) that are virulent to an R gene could affect the durability of (*Vf*) resistant cultivars (Tenzer and Gessler, 1997). Though natural gene flow is highly restricted, human activity has been an important factor in distributing races around the globe. Spores could potentially be moved on infected fruit from orchard to orchard and even further along the international supply chain. Though not well studied, the movement of infected budwood, rootstock, and nursery trees may also play a role. Furthermore, landscape trees (crabapples) and home fruit cultivars can be sources of inoculum that bridge the movement of the pathogen along the highly fragmented landscape of apple orchards.

The population structure of *V. inaequalis* in Minnesota specifically has not been documented although races 1-5 are present in the United States. The population structures in several geographic regions have been the focus of studies in Europe, Asia, and Brazil (Martinez-Bilbao and Murillo, 2005; Melounova et al., 2004; Sandskär and Liljeroth, 2005; Schenato et al., 2008, Xu et al., 2008). Difficulties arise with quantifying

the races present and virulence characteristics of the races due to inadequate sampling strategies. To date, the approach has been one of surveying the populations present and using genetic variation as an important tool for monitoring the pathogen.

Several *V. inaequalis* linkage maps have been created, although the majority of genetic work has focused on the host and not on the pathogen (Sierotzki and Gessler, 1998; Xu et al., 2009). The development of a draft genome sequence for the pathogen is said to be complete, however it has yet to be published (Rees et al., 2010). Molecular markers can be useful in assessing genetic variation in a population, but may not be able to identify specific races. Tenzer et al. (1999) observed significant variation within 11 *V. inaequalis* populations, but little variation between different populations. Races are defined by their ability to cause an infection (i.e. overcome resistance) and may do this through different genetic mechanisms and yet have the same phenotype. One cannot determine if rare alleles that are shared between populations are identical by decent and the result of genetic drift, or if they have independently evolved (Tenzer et al., 1999). RFLP markers have been used to demonstrate significant diversity within populations, which has been attributed to the movement of diseased plants and fruits (Tenzer and Gessler, 1999).

Hypotheses and Objectives

The key objective of the current study was to identify the genes(s) in 'Honeycrisp' that confer resistance to apple scab. In order to do this, experiments were conducted to provide support for genetic resistance, including the development of a 'Honeycrisp' genetic map, characterization of the defense response in 'Honeycrisp', and

the collection and curation of a population of single spore isolates. The research described in this dissertation was guided by the following hypotheses and objectives.

1) A 'Honeycrisp' SNP linkage map will be a useful tool for genetic studies in apple for major gene and QTL mapping. The linkage map can provide insight into areas of high recombination and assist in known issues with the current assembly of the physical map.
2) The defense response in 'Honeycrisp' to infection with *V. inaequalis* will be characterized macro- and microscopically.
3) The observed resistance reactions will be heritable and traceable through the 'Honeycrisp' pedigree.
4) 'Honeycrisp' seedling populations will segregate for disease resistance and support either 1 or 2 R genes in this important parent.
5) SNP and SSR haplotypes will be identified which span the resistance loci and can be used in MAB, especially in identifying pyramided R genes.
6) The population of *V. inaequalis* isolates collected in Minnesota will represent the diverse genetic background of this pathogen due to the range of hosts sampled and geographic locations.

Summary

Chapter 1. A consensus 'Honeycrisp' apple (*Malus* × *domestica*) genetic linkage map from three full-sib progeny populations

Linkage maps allow the ordering of genetic markers along linkage groups to represent the physical positions of genes on a chromosome. These are utilized in QTL mapping and for understanding the structure of the genome. The majority of linkage maps developed in *Malus* have been constructed from SSR and other early molecular marker platforms. Although dense, the SSR map developed by Fernández–Fernández et al. (2012) or the SSR and SNP map by Antanaviciute et al. (2012) were developed from apple rootstock progenies and may not represent important fruit cultivars. The development of a consensus 'Honeycrisp' genetic linkage map from three unique parental maps of important cultivars and breeding parents ('Honeycrisp' × 'Monark', 'Honeycrisp' × 'Gala', and 'Honeycrisp' × MN1764) will provide geneticists an important tool for QTL mapping and MAS. A consensus 'Honeycrisp' map was developed with 1091 SNP markers that are spaced on average 1.36 cM across the genome. The consensus map was in general agreement with the 'Golden Delicious' reference genome sequence although some markers mapped to linkage groups other than the pseudo-chromosome from which they were developed. In addition to supporting breeders and geneticists working with apple cultivars, this linkage map should inform genomicists on potential flaws in the physical assembly.

Chapter 2. Characterization of the defense response to *Venturia inaequalis* in 'Honeycrisp' apple, its ancestors, and progeny

The resistance of 'Honeycrisp' to apple scab was unknown until reports of field resistance in organic orchards (Berkett et al., 2008). In order to characterize the defense response in 'Honeycrisp', greenhouse experiments were conducted to evaluate this cultivar and its pedigree to gain insight on the heritability of this trait. Macro- and microscopic characterizations demonstrate that 'Honeycrisp', its parent 'Keepsake', and grandparents 'Frostbite' and 'Northern Spy' all demonstrated genetic resistance to inoculation with mixed isolates. Responses ranged from 0 (no reaction) to chlorotic flecking, stellate chlorosis, necrotic flecking, and sporulation, respectively. No hypersensitive response was observed. The resistance response occurred as early as 7 days post inoculation (dpi). A progeny population of 'Honeycrisp' × 'Twin Bee Gala', segregated for the resistance response, but showed symptoms to 10-14 dpi possibly due to variable greenhouse conditions. The variation in resistance responses, the detection of resistance in both grandparents, and a 3:1 segregation in the progeny population provides evidence for the presence of two resistance loci in 'Honeycrisp'. The variation within the progeny population demonstrates the interaction of small effect genes, which may be in the downstream signal pathway of the resistance gene(s) that result in varied phenotypes.

Chapter 3. Identification of unique sources of apple scab resistance (*Rvi19* and *Rvi20*) in the apple (*Malus* x *domestica*) cultivar 'Honeycrisp'

In order to map the genetic resistance in 'Honeycrisp' to apple scab, a progeny population of 'Honeycrisp' × 'Twin Bee Gala' was screened for the disease in replicated

greenhouse experiments using two fungal isolates. This population (and several others tested) segregated for the resistance response 3:1, suggesting the presence of 2 R genes. This 'Honeycrisp' × 'Twin Bee Gala' population was genotyped using the IRSC 8K SNP array, and an iterative mapping approach was utilized to position the resistance loci using linkage analysis and QTL mapping approaches. *Rvi19* and *Rvi20*, map to linkage groups 1 and 15 respectively. Following mapping with SNP markers, SSRs were tested in the region, and mapped to provide a highly repeatable, affordable marker haplotype that flanked both loci. The haplotypes defined by SSR markers and SNPs that flank the loci have been developed to provide breeders with reliable tools for marker assisted selection. These SSRs were then utilized in screening advanced selections in the University of Minnesota breeding program which allowed the detection of the presence of these loci individually, or pyramided with other R genes.

The *Rvi19* haplotype spans 2.3 Mb which is ~12.6 cM and includes the well characterized *Rvi6* locus (*Vf*). The gbrowser available at the Genome Database for Rosaceae (GDR; www.rosaceae.org) was used for comparative mapping of the physical and genetic positions of these loci as well as for identifying candidate genes. The *Rvi19* haplotype consists of 263 consensus genes, 18 of which contain a leucine rich repeat region (LRR). The *Rvi20* haplotype spans 3.2 Mb (~10.1 cM) and comprises 460 consensus genes, 23 of which encode resistance gene motifs. The candidate genes identified are excellent targets for future studies in identifying the functional variants responsible for disease resistance.

Chapter 4. Population genetic structure of Minnesota isolates of *Venturia inaequalis* collected from diverse *Malus* species and cultivars

Over 80 isolates of *V. inaequalis* were collected from six locations across Minnesota including from the University of Minnesota: a collection of *Malus* at Minnesota Landscape Arboretum (Chaska, MN), research orchards at the Horticultural Research Center (HRC; Excelsior, MN) and the North Central Research and Outreach Center (Grand Rapids, MN), and from landscape trees at the University of Minnesota-Twin Cities campus (St. Paul, MN), and in a residential area of Minneapolis, MN. Isolates were collected from fruit or leaf tissue and included trees in managed and residential plantings, cultivars, breeding lines, and crab apple species. Twelve SSR markers were utilized for screening the genetic diversity of the isolates. The majority of allelic variation was within rather than among populations, but there was no population stratification detected in several of the analyses, including Structure and other cluster analyses. Nei's pairwise distance matrix separated the Grand Rapids, MN collection location from the other University of Minnesota sites. The AMOVA using genetic distance matrices resulted in Φ and R_{st} values that suggest gene flow among the populations. There was no spatial autocorrelation detected between the genetic distance matrices and geographic distance matrix. The diverse isolates should be screened with a differential set of apple cultivars to assign physiological race. The isolates evaluated in this study provide a snapshot in time of the genetic diversity of *V. inaequalis* in Minnesota and may be useful in seeing the effect of *Rvi19* and *Rvi20* in the landscape.

Table 1 List of reported apple scab resistant genes (old and new nomenclature) and corresponding resistance reaction. Also listed are the reported differential hosts and/or source of the resistance genes. (As modified from Bus et al., 2011, Jha et al., 2009).

Old Name	New Name	Reaction[z]	Differential Host/Source[y]
susceptible		4	Royal Gala
Vg	*Rvi1*	2	Golden Delicious
Vh2	*Rvi2*	2	TSR34T15
Vh3	*Rvi3*	2-SN	Q71, Geneva
Vh4=Vx=Vr1	*Rvi4*	1	TSR33T239
Vm	*Rvi5*	1	9-AR2T196 /*M. micromalus* 245-38; *M. atrosanguinea* 840
Vf	*Rvi6*	0 to 3b	Priscilla
Vfh	*Rvi7*	1	*M. floribunda* 821
Vh8	*Rvi8*	2	*M. sieversii* W193B, B45
Vdg	*Rvi9*	SN	K2, J34, Dolgo
Va	*Rvi10*	1	Antonovka PI 172623
Vbj	*Rvi11*	0 to 3b	*M. baccata jackii*
Vb	*Rvi12*	2 to 3b	Hansen's baccata #2
Vd	*Rvi13*	2	Durello di Forli
Vdr1	*Rvi14*	2	Dülmener Rosenapfel
Vr2	*Rvi15*	0 to 2	GMAL 2473
Vmis	*Rvi16*	0 to 2	MIS op 93.051 G07
Va1	*Rvi17*		Antonovka APF22
Vd3		1 to 2	1980-015-025
---	*Rvi19*	0, 2, 3a, 3b, SN	Honeycrisp[x]
---	*Rvi20*	0, 2, 3a, 3b, SN	Honeycrisp[x]

[z] Phenotypic resistance reaction conferred by the R gene against infection by *V. inaequalis* on apple. No lesions (0), hypersensitive response (1), chlorotic lesions (2), necrotic/chlorotic lesions with slight sporulation (3a-weak resistance), necrotic/chlorotic lesions with sporulation (3b-weak susceptibility), stellate necrosis (SN), and susceptible lesions with high sporulation (4).

[y] Apple cultivar or accession from which the *R* gene has been identified

[x] Two new resistance loci identified in 'Honeycrisp' reported here.

Figure 1 Lesions caused by *Venturia inaequalis* on a mature fruit of a susceptible cultivar showing the damaging effects of the pathogen that result in misshapen, undesirable, and often unsaleable fruit that would not hold-up in cold storage.

Figure 2 Disease cycle of *Venturia inaequalis* on apple is initiated in the spring by the release of ascospores from pseudothecia in the leaf litter at the time of apple bud break. Secondary infections occur throughout the growing season on new leaf tissue. Infected leaves fall to the ground, over winter, and are the source for sexual spore development in winter and early spring.

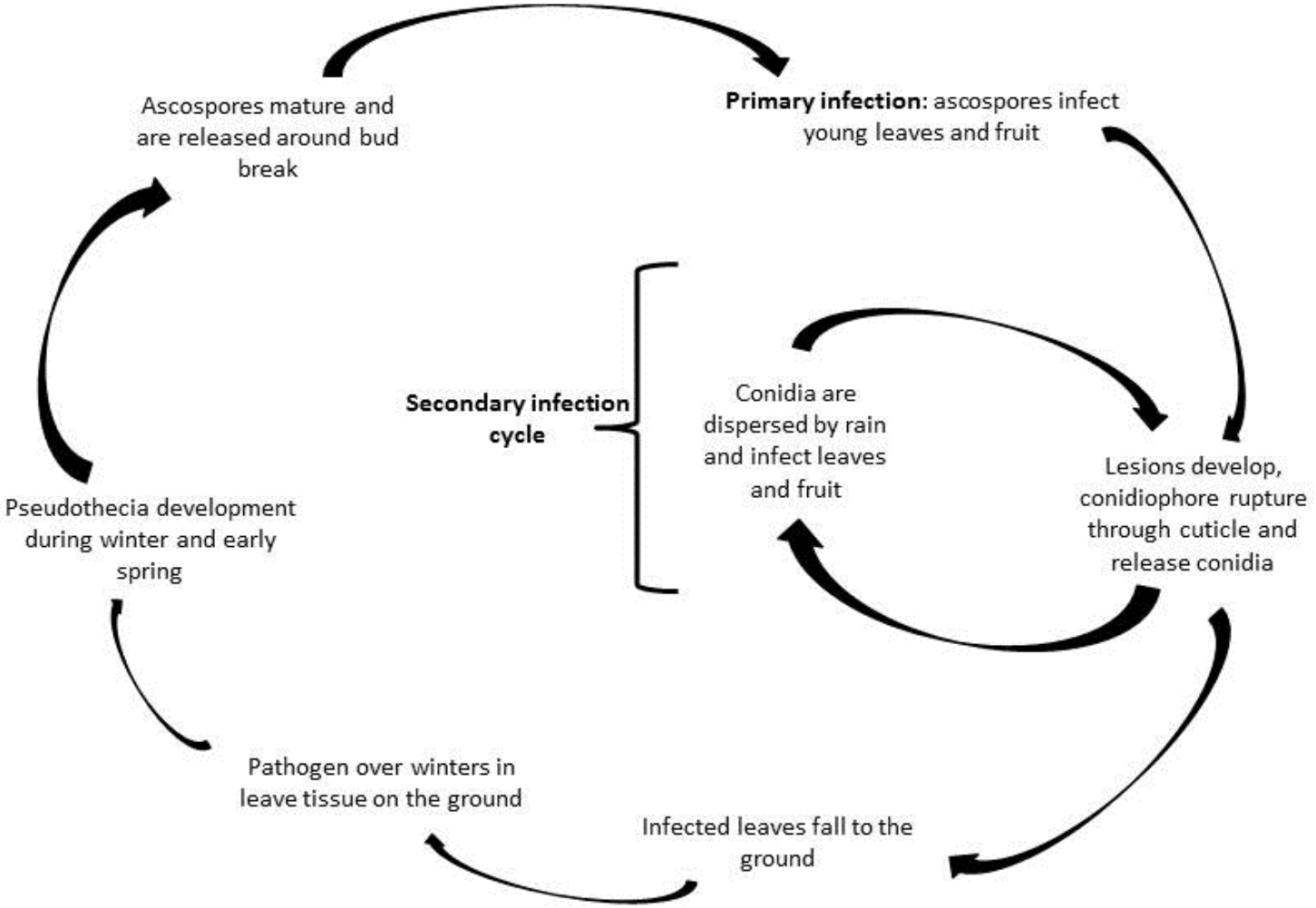


Figure 3. Punnett squares showing the 3:1 segregation of 2 independent resistance genes in the F_1 generation between cross (A) $R_1r_1R_2r_2 \times r_1r_1r_2r_2$ and cross (B) $R_1r_1r_2r_2 \times r_1r_1R_2r_2$. 'Honeycrisp' is proposed to have the $R_1r_1R_2r_2$ genotype for the scab resistance genes *Rvi19* and *Rvi20*

A

	R_1R_2	R_1r_2	r_1R_2	r_1r_2
r_1r_2	**$R_1r_1R_2r_2$**	**$R_1r_1r_2r_2$**	**$r_1r_1R_2r_2$**	$r_1r_1r_2r_2$
r_1r_2	**$R_1r_1R_2r_2$**	**$R_1r_1r_2r_2$**	**$r_1r_1R_2r_2$**	$r_1r_1r_2r_2$
r_1r_2	**$R_1r_1R_2r_2$**	**$R_1r_1r_2r_2$**	**$r_1r_1R_2r_2$**	$r_1r_1r_2r_2$
r_1r_2	**$R_1r_1R_2r_2$**	**$R_1r_1r_2r_2$**	**$r_1r_1R_2r_2$**	$r_1r_1r_2r_2$

B

	R_1r_2	R_1r_2	r_1r_2	r_1r_2
r_1r_2	**$R_1r_1r_2r_2$**	**$R_1r_1r_2r_2$**	$r_1r_1r_2r_2$	$r_1r_1r_2r_2$
r_1r_2	**$R_1r_1r_2r_2$**	**$R_1r_1r_2r_2$**	$r_1r_1r_2r_2$	$r_1r_1r_2r_2$
r_1R_2	**$R_1r_1R_2r_2$**	**$R_1r_1R_2r_2$**	**$r_1r_1R_2r_2$**	**$r_1r_1R_2r_2$**
r_1R_2	**$R_1r_1R_2r_2$**	**$R_1r_1R_2r_2$**	**$r_1r_1R_2r_2$**	**$r_1r_1R_2r_2$**

Chapter 1

A consensus 'Honeycrisp' apple (*Malus* × *domestica*) genetic linkage map from three full-sib progeny populations[1]

The apple cultivar Honeycrisp is emerging in North American markets due to its outstanding eating quality. A set of three 'Honeycrisp' progeny populations from the University of Minnesota apple breeding program were utilized to construct parental and consensus 'Honeycrisp' linkage maps to enable marker assisted breeding. Two populations segregated for fruit texture traits and a third was of interest in examining disease resistance. All available individuals were genotyped with the International RosBREED SNP Consortium (IRSC) apple 8K SNP array v1, for a total of 318 progeny individuals. Three unique 'Honeycrisp' parental maps ('Honeycrisp' × 'Monark', 'Honeycrisp' × 'Gala', and 'Honeycrisp' × MN1764) were developed, consisting of 1018, 1042, and 1041 SNP markers, respectively. Among all three 'Honeycrisp' parental maps, 951 SNP markers were in common. Combining these maps with the MergeMap tool, a consensus 'Honeycrisp' linkage map with 1091 SNP markers was developed with an average distance of 1.36 cM between consecutive markers. The 'Honeycrisp' consensus map was largely in agreement with the physical position of markers in the 'Golden Delicious' reference genome sequence (v1.0, as of February 2013). The consensus linkage map is informative for an elite cultivar that is being utilized in breeding programs worldwide for its superb fruit quality traits.

[1] A modified version of this manuscript is accepted with revisions for publication in Tree Genetics and Genomes, and is co-authored with equal contributions by C.A. Schmitz (2013).

Introduction

'Honeycrisp' is an emerging apple cultivar with increased importance in North America due to its outstanding flavor and textural traits (Hoover et al., 2000; Luby and Bedford, 1992; Tong et al., 1999). Although it is prone to some storage disorders, 'Honeycrisp' can maintain crispness for 6-9 months in storage (Luby and Bedford, 1992; Tong et al., 1999). 'Honeycrisp' has been shown to exhibit field resistance to foliar apple scab infection when grown under organic disease management practices (Berkett et al., 2008), a characteristic important for growers who may be able to reduce fungicide inputs in their orchards. For the apple breeder, using 'Honeycrisp' as a parent offers the genetic background for superb fruit quality and disease resistance traits that should be leveraged in breeding (McKay et al., 2011). Identifying the marker-locus-trait associations in 'Honeycrisp' progeny will give plant breeders additional tools for marker assisted breeding (MAB), in developing new cultivars. The development of a 'Honeycrisp' linkage map will add to the toolbox available to apple breeders and geneticists.

Fruit quality traits are among the most important characteristics evaluated and the most crucial component of a breeding project as the fruit are the saleable product driven by consumer demand. These quality traits include texture (King et al., 2000) and its components firmness (Pre-Aymard et al., 2005), juiciness, and crispness. The development of scab resistant cultivars faces genetic challenges (linkage drag) and marketing challenges. Any new cultivar must be an outstanding alternative or replacement to an existing, consumer-recognized cultivar. Consumer familiarity with a

cultivar, and previous purchase of a particular apple cultivar rank as top determinants in selecting fruit to purchase (Kelley et al., 2010).

Cultivar development is hindered by long juvenility and self-incompatibility which constrain crossing decisions. The development of a single cultivar can take as many as 20-25 years. Due to the large size of mature trees, orchard space is limiting, and the maintenance of individual trees from juvenility to fruit-bearing age is expensive and requires a large amount of space. The development of genetic markers to screen important traits at the seedling stage and for parental selection will result in the enrichment of the target trait among seedlings that are grown to maturity for phenotypic evaluation. Accurate phenotyping of the traits of interest predicates detection of robust marker-trait associations to enable MAB (Luby and Shaw, 2001). The traits must be well defined and also objectively measurable.

Due to its highly heterozygous genome, high levels of inbreeding depression, and self-incompatibility, genetic studies in apple can be challenging (Lawson et al., 1995). Linkage maps for self-incompatible species, including apple, are created using the two way pseudo test-cross method within a single progeny (Grattapaglia and Sederoff, 1994). In this approach, a map for the first parent is made using makers heterozygous in the first parent and homozygous in the second, and conversely the second parental map consists of markers homozygous in the first parent and heterozygous in the second. These maps can then be integrated using markers heterozygous in both parents, creating a population map. Genetic mapping using bi-parental mapping populations is common in apple genetics, especially in developing molecular markers for monogenic traits such as disease

resistance (Schenato et al., 2008; Tartarini and Sansavini 2003). A number of linkage maps have been developed and were used to detect quantitative trait loci (QTLs) and map genes for a range of important traits including disease resistance (*Vf* for apple scab (Gianfranceschi et al., 1996)), acidity (*Ma*; Maliepaard et al., 1998), and growth habit and developmental traits (Lawson et al., 1995). Mapping populations typically use parents divergent for an important trait. This approach is illustrated by recently published microsatellite and single nucleotide polymorphism (SNP) maps in *Malus* species (Antanaviciute et al., 2012; Fernández-Fernández et al., 2012; Wang et al., 2011). The advantage of a consensus 'Honeycrisp' linkage map, constructed across different populations, is that marker alleles in this cultivar would yield a novel map that would be informative for MAB in breeding programs using this cultivar.

Recently, the apple genome was sequenced (Velasco et al., 2010), and additional supporting tools have been developed, including a physical map, BLAST search engine, and genome browser available on Genome Database for Rosaceae (GDR; http://www.rosaceae.org, Jung et al., 2008). High throughput SNP genotyping allows for efficient genotyping of large numbers of individuals or populations with a relatively low cost per marker. An 8K SNP array v1 was developed by the International RosBREED SNP Consortium (IRSC). Based on the Illumina Infinium platform, the BeadChip is a small, portable, highly repeatable assay that allows for rapid scoring of individuals, providing even coverage throughout the apple genome, including SNPs within putative expressed genes (Chagné et al., 2012). The array was designed using a clustering strategy with a cluster of 4-10 closely positioned SNPs spaced at 1cM intervals between

clusters (Chagné et al., 2012). Clustered markers should provide local information for diverse apple populations representing unique haplotypes, and recombination is rarely expected within a cluster. The result is a SNP array that is not population dependent and is applicable across cultivars and progeny populations (Micheletti et al., 2011).

Linkage maps with dense marker coverage and with markers evenly spaced across the genome are ideal for QTL analysis. Increased density and coverage of markers helps increase power and precision of QTL analysis, thereby helping in gene discovery. Khan et al. (2012) created a highly saturated map of apple by merging five bi-parental maps by use of single sequence repeat (SSR) and SNP markers shared among the linkage maps. The construction and analysis of genetic linkage maps provide support for the placement of molecular markers into the correct order and position. The correct order and position is very important to precisely locate QTLs. A constraint in map construction is marker checking to validate and correct automated SNP genotyping calls, especially in cases of expected paralogous regions from local or whole genome duplication events, which are common in plant genomes, including those of *Malus* species (Velasco et al., 2010). A comparative analysis of maps from different populations and development of a consensus map help to determine whether large genome rearrangements are present and to establish consensus order and positions of mapped markers.

The objective of this study was to develop a high-density, SNP consensus linkage map for ‘Honeycrisp’ utilizing several ‘Honeycrisp’ full-sib progeny populations that segregate for fruit quality and apple scab resistance. This map will provide the framework for future genetic studies in ‘Honeycrisp’-specific progeny to identify marker-

locus-trait associations for important fruit quality and disease resistance traits, thus enabling MAB. It will also provide additional support in map construction (marker order and position) for pedigree-based analysis and in resolving potential issues in the apple physical map v1 (GDR database: http://www.rosaceae.org; Jung et al., 2008).

Materials and Methods

Plant Materials

A portion of the genotypic data in this study was produced as part of the RosBREED crop reference set (rosbreed.org). The corresponding apple genotypes, hereafter referred to as "RosBREED samples", included the parents ('Honeycrisp', 'Gala', 'Monark', MN1764, and 21 individuals of the 'Honeycrisp' × 'Monark' population (described below). The majority of individuals described in this paper were genotyped independently of the RosBREED crop reference set and these individuals are hereafter referred to as "UMN samples".

The UMN samples comprise three full-sib families sharing 'Honeycrisp' as a common parent and were utilized in the development of the 'Honeycrisp' consensus map. Two *ad hoc* populations 'Honeycrisp' × MN 1764 (n=130) and 'Honeycrisp' × 'Monark' (n=88)] were selected from breeding populations growing at the University of Minnesota Horticultural Research Center (Excelsior and Chanhassen, MN) that were developed from crosses made in 1992-1998. These *ad hoc* populations have been described previously McKay et al. (2011). A third population was created in 2010 from a cross of 'Honeycrisp' × 'Twin Bee Gala' (n=128; this population is referred to as 'Honeycrisp' ×

'Gala' throughout) and grown in greenhouses at the University of Minnesota-Twin Cities (St. Paul, MN).

DNA Extraction Protocol

For RosBREED samples, stems with newly expanding leaf tissue were collected in the field in 2010 and 2011 and placed in labeled plastic bags on ice. Thirty to 50 mg of leaf tissue were later harvested into a cluster tube (Corning, Tewsbary, MA). These RosBREED tissue samples were frozen in liquid nitrogen and held at -80°C until DNA extraction. For the UMN samples, newly expanding or youngest leaves were collected from individual trees in 2012, frozen at -80° C, lyophilized, and held at -80° C until DNA extraction. Approximately 10 to 15 mg of lyophilized leaf tissue from each sample was placed into a cluster tube.

The day of DNA extraction, leaf tissue was homogenized by grinding lyophilized (UMN) or frozen (RosBREED) samples. A 4 mm stainless steel bead (McGuire Bearing Company, Salem, OR) was added to each cluster tube, caps applied, and the 96-tube rack was submerged in liquid nitrogen. The rack was then placed into a Retsch MM301 Mixer Mill (Retch, Haan, Germany) and shaken for 30 seconds. Sample racks were re-submerged in liquid nitrogen and shaken two additional times, disrupting the leaf tissue into a fine powder. The homogenized RosBREED and UMN tissue was stored at -80° C until 10 minutes prior to extraction.

Extraction was conducted using the E-Z 96® Plant DNA Kit (Omega Biotek, Norcross, GA) with modifications (Gilmore et al., 2011). Modifications included using SP1 solution equilibrated to 65° C in a water bath. The supernatant (580 µL) for each

sample was transferred in one step to a new cluster tube containing 10 µL RNase solution (2.5 µL RNase and 7.4 µL TE pH8). After the drying step, DNA was eluted in 100 µL elution buffer, and samples were stored at 4° C and quantitated within seven days, or stored at -20° C. DNA samples were quantified using the Quant-iT ™ PicoGreen® dsDNA assay kit (Invitrogen, Eugene, OR) and a Victor multi-plate reader (Perkin Elmer Inc., San Jose, CA, USA).Samples with DNA concentrations > 100 ng/µL were diluted with the addition of an equal volume of TE to achieve concentrations between 50-100 ng/µL. Fifteen µL of each DNA sample were aliquoted into PCR plates comprising 96-0.2 mL wells, sealed with adhesive aluminum foil seals, and shipped to the genotyping facility on dry ice.

Marker Data Generation and Analysis

The UMN DNA samples were submitted to the SNP Genotyping Facility at Michigan State University (East Lansing, MI). The RosBREED samples were analyzed at the University of Western Cape, South Africa. Using previously published protocols (Illumina, 2006) samples were hybridized onto the IRSC 8K SNP array (Chagné et al., 2012) following a whole genome amplification reaction. BeadChips were imaged by the iSCAN system and converted into intensity data. The intensity data from the two data sets were combined for analysis and interpretation in the Genotyping Module of GenomeStudio for genotype clustering (Illumina Inc., 2010a).

The iSCAN data from both genotyping facilities were loaded into a single project file for data analysis. SNP genotype scoring employed the Genotyping module of GenomeStudio (Illumina Inc., 2010b) software version v2010.3.0.30128. The software

normalizes the intensity values across BeadChips to allow for uniformity in allele-calling. To ensure high quality reads, stringent initial parameters were set as: GenTrain > 0.60 and AB Freq from 0.45 to 0.55. The SNPs were clustered by marker locus using the clustering algorithm Gentrain2 (Illumina Inc.,. 2010c) and all SNPs were visually examined for an expected maximum of three clusters (AA, AB, and BB) and then classified as failed, monomorphic, or polymorphic.

Automated allele calling with visual checking to confirm clustering of individuals into appropriate classes was utilized. Manual clustering was performed for some markers, when automated clustering was not satisfactory. Markers with more than three distinctly-spaced clusters, presumably the result of annealing to more than one genomic region (i.e. paralogs), were excluded. The 'Honeycrisp' × MN1764 population, with the largest number of progeny, was utilized to select nearly 2000 high quality markers for the development of a saturated linkage map as suggested by Micheletti et al. (2011). A preliminary map was developed to evaluate genome coverage and relative positions in comparison to the physical map (Clark et al., 2013). For the preliminary map, the default settings of the maximum likelihood method in JoinMap 4.1 (Kyazama B.V. Wageningen, Netherlands; Van Ooijen, 2006) were used to map 1952 SNP markers. Marker grouping during map construction utilized a published SNP map (Antanaviciute et al., 2012). These ~2000 markers were then scored for the 'Honeycrisp' × 'Gala' and 'Honeycrisp' × 'Monark' populations.

Marker loci at which missing parental genotypes could not be positively determined based on progeny segregation in two or more families were removed.

Markers with >10% missing data were eliminated. Progeny that did not conform to the parental genotypes were removed, as they were expected to be outcrosses, non-progeny, or contaminated samples. Progeny genotypic scores identified as genotyping error were considered as missing. The identity by descent (IBD) analysis program within FlexQTL (Bink et al., 2008) was used to identify miscalled alleles and impute parental genotypes using the 'Golden Delicious' physical map positions. This tool allowed for the aggressive detection of errors (missing markers, null-alleles, other anomalies), but required additional manual correction or imputation of parental genotypic scores based on the progeny SNP calls.

Linkage Mapping

The codominant SNP markers from each outbreeding, full-sib population were coded for linkage map construction according to JoinMap 4.1 conventions as heterozygous in either first or second parent (<nn x np>, <lm x ll>) or both parents (<hk x hk>) (Van Ooijen, 2011). Initially, the three populations were mapped separately. The initial grouping procedure in JoinMap was completed using the published M432 progeny linkage map (Antanaviciute et al., 2012), resulting in a large proportion of the called SNPs remaining ungrouped. The strongest crosslink values (SCL) were applied repeatedly using restrictively lower values in an iterative process to assign ungrouped loci to the correct linkage group (Van Ooijen, 2006). Markers with suspect linkage (recombination frequency estimate >0.6) were removed before mapping. Then, map order was calculated using the maximum likelihood option which calculates both parental

maps. For this study, only the single parent 'Honeycrisp' map from each population was used for the construction of the consensus map.

Each of the three 'Honeycrisp' maps and corresponding progeny genotypic data set were assembled for analysis in FlexQTL (Bink et al., 2008) to detect observed double recombinants versus expected double recombinants provided the newly constructed linkage map. The FlexQTL program calculated observed double recombinants (oDR) minus the expected double recombinants (eDR) for the two parents at each marker position. This helped to identify markers that had high genotyping error rates or that are misplaced by the mapping algorithm. Markers with oDR - eDR $\geq$ 0.03 were removed from the subsequent round of JoinMap mapping, eliminating 100 ('Honeycrisp' × MN1764), 80 ('Honeycrisp' × 'Gala'), and 105 ('Honeycrisp' × 'Monark') spurious markers. After two rounds of mapping and removal of suspect markers identified with FlexQTL, maps were inspected for large gaps (> 15cM) and markers creating unusual large gaps were identified. Markers creating unusually large gaps at linkage group ends were referred to as "lone wolf" markers as the gaps suggest poor linkage to the marker group. If a large gap existed at the end of a LG in a single population map and the causative marker was not found in the corresponding LG in either of the other two maps, it was removed. After marker removal from any map, the map was recalculated in JoinMap 4.1. The resulting three 'Honeycrisp' maps were combined into a consensus map with the MergeMap (2012 version) software tool (Wu et al., 2011). Maps were weighted based on population size ('Honeycrisp' × 'Monark'; 0.255, 'Honeycrisp' × 'Gala'; 0.393, 'Honeycrisp' × MN1764; 0.352).

The consensus ‘Honeycrisp’ linkage map was compared to the available physical map of apple. SNP map positions for each of the 17 linkage groups were plotted against marker positions in the respective pseudo-chromosomes of the ‘Golden Delicious’ genome sequence with R v2.15.1 (R Core Team, 2012). Base pair positions were those of the mapped IRSC apple markers and these data are available at the Genome Database for Rosaceae (http://www.rosaceae.org; accessed 28 Feb 2013). Each marker included in the consensus ‘Honeycrisp’ map was checked for significant segregation distortion (χ^2, $p < 0.005$) in each of the three families using JoinMap.

Results

A diagram describing the work flow and remaining high quality SNP markers at each phase are shown in Figure 1-1. For each of the populations, individuals were removed whose genotype did not conform to the parental genotypes, suggesting it was an outcross, non-progeny, or contaminated sample (‘Monark’; 7 , ‘Gala’; 3, and MN1764; 18). Three ‘Honeycrisp’ linkage maps were constructed which varied due to different levels of heterozygosity and recombination frequencies in the alternate parent. Figure 1-2 details heterozygosity for each parent (‘Honeycrisp’ is heterozygous at each marker position) along the consensus map. MN1764 had the lowest proportion of heterozygous markers (34.5%) in the corresponding ‘Honeycrisp’ parental map, and MN1764 additionally had the lowest proportion of heterozygous markers in the consensus map (32.9%; Table 1-1). The highest proportion of heterozygous markers was in the ‘Honeycrisp’ × ‘Monark’ population with 48.4% in the parental map and 45.2% in the

consensus map. The ‘Honeycrisp’ × ‘Gala’ population had 33.0% heterozygous markers in the parental map and 31.0% in the consensus map.

Parental Linkage Maps

Three ‘Honeycrisp’ (single parent) linkage maps were constructed from segregating populations using SNP markers (Figure 1-3; Supplemental Resource 1). The maps each contain 17 linkage groups representing the 17 known chromosomes that comprise the *Malus* × *domestica* genome. The shortest map was 1097.55 cM and was constructed from the ‘Honeycrisp’ × ‘Gala’ population from 1042 markers with an average spacing of 1.05 cM between markers. The next longest map was 1340.20 cM and was constructed from the ‘Honeycrisp’ × ‘Monark’ population with 1018 SNP markers and an average marker spacing of 1.32 cM between markers (Table 1-2). The ‘Honeycrisp’ × MN1764 map was 1350.29 cM in length and was constructed from 1041 SNP markers, with an average marker spacing of 1.30 cM. The marker coverage for the linkage groups ranged from 23 markers [LG7 (‘Honeycrisp’ × MN1764)] to 88 markers [LG4 (‘Honeycrisp’ × MN1764 and ‘Honeycrisp’ × ‘Monark’)]. The maximum gap size for any linkage group ranged from 5.13 cM (LG9 ‘Honeycrisp’ × ‘Gala’) to 129.64 cM (LG17 ‘Honeycrisp’ × ‘Monark’). The “lone wolf” marker on LG17 (refer to Figure 1-3) was retained as it met the parameters described above and was resolved in the consensus map.

Consensus Linkage Map

The three ‘Honeycrisp’ linkage maps were merged to create one consensus linkage map comprising markers segregating in one or more of the ‘Honeycrisp’ mapping

populations (Figure 1-4). The consensus map was constructed using 1091 SNP markers (13.9% of the IRSC 8K SNP array v1; Table 1-2; Supplemental Resource 2). Figure 1-5 details the 951 markers in common across all three populations, and the 140 SNP markers segregating in only one or two populations. The consensus map is 1481.72 cM with an average distance of 1.36 cM between markers (Table 1-2). The sizes of the linkage groups range from 61.58 cM (LG8) to 130.48 cM (LG15). The largest gap in the consensus map was 34.21cM on LG7.

Comparison of Genetic Positions to Physical Map

The genetic positions of markers in the consensus 'Honeycrisp' map were plotted against the physical positions of marker loci on the 'Golden Delicious' genome (Figure 1-6). Generally, there was agreement in the placement of the markers between the 'Honeycrisp' map and the genome sequence as evidenced by the linearity in the plots. The majority of the markers revealed direct correspondence between the linkage groups and the 'Golden Delicious' pseudo-chromosomes. Across the linkage map, 110 (10.1%) markers mapped to linkage groups other than the corresponding pseudo-chromosome. Eight markers that were placed in the consensus 'Honeycrisp' map were classified as "unanchored" in the physical map. Areas of high recombination, indicated by large horizontal gaps in the Figure 1-6, were detected along several of the LGs including LG 1, 6, 7, and 10. Areas of low recombination are also evident as marker clusters.

Segregation Distortion

Of the markers included in the consensus linkage map, 57 showed significant ($p < 0.005$) segregation distortion in the 'Honeycrisp' × 'Gala' progeny, 58 were significantly

distorted in the 'Honeycrisp' × MN1764 progeny, and 41 were significantly distorted in 'Honeycrisp' × 'Monark' progeny. In total, only nine markers showed significant segregation distortion in two families (black points, Figure 1-6) and 138 markers showed significant segregation distortion in only one family (gray points, Figure 1-6). None of the markers of the consensus map showed significant segregation distortion at the 0.005 level in all three progenies. Because segregation distortion was not used as a quality control measure during marker selection or map construction, 13.5% of mapped markers showed significant segregation distortion in at least one of the three families. Significant distortion was primarily clustered to regions on LGs 2, 5, 6, 13, 14 and 17.

Discussion

We have developed a consensus 'Honeycrisp' linkage map spanning 17 linkage groups representing the 17 chromosomes in the apple genome using the high-throughput IRSC 8k SNP array v1 (Chagné et al., 2012) and three mapping populations. The strategy utilized stringent data checking steps to ensure quality marker data including: selection of high quality SNP reads, removal of markers demonstrating a high frequency of double recombination, and examination of "lone wolf" markers. FlexQTL identified problematic markers that exceeded the threshold for observed double recombinants based on the expected frequency in each of the families. We were not able to position these markers elsewhere in the map using Joinmap. The double recombination pattern was visualized in Map Chart v2.2 (Voorrips, 2002) and also provided a quick, graphical interpretation after each round of mapping. This method was convenient and intuitive without the added complexity of graphical genotyping for ordering markers and

identifying spurious markers. This methodology utilizes files that can be used in QTL analysis with FlexQTL, thus reducing the burden of creating new files or data for other interfaces.

The mapping approach outlined here drastically reduced the number of SNP markers to only 13.8% of those on the 8K IRSC SNP array. The first reduction to ~2000 SNP markers was based on stringent parameters to identify high quality reads with visually distinguishable clusters in the GenomeStudio software. These markers were then scored for all three populations in accordance with other reports using similar numbers of markers for linkage mapping in apple (Antanaviciute et al., 2012; Micheletti et al., 2011). The FlexQTL inheritance checking algorithm efficiently identified problematic markers or inheritance errors. Data free of genotyping errors are very important for construction of genetic maps to ensure proper marker ordering.

The detection of functional 'Honeycrisp' haplotypes will provide utility in genetic studies of progeny populations with the aim of identifying genetic contributions specific to this parent. The consensus map has an average interval of 1.36 cM between markers, a much higher marker density than has been achieved with SSR linkage maps, and provides sufficient marker coverage for moderate sized QTL mapping populations. The often touted advantage of a high-throughput SNP array is the reduced price per marker. But marker quality and usefulness are not uniform across all loci. Homozygosity at a marker locus, genotyping quality, and genotyping errors all contribute to increasing the cost per informative marker. Chagné et al (2012) showed that of the 8K array, only 70.6% of the markers were polymorphic in the > 1600 individuals, accessions and segregating

populations that were evaluated. The development of a reduced array that retains those highly informative, polymorphic markers across the genome could increase the efficiency for MAB.

Linkage mapping in JoinMap 4.1 utilizing the published M432 map (Antanaviciute et al., 2012) for the grouping step was computationally efficient. Using a previously published map for group assignment may result in incorrectly grouped markers for those SNPs shared between maps. Furthermore some markers may map to one genomic location in one segregating population and map to a paralog in a different population. The method employed here was designed to select markers for consensus map construction that consistently placed SNPs to common groups. The multipoint maximum likelihood method for mapping was faster than the regression mapping and was thus utilized in this study of outcrossing populations (Van Ooijen, 2011). The construction of two parental maps and an integrated map for each population was useful in determining the fate of "lone wolf" markers although only the 'Honeycrisp' parental map was retained for consensus map construction.

The three 'Honeycrisp' parental maps each comprised shared and unique markers due to observed differences in heterozygosity in the parents and the quality of SNP calls. For example, in the consensus map, the distal end of LG15 was greatly extended by the inclusion of the 'Gala' and 'Monark' populations since the MN1764 population was uninformative in that region (Figure 1-2). Heterozygous markers may have been discarded differently within GenomeStudio among the populations due to the quality of the reads. Low levels of heterozygosity were observed even in the consensus map in

some areas such as LG7, similar to the M432 map (Antanaviciute et al., 2012). To increase the coverage in these regions, one could return to GenomeStudio and use less stringent quality parameters for SNP calls. Additionally, markers developed specifically from pseudo-chromosome 7 could be scored and added to the maps. Genomic regions with high levels of homozygosity shared among cultivars could be an artifact of domestication, genetic drift, other intentional or unintentional selection, or a bottleneck. An exploration of these genome areas among other cultivars and *Malus* species linkage maps could provide insight into the genes that reside in these areas.

The clustering strategy that was utilized in the development of the IRSC 8k SNP array resulted in many SNP markers mapping to the same locus. Low recombination in these areas makes it difficult to assign the correct map order. Observed differences in local homology between the parental maps may be the result of within cluster ordering. Using the physical map to order the markers would be one strategy to resolve this issue, however the ordering of the physical map may also be incorrect. Additionally, the physical order of markers may be different between the three populations due to disruption in micro-synteny and structural variations (Khan et al., 2012). Because the recombination frequency is so small within a cluster or tightly mapped clusters/markers, the precise order may not serve as a barrier to QTL detection. This is especially true in a pedigree-based approach, in which markers within a cluster may have different utility for individuals of different subpopulations. That is, any given individual SNP marker within a cluster at a single marker locus may segregate for some individuals or subpopulations and not others, but the map position is not lost for the entire pedigreed population.

Additionally, local marker order may not be important in establishing functional haplotypes in a cluster in which low frequencies of recombination events occur.

Antanaviciute et al. (2012) compared map positions of an integrated apple rootstock linkage map to the 'Golden Delicious' genome sequence, reporting that 13.7% of genetically mapped markers did not associate with the predicted pseudo-chromosome. Our results are consistent with this finding, but may be influenced by our use of the M432 map for grouping of markers. Over 10% of markers in the consensus map were placed to linkage groups other than the corresponding pseudo-chromosome. These markers should be evaluated for known homology in the *Malus* × *domestica* genome (specifically, known genome duplications and possible misalignments of contigs in the development of the 'Golden Delicious' genome sequence). Of the 110 markers of the consensus map (1091 total SNPs) that mapped to alternate pseudo-chromosomes, only 10 (9.1%) were associated with potential homeologous chromosomes from the genome wide duplication event (Velasco et al., 2010).

To test bias in the grouping strategy, the 'Honeycrisp' × 'MN1764' population was grouped *de novo* in JoinMap 4.1 using the high-quality 1785 SNPs that remained after suspect markers had been removed. 13.5% of the SNP markers were grouped to positions other than the predicted pseudo-chromosome. Of these, 14.7% were predicted to potential homeologous regions. For instance, a cluster of markers initially associated with pseudo-chromosome 9 of 'Golden Delicious' maps to the top of LG4 in both the M432 and 'Honeycrisp' maps. However, had our data not supported these placements, it

is likely the markers would have been identified as "suspect linkages" during mapping and thus been discarded.

Significant segregation distortion was observed for 13.5% of the markers in the final 'Honeycrisp' consensus map when no quality control measures regarding segregation distortion were used during marker checking or linkage map construction. The choice not to use segregation distortion as a quality control measure was made because marker segregation distortion could represent real, biologically relevant segregation distortion and the inclusion of these markers could be useful in QTL detection. Largely supporting this hypothesis is the observation that markers exhibiting segregation distortion mapped in cohesive clusters along only several linkage groups. Biological reasons for segregation distortion are those that impose selection upon the population such as selective fertilization (apple's gametophytic self-incompatibility), abortion of gametes (Liebhard et al., 2003b), and other unavoidable natural selective pressures such as field environment (e.g. winter hardiness) that are inadvertently imposed upon the breeding populations (i.e. the *ad hoc* mapping populations utilized in this study). Markers with observed segregation distortion need not be within the survival gene, and they may be linked with the gene conferring survivorship. Segregation distortion observed in this study was not found in the same linkage groups as that reported by Antanaviciute et al. (2012) with the exception of that on LG17 which contains the *S*-locus (Maliepaard et al., 1998).

The GenomeStudio software and manual calling of SNPs into biallelic clusters (AA, AB, BB) is constrained by the quality of reads. Inherent in difficulty with read

quality are errors resulting from DNA quality, contamination, DNA hybridization and extension, and fluorescence signal. Recent whole genome duplication, segmental duplication, and a high degree of homology between some markers results in SNP markers exhibiting segregation behavior similar to that of polyploids in the cluster plots (Voorrips et al., 2011; personal observation). DNA from different genomic regions may hybridize to the same marker, typically resulting in more than three clusters. However, not all of these occurrences may be detected manually or within the automated calling. The spread of a cluster in automated/manual calling of multiple populations (pedigrees, diverse sets) may provide statistical support of a single cluster, but may mask the presence of more than three clusters within a single population that would have been identified as a potential homolog and removed.

A high degree of colinearity was observed between the consensus map and the physical positions along the 'Golden Delicious' pseudo-chromosomes. Large linkage gaps were observed in regions of low marker coverage, presumably centromeric and telomeric regions. Colinearity supports the physical ordering of markers, and strengthens the development of meaningful haplotypes that represent true chromosome position. Markers that do not align may result in haplotypes that are a mosaic of different chromosome segments.

The consensus 'Honeycrisp' linkage map developed from three progeny populations consists of 1091 SNP markers distributed across the apple genome. These markers were developed from exonic regions from the 'Golden Delicious' genome sequence which adds to their utility in predicting function in marker-locus-trait

associations (Chagné et al., 2012). More importantly, these markers are informative in an elite cultivar that is being utilized in breeding programs worldwide for its superb fruit quality traits. QTL analysis in 'Honeycrisp' will focus on identifying the haplotypes associated with crispness, firmness, and juiciness, but will also focus on identifying deleterious associations with post-harvest disorders such as soft scald, internal browning, and bitter pit, to which 'Honeycrisp' is prone.

Tables

Table 1-1 Number and percentage of heterozygous markers of the non-'Honeycrisp' parent for its corresponding parental map and in the consensus map for three mapping populations ('Honeycrisp' × 'Gala', 'Honeycrisp' × MN1764, and 'Honeycrisp' × 'Monark'). 'Honeycrisp' is heterozygous at all mapped loci.

Parent	Heterozygous Markers in Parent	Markers in Parental Map	Proportion of Parental Map (%)	Proportion of Consensus Map (%) (1091 markers)
Gala	448	1042	43.0	41.1
MN1764	359	1041	34.5	32.9
Monark	493	1018	48.4	45.2

Table 1-2 Details from the genetic linkage maps of three 'Honeycrisp' parental maps from three full-sib populations ('Honeycrisp' × 'Gala', 'Honeycrisp' × MN1764, and 'Honeycrisp' × 'Monark'). Number of markers per linkage group, map size (cM), density, and largest gap are given. Details from the consensus map constructed from the integration of the three 'Honeycrisp' parental maps are also shown in bold.

		LG1	LG2	LG3	LG4	LG5	LG6	LG7	LG8	LG9
Number of Markers	Gala	55	77	48	85	82	51	24	56	90
	MN1764	54	80	50	88	82	51	23	55	84
	Monark	54	77	49	88	69	49	24	51	87
	Consensus	**56**	**83**	**53**	**90**	**83**	**52**	**26**	**57**	**91**
Size (cM)	Gala	57.6	53.95	71.32	50.36	83.08	66.91	66.47	45.98	40.18
	MN1764	50.99	74.21	134.16	125.03	94.46	64.73	73.84	37.32	56.76
	Monark	63.53	51.35	84.02	51.86	76.54	101.9	94.16	51.16	59.37
	Consensus	**71.34**	**78.86**	**112.4**	**84.11**	**108.66**	**78.39**	**89.14**	**61.58**	**72.23**
Average marker distance (cM)	Gala	1.05	0.7	1.49	0.59	1.01	1.31	2.77	0.82	0.45
	MN1764	0.94	0.93	2.68	1.42	1.15	1.27	3.21	0.68	0.68
	Monark	1.18	0.67	1.71	0.59	1.11	2.08	3.92	1	0.68
	Consensus	**1.27**	**0.95**	**2.12**	**0.93**	**1.31**	**1.51**	**3.43**	**1.08**	**0.79**
Maximum gap size (cM)	Gala	11.66	7.78	13.54	7.77	11.66	9.68	31.18	5.94	5.13
	MN1764	8.76	7.71	13.21	7.7	13.21	12.43	38.63	8.76	10.94
	Monark	12.57	8.04	25.14	61.24	14.2	41.96	33.15	11	8.02
	Consensus	**10.87**	**6.88**	**12.24**	**12.92**	**12.39**	**9.48**	**34.21**	**6.33**	**7.91**

Table 1-2 continued (2 of 2)

		LG10	LG11	LG12	LG13	LG14	LG15	LG16	LG17	Total
Number of Markers	Gala	66	59	50	48	42	84	57	68	1042
	MN1764	67	62	51	50	38	84	55	67	1041
	Monark	68	60	51	48	38	85	55	65	1018
	Consensus	**72**	**64**	**52**	**51**	**44**	**91**	**58**	**68**	**1091**
Size (cM)	Gala	63.71	51.61	58.51	104.7	65.63	99.83	54.64	63.07	1097.55
	MN1764	90.56	84.22	63.48	65.05	56.19	134.47	77.85	66.98	1350.29
	Monark	81.72	83.35	60.34	66.34	64.07	89.72	63.11	197.66	1340.2
	Consensus	**91.54**	**97.18**	**75.51**	**97.6**	**75.95**	**130.48**	**74.12**	**82.63**	**1481.72**
Average marker distance (cM)	Gala	0.97	0.87	1.17	2.18	1.56	1.19	0.96	0.93	1.05
	MN1764	1.35	1.36	1.24	1.3	1.48	1.6	1.42	1	1.3
	Monark	1.2	1.39	1.18	1.38	1.69	1.06	1.15	3.04	1.32
	Consensus	**1.27**	**1.52**	**1.45**	**1.91**	**1.73**	**1.43**	**1.28**	**1.22**	**1.36**
Maximum gap size (cM)	Gala	15.87	9.68	8.72	53.36	10.66	16.98	11.66	6.85	
	MN1764	14.38	16.82	12.06	12.06	10.94	20.72	16.82	15.59	
	Monark	14.12	15.85	9.49	14.2	9.49	11	6.59	129.64	
	Consensus	**13.34**	**23.54**	**10.09**	**22.26**	**8.68**	**16.77**	**12.19**	**5.82**	

Figure 1-1 Work flow describing the mapping in process including the number of SNP markers retained at each stage.

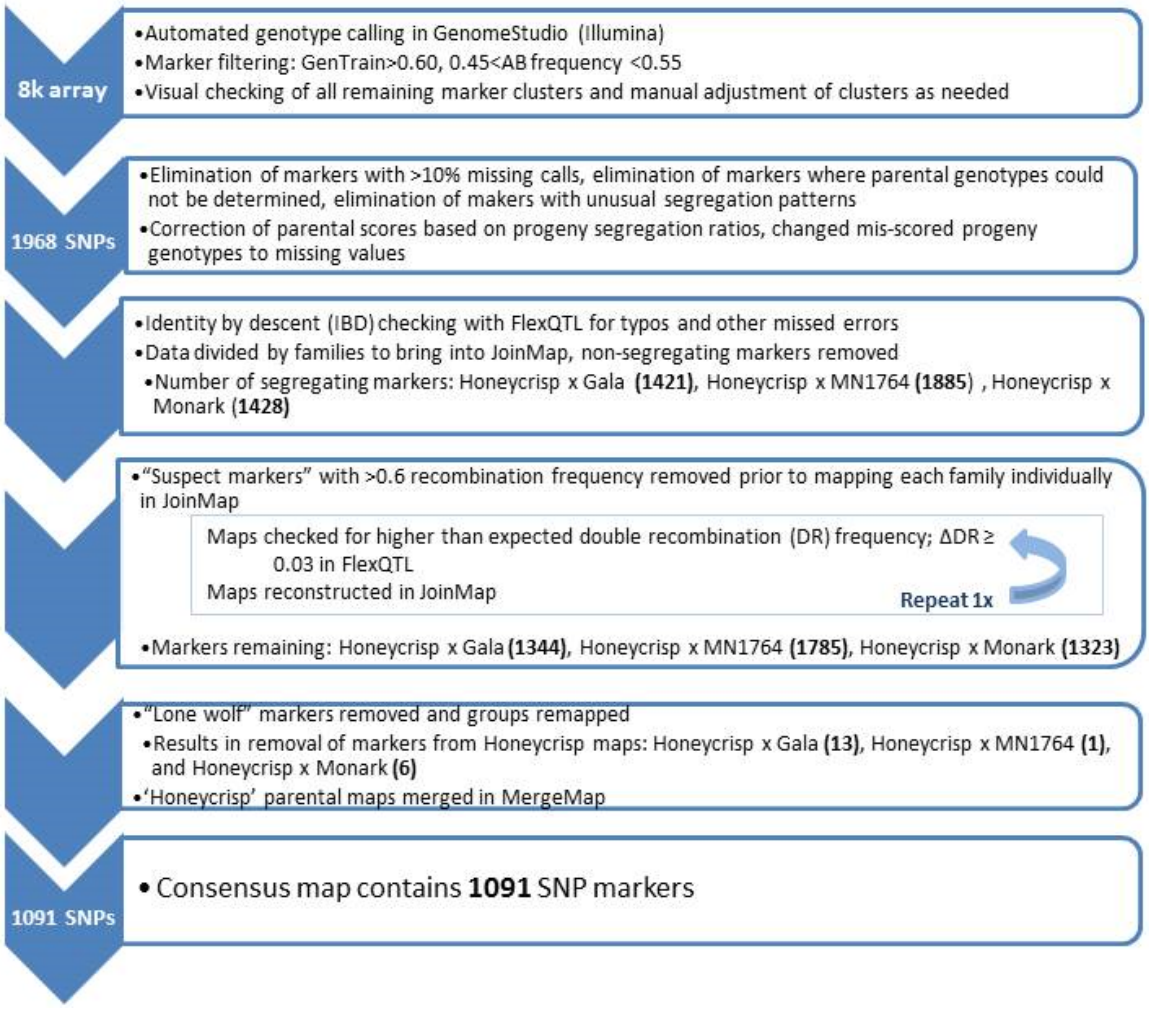


Figure 1-2 Heterozygosity plot indicating polymorphism in the parents from the three mapping populations ('Honeycrisp' × 'Gala', 'Honeycrisp' × MN1764, and 'Honeycrisp' × 'Monark') plotted on the consensus map (X-axis). 'Honeycrisp' is heterozygous at all loci. Multiple open circles at a locus indicate more than one SNP marker mapped to that locus for the given parent.

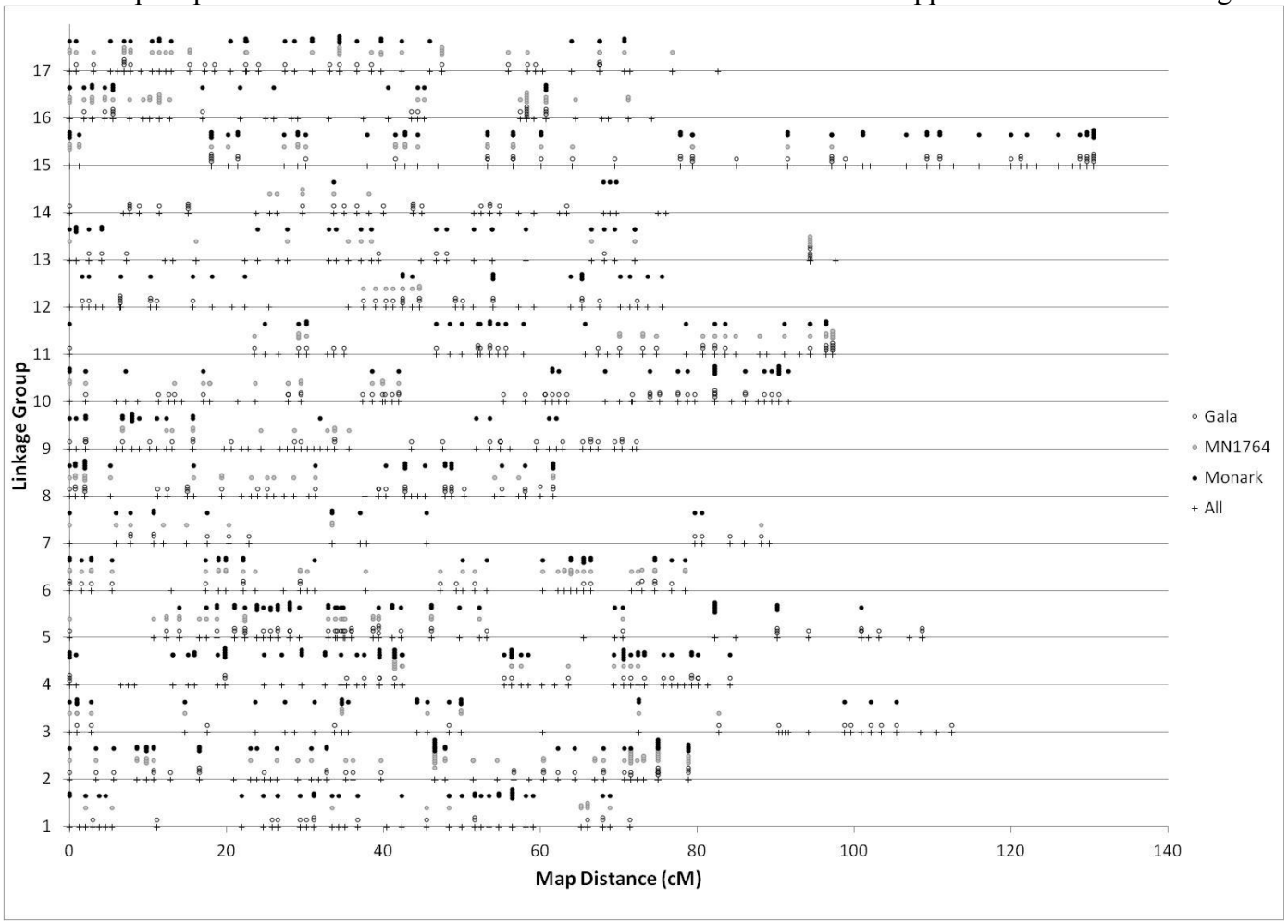


Figure 1-3 Three ‘Honeycrisp’ parental maps (‘Honeycrisp’ × ‘Gala’, ‘Honeycrisp’ × MN1764, and ‘Honeycrisp’ × ‘Monark’) utilized in consensus map construction. Lines between linkage groups show homology between maps within that linkage group.

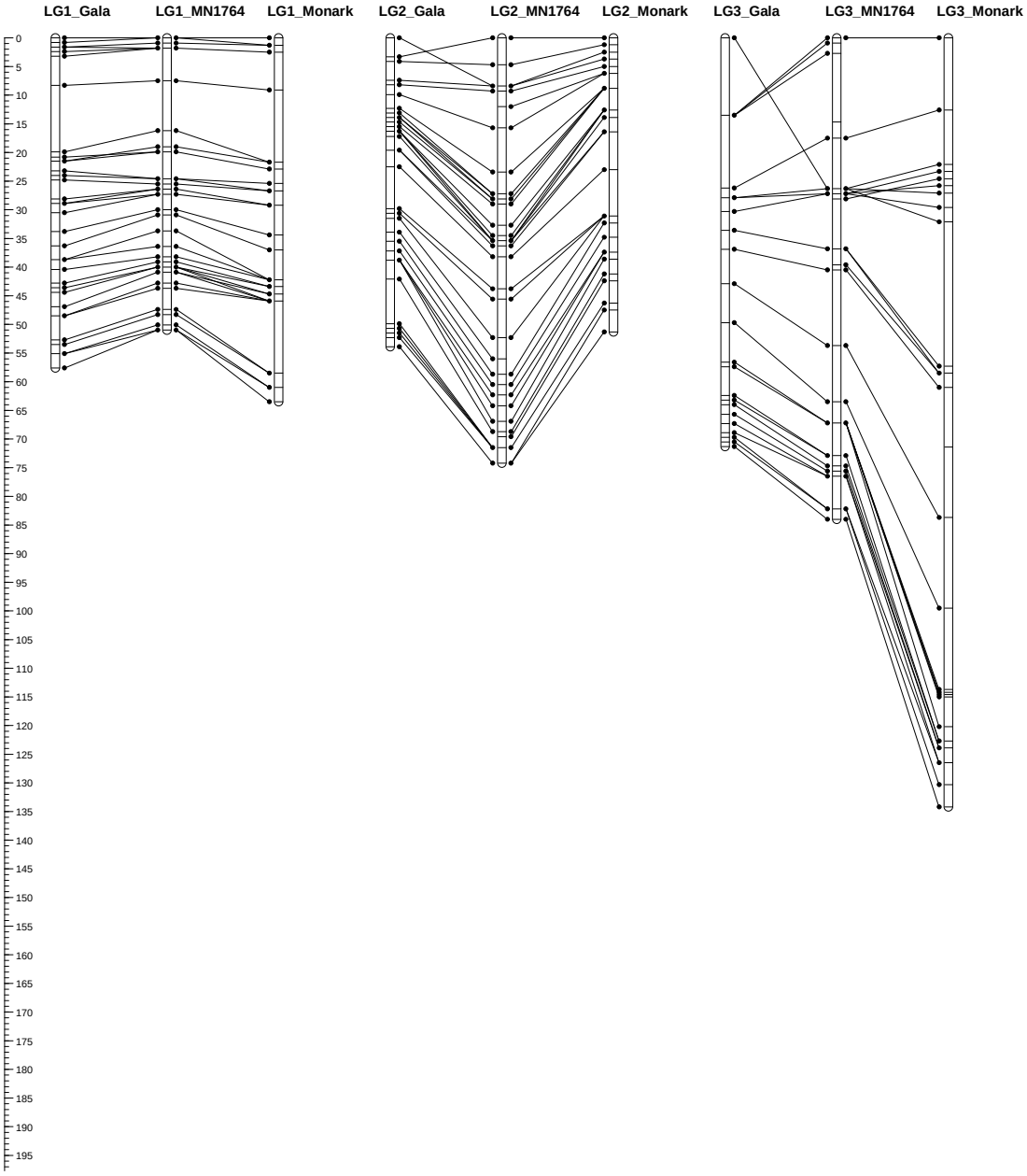


Figure 1-3 continued (2 of 6)

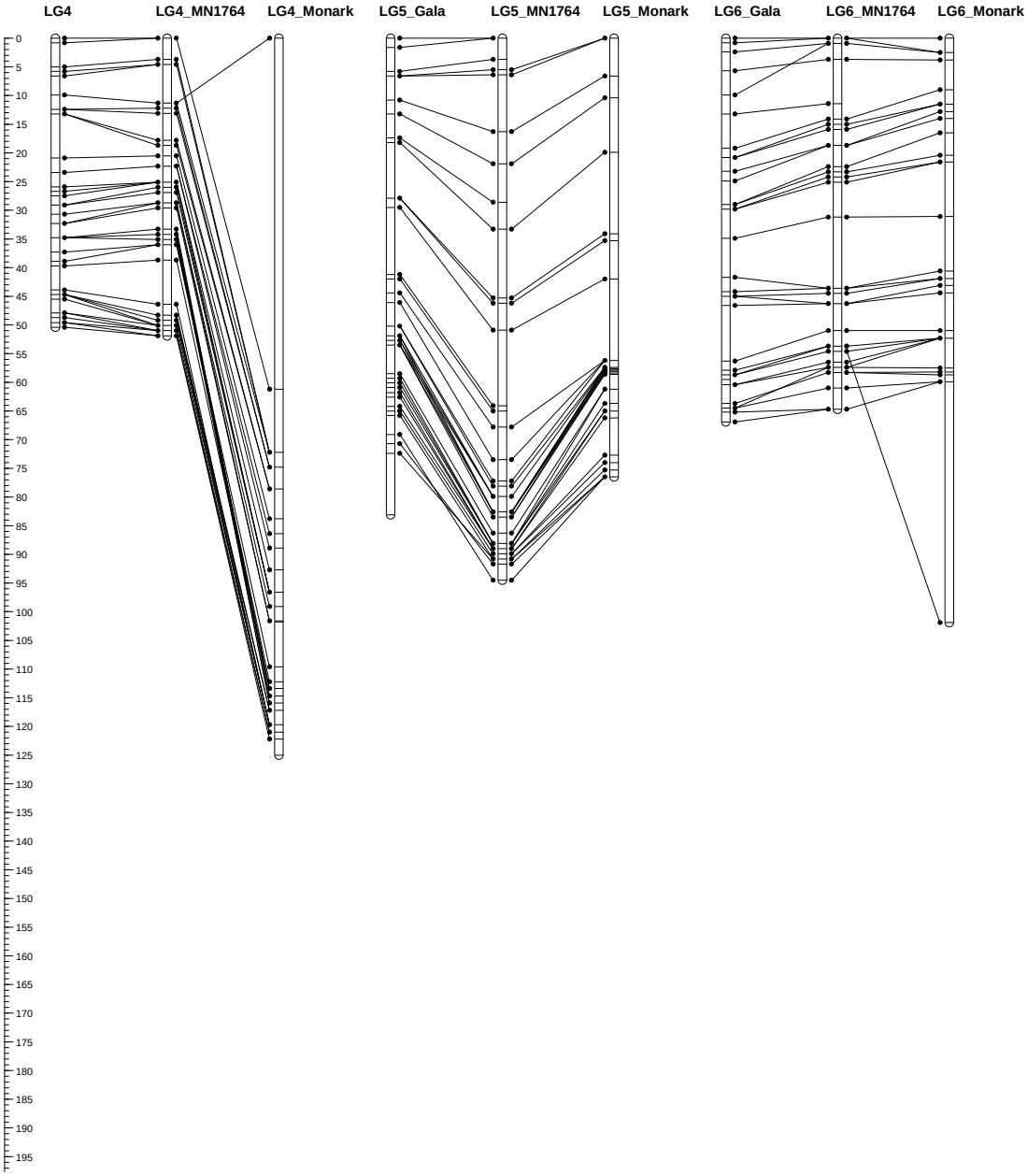


Figure 1-3 continued (3of 6)

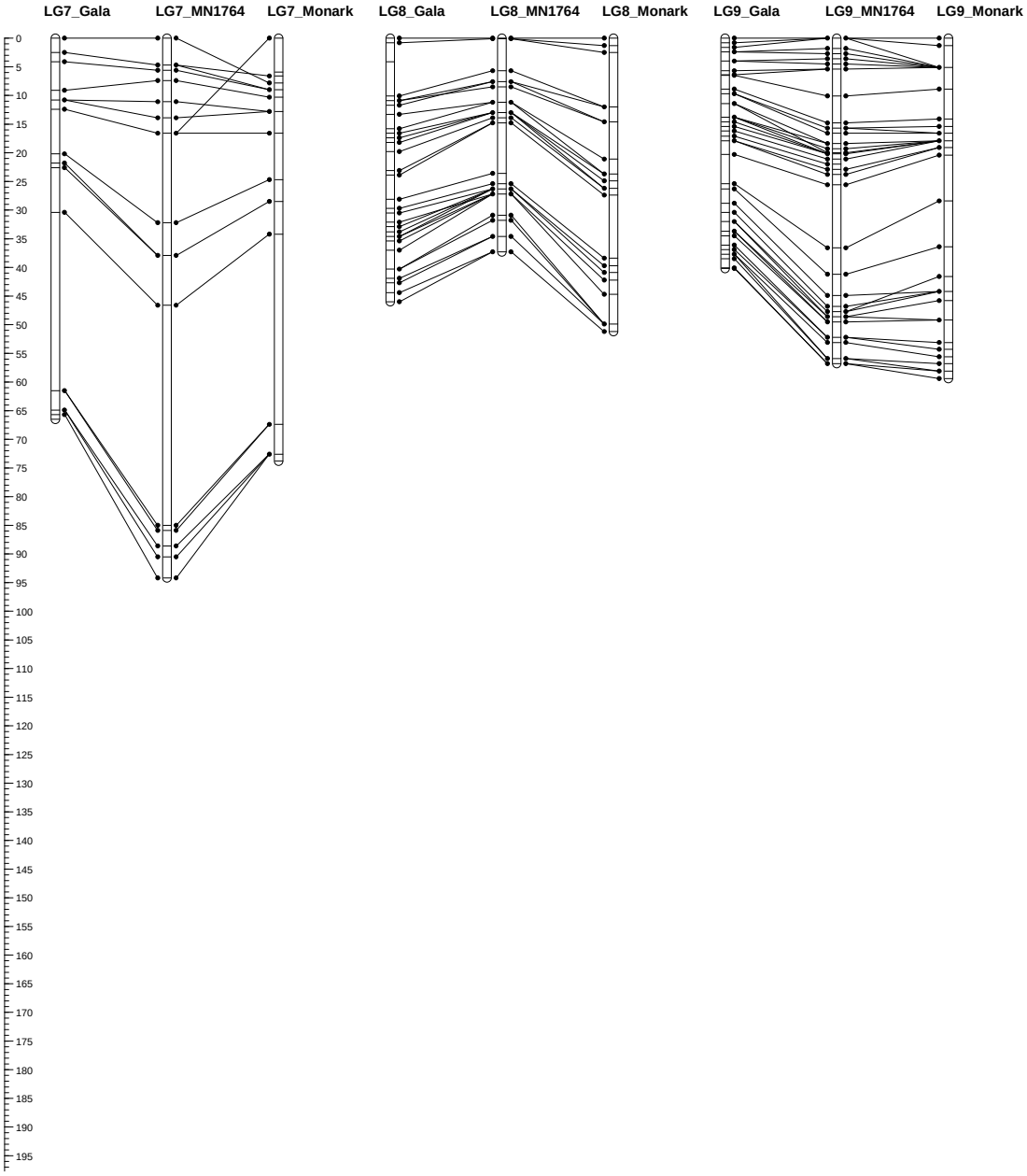


Figure 1-3 continued (4 of 6)

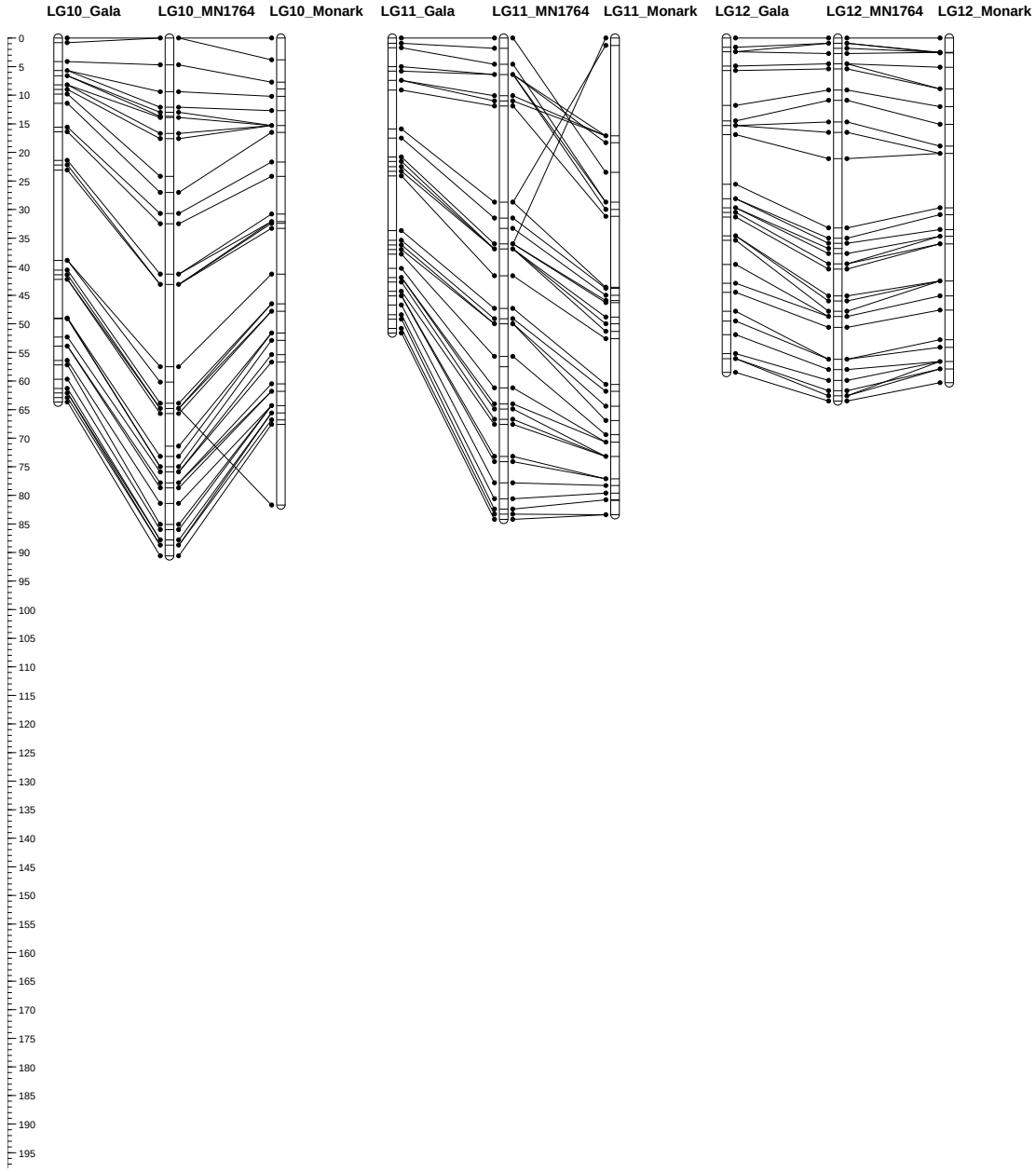


Figure 1-3 continued (5 of 6)

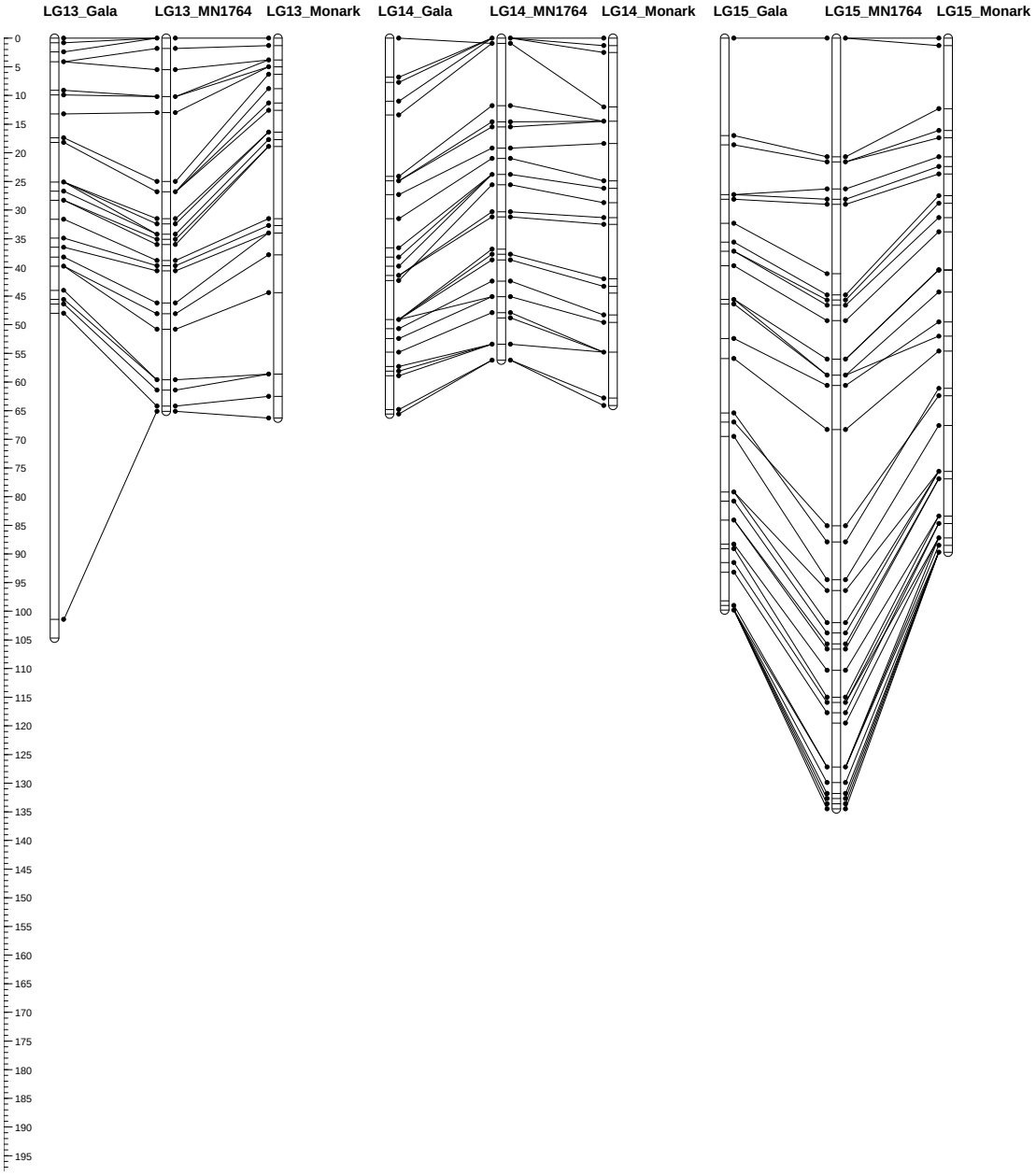


Figure 1-3 continued (6 of 6)

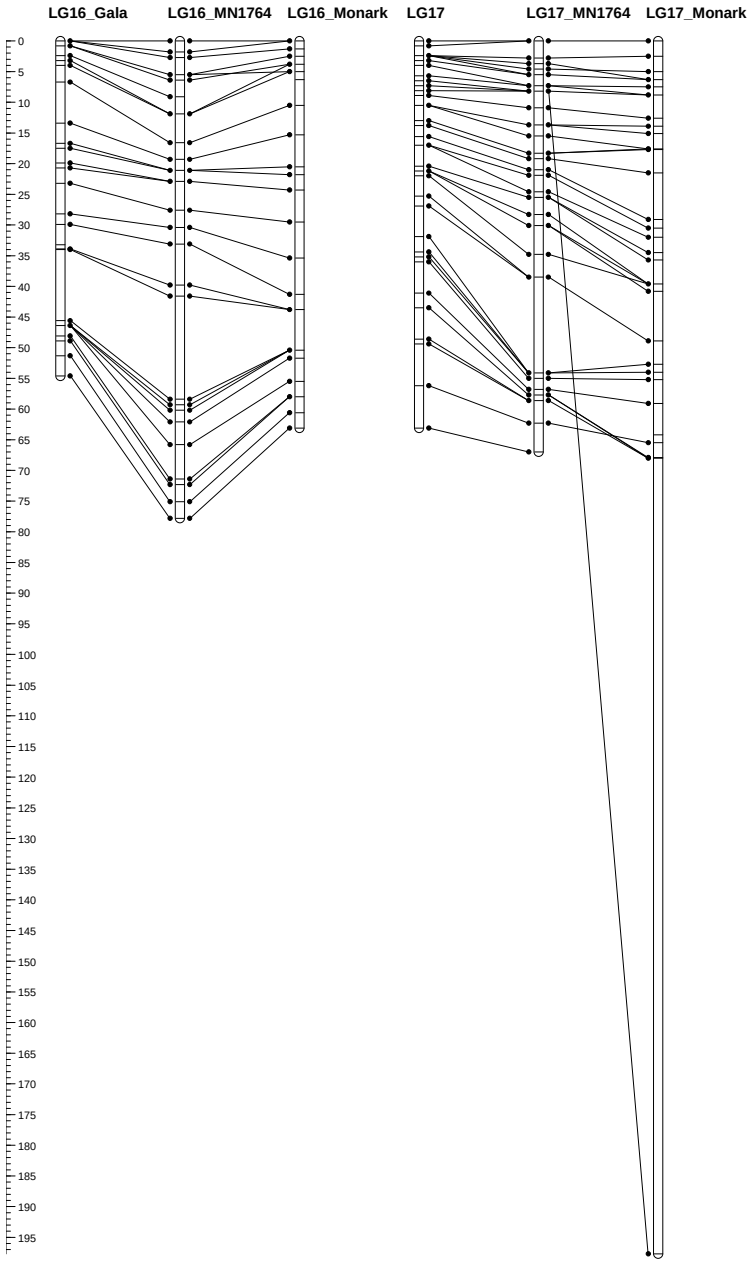


Figure 1-4 Consensus ‘Honeycrisp’ linkage map constructed from three ‘Honeycrisp’ parental maps (‘Honeycrisp’ × ‘Gala’, ‘Honeycrisp’ × MN1764, and ‘Honeycrisp’ × ‘Monark’). Markers shown in blue (bold) were not common to all three parental maps.

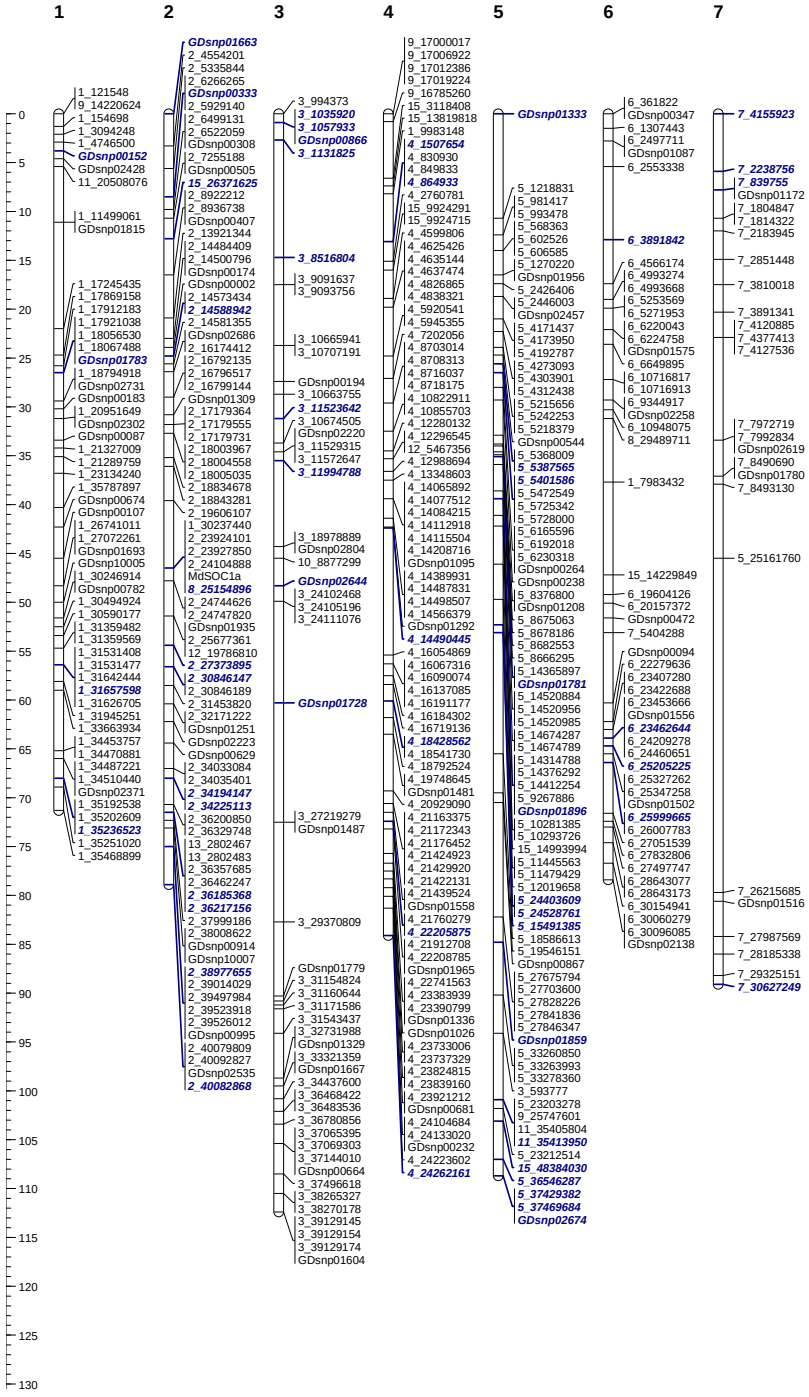


Figure 1-4 continued (2 of 3)

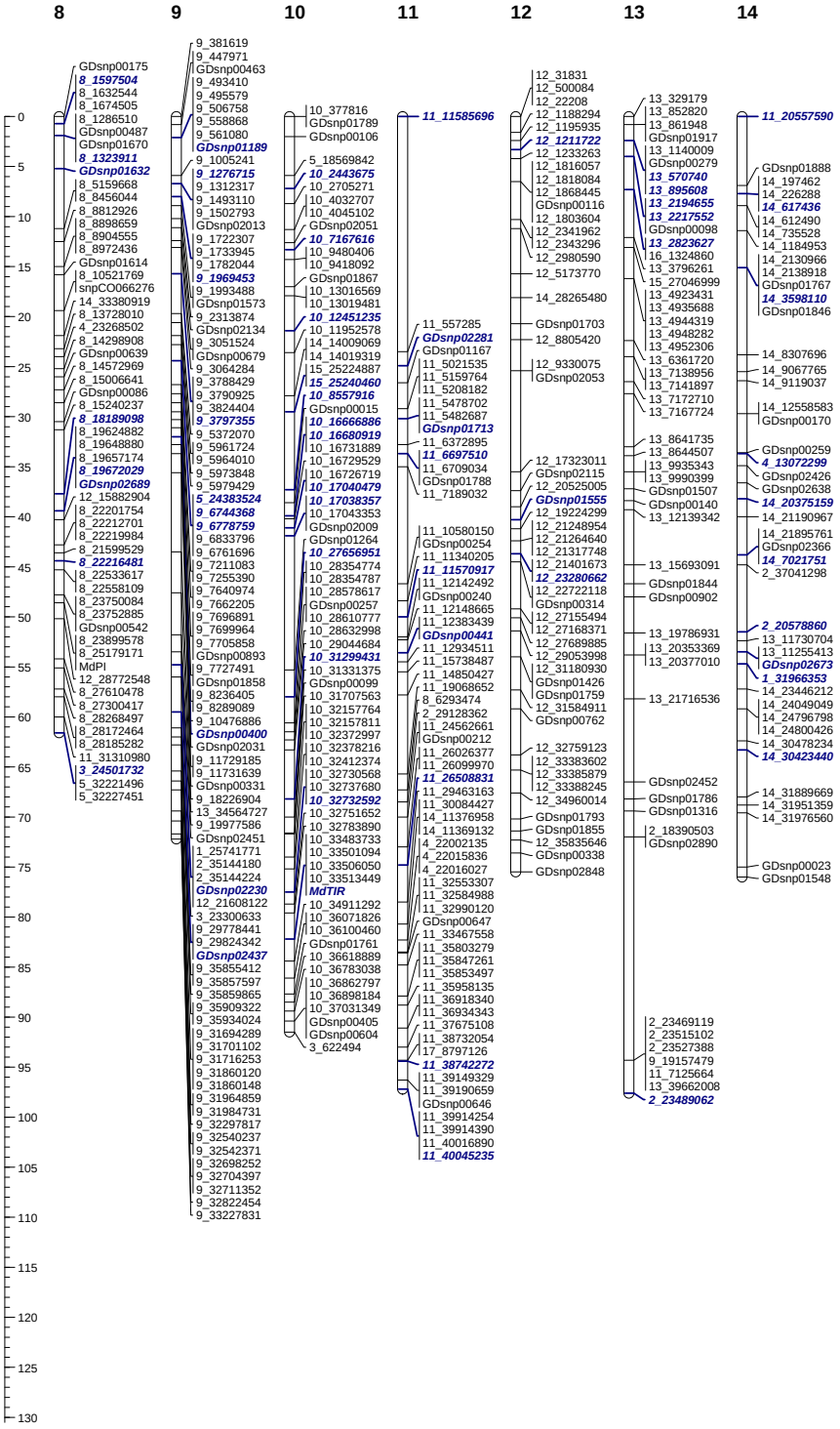


Figure 1-4 continued (3 of 3)

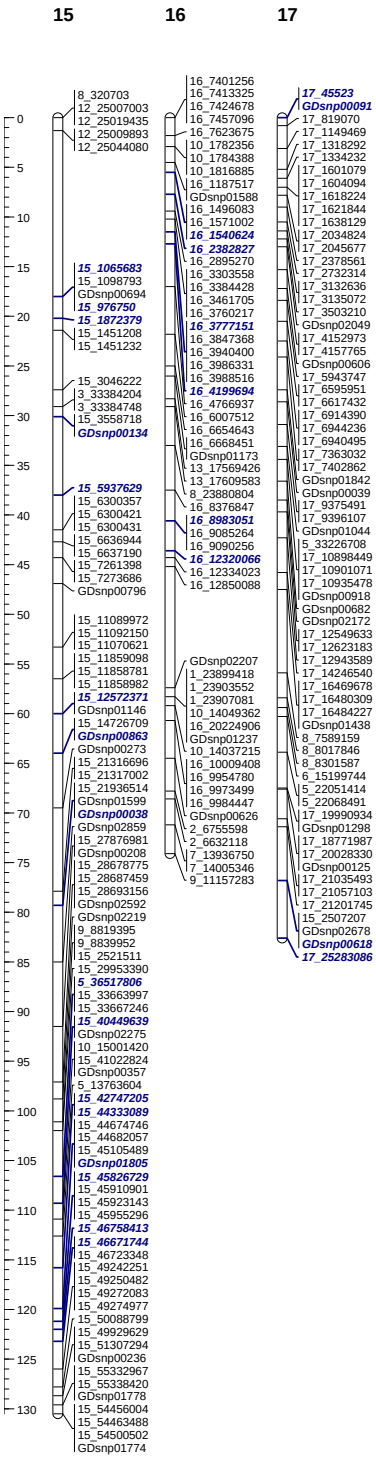


Figure 1-5 Venn diagram showing the number of markers shared in the 'Honeycrisp' consensus map (1091 total SNP markers) and those unique to each population.

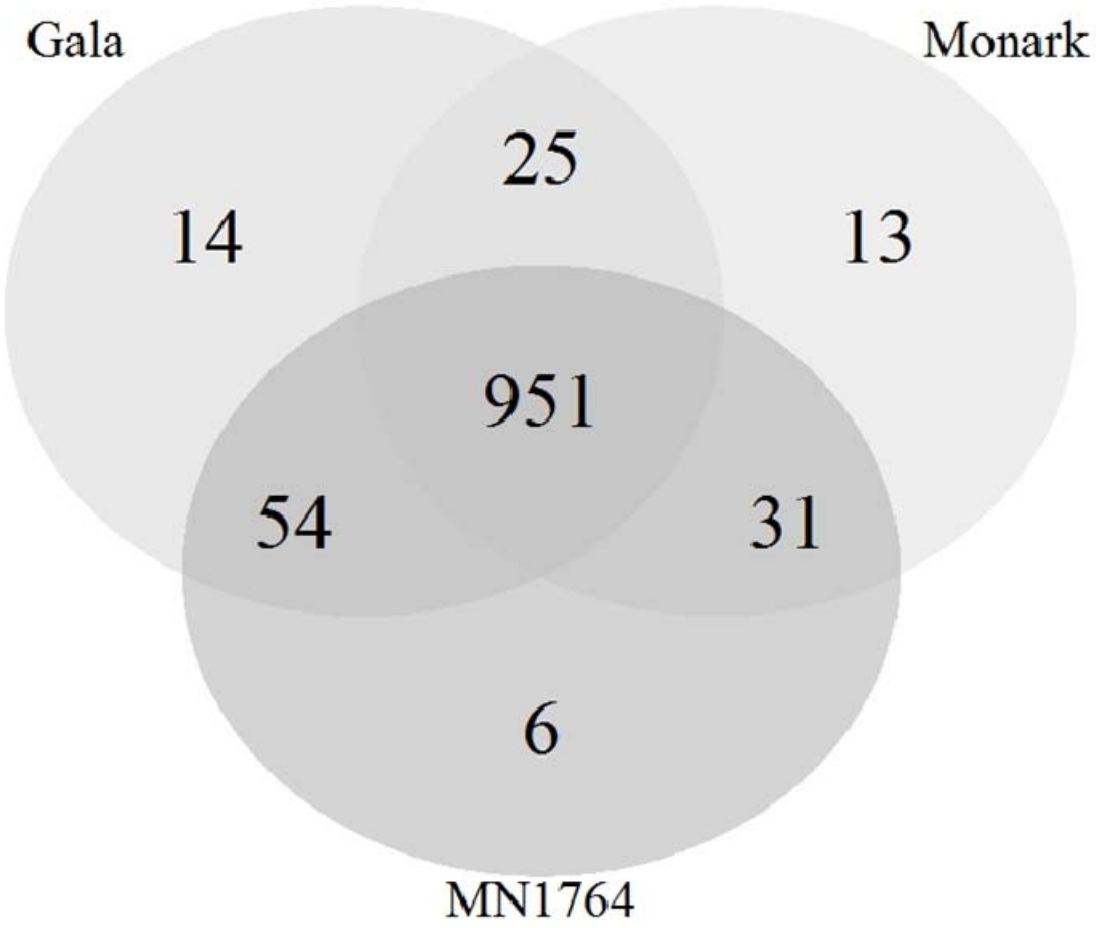


Figure 1-6 Comparison of 'Honeycrisp' consensus map to physical position on 'Golden Delicious' genome sequence for each of the 17 linkage groups. Each plot directly compares the linkage group (LG1- LG17) to the pseudo-chromosome (1-17) available in the Genome Database for Rosaceae (www.rosaceae.org). Markers showing segregation distortion (P-value 0.005) are indicated as follows: open circles (○) no significant distortion in any of the three families, gray filled circles (●) significant distortion in one family, and black filled circles (●) significant distortion in two families. No markers in the consensus 'Honeycrisp' linkage maps showed significant segregation distortion in all three of the mapping populations.

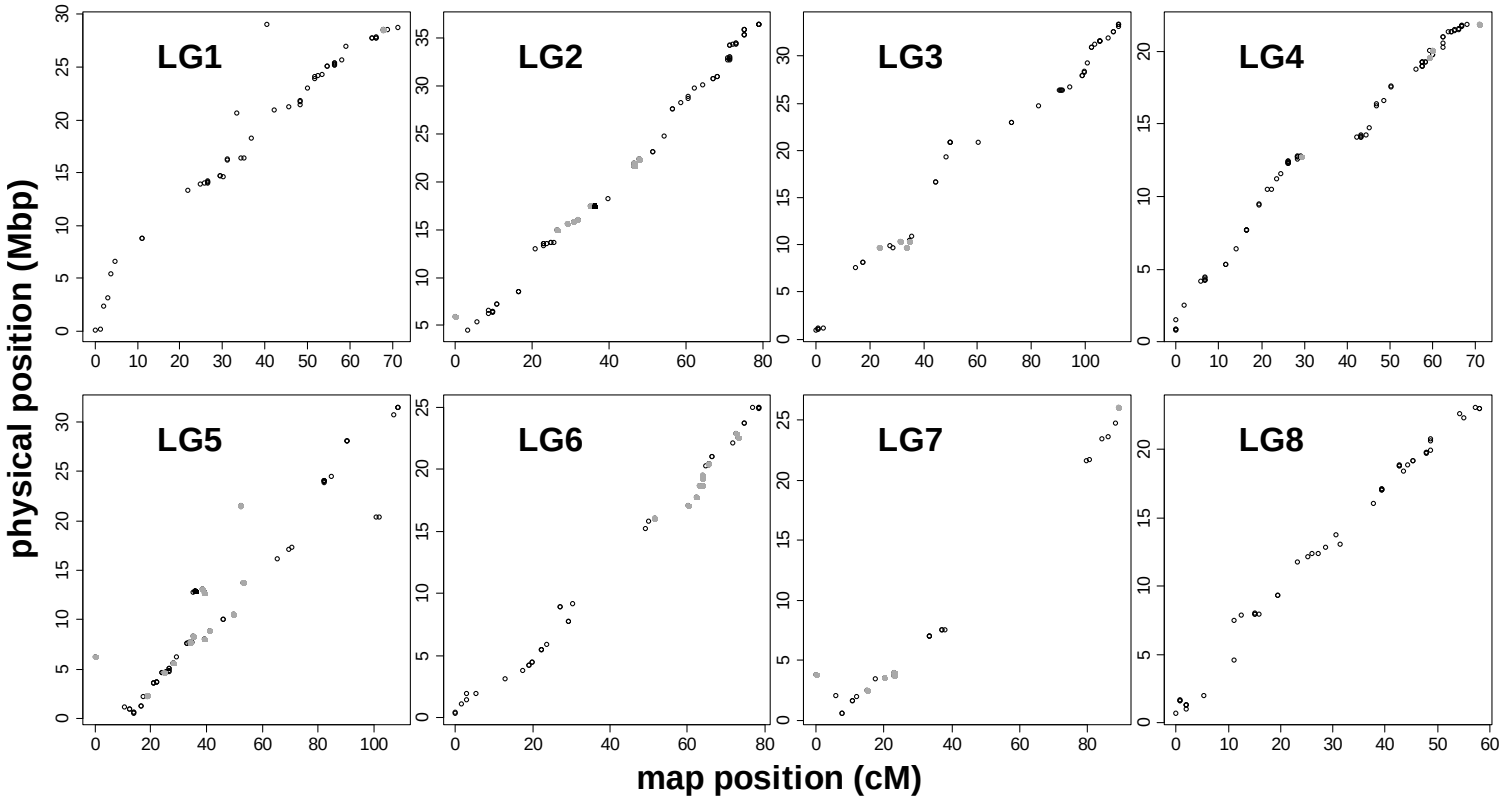
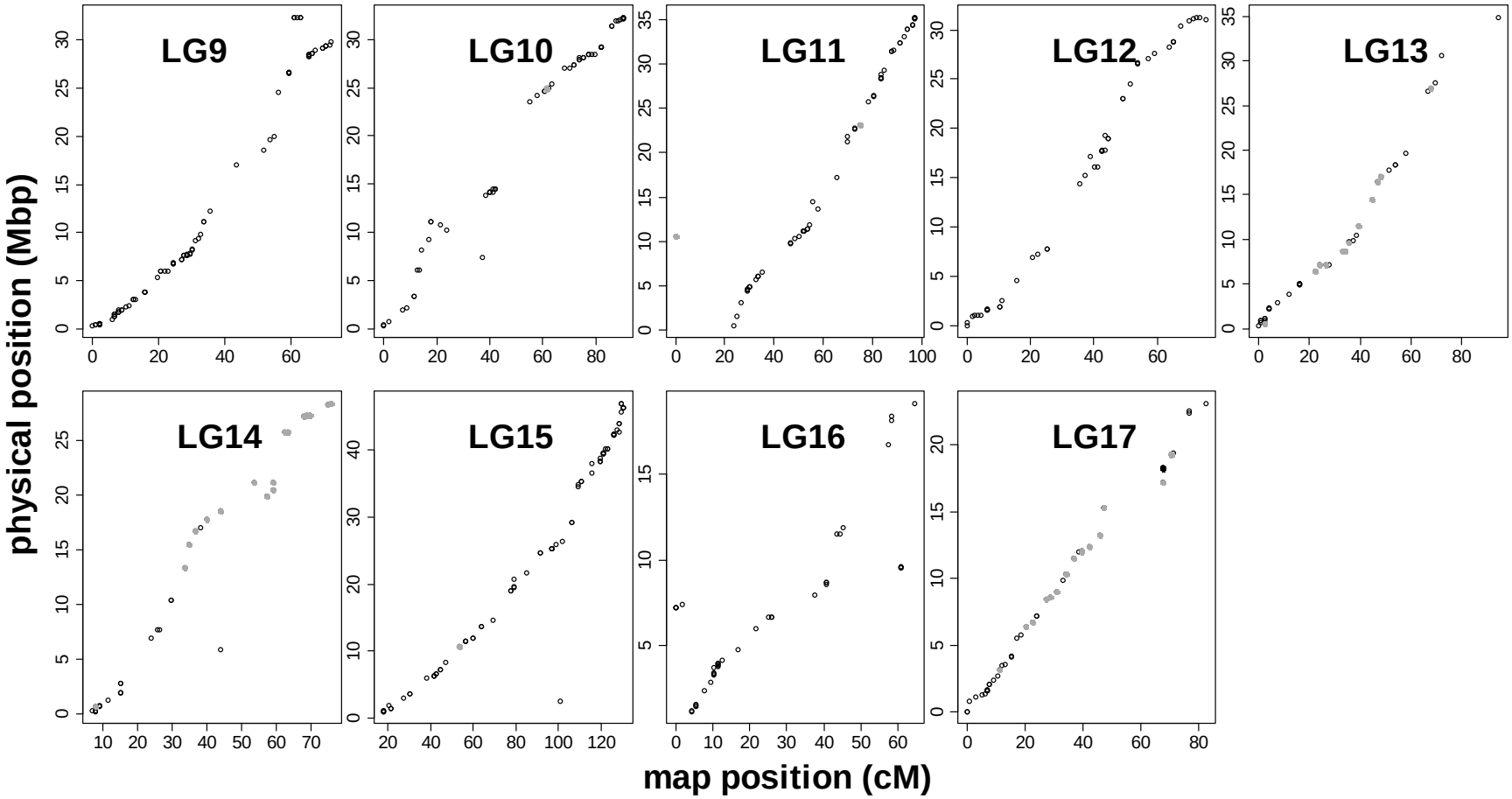


Figure 1-6 continued (2 of 2)



Chapter 2

Characterization of the defense response to *Venturia inaequalis* in 'Honeycrisp' apple, its ancestors, and progeny

The apple cultivar Honeycrisp exhibits genetic resistance to apple scab. The characterization of the macroscopic and microscopic responses in leaves infected by the pathogen *Venturia inaequalis* are described. The macroscopic resistance reactions observed in 'Honeycrisp', its parent 'Keepsake', and grandparents 'Frostbite' and 'Northern Spy' ranged from 0 (no reaction) to chlorotic flecking, stellate chlorosis, necrotic flecking, and sporulation. No hypersensitive response was observed. The resistance response occurred as early as 7 days post inoculation (dpi) at the same time that susceptible plants exhibited macroscopic signs of the disease. The resistance reactions were similar in the progeny population of 'Honeycrisp' × 'Twin Bee Gala', although they were delayed to 10-14 dpi possibly due to variable greenhouse conditions. This population segregated 3:1, which provides additional evidence for the presence of two genes in 'Honeycrisp'. The variation within the progeny population demonstrates the interaction of small effect genes, which may be in the downstream signal pathway of the resistance gene(s) that result in varied phenotypes. Different responses within 'Honeycrisp' to mixed inoculum suggest differential recognition of pathogen effectors.

Introduction

The ascomycete *Venturia inaequalis* (Cke.) Wint. is a plant pathogen of apple (*Malus* spp.) in production areas worldwide. The disease caused by the fungus, commonly known as apple scab, is evident on leaves and can negatively affect plant health, habit, and growth. More importantly, symptoms develop on fruit which render them unsalable to many fresh markets. In the orchard, the symptoms first develop in early spring from a primary infection caused by ascospores that are released from pseudothecia in leaf litter. The first ascospore release often coincides with bud break when the host tissue is most vulnerable and the environmental conditions are highly favorable. The ascospores are moved into air currents during rain events. With appropriate weather conditions, the ascospores germinate on young leaves, penetrate the cuticle, and develop into subcuticular stroma that eventually produce conidiophores which rupture the cuticle. Mycelia, conidiophores, and asexual spores called conidia develop resulting in circular green to brown "scab" lesions on infected leaves. The conidia are capable of causing secondary infections on leaves and fruit, and are dispersed throughout the tree canopy through rain and water droplets. As in many host-pathogen associations, resistance in the host can be triggered by host recognition of the pathogen resulting from direct or indirect interaction of host resistance (R) gene products and pathogen effector molecules. Recently, Berkett et al. (2008) identified resistance in the emerging apple cultivar Honeycrisp grown under organic disease management, findings

which led to the current microscopic and macroscopic investigation of the resistance reaction.

To date, 17+ R genes mediating *Malus*-*V. inaequalis* interactions have been described (Gessler et al., 2006; Bus et al., 2011; Jha et al., 2009). These genes each impart pathogen race-specific resistance following the gene-for-gene model first described by Flor (1956). The model defines the incompatible interaction in which the R locus is present in the host and the cognate *Avr* locus is present in the pathogen, resulting in a host phenotype disease resistance (Dangl and Jones, 2001). Vanderplank (1978) described the model as a protein-for-protein interaction which more accurately describes the interaction of the pathogen effector's AVR protein and the host R gene protein(s). Resistant and susceptible plants are each capable of mounting a defense response, however, the lack of detection of the pathogen effector allows the fungus to colonize susceptible leaves and fruit (Boone, 1971).

A key feature of many R proteins is an association with the plasma membrane and possession of extracellular domains involved in protein-protein interactions. The leucine rich repeat (LRR) domain has been shown to be under evolutionary pressure to adapt to changes in pathogen Avr proteins and even minor mutations in the LRR domain can lead to altered specificities in protein-protein interactions, sometimes altering the host resistance phenotype (Dangl and Jones, 2001). The most well characterized apple scab resistance locus in apple, *Rvi6* (*Vf*), is a cluster of four paralogous genes (Vinatzer et al., 2001; Xu and Korban, 2002). In *Rvi6*, the four R-gene paralogs within this cluster show divergence in the LRR (Xu and Korban, 2002) which is attributed to somatic variation

(Xu and Korban, 2004). Initially, two of the paralogs, *Vfa1* and *Vfa2*, appeared to provide functional resistance to apple scab when transformed into susceptible lines (Malnoy et al., 2008). In a later study, only *HcrVf2* (= *Vfa2*) was demonstrated to confer resistance (Joshi et al., 2011)

R gene nomenclature for the *Malus-Venturia* pathosystem has been updated to provide a concise system for describing the gene-for-gene interaction (Bus et al., 2009, 2011). In the *Malus-Venturia* pathosystem, the avirulence factors are named based on the gene-for-gene model according to the cognate R gene (e.g. *AvrRvi6* corresponds to *Rvi6*) (Bus et al., 2011). Several of the *Avr* genes have been genetically mapped in *V. inaequalis* (Broggini et al., 2010). Proposed *avr* factors involved in pathogenesis in *V. inaequalis* include cellulase, polygalacturonase, and cell wall degrading enzymes (MacHardy, 1996; Kollar, 1998). To date, eight known physiologic races of the pathogen have been described based on patterns of infection and disease development on a differential set of apple cultivars and genotypes (Bus et al., 2005a).

The initial stages of infection starting with spore germination, appressoria development, penetration of the cuticle, and hyphal development are similar in resistant and susceptible cultivars (Nicholson, 1977; Komjanc et al., 1999). The cuticle is not a barrier to the pathogen (Valsangiacomo and Gessler, 1988). Any subsequent resistance reaction falls on a continuum from complete resistance [hypersensitive response (HR)] to complete susceptibility (sporulating lesions) depending on the resistance genes, pathogen race, and incubation conditions (Shay and Hough, 1952). Ontogenic resistance in older leaves prevents infection in these tissues, and the development of a stroma and

colonization and sporulation are slowed in progressively older leaves, although spore germination and appressoria development occur at the same rate (Gessler and Stumm, 1984).

The HR in apple is known to be conditioned by several previously described R genes including *Rvi4* (*Vh4=Vx=Vr1*), *Rvi5* (*Vm*), *Rvi7* (*Vfh*), *Rvi10* (*Va/Va2*), *Rvi15* (*Vr2*) and *Rvi16* (*Vmis*) (Win et al., 2003; Galli et al., 2010; Bus et al., 2011). This "pin-point" reaction results from the rapid death and subsequent collapse of cells immediately surrounding the penetration site of the spore (Goodman and Novacky, 1994). The HR is visible to the naked eye as small depressions on the leaf surface that extends from the upper to lower leaf surface and are visible on abaxial and adaxial surfaces (Nicholson et al., 1973). The HR has been well characterized in fixed and cleared leaf tissue (Chevalier et al., 1991; Galli et al., 2010). Although classically understood to be an immediate host response to pathogen attack, the HR observed in *Malus-Venturia* pathosystem has varied rates of reaction depending on the R gene present. Reaction times range from 2 to 11 days (Shay and Hough, 1952; Galli et al., 2010; Bowen et al., 2011). The HR prevents colonization through programmed cell death (PCD) and may involve cell to cell signaling mediated in part by reactive oxygen species. Not all components of the PCD are known but various phytoalexins and the accumulation and oxidation of other phenolic compounds (phloridzin and phloretin) have been implicated in the HR (Nicholson, 1977).

Early studies of apple leaf and peel tissues to infection by *V. inaequalis* and *Podosphaera leucotricha* (powdery mildew) exhibited increased concentrations of fluorescent phenolic compounds in both resistant and susceptible interactions which are

attributed to the host response (Barnes and Williams, 1960). Investigations of plant-pathogen interactions in other pathosystems provide additional support that the phenolic compounds that auto-fluoresce under ultraviolet light provide a convenient research tool to document the presence of the HR (Heath et al., 1997). HR also results in structural cellular changes including granulation of the cytoplasm (Nicholson, 1977). The microscopic wound caused by PCD can leave the leaf vulnerable to other opportunistic secondary infections, and it is proposed that fluorescence may be due to the accumulation of rigid chemical compounds such as phyotalexins involved (Vanderplank, 1982). Lignification of cell walls also occurs in cells surrounding an infection site, reducing the risk of water loss and secondary infections (Holzapfel et al., 2011).

In addition to the HR, other resistance phenotypes have been observed in the *Malus-Venturia* pathosystem. These include chlorosis (chlorotic flecking), stellate chlorosis, and stellate necrosis. Necrotic flecking is also observed in some reactions (Bénaouf and Parisi, 2000; Dunemann and Egerer, 2010). Chevalier (1991), following earlier phenotypic scales for evaluating resistance (Shay and Hough, 1952), developed a six point macroscopic scale to describe resistance induced by Rvi6 (*Vf*). The symptoms range from no lesions (0), HR (1), chlorotic lesions (2), necrotic/chlorotic lesions with slight sporulation (3a-weak resistance), necrotic/chlorotic lesions with sporulation (3b-weak susceptibility), and lesions with high sporulation (4). The differences observed in a progeny population among those containing the R gene is the result of isolate virulence and minor or modifying genes that are also segregating in the host population (Lamb and Hamilton, 1969; Williams and Kuc, 1969; Gardiner et al., 1996).

Here we report on the resistance reaction in 'Honeycrisp' and its putative ancestors to inoculation with a mixed conidial suspension of Minnesota *V. inaequalis* isolates in a greenhouse environment. A subset of a seedling progeny population of 'Honeycrisp' × 'Twin Bee Gala' was also evaluated using single spore isolates in the greenhouse.

Materials and Methods

Two key experiments were conducted to elucidate and describe the defense responses in 'Honeycrisp' and its relatives. In the first experiment, the resistance reaction in the 'Honeycrisp' pedigree, including one seedling offspring, 'Minneiska' were evaluated. Figure 2-1 displays the putative 'Honeycrisp' pedigree (Cabe et al., 2005), including one progeny population ('Honeycrisp' × 'Twin Bee Gala'; n=125), and other individuals utilized in this study. Known R genes carried by these individuals are specified. Reference accessions screened in this study were 'Golden Delicious', 'Pristine', *M. floribunda* 821, and 'Budagovsky 9'. 'Minnewashta' and 'Royal Gala' served as susceptible host checks.

Experiment 1

The cultivars utilized in this study were grown on 'Budagovsky 9' rootstock and maintained in greenhouses at the University of Minnesota-Twin Cities Campus, St. Paul, MN. Single spore *V. inaequalis* isolates of unknown race (Table 2-1) derived from infected leaves and fruit from unknown crab apples, advanced breeding material, and named cultivars (Minnewashta and Minneiska) from orchards at the Horticultural Research Center, Excelsior and Chaska, MN, the North Central Research and Outreach

Center, Grand Rapids, MN, and a residential neighborhood in Minneapolis, MN, were grown in axenic conditions on potato dextrose agar (39 g L^{-1}; Oxoid, LTD, Basingstoke, Hampshire, England) modified with 50 mg L^{-1} Rifamycin SV sodium salt (MP Biomedicals, LLC, Solon, OH, USA) (Parker et al., 1995; Barbara et al., 2008). Isolates were tested separately for virulence and then bulked-up on susceptible young seedlings and stored on dried leaves at -20°C (Bus et al., 2005a,b). A mixed inoculum was made with equal spore concentrations of each isolate in deionized water and brought to 10^5 conidia ml^{-1}. A positive control consisted of a single spore isolate (10^5 conidia mL^{-1}) derived from 'Honeycrisp' leaves collected at the Horticultural Research Center, Chaska, MN. Conidia were harvested from this isolate directly from sporulating plates and not tested on seedlings.

Shoots with newly unfurled leaves (2-4 leaves) were inoculated by hand spraying until run-off with a conidial suspension (10^5 conidia /mL) of mixed *V. inaequalis* isolates. Plants had multiple stems and replicate trees were used, when available, for each inoculum treatment. Trees were placed into a dew chamber at ~20°C with relative humidity near 100% for 48 hours to maintain leaf wetness. Following this infection period, trees were maintained in a greenhouse with intermittent mist to favor disease conditions (RH ~80%). Insect pests (thrips and spider mites) were controlled with applications of insecticides prior to inoculation. The two youngest leaves from day 0 were collected from each genotype at 1, 3, 5, 7, 8, 9, 10, and 14 days post inoculation (dpi) and the micro- and macroscopic evaluations were conducted as described below. This experiment was commenced on 21 April 2013 and replicated on 02 June 2013. A

third replicate was conducted on 13 April 2013 with a limited number of genotypes ('Frostbite', 'Northern Spy', 'Keepsake', 'Honeycrisp' and 'Minnewashta') to elicit and evaluate a response in 'Keepsake', which had been infected with other pathogens in replications 1 and 2.

Leaves were evaluated macroscopically for the presence of resistance reactions and/or evidence of mycelium or sporulation at each time point post inoculation. Leaves with evidence of insect feeding damage were not included in evaluations. Photographs of whole leaves were taken to document disease progression and resistance reactions. A binocular dissecting microscope was utilized to photograph macroscopic symptoms with increased magnification (Nikon SMZ800 with DS-2 camera and Digital Sight DS-L2; Nikon Instruments Inc., Melville, NY). Leaf samples were cleared and stained in 1.6 mL microcentrifuge tubes following the protocol described by Galli et al. (2010). Cleared leaf samples were stored and mounted in 50% ethanol: 50% lactic acid for observation under a light microscope and in the auto-fluorescence interference blue range using the UV lamp (420-490 nm excitation filter, dichroic mirror 510 nm, barrier filter 515 nm (Ernst Leitz Wetzlar 307-143.004 microscope; Ernst Leitz Wetzlar GmbH, Germany). Photographs were obtained using a SPOT Insight 4 camera and accompanying SPOTBasic software (SPOT Imaging Solution, Sterling Heights, MI). Photographs were compiled and scale bars inserted in ImageJ software (Schneider et al., 2012).

Experiment 2

A seedling mapping population (AE1022, n=121) from the cross of 'Honeycrisp' × 'Twin Bee Gala' was evaluated for macroscopic resistance reactions (pedigree shown

in Figure 2-1). Seedling trees were inoculated with a single-spore conidial suspension (7.5×10^5 conidia /mL) by hand spraying until leaf wetness resulting in run-off with single spore isolates GR19142b and 1914D (Table 2-1). Trees were placed into a dew chamber at ~20°C with relative humidity near 100% for 48 hours to maintain leaf wetness. Following this infection period, trees were maintained in a greenhouse with intermittent mist to favor disease conditions (RH ~80%). After 10-14 days, symptoms were assessed using a 6 point scale: (0), HR (1) chlorotic lesions (2), necrotic/chlorotic lesions with slight sporulation (3a-weak resistance), necrotic/chlorotic lesions with sporulation (3b-weak susceptibility), and lesions with high sporulation (4) (Chevalier et al., 1991). Segregation ratios were calculated for this and other 'Honeycrisp' × susceptible seedling populations (see Chapter 3 for more details). Whole leaf photographs were taken from a subset of the AE1022 progeny and were selected for their distinct resistance reaction to document the range of resistance reactions observed.

Results

Experiment 1

Microscopic observations of leaves at 1-5 dpi showed the presence of conidia from the inoculum on the leaf surfaces, including the development of the germ tubes and appressoria in both compatible and incompatible reactions in inoculations from the mixed isolate suspension only. There was no evidence of germination or infection in the single-spore isolate control inoculation throughout the experiment despite the confirmed microscopic presence of conidia. The lack of pathogenicity may be due to changes which occur in the pathogen while in culture (Nusbaum and Keitt, 1938). Alternatively, conidia

may have been dead in this isolate. Macroscopic observations yielded no signs or symptoms of the pathogen until 7-8 dpi when scab lesions were detected on compatible host plants and a resistance reaction was evident on some incompatible host plants. Table 2-2 describes the reaction for each of the cultivars in the study.

The putative parent 'Keepsake' did not demonstrate any reaction in the first replication of the experiment; which may be attributed to heavy powdery mildew (*Podosphaera leucotricha*) infections. The second replication of the experiment resulted in numerous older leaves showing symptoms of bacterial infection in that cultivar. Leaves for observation in replication two were chosen based on limited or no bacterial lesions on the shoot or specific leaf. In the second and third replications, 'Honeycrisp' and its ancestors all displayed resistance reactions including 'Keepsake'.

The macroscopic resistance reactions observed for 'Honeycrisp', 'Keepsake', 'Frostbite' and 'Northern Spy' were characterized as 2-3a (chlorotic flecking, stellate chlorosis, slight sporulation) as early as 7 dpi in the second replication of the experiment and 8 dpi in the first replication. Visible browning, an indication of possible necrosis of leaf tissue was apparent after 10 dpi in 'Honeycrisp' and increased by 14 dpi. The 'Northern Spy' reaction had evidence of stellate necrosis (SN) at 7 dpi, although not as pronounced as in other SN reactions, such as those attributed to *Rvi8* (*Vh8*) (Bus et al., 2005a). The susceptible cultivars Gala, Minnewashta, and Minneiska showed no resistance reaction, only sporulating lesions.

At 1-5 dpi, no auto-fluorescence was observed under ultraviolet light, even at the fungal penetration site. The susceptible reaction on 'Royal Gala' was primarily on the

abaxial leaf surface. Figure 2-2 shows representative resistant (A-H) and susceptible (I-J) reactions of leaf tissue to the presence of the pathogen at several time points under brightfield and auto-fluorescence blue microscopy. At 10 dpi, small brown areas, presumably necrosis of the epidermal cells, were observed in brightfield observations of the resistance reactions. In a few localized lesions, extensive sporulation was also visible on 'Honeycrisp' under the dissecting microscope and in the cleared tissues at higher magnification (Figure 2-3A, B; Figure 2-4B).

As early as 7 dpi and continuing through 14 dpi, the 'Honeycrisp', 'Keepsake', 'Frostbite' and 'Northern Spy' resistance reactions varied in size and shape and were distinguished by auto-fluorescence primarily of epidermal cells surrounding the penetration site. Auto-fluorescence in the resistance reactions was independent from fluorescence in vascular tissue and cell walls normally produced by the plant. The reactions in 'Keepsake', 'Frostbite' and 'Northern Spy' were similar, browning of epidermal and mesophyll cells along the infection zone with stellate mycelial growth extending beyond the visible resistance reaction. Similar resistance responses were observed macro- and microscopically, except for 'Honeycrisp'. In the auto-fluorescence observations (Figure 2-5), the 'Honeycrisp' resistance reactions were generally circular, with some evidence of necrotic tissue or browning of cells but not as extensive as typically observed in the classical HR. In addition to responses that completely restricted fungal invasion, there was evidence of some conidia establishing an infection site leading to sporulation. In cases where sporulation was visible 14 dpi on 'Honeycrisp' leaves, autofluorescence was also detected (Figure 2-3A, E). In addition to lesions caused by

conidia, there was evidence that mycelia from the spore suspension were capable of causing resistance reactions in the incompatible genotypes.

Experiment 2

In numerous observations on two fungal isolates derived from 'Minneiska', the 'Honeycrisp' × 'Twin Bee Gala' progeny population demonstrated a range of resistance reactions along the six point scale (Chevalier et al., 1991). Figure 2-6 shows examples from a subset of resistance and susceptible phenotypes. The classic HR response was not observed in any of the progeny. Class 0 (no reaction) was common, even when individuals were screened numerous times with the same isolate to capture potential 'escapes' from the screening protocol. Stellate chlorosis and SN were observed in some of the progeny. The progeny population segregated 1:1 when class 3b was considered susceptible and 3:1 when class 3b was considered as resistant (see Chapter 3, Table 3-2).

Discussion

The visible resistance reactions observed in 'Honeycrisp' appear approximately 7 dpi, at about the same time that susceptible reactions appear macroscopically. Up to that point, there are no observable macroscopic differences between compatible and incompatible reactions. From 1-5 dpi, germinating conidia and the development of appressoria were observed microscopically. In contrast to observations of Galli et al. (2010), in the current study there was no evidence of auto-fluorescence directly at the infection site or along subcuticular mycelium in either reaction type before 7 dpi. The mixed inoculum was effective in eliciting compatible and incompatible reactions, which resulted in several resistance classes in 'Honeycrisp', including 2-3a with chlorotic

flecking, stellate chlorosis and necrotic flecking, to 3b with sporulation. These resistance reactions are also present in the ancestors and offspring. Stellate necrosis was present in 'Northern Spy' and in several of the 'Honeycrisp' × 'Twin Bee Gala' offspring. Resistance reactions were generally limited to the adaxial surface. What appeared to be necrotic flecking macroscopically was evidenced by browning and possible cell death of epidermal cells. The outer edges of epidermal cells and some mesophyll cells exhibited fluorescence, a key indicator of a defense response (Bus et al., 2005a).

The growth of subcuticular mycelia in incompatible reactions on 'Honeycrisp' was generally constrained to a circular resistance response. However incompatible sporulating lesions (3b) and compatible (4) reactions were also observed. The reactions in 'Keepsake', 'Northern Spy' and 'Frostbite' exhibited stellate hyphal growth of the pathogen with corresponding resistance reactions along the growth front. The 'Honeycrisp' × 'Twin Bee Gala' progeny population segregated along the compatible-incompatible spectrum, except that no macroscopic class 1 (HR) was observed. The interaction also required more time before macroscopic reactions were evident. This could be due to the different single spore isolates utilized as well as differences in greenhouse conditions between experiments.

Mixed inoculum was utilized to elucidate the resistance response, as to date there is no information about which of the physiologic scab race(s) are capable of causing infection in 'Honeycrisp'. This mixture was derived from a suspension of several single-spore isolates from varied host genotypes, which had been bulked-up on whole plants, from which leaves were stored at -20ºC. These isolates had demonstrated efficacy in

inciting infections and resistance reactions. To date, these isolates have not been assigned to physiologic races, limiting interpretation of the resulting incompatible plant-pathogen interactions within the gene-for-gene (or genes) model. The positive control isolate, although derived from a 'Honeycrisp' fruit lesion, was not capable of inciting a compatible or incompatible reaction. Spores were present 1-3 dpi, but failed to germinate or develop appressoria and may have been dead. The single spore isolate suspension collected from 'Honeycrisp' was produced in axenic culture and had not been tested previously for its ability to cause infection. This isolate was intended to be the positive control, but in fact functions as a negative control (mock inoculation) for this experiment. No sporulation, resistance reaction, or autofluorescence was observed using this isolate.

The distinctly different resistance reactions in the 'Honeycrisp' ancestors ('Frostbite' 2-3a, 'Northern Spy' 2-3 with SN) provides evidence for the possibility of two different major R genes being inherited in 'Honeycrisp' through the known pedigree. However, mapping experiments will be needed to test the hypothesis and trace the inheritance (see Chapter 3 for such experimetns). In addition to different reaction types due to race specificity, other genetic factors within 'Honeycrisp' may be contributing to the different resistance reactions observed in 'Honeycrisp' × 'Twin Bee Gala' progeny population. Macroscopic resistance reactions were difficult to categorize in some seedlings, whereas others exhibited clear, classic responses as described by Aldwinckle et al. (1976). The designation of reaction phenotypes into intermediate classes from 3a to 3b to 4 macroscopically was difficult in seedlings. The class 0 reactions observed in the progeny may in fact be HR that affect a single or a very limited number of cells and do

not lead to the pinpoint reaction that is generally associated with HR in *Malus*. Additional microscopic observations of the resistance reactions in the progeny population may provide better quantification of this trait. However, microscopic observations are labor intensive and would be prohibitive for determining segregation of phenotypes in progeny populations for genetic mapping. Resistance itself is often treated as a binary trait for genetic mapping, but an appropriate phenotyping schema may allow for the elucidation of minor effect genes within this quantitative trait. A recent report demonstrates the effectiveness of quantitative real-time polymerase chain reaction for detecting and quantifying the pathogen on leaf material and may serve as high-throughput tool for screening progeny (Gusberti et al., 2012).

The classification of 'Honeycrisp' and its ancestors and progeny into several resistance classes is similar to the diverse responses observed in *Rvi6*, *Rvi11* and *Rvi12*, which also range from 0 to 3b (Table 2-2). The lack of a HR as typical of resistance conditioned by *Rvi4*, *Rvi5*, and *Rvi7* provides evidence that the resistance genes in 'Honeycrisp' are possibly unique. The time course from inoculation to the observation of macro- and microscopic reactions was also not as fast as the classic HR response, but was similar to reactions observed in *Rvi15* which becomes visible at 7 dpi (Galli et al., 2010). In the *Rvi15* resistance, fluorescence is limited to the browning epidermal and mesophyll cells that constitute the HR reaction (Galli et al., 2010). Stellate necrosis is associated with *Rvi2*- and *Rvi9*-mediated resistance, and was first documented as an intermediate reaction by Shay and Hough (1952) at 4-6 dpi (Bus et al., 2005a). The stellate necrotic reaction observed in this experiment became visible after 7 dpi. The radial growth

pattern of the pathogen was very apparent, as well as the resistance response along the lesion edge which included necrosis and fluorescence (Figure 2-3B, E).

The range of resistance reactions, including sporulating lesions, on 'Honeycrisp' from a mixture of single spore isolates highlights the importance of considering R genes within the gene-for-gene model. Furthermore, a single R gene cannot be considered a "silver bullet" providing permanent, i.e. durable, host immunity as the pathogen population is under selection pressure to evolve and circumvent host resistance. Consequently, the different isolates utilized in the experiments led to a range of defense responses within the same host.

The 'Honeycrisp' resistance reaction is not a classic HR nor is there strong evidence for cell death beyond browning of epidermal cells in some reactions. These experiments support the model that resistance reactions are induced after penetration of the cuticle as the 'Honeycrisp' defense response initiated by the recognition of the pathogen effectors (AVR proteins) occurs 7 dpi well after the development of the fungal germ tube and appressorium. The variation in responses observed across the progeny population is evidence for minor genes or QTL that determine the resistant phenotype. These genes may result in different recognition mechanisms, recognition of non-specific elicitors, have altered signaling pathways or other genetic differences that alter the efficacy of pathogen recognition or the downstream response pathways (Heath, 2000). Signal molecules that may play a role include salicylic acid, jasmonic acid, reactive oxygen species, and nitric oxide (Richberg et al., 1998). The *LRPKm1* gene in apple is involved in either detecting pathogen effectors or a plant signal that is the response of the

defense cascade (Komjanc et al., 1999). Defense responses are more well-defined in model species like *Arabidopsis thaliana* (Glazebrook, 2001), in which the defense cascade and pathogen-induced gene regulation have been studied in gene expression experiments (Glazebrook et al., 2003). Resistant and susceptible apple cultivars have been shown to have differential gene expression when inoculated with *V. inaequalis* (Holzapfel et al., 2011). In infected apple leaves, the increased transcripts related to reactive oxygen species and oxidative burst are often implicated in PCD (Paris et al., 2009).

In addition to PCD, plants may inhibit the pathogen directly to prevent colonization. Pathogenesis related (PR) proteins including chitinase, β-1,3-glucanase, and cysteine-like protease, are similarly deployed in response to attack by *V. inaequalis* as well as *P. leucotricha* (powdery mildew; Gau et al., 2004). The resistant cultivar Remo demonstrated constitutive production of PR proteins in its leaves that were also detected in the susceptible cultivar Elstar only after it was inoculated with *P. leucotricha* and *V. inaequalis* (Gau et al., 2004).

Auto-fluorescence under ultraviolet light has been used in several studies of the *Malus*-*Venturia* pathosystem to describe the incompatible reaction (Win et al., 2003; Bus et al., 2005a, 2010; Galli et al., 2010). Despite the clear visual clues provided by auto-fluorescence, little is known about the compounds involved in the resistance response. Fluorescent compounds often have rigid structures and are associated with phenolic compounds in defense response (Nicholson and Hammerschmidt, 1992; Davidson, 1996). Auto-fluorescence has been observed in other pathosystems and has been attributed to

phytoalexin, amino acids, flavonoids, callose, and phenolic acids (Nicholson, 1977; Nicholson and Hammerschmidt, 1992; Dai et al., 1996; Jeun and Lee, 2005). In lettuce leaves infected with downy mildew, auto-fluorescence was attributed to the accumulation of ester-linked syringaldehyde and caffeic acid on plant cell walls, as well as the release of preformed phenolics from the vacuole in the HR (Bennett et al., 1996). Peroxidase plays a role in cell auto-fluorescence and browning that accompany HR as the result of oxidation of phenolic materials (Heath, 1998).

R proteins are defined as those involved in the recognition of the AVR signals (effectors) and activation of downstream responses through a signal cascade that is distinct from the R protein itself. The selection and breeding for an R gene may not capture the full effect of resistance reaction, but only the gene involved in recognition and R-mediated signaling. This may explain some of the differences observed in *Rvi6* as well as 'Honeycrisp' progeny populations. However, Gardiner et al. (1996) rejected the hypothesis that the range in *Rvi6* (*Vf*) phenotypes was a result of the loss of closely linked R genes as large portions of the donor genome (*M. floribunda* 821) were still intact around the locus after several generations of introgression into breeding lines. These "modifying genes" as well as broad spectrum resistance loci involved in signaling pathways may explain differences in resistance classes for a given R gene that is observed in a progeny population (Durel et al., 2004). A 3:1 segregation ratio in the AE1022 population supports a model for two independent resistance genes being inherited from 'Honeycrisp'. Differences in the AE1022 population and in 'Honeycrisp' may be due to the monitoring of different effector proteins in the host by each R gene.

Additional research will be required to elucidate the gene-for-gene interaction involved with these potentially novel resistance genes.

Micro- and macroscopic characterization of the 'Honeycrisp' resistance reaction provides information for apple breeders, growers, and pathologists. This study demonstrated the cellular response in 'Honeycrisp' and its ancestors, and supports the presence of two underlying R genes, although it is unclear if these are contributed from 'Northern Spy' and/or 'Frostbite'. The deployment of 'Honeycrisp' and its R genes on the landscape may impact pathogen populations. By documenting the resistance reaction in 'Honeycrisp', attempts can be made to monitor the development of virulent pathogens in 'Honeycrisp' orchards and the breakdown of resistance over time. Steps will need to be taken to identify the single gene-for-gene interaction using differential hosts to elucidate the pathogen races capable of inciting infection on 'Frostbite' and 'Northern Spy'. These cultivars could be utilized in trap orchards in monitoring pathogen populations for the development of new races around the world as part of a collaborative monitoring program (www.vinquest.ch). The introgression of the 'Honeycrisp' R genes into new cultivars will require screening with molecular markers to capitalize efficiently on the combinations of major and minor effect genes that impart the greatest levels of resistance. Pyramiding 'Honeycrisp' R genes with other broad-spectrum genes may provide durability in resistance to *V. inaequalis*.

Tables

Table 2-1 *Venturia inaequalis* monoconidial isolates, originating lesion tissue, host genotype, collection location, and collection year for isolates pooled into mixed inoculum utilized in Experiment 1 for characterizing the apple scab resistance reaction in 'Honeycrisp'.

Isolate	Origin	Genotype	Location	Year
1731	Fruit	MN1731	Grand Rapids, MN	2010
1914C	Leaf	Minneiska	Chaska, MN	2010
1914D*	Leaf	Minneiska	Chaska, MN	2010
80WA	Fruit	unknown	Excelsior, MN	2010
GR19142b*	Fruit	Minneiska	Grand Rapids, MN	2010
GR1914a	Fruit	Minneiska	Grand Rapids, MN	2010
Manchurian	Leaf	Manchurian	Chaska, MN	2010
MplsCrabd	Leaf	unknown	Minneapolis, MN	2010
ZestarFruit	Fruit	Minnewashta	Chaska, MN	2010
HC12a**	Fruit	Honeycrisp	Chaska, MN	2012

*Single spore isolates also used in Experiment 2 for screening 'Honeycrisp' × 'Twin Bee Gala' progeny population.

**Single spore isolate used as a control.

Table 2-2 Macro- and microscopic characterization of genotypes screened in the greenhouse with a mixed inoculum of *Venturia inaequalis* single spore isolates observed at several time points over two replications. The symptoms range from no lesions (0), hypersensitive response (1), chlorotic lesions (2), necrotic/chlorotic lesions with slight sporulation (3a-weak resistance), necrotic/chlorotic lesions with sporulation (3b-weak susceptibility), and lesions with high sporulation (4). Fl = auto-fluorescence, SC= stellate chlorosis, SN = stellate necrosis. *Observations of the cultivar Keepsake were made during the second and third replications.

	Days Post Inoculation					
Cultivar	**1-3-5**	**7**	**8**	**9**	**10**	**14**
Frostbite	Germinated, appressorium	2-3a-SC, Fl	2-3a-SC, Fl	2-3a-SC, FL	2-3a-SC, Fl	2-3a-SC, Fl
Northern Spy	" "	2-3a-SN, Fl	2-3a-SN, Fl	2-3a-SN, Fl	2-3a-SN, Fl	2-3a-SN, Fl
Keepsake*	" "	Small lesions 2-3a-SC, Fl	2-3a-SC, Fl	2-3a-SC, Fl	2-3a-SC, Fl	2-3a-SC, Fl
Honeycrisp	" "	2-3a-SC, Fl	2-3a-SC, Fl	2-3a-SC, Fl	2-3a-SC; 3b-4; necrosis , Fl	2-3a-SC; 3b-4; necrosis, Sporulating, Fl
Minnewashta	" "	Sporulating	Sporulating	Sporulating	Sporulating	Sporulating
Minneiska	" "	Sporulating	Sporulating	Sporulating	Sporulating	Sporulating
Royal Gala	" "	Sporulating	Sporulating	Sporulating	Sporulating	Sporulating
Golden Delicious (*Vg*; *RVi1*)	" "	2-3b, Fl	2-3b, Fl	2-3b, Fl	2-3b, Fl	2-3b, Fl Sporulating
M. floribunda-821 (*Rvi6*, *Rvi7*)	" "	0	0	0	1, Fl	1, Fl
Budagovsky 9	" "	Sporulating	Sporulating	Sporulating	Sporulating	Sporulating
Pristine (*Rvi6*)	" "	0	0	0	0	No visible reaction

Figures

Figure 2-1 'Honeycrisp' pedigree including the 'Honeycrisp' × 'Twin Bee Gala' population (AE1022) utilized in mapping the resistance gene(s) in 'Honeycrisp'. Highlighted individuals indicate those with a known resistance gene (*Rvi* indicated) or a proposed resistance under investigation. The AE1022 population segregates for resistance. Individuals separate from the pedigree were included in the experiment for phenotypic observations (Pedigree constructed using Pedimap (Voorrips et al., 2012)).

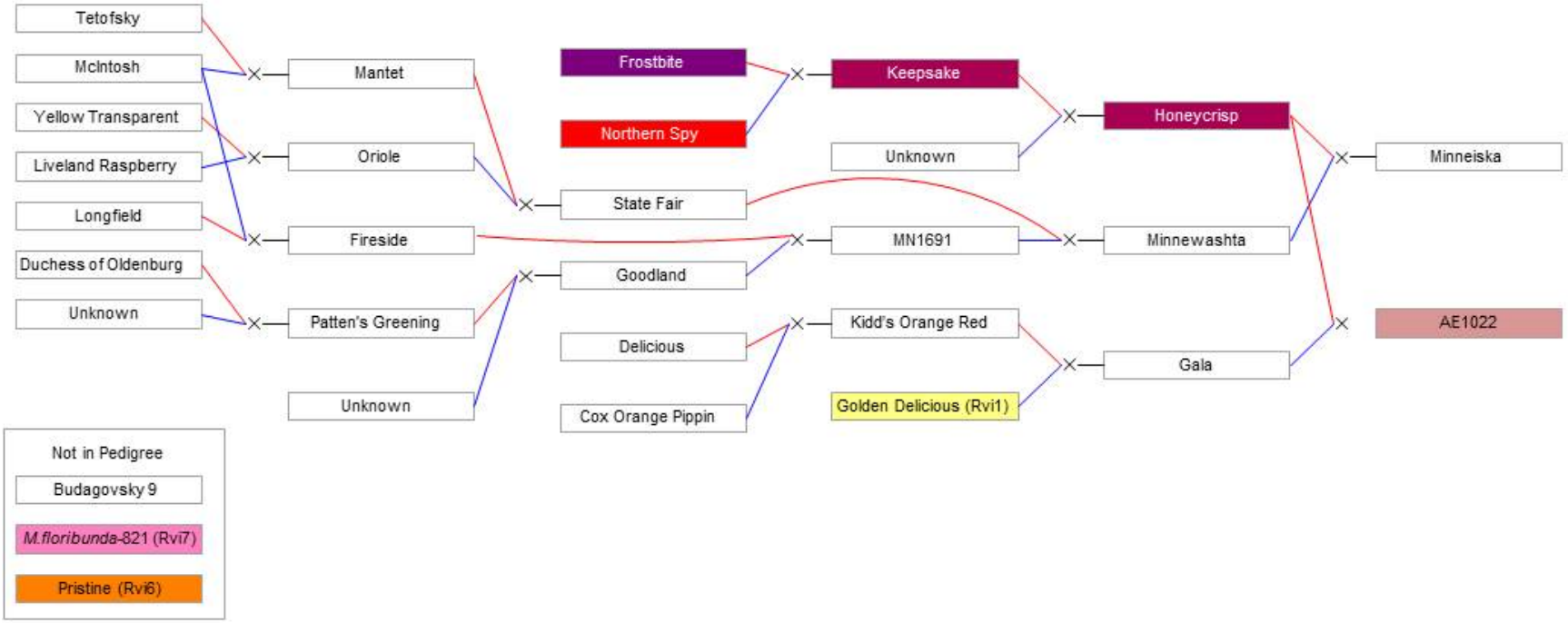


Figure 2-2 Susceptible and resistance responses shown with brightfield (A,B,C) and fluorescent (D,E,F) microscopy. No difference in spore germination and appressoria development at 1, 3, and 5 days post inoculation (dpi) between resistant and susceptible cultivars (A, D; 'Honeycrisp' shown at 5 dpi). Conidia (c), appressorium (a), germ tube (g), and hyphae (h) are labeled. Typical circular resistance response (B) in 'Honeycrisp' 8 dpi including browning of some epidermal cells (e) and auto-fluorescence of primarily epidermal cells (E). The compatible genotype 'Minnewashta' at 8 dpi exhibits copious sporulation of the pathogen stained blue (C) and only exhibits auto-fluorescence of vascular tissue and no resistance reaction (F).

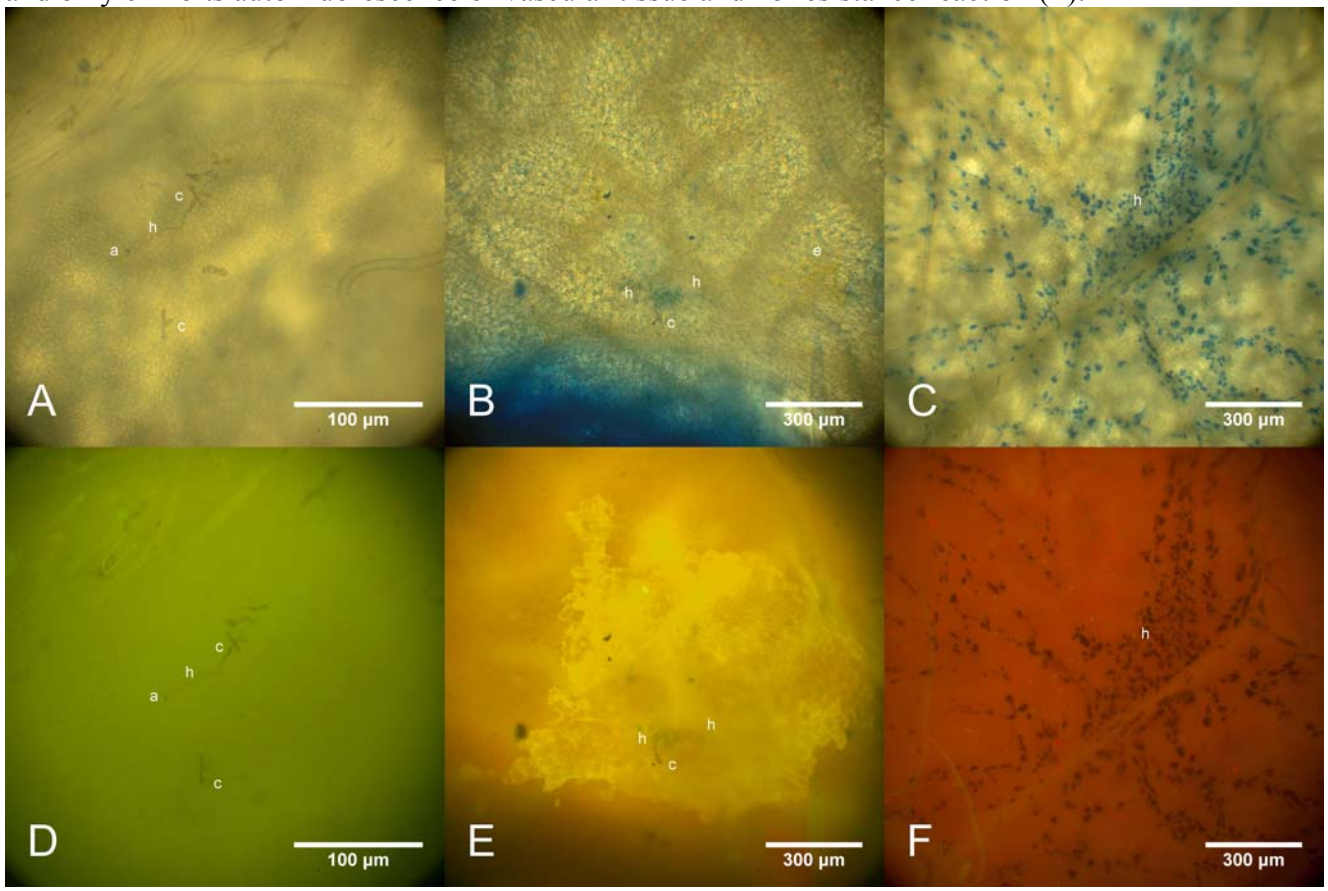


Figure 2-3 Resistance reactions were varied within and across genotypes including sporulating lesions in 'Honeycrisp'. (A) Hyphae and conidia present (stained blue) in addition to browning of epidermal cells in 'Honeycrisp'. (D) Auto-fluorescence was evident in the host although sporulation was present. This may be due to mixed isolates which resulted in both compatible and incompatible reactions. (B/E) Stellate growth of the pathogen is apparent in 'Northern Spy', as well as a resistance reaction along the growth front as evidence by fluorescing cells. (C/F) Sporulating lesion in the susceptible cultivar Minnewashta shows numerous conidia and no evidence of auto-fluorescence.

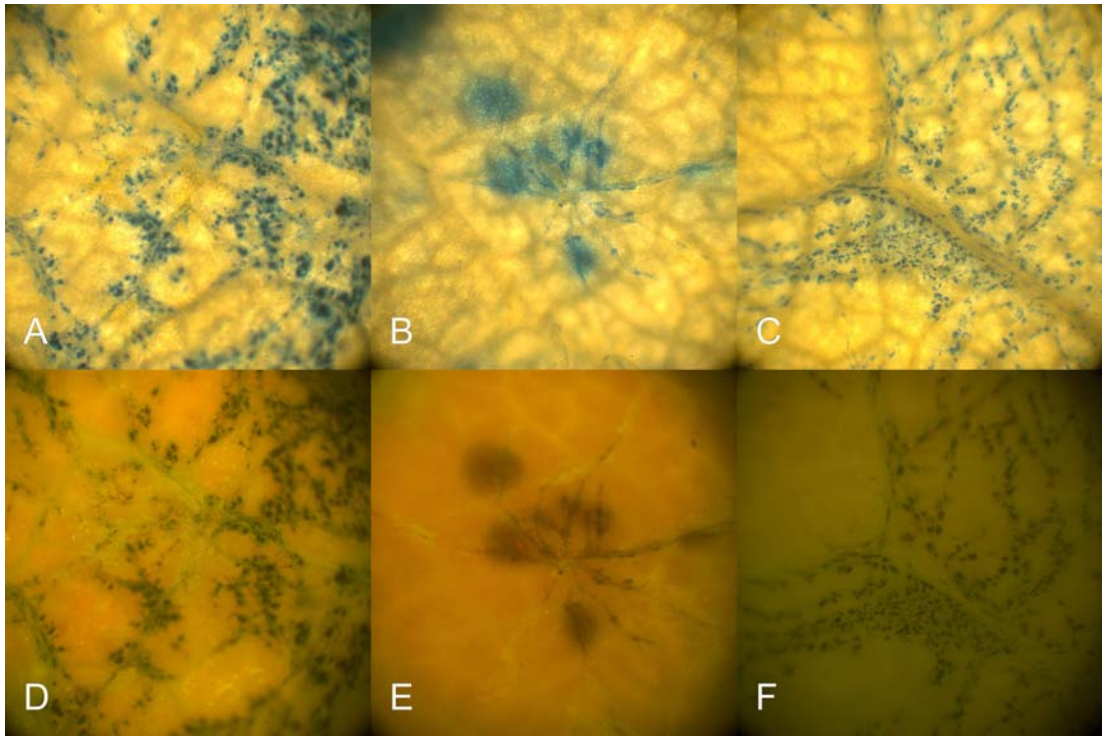


Figure 2-4 Dissecting microscope photographs of resistance reaction in host plants 15 days post inoculation with mixed isolates of *Venturia inaequalis*. (A) 'Honeycrisp' with chlorotic flecking and necrosis. (B) 'Honeycrisp' with sporulating lesion and resistance reaction including necrosis. (C) Dense sporulating lesions in susceptible cultivar Minnewashta. (D) Resistance reaction in 'Northern Spy'. (E) 'Keepsake' exhibiting small lesions more macroscopically similar to the hypersensitive response with some necrosis. (F) Resistance reaction in 'Golden Delicious'.

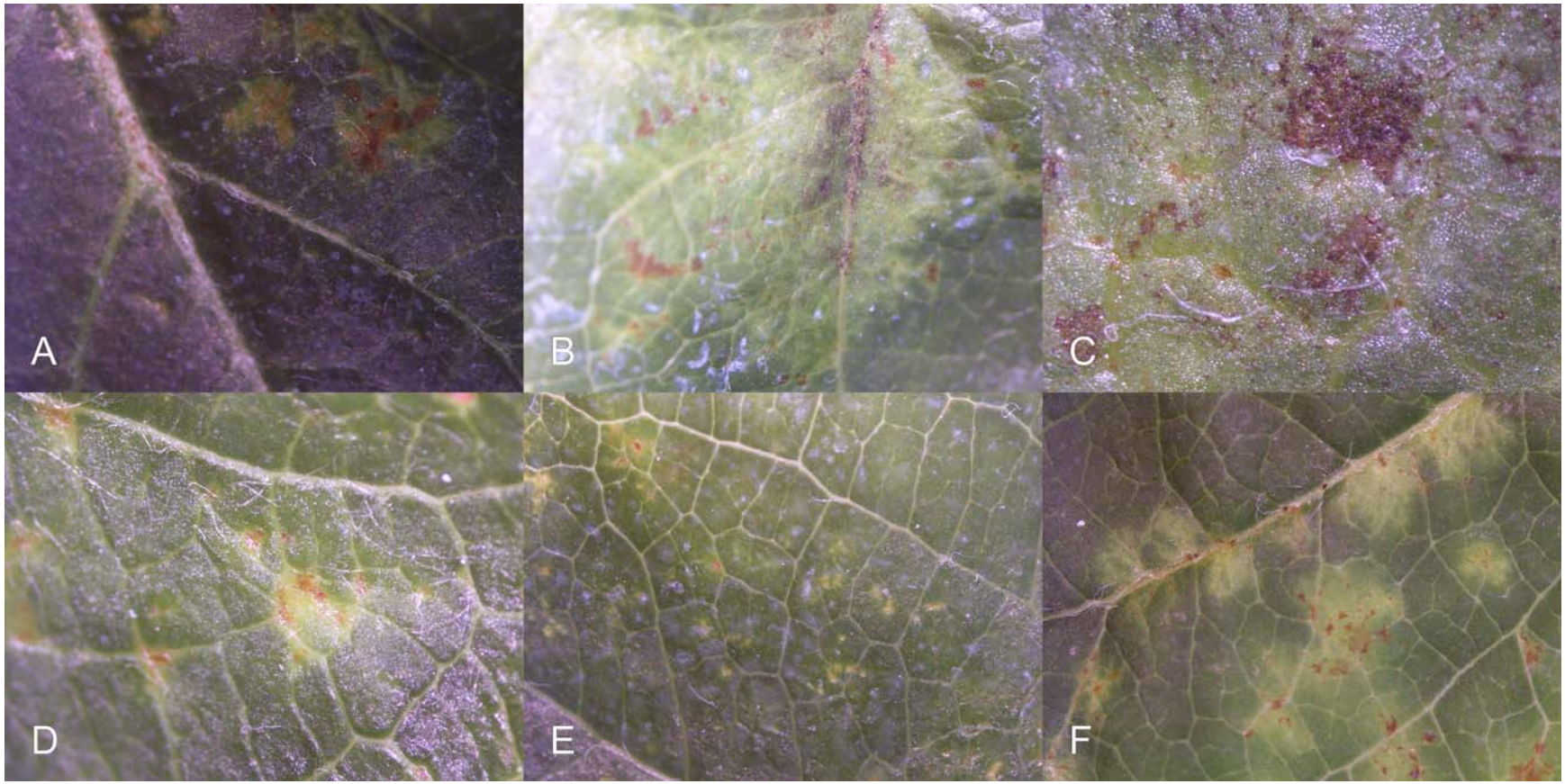


Figure 2-5 Resistance reaction in 'Honeycrisp' to a suspension of mixed isolates of *Venturia inaequalis* 9 days post inoculation. Evidence of germinated conidia (c) and mycelia (m; stained with aniline blue) that extends beyond the resistance reaction. (A) Brightfield image of conidia and brown epidermal cells. (B) Auto-fluorescence of epidermal cells at the infection site under interference blue microscopy.

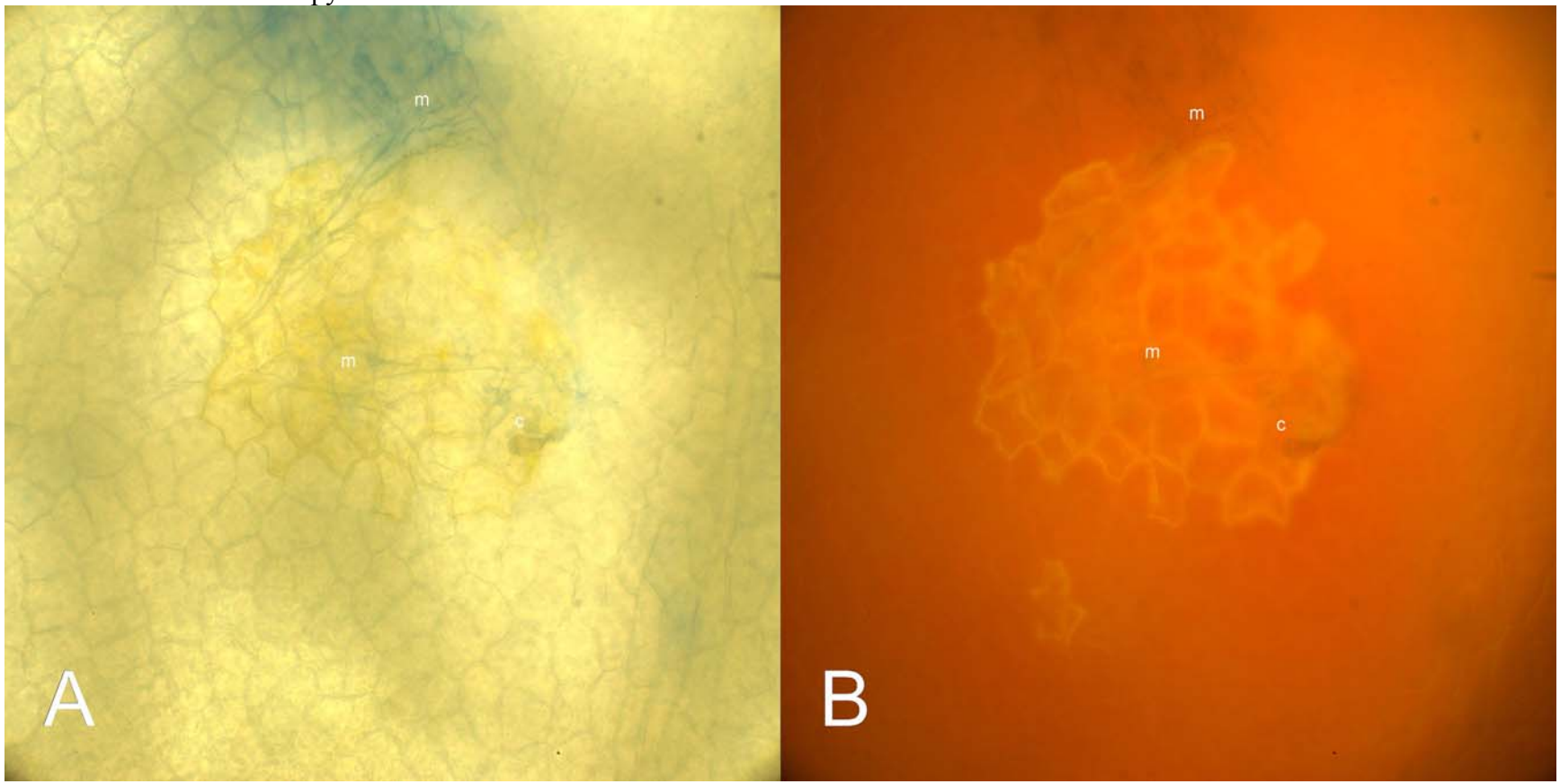
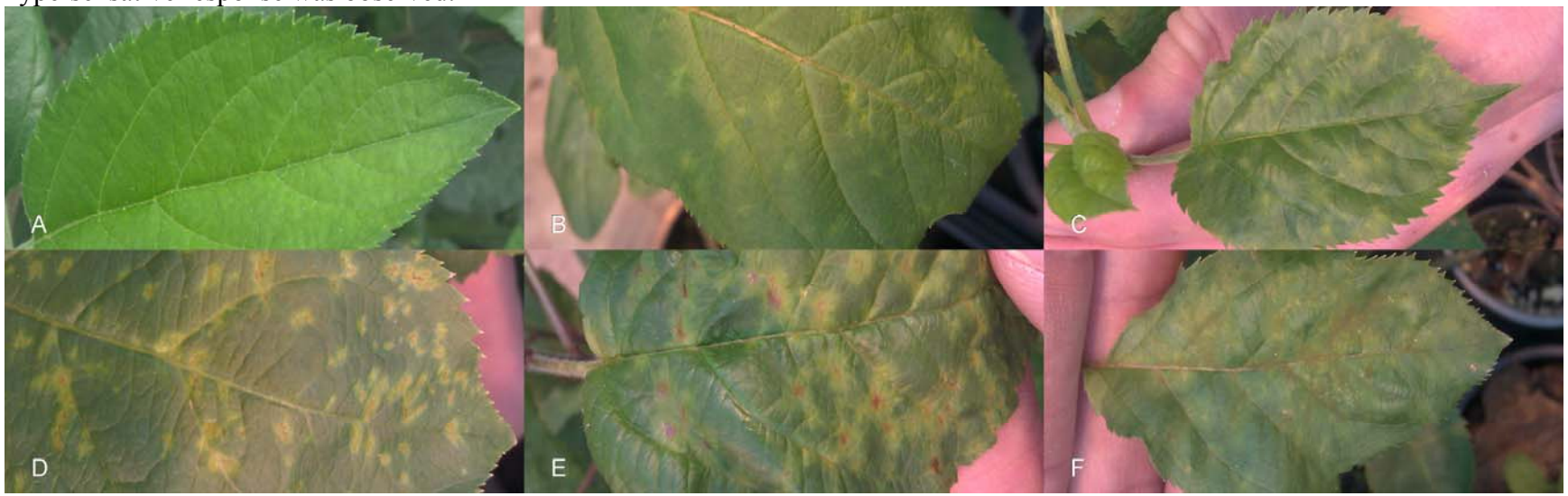


Figure 2-6 Images demonstrating the range of observed phenotypic reactions classified according to Chevalier et al. (1991) in seedlings of a 'Honeycrisp' × 'Twin Bee Gala' population utilized in mapping the 'Honeycrisp' resistance gene(s) to a single spore isolate (derived from 'Minneiska'). (A) Class 0 escape or no symptoms, (B) Class 2 chlorotic flecking , (C/D) Class 3A chlorosis, necrosis, light sporulation, (E) Class 3B sporulation with cholorosis and necrosis, (F) Class 4 susceptible. No classic pin-point hypersensative response was observed.



Chapter 3

Identification of unique sources of apple scab resistance (*Rvi19* and *Rvi20*) in the apple (*Malus* x *domestica*) cultivar Honeycrisp

Two apple scab resistance loci have been identified in the emerging cultivar Honeycrisp. Apple scab, incited by the fungal pathogen *Venturia inaequalis,* is one of the most economically important diseases in apple. Novel resistance loci, *Rvi19* and *Rvi20*, were mapped in a seedling progeny population (n=125) of 'Honeycrisp' × 'Twin Bee Gala'. This population was screened in the greenhouse with two monoconidial isolates of unknown race. *Rvi19* maps to linkage group 1 and is inherited from 'Frostbite' through 'Keepsake'. *Rvi20* maps to linkage group 15 and is inherited from the 'Honeycrisp' parent that is unknown to date. Haplotypes defined by microsatellite and single nucleotide polymorphic (SNP) markers that span the loci have been developed to provide breeders with reliable tools for marker assisted selection. The *Rvi19* haplotype spans 2.3 Mb which is ~12.6 cM and includes the well characterized *Rvi6* locus (*Vf*). A simple sequence repeat (SSR) marker, Ch-Vf1, mapping 3.8 cM from the resistance locus has an allele associated with resistance that is 138 bp, the same as in *Rvi17*. The *Rvi19* haplotype consists of 263 predicted genes, 24 of which encode motifs common in resistance genes. The *Rvi20* haplotype spans 3.2 Mb which is ~10.1 cM. This haplotype is identical by state with 'Golden Delicious', which appears to be susceptible. In the *Rvi20* haplotype, 460 predicted genes were identified with 23 encoding common R gene motifs, making these genes prime candidates for the R gene at this locus.

Introduction

One of the goals of most plant breeding programs is the development of cultivated varieties with resistances to biotic and abiotic stress. Apple scab, caused by *Venturia inaequalis,* is one of the most economically important diseases in apple (*Malus* x *domestica* Borkh.), the most important temperate fruit crop with production exceeding 75.5 million metric tonnes in 2011 worldwide (FAOSTAT, 2013). This pathogen can have devastating effects on apple trees and fruit. The disease greatly impacts grower safety and orchard profitability with the number of applications of fungicidal sprays needed to manage the disease. Losses from apple scab generally come from unsightly fruit blemishes, which results in unsalable fruit to most fresh markets. However, infection can also effectively reduce overall fruit set, affect the growth of young plants resulting in long-term structural damage, contribute to defoliation, reduce fruit bud formation, and reduce overall plant health and vigor.

The development of disease resistant apple cultivars is intended to reduce the number of chemical inputs required for disease management and to increase the sustainability of apple production. Very few apple scab resistance (R) genes have been identified in the cultivated apple. In the early to mid-20th Century, apple breeders in the United States began examining collections of related *Malus* spp. (crab apples) for field resistance to apple scab (Janick, 2006). Apple breeders from Purdue, Rutgers, and Illinois (PRI) have cooperatively developed a number of commercial cultivar releases with scab resistance (Crosby et al., 1992; Janick, 2006).

Early research conducted by the PRI consortium on scab resistance identified a

number of selections of *Malus* species generally grown as ornamental specimens (i.e. flowering crabapples). These species included *M. baccata*, *M. floribunda M. hupenhensis, M. micromalus, M. prunifolia*, and interspecific hybrids that displayed field resistance at research stations throughout the United States (Shay and Hough, 1952). Several of these species were used to introduce the resistance factors (i.e. the R genes) into commercial breeding programs (Shay and Hough, 1952). The resulting scab resistant interspecific hybrid cultivars have not been widely accepted because of their lower fruit quality, but they remain important in breeding programs (Crosby et al., 1992). Related *Malus* species, especially those collected from the Asian center of diversity, are important resources for introgressing important R genes for scab (Luby et al., 2001; Bus et al., 2002) and fireblight (*Erwinia amylovora*) (Luby et al., 2002) not found currently in breeding programs.

To date, seventeen major R genes imparting scab resistance have been identified and mapped (Boone, 1971; Gessler et al., 2006; Jha et al., 2009; Bus et al., 2011). This excludes Rvi18 which is currently being investigated, and *Rvi19* and *Rvi20* which we report in this study. The first gene implicated in resistance to apple scab was identified from *M. floribunda* 821 and in crosses of this genotype with 'Rome Beauty' initially made in 1914 (Crandall, 1926). Known as *Vf* (*Rvi6*), this allele has been utilized in apple breeding programs worldwide with over 70 cultivars released, although none are planted extensively (Janick et al., 1999). The *Vf* allele offers strong resistance to races 1-5 of *V. inaequalis* (Lespinasse et al., 2002). However, the use of *Vf* resistance in cultivars quickly resulted in emergence of a race (6) that can overcome this gene (Parisi et al., 1993). The new race is not able to overcome the resistance in *M. floribunda* 821 which

suggests that *Vf* is a single gene and that another gene *Vfh* (*Rvi7*) in the original source has since been lost in cultivar development and segregates independently (Parisi et al., 1993; Bénaouf and Parisi, 2000).

The *Vf* gene has been mapped by several research groups using different genetic marker platforms and populations (Gardiner et al., 1996; King et al., 1998; Maliepaard et al., 1998; Tartarini et al., 1999). Xu and Korban (2000) saturated the *Vf* region to identify closely linked amplified fragment length polymorphism (AFLP) markers. Closely linked markers can be utilized to identify just the *Vf* gene and to select against closely linked, deleterious genes from *M. floribunda* which are responsible for the genetic drag which limits cultivar development (Tartarini, 1996). Other linked markers have been developed to improve linkage maps and to examine introgression of *Vf* in *Malus* accessions and cultivars (King et al., 1999).

The Russian apple 'Antonovka' was identified as scab resistant (Schmidt, 1938) and utilized in breeding due to its large fruit size, which sets it apart from other resistant crabapple species. Although different 'Antonovka' genotypes were utilized in breeding efforts, two major genes, *Va1* (*Rvi17*) and *Va/Va2* (*Rvi10*), have been mapped on linkage group (LG) 1 which can be traced to the Schmidt clone of 'Antonovka' (Bus et al., 2012). *Va1* (*Rvi17*) maps closely to *Vf*, and the resistance phenotype (chlorosis) co-segregates with a 138bp fragment of the Ch-Vf1 SSR marker (Dunemann and Egerer, 2010). The *Rvi10* locus maps to ~ 24 cM above *Vf* and exhibits the hypersensitive response (HR; Dayton and Williams, 1968; Hemmat et al., 2003).

Early descriptions of polygenic resistance to known races were published by Williams and Kuc (1969). Polygenic resistance comprising multiple partial resistances is

theoretically more durable than a single major resistance gene. Polygenic resistance results in variation in *V. inaequalis* sporulation across apple genotypes (MacHardy, 1996). Partial, polygenic resistance has been observed in some apple cultivars (Liebhard et al., 2003b; Calenge et al., 2004). Polygenic resistance is more challenging to breed for and has increased sensitivity to the environment (Ignatov and Bodishevskaya, 2011).

Genetic mapping using bi-parental mapping populations is common in apple genetics, especially in developing molecular markers for monogenic traits like disease resistance (Tartarini and Sansavini, 2003; Schenato et al., 2008). A number of linkage maps have been developed to detect quantitative loci (QTLs) and map genes for a range of important traits including disease resistance (*Vf* for apple scab), acidity (*Ma*), and growth habit and developmental traits (Lawson et al., 1995; Maliepaard et al., 1998). More recently the apple genome was sequenced, providing researchers with additional tools for genetic studies (Velasco et al., 2010). The development of an 8K SNP array (Chagné et al., 2012) for apple has allowed high throughput genotyping, and the construction of dense linkage maps which should be useful in mapping major genes and QTL (Clark et al., accepted; Antanaviciute et al., 2012).

Selection based on DNA markers can be used as a substitute for a disease screen, to reduce linkage drag, and to select for linked resistance loci in a population (Michelmore, 1995). Traditional screening of seedlings with scab inoculum is equally effective in identifying resistant progeny with *Vf* (or other single R gene). However, marker assisted selection (MAS) can also be efficient for identifying multiple R genes within a single individual (in gene pyramiding) and for selecting for other traits (Maliepaard et al., 1998). Specific PCR primers designed to anneal to genome regions of

interest and flanking markers can be used to screen for recombinants adjacent to the R genes. Using markers tightly linked to an R gene, one can identify genotypes possessing the R gene but without other nearby genes deriving from the less desirable parent (Gianfranceschi et al., 1996).

The apple cultivar Honeycrisp has outstanding flavor and textural traits (Luby and Bedford, 1992; Tong et al., 1999; Hoover et al., 2000) that have led to its rising importance in North American apple production. 'Honeycrisp' has also been shown to exhibit field resistance to foliar apple scab infection when grown under organic disease management practices (Berkett, 2007). This characteristic is important for 'Honeycrisp' growers who may be able to reduce fungicide inputs in their orchards. For the apple breeder, using 'Honeycrisp' as a parent offers the genetic background for superb fruit quality traits and disease resistance traits that should be exploited. Identifying the R gene(s) in 'Honeycrisp' will give plant breeders additional tools for MAS and in developing resistant cultivars.

The parentage of 'Honeycrisp' is unknown; however DNA fingerprinting using SSR markers (Cabe et al., 2005) has been utilized to provide putative paternal ancestry for two generations (Figure 3-1) that is further supported by SNP genotyping across the genome (data not shown). The resistance responses to *V. inaequalis* in 'Honeycrisp', and in the cultivars within the 'Honeycrisp' pedigree have been recently characterized (Chapter 2; Clark et al., submitted). One hypothesis is that the scab resistance observed in 'Honeycrisp' was derived from the paternal ancestors 'Frostbite' (MN447) and 'Northern Spy', each of which contributed an R gene to 'Keepsake'.

The objectives of this study are to describe the genetic control of scab resistance

in ‘Honeycrisp’ and the inheritance of the resistance through its pedigree. Additionally, candidate genes are identified which should be targets for molecular marker development and further characterization of the resistance loci. Several mapping populations using ‘Honeycrisp’ as the resistant parent were screened in the greenhouse to assess the segregation ratios in the offspring. One population, ‘Honeycrisp’ × ‘Twin Bee Gala’ (AE1022), was used for genetic mapping using both single nucleotide polymorphism (SNP) markers and microsatellite simple sequence repeat (SSR) markers to accurately position two R gene loci which we refer to as *Rvi19* and *Rvi20*. This is the first report of a scab R gene on LG 15 of apple. SSR markers were utilized to validate the position of these loci in an independently screened second population. The SSR marker haplotypes across the R gene loci were used to screen University of Minnesota advanced disease resistant selections to identify those with pyramided resistance genes.

Materials and Methods

Plant materials and *V. inaequalis* isolates

In the USA, three bi-parental mapping populations were developed to study the genetics of scab resistance of ‘Honeycrisp’ by crossing it with the susceptible genotypes ‘Twin Bee Gala’, MN1940, and MN1789. The ‘Twin Bee Gala’ progeny population (AE1022) was subdivided into two sub populations and the pedigree is shown in Figure 3-1. A fourth population, ‘Honeycrisp’ × A248R22T004 (population 2009-07), was used in the Netherlands to validate the genetic marker developed in AE1022. A248R22T004 is a ‘Splendour’ × Russian apple R12740-7A progeny. Seedlings were raised and maintained in the greenhouse for controlled inoculation experiments.

Three single spore isolates in the USA and four isolates in the Netherlands were utilized for screening the respective progeny populations (Table 3-1). Isolates were cultured on potato dextrose agar and multiplied on infected susceptible leaves that were air dried and stored at -20°C (Bus et al., 2005).

Inoculation and screening

The USA seedling trees were inoculated with a conidial suspension ($7.5x10^5$ conidia /mL) of one of the isolates (Table 3-1) by hand spraying until leaf wetness resulting in run-off. Trees were placed in a dew chamber at ~20°C with relative humidity near 100% for 48 hours to maintain leaf wetness. Following this infection period, trees were maintained in a greenhouse with intermittent mist to favor disease conditions (~20°C and RH ~80%) for 10-14 days until observations. Insect pests (thrips and spider mites) were controlled with applications of insecticides as needed. For population AE1022-b, inoculation experiments were replicated for each isolate; isolate 1914D (8 July 2011, 26 January 2013, and 11 March 2013), and isolate GR19142b (20 September 2011, and 13 October 2011). A consensus score for each seedling-isolate combination was assigned as the most severe (susceptible) rating over replications.

In the Netherlands, plants were inoculated by placing 150 µL droplets of a spore suspension at 10×10^4 conidia/mL on a leaf using an inoculation chamber (Bus et al., 2005a, b). The plants were kept in the dark in plastic tents for two days at 18 °C to establish infection. After two days the plants were placed in a greenhouse chamber with a day temperature of 20°C, and a night temperature 14° C , regulated by automated roof venting or heating. Relative humidity was set at 80%. Symptoms were observed two and three weeks after inoculation.

Disease symptoms were assessed using a 6 point scale: no symptoms (0), HR (1) chlorotic lesions (2), necrotic/chlorotic lesions with slight sporulation (3a-weak resistance), necrotic/chlorotic lesions with sporulation (3b-weak susceptibility), and lesions with high sporulation (4) (Chevalier et al., 1991). In order to properly identify escapes from class 0, replicate inoculations were conducted. For characterization as a binary trait, classes 0-3a and 0-3b were each considered as resistant and each tested for segregation ratios using the χ^2 test of independence in Microsoft Excel (Table 3-2).

DNA extraction

DNA was extracted from the 'Honeycrisp' × 'Gala' population and its ancestors using the E-Z 96® Plant DNA Kit (Omega Biotek, Norcross, GA) with modifications (Chapter 1; Clark et al., accepted; Gilmore et al., 2011). Additional DNA samples were extracted from parents and ancestors for microsatellite marker analysis using the silica bead extraction method (Edge-Garza et al., submitted) with modifications from a 96-well plate. Newly expanding leaf tissue (~50 mg) for each sample was desiccated in 1.6 mL microcentrifuge tube containing ~0.2 g of PillowPak silica bead desiccant (Dessicare, Inc., Reno, NV) for a minimum of 12 hours. Samples (leaf tissue and silica beads) were ground in the microcentrifuge tube using a Retsch MM301 Mixer Mill (Retch, Haan, Germany) for 3 minutes. The remaining extraction steps used individual microcentrifuge tubes.

Molecular marker analysis and R gene mapping

A multi-step mapping approach was taken to accurately estimate linkage between the resistance gene loci and molecular markers. The AE1022 population was genotyped using the International RosBREED SNP Consortium (IRSC) apple 8K SNP array V1

(Chagné et al., 2012) as previously described (Chapter 1; Clark et al., accepted). The resistance phenotypes were first mapped in JoinMap4.1 (Van Ooijen, 2011) by coding susceptible phenotypes as homozygous and resistant phenotypes as heterozygous (ll x lm). Linkage calculations utilized all polymorphic SNP markers previously mapped in 'Honeycrisp' × 'Twin Bee Gala' (Chapter 1; Clark et al., accepted). Markers were grouped using the 'Honeycrisp' integrated linkage map (Chapter 1; Clark et al., accepted). The maximum likelihood method was used with default settings (Van Ooijen, 2011).

An approach was taken to assess allele frequency differences between *in silico* pooled DNA samples of resistant and susceptible genotypes at each SNP along the consensus map. The allele frequency differences between pools of resistant and susceptible seedlings were calculated and plotted in Microsoft Excel to identify potential genomic regions for further investigation.

Using the integrated SNP 'Honeycrisp' linkage map constructed by Clark et al. (accepted), Kruskal-Wallis (KW; rank sum) and interval mapping (IM) mapping approaches were conducted in MapQTL®5 (Van Ooijen, 2004) to identify genomic regions associated with resistance. Phenotypic data for isolates GR19142b and 1914D were used with the Chevalier et al. (1991) scale converted to a quantitative scale [0; 1; 2; 3(3a); 3.5 (3a-3b); 4 (3b); 4.5(3b-4); 5)].

SNP haplotypes were constructed in Microsoft Excel and Pedimap (Voorrips et al., 2012) for each of the progeny and ancestors in order to assign resistance to a 'Honeycrisp' haplotype and to trace inheritance through the pedigree. Identified loci were examined with χ^2tests to assess segregation ratios. An Excel-based haplotyping tool

developed by Cameron Peace (personal communication) was used to deduct the SNP haplotypes at LGs 1 and 15 for a number of North American breeding parents, selections, and seedlings that were previously genotyped as part of RosBREED using the IRSC SNP array. The haplotypes were constructed using the physical position (Velasco et al., 2010) and the genetic positions in the 'Honeycrisp' × 'Twin Bee Gala' map.

SSR marker and SNP mapping

Microsatellite (SSR) markers were selected for saturating the observed QTL regions from a database of markers (www.hidras.unimi.it) or based on SSRs mapped in the M432 mapping population (Antanaviciute et al., 2012). The polymerase chain reaction (PCR) primers, size ranges, and annealing temperatures for each QTL are given in Table 3-3. Using apple genomic sequences spanning the QTL [Genome Database for Rosaceae (GDR); www.rosaceae.org; Jung et al., 2008], a novel SSR locus was identified on contig MDC012480.81[the same contig on which ss475882286 (GDsnp10005) is located], and PCR primers were developed using msatcommander v.0.8.2 (Faircloth, 2008) and Primer3 (Rozen and Skaletsky, 1999). To develop new, robust primers at the CH03b06 locus, a Blast sequence alignment of this locus in GDR identified the CH03b06 sequence on contig MDC022202.499. Novel primers were designed with Primer3. All forward primers included an 18-bp M13 (TGTAAAACGACGGCCAGT) sequence tag to allow for incorporation of an M13-tagged fluorescent dye (Hex, Ned, 6-Fam) used in capillary electrophoresis (Schuelke, 2000).

PCR reactions were carried out in 13µL reaction volumes consisting of 1µL DNA template, 6µL GoTaq® Colorless Master Mix (M714; Promega Corporation, Madison, WI, USA), 0.6 µL volume of each primer (10 mM), 0.6 µL fluorescent label (10 mM) in

the GeneAMP® 2700 thermal cycler (Applied Biosystems, Foster City, CA) or the MWG-Biotech Primus96 Plus thermal cycler (MWG-Biotech Huntsville, AL). Thermal cycler conditions were: 1 cycle of 3 min at 94°C, 30 cycles of 94°C for 45s, 58°C for 45s, 72°C for 45s; 9 cycles of 94°C for 40s, 53°C for 45s, 72°C for 45s; a final extension at 72°C for 30 min; and products held at 4°C.

For each PCR plate, a subsample of PCR products was separated on a 3% agarose gel (0.5x sodium borate buffer, 250V, 30 m) to confirm the presence of amplicons for each primer pair within a multiplex. The plates were closed with aluminum foil seals and wrapped in foil to prevent degradation from ambient light. The PCR products were analyzed using the Applied Biosystems 3730xl Genetic Analyzer at the University of Minnesota Genomics Center (St. Paul, MN) using the internal size standard ROX. The electropherograms were analyzed using the PeakScaner ™ v1.0 software (Applied Biosystems, Foster City, CA) and manually binned using Microsoft Excel 2010 (Microsoft, Redmond, WA).

SSR markers were mapped along with SNP markers for LG1 and LG15 using the maximum likelihood default settings in JoinMap4.1 (Van Ooijen, 2011). Markers were grouped using the 'Honeycrisp' integrated map (Clark et al., accepted) and the new maps for LG1 and LG15 were compared for homology with the SNP map using MapChart®5 (Voorrips, 2002). Seedlings with marker scores identified as possible double recombinants or with phenotype incongruence (Gygax et al., 2004) had those marker scores removed for final map creation. The final maps with SSR and SNP markers on LG1 and LG15 were used to re-map the QTL for the 'Honeycrisp' × 'Gala' population using KW and IM approaches in MapQTL (Figures 3-2 and 3-3). The 2-LOD QTL

interval, which explains ~95% confidence interval around the QTL, was calculated automatically in MapChart. Individual haplotypes were visualized in Microsoft Excel and χ^2 tests were used to confirm association of the haplotype with the resistance phenotype. The SSR and SNP haplotypes were also visualized in Pedimap (Voorrips et al., 2012) to trace inheritance through the 'Honeycrisp' pedigree (Figures 3-4 and 3-5).

Validation of the resistance in a New Zealand population

For population 2009-077, DNA was extracted using a CTAB protocol. SSR markers were amplified using the parameters outlined above for haplotypes on LG 1 and LG15. The SSR haplotypes were visualized in Excel and χ^2 tests were utilized to assess cosegregation of the resistance phenotype and 'Honeycrisp' alleles.

Candidate genes

The SSR and SNP haplotypes for *Rvi19* and *Rvi20* were compared to their physical positions using the genome browser available at GDR and by Blast sequence alignment of known SSR loci sequence (when available) or their corresponding primer sequence. The haplotype at *Rvi19* did not include marker Md-Exp7 as it maps to ~13cM above the region of interest. The *Rvi19* haplotype was flanked on the top by SNP ss475882285 to enable visualization of local rearrangement and to capture additional candidate genes in the region. The predicted gene set, consisting of predicted gene models (mRNA) across each haplotype, were downloaded directly from the genome browser for the window spanning each haplotype. The annotated output included gene ontologies and predicted protein models that were searched for resistance gene motifs including Toll-Interleukin-1 Receptor (TIR), nucleotide binding (NB-arc, NBS), protein

kinase, leucine rich repeat (LRR), and LRR with N-terminal domain (LRRNT; Velasco et al., 2010).

The peptide sequences for all of the predicted genes were submitted as a batch sequence search for Pfam-A (protein family) matches at pfam.janelia.org (Punta et al., 2012). Sequences were analyzed with a moderately restrictive E-value (0.00001) to identify candidate resistance gene motifs. The Pfam search output were evaluated for the R gene motifs by identifying those with the corresponding Pfam family identifier: [protein kinase (PF00069.20), TIR (PF01582.15), NB-arc (PF0931.17), LRR (PF12799.2; PF13855.; PF07725.7), and PF08263.7(LRRNT)]. Predicted genes residing within the genome region flanked by markers associated with the observed resistance QTL and encoding one or more R gene domains were considered to be *bona fide* candidate genes. Candidate genes were plotted onto a physical map representation relative to SSR and SNP markers utilized in this study (Figures 3-6 and 3-7). Tables 3-6 and 3-7 shows the predicted genes with Pfam identifier, gene ontologies annotated at GDR, and physical start and end positions for each predicted gene across both haplotypes.

Gene pyramiding in disease resistant selections

SSR markers linked to resistance loci in our mapping populations were used to screen advanced apple selections of the University of Minnesota breeding program shown through field observations to have some level of scab resistance. These selections had at least one known scab resistant parent other than 'Honeycrisp' (Table 3-8). Greenhouse screening was conducted using the inoculation protocols outlined above using isolate 1914D and replicated at three time points. Other advanced selections,

cultivars, breeding parents, and germplasm accessions (including several other known scab resistant cultivars) were also genotyped with SSR markers on LG1 and LG15. DNA was extracted using the silica bead method and PCR markers were amplified and analyzed following the protocols described above.

Results

The three 'Honeycrisp' × scab susceptible populations screened with single spore isolates in the greenhouse varied in χ^2 square probabilities for 1:1 or 3:1 segregation ratios (Table 3-1). Population AE0908 segregated 3:1 when intermediate phenotypic classes 3a or 3b were considered resistant with additional support for the latter (χ^2 = 0.66). Population AE0910-b had a 3:1 segregation ratio (χ^2= 0.91) when 3b was considered resistant. The population AE1022 was screened with two *V. inaequalis* isolates and the characterization of 3a as resistant supported a 1:1 segregation, whereas the classification of 3b as resistant resulted in a 3:1 segregation ratio for both isolates. Overall, the three populations support a 3:1 segregation ratio, i.e. a model with two segregating R genes.

A genome wide linkage mapping approach treating resistance as a qualitative trait identified two genomic locations conditioning resistance. Resistance mapped with strong association to LG1 near marker ss475882286 and to LG15 near SNPs ss475883045 and ss475882502 (Figures 3-2 and 3-3, red text). Two distinct peaks were plotted in the genome wide analysis of allele frequency differences between resistant and susceptible pools. These peaks were the same markers most highly associated with resistance that were identified through linkage mapping (Figure 3-8).

Interval mapping using 1,324 SNP markers positioned across the genome identified significant QTL conditioning resistance against the single spore isolate 1914D on LGs 1 and 15 (which we named *Rvi19* and *Rvi20*, respectively; Table 3-4). No significant QTL were detected when the population was screened with isolate GR19142b using KW and IM approaches (Figure 3-2 and 3-3). QTL identified by only the KW or IM mapping approach (but not by both approaches) and QTL whose detection relied on a single marker were conservatively deemed spurious and were removed from our analyses.

The reconstruction of the AE1022 LG1 and LG15 genetic maps by incorporation of SSR markers resulted in some SNPs being dropped from the original linkage maps and some local rearrangement. However, the relative order of the SSR markers along the LG1 haplotype was conserved among published maps (Celton et al., 2009; Costa et al., 2008). Relative SSR marker order was also conserved on LG15 (Fernández-Fernández et al., 2008; Gasic et al., 2009). SNP with SSR mapping of resistance QTL using MapQTL resulted in similar QTL probability peaks (Figures 3-2b and 3-3b), LOD scores, and estimates of total phenotypic variance (R^2) compared with SNP mapping alone (Table 3-5).

Several 'Honeycrisp' × 'Gala' seedling haplotypes visualized in Pedimap show the marker allele calls traced through the pedigree (Figures 3-4 and 3-5). The resistant LG1 haplotype (associated with *Rvi19*) traces from 'Frostbite' to 'Keepsake' to 'Honeycrisp' and to resistant progeny (Figure 3-4). The resistant LG15 haplotype (associated with *Rvi20*) traces from the unknown parent to 'Honeycrisp' and to resistant progeny (Figure 3-5). Seedlings were identified carrying both *Rvi19* and *Rvi20* resistant

haplotypes. The cosegregation of marker haplotypes with observed phenotypes supported a 1:1 segregation at each locus and a 3:1 segregation across both loci.

New Zealand population 2009-77 segregated for resistance against fungal isolate NL05 with χ^2 values that supported 3:1 (0.14) and 7:1 (0.22) segregation, suggesting two or three resistance genes in the population. In this population, validation of the SSR haplotypes assumed that two genes were inherited from 'Honeycrisp' and a third unmapped gene was inherited from A248R22T004 (Russian apple x 'Splendour'). In this three gene model, resistance cosegregated 1:1 with the 'Honeycrisp' SSR haplotype at both *Rvi19* and *Rvi20*, and in the overall population 3:1. As expected, a number of resistant individuals carried the susceptible haplotype at both *Rvi19* and *Rvi20*, providing support for the postulated third resistance locus. Few seedlings carried the susceptible haplotype at both loci in this population.

The *Rvi19* haplotype (including ss475882285) spans 2.3 Mb which is ~12.6 cM on the 'Honeycrisp' × 'Twin Bee Gala' linkage map (n=125) utilized for mapping the resistance loci. This haplotype includes the well-characterized *Rvi6* locus (*Vf*). Comparison of the physical and genetic positions of *Rvi6* and *Rvi19* is shown in Figure 3-6 along with candidate genes. The order of the markers in the linkage maps and their physical positions demonstrate some incongruence. The *Rvi19* haplotype comprises 263 predicted genes, 24 of which encode motifs common to R proteins (Figure 3-6; Table 3-6). Eighteen encode the LRR (or LRRNT) motif. These are clustered into six regions along this haplotype. Four candidate genes cluster near the Ch-Vf1 microsatellite marker and we proposed these to be the cluster of R gene paralogs associated with *Rvi6*

resistance (*HcVf1* to *HcVf4*; Vinatzer et al., 2004; Boudichevskaia et al., 2009). It is unlikely that any of these four are *Rvi19*, as the genetic map places *Rvi6* over 3 cM away.

The *Rvi20* haplotype spans 3.2 Mb (~10.1 cM) and comprises 460 predicted genes, 23 of which encode resistance gene motifs (Figure 3-7; Table 3-7). Three of these predicted genes contained the LRR motif. More common in this region were protein kinases (14) or TIR (7) domains.

The Excel-based haplotyping tool was used to deduct *Rvi20* SNP haplotypes in a wider germplasm set. The SNP haplotype associated with resistance in 'Honeycrisp' on LG15 is common in apple germplasm. This is due to the widespread presence of a haplotype that is identical by state (IBS) from 'Golden Delicious', a common ancestor common in much of the germplasm. Another individual sharing this haplotype is 'Splendour'. A similar haplotype was deduced in 'Northern Spy'.

Advanced selections in the University of Minnesota breeding program carrying SSR haplotypes associated with more than one scab resistance gene were identified (Table 3-7). This included pyramiding of haplotypes on LG1 (*Rvi6* and *Rvi19*, *Rvi17* and *Rvi19*) and between LG1 and LG15 (*Rvi6* and *Rvi20*, *Rvi19* and *Rvi20*). Two individuals were identified with haplotypes suggesting resistance conditioned by *Rvi6*, *Rvi19*, and *Rvi20* warranting further investigation. Other breeding selections genotyped, including the cultivar Sweet Sixteen, demonstrated inheritance of *Rvi19* directly from 'Frostbite'. The *Rvi19* SSR haplotype, which is IBS with the *Rvi17* SSR haplotype, was identified in the cultivars Duchess (PI58801), Red Baron, and Wildung (sold under the trademark SnowSweet). The *Rvi17*/*Rvi19* haplotype was also identified in four 'Antonovka' cultivars [Antonovka 1.5 pound (PI107196), Antonovka 17260-B (PI589956), Antonovka

Monasir (PI588784), and BVIII.33.8 (PI172612)]. Two genotypes identified as Dolgo (PI58870 and an unknown accession) carried the 158 bp allele at Ch-Vf1 (*Rvi6*), but lacked other markers alleles that define the *Rvi6* haplotype in donor *M. floribunda*-821.

Discussion

Two new apple scab resistance loci have been identified in the important cultivar Honeycrisp. This cultivar has become prominent in breeding programs worldwide because of its superb fresh eating quality and long-term storage traits. Genetic studies were conducted to understand the inheritance of resistance in this cultivar. Mapping approaches placed these genes onto linkage groups 1 and 15 and we have named them *Rvi19* and *Rvi20*, respectively, following the nomenclature scheme devised for the *Malus*-*Venturia* pathosystem (Bus et al., 2009).

The segregation ratios in the three Minnesota populations and one New Zealand population supported a 3:1 segregation ratio, when the phenotypic class 3b was classified as resistant. Although the 3b reaction includes sporulation capable of causing secondary infection, there is an observable defense reaction in the host tissue to the presence of the pathogen. The 3b reaction is evidence for genetic resistance, but the efficacy of the host defense response is impacted by other genes segregating in the population. Similar to the progeny of *Rvi6* resistant cultivars, the populations in this study exhibit resistance reactions across the Chevalier (1991) scale with the exception of no observable HR (class 1) in the 'Honeycrisp' resistance. However, a number of individuals were scored as 0, which may suggest HR with no visible macroscopic symptoms. The 'Honeycrisp' ancestors do not exhibit the HR when inoculated with a mixed isolate suspension of the

pathogen but do display classes 2-3b, including stellate necrosis (Chapter 2, Clark et al., submitted).

One difficulty in accurately phenotyping using the Chevalier et al. (1991) scale is establishing a threshold for delineation of the intermediate resistance classes 3-3a-3b. New, high-throughput phenotyping techniques like real-time PCR may offer improved quantification of infection to increase the precision in QTL mapping (Gusberti et al., 2012). There is no distinguishing resistance phenotype between *Rvi19* or *Rvi20* in populations AE1022 or 2009-77.

The four mapping approaches utilized in this study (linkage mapping, allele frequency differences of pooled phenotypic classes, Kruskal-Wallis analysis, and interval mapping) resulted in consistent placement of two resistance loci supported by a 3:1 segregation ratio. Because disease resistance to scab in 'Honeycrisp' can be categorized along a quantitative scale and as a binary trait, the best schema was utilized for the appropriate mapping strategy for each data set.

By adding SSR markers to the SNP QTL regions, functional haplotypes were assigned to the pedigree that could be used to validate the resistance in in the 2009-77 population. SSR markers are an appropriate tool for MAS as they are affordable and highly repeatable across labs. The creation of a 'Honeycrisp' × 'Twin Bee Gala' SNP and SSR linkage map for the two regions allowed positioning the CH-Vf1 locus to ~3.5 cM above ss475882286 (GDsnp10005), with the placement of the new marker SSR MDC012480.81 between them. A new SSR marker (MDC022202.499) for the Ch03b06 locus was developed in this study and is a good alternative to Ch03b06, especially for

multiplex reactions since the larger fragment size works well in combination with other markers.

Marker haplotypes support the inheritance of *Rvi19* from 'Keepsake' and 'Frostbite', and suggest *Rvi20* was inherited from the unknown 'Honeycrisp' parent. However, the *Rvi20* SSR and SNP haplotype associated is IBS between the resistant 'Honeycrisp' and the susceptible 'Golden Delicious'. This haplotype is inherited in 'Gala' and our segregating population (as a susceptible allele). 'Gala' has long been described as highly susceptible and is utilized in a differential set of apple cultivars for race identification and detection of avirulence loci of *V. inaequalis* isolates (Bus et al., 2011). An approach was taken to screen the RosBREED apple reference germplasm set to identify individuals sharing the SNP haplotype. The *Rvi20* haplotype is common due to frequent use of 'Golden Delicious' in North American breeding programs. The SNPs and SSRs do not provide a high enough resolution to identify sequence variants responsible for resistance.

The 138bp allele at Ch-Vf1 is associated with the apple scab resistance *Rvi17* (*Va1*) from the cultivars known as Antonovka and in *Rvi19* in 'Honeycrisp'. The SSR haplotypes of several 'Antonovka' accessions at *Rvi19* are identical with that of 'Honeycrisp'. *Rvi17* previously mapped ~1cM away from *Rvi6* suggesting that *Rvi17* resistance is not allelic to *Rvi6* (Dunemann and Egerer, 2010). In the current study, *Rvi19* maps ~ 3.8 cM away from *Rvi6*. The SSR and SNP haplotypes for *Malus floribunda*-821 at *Rvi19* provide additional support that *Rvi17*/*Rvi19* are not allelic with *Rvi6*. Markers employed in the current study cannot distinguish *Rvi19* from *Rvi17*. Gaps in the known 'Honeycrisp' pedigree limit the study of inheritance of both *Rvi19* and *Rvi20*. The joint

utility of Ch-Vf1 for detecting *Rvi17/Rvi19* and *Rvi6* make this marker especially useful, and allowed for the identification of advanced selections with single or pyramided resistance genes. Like 'Honeycrisp', these selections will be ideal parents due to their fruit quality and scab resistance traits. Indeed, many of these selections are already in use in our breeding program. Cultivars with pyramided resistance may be especially attractive to organic and sustainable apple producers.

The cultivar Wildung (sold under the trademark SnowSweet) exhibits exceptional field resistance to apple scab when grown under no-spray conditions at the University of Minnesota North Central Research and Outreach Center in Grand Rapids, MN (personal observation). The shared *Rvi17*/*Rvi19* haplotype is present in this cultivar and is proposed to be inherited through 'Sharon', an offspring of the resistant cultivar 'Longfield' (a Russian apple sometimes referred to as 'Pepinka Letovskay'). Additional research will be needed to determine if the resistance in 'Wildung' is conferred by the haplotype defined here, and if it is unique or allelic to *Rvi17* or *Rvi19*. The advanced selection MNY35 carries the 138bp allele at Ch-Vf1 and is descended from 'Discovery' which has been used in mapping qualitative scab resistance, although none mapped to LG1 (Liebhard et al., 2003b). Additional genotyping of MNY35 and 'Discovery' is warranted to determine the parentage of this selection and to investigate 'Discovery' for the *Rvi19* resistance haplotype.

Because *Rvi6*, *Rvi17*, and *Rvi19* seem to occupy a very small genetic and physical space, precise genic markers could allow the detection of recombination events that place these alleles onto the same chromatid. Along LG1 are several other important loci used in parental and seedling selection in apple. *Md-Exp7* is an expansin gene ~17 cM above

the *Rvi19* haplotype which was mapped in this experiment. *Md-Exp7* is known to be involved in fruit softening during storage (Costa et al., 2008). The 210 bp allele in 'Honeycrisp' through 'Keepsake' is relatively rare in *Malus* germplasm and may contribute to the long storage life of this cultivar. Recently, a QTL related to fructose content in apple at harvest and two storage time points was mapped adjacent to the *Rvi19* haplotype (Guan, 2013). The SNP ss475876885 is shared between the scab resistance and fructose QTL haplotypes and maps ~6.6 cM below Ch-Vf1. Breeders will need to be cognizant of the desirable tight linkage between the very high fructose haplotype associated with 'Honeycrisp', *Rvi19*, and *Md-Exp7*, which are all on this chromatid.

The incongruence between the genetic positions (linkage map) and physical positions demonstrate the inherent difficulties in statistical approaches in high-throughput genotyping, mapping, and genomics. Local rearrangements have been observed in a number of linkage maps including between those of higher densities (Fernández-Fernández et al., 2008; Antanaviciute et al., 2012; Clark et al., accepted). In *Malus*, the clustering strategy used in development of the IRSC SNP array results in linkage maps within JoinMap4.1 with alternative models. Because there is low recombination within a cluster, errors in genotype calls may bias local arrangement along a linkage group. The apple physical map also contains errors with ~10% of markers developed from one pseudochromosome mapping to another linkage group (Clark et al., accepted). For example, two SNPs within the haplotype for *Rvi20* on LG15 were originally developed from contigs aligned to pseudochromosome 3. Short sequence reads utilized in genome sequencing, especially those with large numbers of repetitive regions may be difficult to assemble correctly (Schatz et al., 2010). Despite these issues, the defined haplotypes will

allow the detection of an association of a trait with a marker (or flanking markers) in > 90% of seedlings due to SSR markers less than 10 cM away.

R genes are known to exist in clusters or R gene complexes throughout plant genomes. In *Malus*, one cluster is on LG1 around Ch-Vf1 (Vinatzer et al., 2004) and two purported clusters are on LG2 (*Rvi15* and *Rvi4*; *Rvi11, Rvi9, Rvi2,* and *Rvi8*) (Bus et al., 2011). Our mapping of candidate R genes suggests additional clusters on the *Rvi19* and *Rvi20* haplotypes. A relatively conservative E-value threshold (0.0001) for identifying protein families using Pfam resulted in few predicted genes encoding LRR domains. Candidate genes serve as good starting points for the development of molecular markers and cloning experiments. Resequencing and alignment of the 'Honeycrisp' candidate genes to the 'Golden Delicious' reference sequence (Velasco et al., 2010) may provide the high-resolution need to identify functional variants responsible for resistance at both loci. LRR encoding genes identified on LG1 should be considered candidates for *Rvi17* and *Rvi19* and tested further tested to confirm they are not allelic.

Several hypotheses should be further explored at the sequence level to decipher differences at *Rvi20* between 'Honeycrisp' and 'Golden Delicious'. The markers in the current study cannot detect a recombination event at this locus to test if these sequences are IBS or IBD. Recombination itself (along with gene conversion) is proposed as one way in which new genetic variation is developed. Unequal crossing over during meiosis results in new haplotypes and alleles that may have a new specificity for monitoring pathogen effectors (Ellis et al., 2000). Tandem duplication from unequal crossing over may also result in gene clusters in which each resistance gene can be under selection (Meyers et al., 2003). The LRR domain is under diversifying selection resulting in

changes in residues that lead to specificity for gene-for-gene interactions with the pathogen (Michelmore and Meyers, 1998).

Although unlikely in the case of 'Honeycrisp' scab resistance, somatic recombination may be a source of novel variation, especially in long-lived crops (McDowell and Simon, 2006). A portion of a tree may have an increased fitness advantage with these somatic variations (Michelmore and Meyers, 1998). Somatic variation has been suggested as one way in which the *Vf* locus has diversified following duplication events as there is no support for the aforementioned exchange of sequences and mispairing (Xu and Korban, 2004).

Additional research will also be necessary to determine which *V. inaequalis* race(s) are compatible with *Rvi19* and *Rvi20*. Well-typed isolates can be screened on seedlings carrying a single resistance gene. These seedlings can then be utilized in a differential set of resistant cultivars and seedlings for monitoring the pathogen. Furthermore, isolates capable of infecting 'Honeycrisp' should be screened with the differential set to investigate the avirulence genes involved in this compatible reaction.

Tables

Table 3-1 *Venturia inaequalis* monoconidial isolates used in this study for screening seedling progeny for apple scab resistance.

Isolate	Sampling date	Country	Accession	Tissue origin
GR19142b	2010	USA	Minneiska	Leaf
ZestarFruit	2010	USA	Minnewashta	Fruit
1914D	2010	USA	Minneiska	Leaf
1639	2001	France	TSR34T15	Apple or leaf
1774-1	unknown	USA	unknown	---
EU-NL05	1998	Netherlands	*Malus floribunda* 821	---
EU-NL24	1998	Netherlands	Prima	---

Table 3-2 Segregation for 5 seedling families screened for apple scab resistance reactions with single spore isolates in the greenhouse in the USA and the Netherlands.

Population	Parent1	Parent2	Isolate[z]	Resistance class	Phenotype[y]		Total	R:S	X^2	*p*
					R	S				
AE0908	Honeycrisp	MN1940	GR19142b	0-3a	141	62	203	3:1	3.33	0.07
				0-3b	155	48	203	3:1	0.20	0.66
AE0910-b	Honeycrisp	MN1789	ZestarFruit	0-3a	69	37	106	3:1	5.55	0.02
				0-3b	79	27	106	3:1	0.01	0.91
AE1022-b	Honeycrisp	Twin Bee Gala	1914D	0-3a	65	56	121	1:1	0.67	0.41
				0-3b	85	36	121	3:1	1.46	0.23
			GR19142b	0-3a	65	49	114	1:1	2.25	0.13
				0-3b	78	36	114	3:1	2.63	0.10
2009-077	Honeycrisp	A248R22T004	EU-NL24		26	44	70	1:1	4.63	0.03
			1774-1		28	41	69	1:1	2.45	0.12
			1639		1	69	70	0:1	0.01	0.90
			EU-NL05		57	12	69	7:1	1.51	0.22
			EU-NL05 (when EU-NL24=S)		30	12	42	3:1	0.29	0.59

[z] See Table 3-1 for the origins of the isolates
[y] R = resistant, S = susceptible

Table 3-3 Microsatellite primer sequences for mapping resistance on LG1 and LG15 of apple including specific annealing temperatures used in this study. Forward primers tagged with an 18bp, M13 sequence (TGTAAAACGACGGCCAGT). All reactions were carried out at an annealing temperature (T_a) of 58 °C and fluorescently labeled with Hex, Ned, or 6-Fam (T_a=53 °C).

Linkage group	Marker	Sequence (5'-3') Forward	Reverse	Size range (bp)	Source
1	Md-Exp7	CATAGAAGGTGGCATGAGCA	TTTCTCCTCACACCCAAACC	129-212	Costa et al., 2008
	CH-Vf1	ATCACCACCAGCAGCAAAG	CATACAAATCAAAGCACAACCC	129-174	Vinatzer et al., 2004
	MDC012480.81	AGTTTCAGCCAAAGCCCAAC	GTGGTTCAAATTCACCTTTGACG	217*	
	NZmsCN879773.z	CCCTCTGTTACTTTGACTCTTCTC	TGGTTTGGGTTGAAAATGGT	139-148	Celton et al., 2008
15	CH03b06	GCATCCTTGAATGAGGTTCACT	CCAATCACCAAATCAATGTCAC	111-136	Liebhard et al., 2002
	MDC022202.499	GTAGATGCCGTGAAGGTGC	GCAGTACACTCAGCTTGCG	344*	
	NZ02b1	CCGTGATGACAAAGTGCATGA	ATGAGTTTGATGCCCTTGGA	212-238	Guilford et al., 1997
	CN908484	CAGGCGCCATTTTTAGAGAG	GGAGTGGCGAATTAGCTGAG	150-275	Gasic et al., 2009
	CH04a06	AGAAAATCTAAGAGCAGCAG	TAAAACTCAAGTCGCCCGTC	106-110	Liebhard et al., 2002

*Expected product size based on 'Golden Delicious' sequence.

Table 3-4 Genome wide QTL mapping results when segregating population AE1022 was screened with *V. inaequalis* isolate 1914D for interval mapping and Kruskal-Wallis mapping approaches using the consensus 'Honeycrisp' map (SNPs only) and the 'Gala' map built with SSR and SNP markers. For the given linkage groups, the SNP marker(s) most associated with resistance are shown, along with logarithm of odds (LOD) score, K-value, and the R^2 value (percentage of the variance explained for by the QTL).

Map	Linkage Group	Map position (cM)	Associated marker	LOD	K-Value[z]	R^2
Consensus	1	50.00	ss475882286	3.72	13.969*****	15.0
Honeycrisp	15	30.07	ss475883045	2.85	11.547*****	12.2
		30.07	ss475882502	2.85	8.458**	10.9
Honeycrisp ×	1	54.28	ss475882286	3.72		15.9
Twin Bee Gala	15	14.86	ss475883045	2.76		12.0

[z] K values same in each map
** 0.05
*****0.001

Table 3-5 Segregation of marker haplotypes and phenotypes following greenhouse *Venturia inaequalis* inoculation tests at each resistance locus (LG1 and LG15) for AE1022 and 2009-077 seedling populations. χ^2 test evaluates the expected distribution of the null hypothesis of 1:1:1:1.

Population[z]	LG	Phenotype:Haplotype combination[y]				Total	X^2	*p*
		R+	R-	S+	S-			
AE1022	1	46	29	8	24	107	27.45	< 0.001
	15	44	31	7	25	107	26.50	< 0.001
2009-077	1	31	23	4	7	65	30.69	< 0.001
	15	25	23	6	6	60	21.73	< 0.001

[z] AE1022 is a USA seedling population of 'Honeycrisp' × 'Twin Bee Gala'. 2009-077 is a New Zealand population of 'Honeycrisp' × A248R22T004.

[y] R = resistant phenotype (0-3b); S = susceptible phenotype; "+" = resistant haplotype; "-" = susceptible haplotype

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Table 3-6 Predicted genes identified in the *Rvi19* haplotype with known resistance gene encoding motifs including: leucine rich repeat (LRR), leucine rich repeat N-terminal domain (LRRNT), nucelotide binding (NB-arc, NBS), protein kinase (Pkinase), and Toll-Interleukin-1 Receptor (TIR). Also shown are start and end site on the apple physical map for this chromosome, gene ontology and Pfam terms.

Predicted Gene	Motif	Start	End	Gene Ontology Term	Pfam Term
MDP0000213589	LRRNT	21877175	21880724	GO:0005515, GO:0005524, GO:0016887	PF08263.7
MDP0000812673	LRR/LRRNT	21902752	21905571	GO:0005515, GO:0005524, GO:0016887	PF08263.7, PF13855.1
MDP0000088815	LRR/LRRNT	22356252	22359302	GO:0005515	PF08263.7, PF12799.2, PF13855.1
MDP0000318241	LRR/LRRNT	22365915	22369013	GO:0005515	PF08263.7, PF12799.2, PF13855.1
MDP0000225282	LRRNT	22502483	22506779	GO:0005515	PF08263.7
MDP0000301494	LRRNT	22522247	22529246	GO:0005515	PF08263.7
MDP0000303490	LRRNT	22548628	22557670	GO:0005515	PF08263.7
MDP0000551940	NBS	22790661	22793731	GO:0005524, GO:0005515, GO:0017111, GO:0006915, GO:0006952	PF00931.17
MDP0000249604	Pkinase	22923293	22924672	GO:0004674, GO:0005524, GO:0006468, GO:0007165, GO:0004672, GO:0004713	PF00069.20
MDP0000870727	Pkinase	22923316	22924695	GO:0004674, GO:0005524, GO:0006468, GO:0007165, GO:0004672, GO:0004713	PF00069.20
MDP0000301393	TIR	22995055	23000274	GO:0005524, GO:0005515, GO:0017111, GO:0004888, GO:0031224, GO:0006952, GO:0045087, GO:0006915, GO:0007165	PF01582.15
MDP0000694809	TIR-NBS-LRR	23155138	23159699	GO:0005524, GO:0005515, GO:0004888, GO:0031224, GO:0045087, GO:0006915, GO:0007165	PF00931.17, PF01582.15, PF12799.2, PF13855.1
MDP0000158470	LRR/LRRNT	23561065	23562865	GO:0005515	PF08263.7, PF12799.2
MDP0000673917	LRR	23574090	23585461	GO:0005515	PF12799.2, PF13855.1
MDP0000160413	LRR/LRRNT	23594914	23597634	GO:0005515	PF08263.7, PF12799.2, PF13855.1
MDP0000212713	LRR/LRRNT	23606297	23609383	GO:0005515	PF08263.7, PF12799.2, PF13855.1
MDP0000380035	LRR/LRRNT	23701908	23703257	GO:0005515	PF08263.7, PF13855.1
MDP0000792424	LRR/LRRNT	23721495	23723975	GO:0005515	PF08263.7, PF12799.2, PF13855.1
MDP0000558833	LRR/LRRNT	23728238	23729713	GO:0005515	PF08263.7, PF13855.1

Table 3-6 continued (2 of 2)

MDP0000322294	LRR-Pkinase	23745625	23749661	GO:0004674, GO:0005524, GO:0005515, GO:0004872, GO:0006468, GO:0004672, GO:0004713	PF00069.20, PF13855.1
MDP0000468697	NBS-arc	23934028	23937342	GO:0005524, GO:0005515, GO:0006915, GO:0006952	PF00931.17
MDP0000473154	LRRNT/LRR-Pkinase	23947943	23952852	GO:0004674, GO:0005524, GO:0005515, GO:0006468, GO:0004672, GO:0004713	PF00069.20, PF08263.7, PF13855.1
MDP0000232662	LRR/LRRNT	23980743	23983899	GO:0004674, GO:0005524, GO:0005515, GO:0006468	PF08263.7, PF13855.1
MDP0000321036	Pkinase	23992169	23998542	GO:0005524, GO:0004672, GO:0004674, GO:0006468, GO:0004713	PF00069.20

Table 3-7 Predicted genes identified in the *Rvi20* haplotype with known resistance gene encoding motifs including leucine reach repeat (LRR), leucine rich repeat N-terminal domain (LRRNT), nucelotide binding (NB-arc, NBS), protein kinase, and Toll-Interleukin-1 Receptor (TIR). Also shown are start and end site on the apple physical map for this chromosome, gene ontology and Pfam terms.

Predicted Gene	Motif	Start	End	Gene Ontology Term	Pfam Term
MDP0000197558	NBS-arc	3266678	3268384	GO:0005524, GO:0005515, GO:0006915, GO:0006952	PF00931.17
MDP0000278586	Pkinase	3486119	3498488	GO:0004674, GO:0005524, GO:0006468, GO:0004672, GO:0004713	PF00069.20
MDP0000220546	TIR	3496250	3496765	GO:0005524, GO:0005515, GO:0004888, GO:0031224, GO:0006952, GO:0045087, GO:0006915, GO:0007165	PF01582.15
MDP0000757615	Pkinase	3502312	3504579	GO:0004674, GO:0005524, GO:0006468, GO:0004672	PF00069.20
MDP0000320418	Pkinase	3784685	3787911	GO:0004674, GO:0005524, GO:0006468, GO:0004672	PF00069.20
MDP0000177665	TIR	4108911	4116403	GO:0003677, GO:0005634, GO:0003700, GO:0004888, GO:0007165, GO:0031224, GO:0043565, GO:0045087, GO:0045449	PF01582.15
MDP0000664885	TIR-NBS	4116972	4122321	GO:0005524, GO:0005515, GO:0004888, GO:0031224, GO:0006952, GO:0045087, GO:0006915, GO:0007165, GO:0031072	PF00931.17, PF01582.15
MDP0000877582	TIR	4119896	4120468	GO:0005524, GO:0004888, GO:0005515, GO:0031224, GO:0006952, GO:0045087, GO:0006915, GO:0007165	PF01582.15
MDP0000481972	TIR	4123775	4124065	GO:0005524, GO:0005515, GO:0004888, GO:0031224, GO:0006952, GO:0045087, GO:0006915, GO:0007165	PF01582.15
MDP0000481973	TIR	4124082	4126271	GO:0005524, GO:0005515, GO:0004888, GO:0017111, GO:0031224, GO:0045087, GO:0007165	PF01582.15

Table 3-7 continued (2 of 2)

MDP0000297052	TIR-NBS	4128703	4133550	GO:0005524, GO:0005515, GO:0004888, GO:0017111, GO:0031224, GO:0006952, GO:0045087, GO:0006915, GO:0007165	PF00931.17, PF01582.15
MDP0000318360	LRRNT/LRR-PKinase	4157067	4160117	GO:0004713, GO:0005524, GO:0005515, GO:0004872, GO:0016021, GO:0006468, GO:0004672, GO:0004674	PF00069.20PF08263.7PF13855.1
MDP0000247898	LRRNT/LRR-Pkinase	4158466	4161517	GO:0004713, GO:0005524, GO:0005515, GO:0004872, GO:0016021, GO:0006468, GO:0004672, GO:0004674	PF00069.20PF08263.7PF13855.1
MDP0000272145	Pkinase	4186482	4187381	GO:0004674, GO:0005524, GO:0006468, GO:0004672, GO:0004713	PF00069.20
MDP0000167752	Pkinase	4187773	4193156	GO:0004674, GO:0005524, GO:0000151, GO:0006468, GO:0006950, GO:0004672, GO:0004713	PF00069.20
MDP0000596128	Pkinase	4203075	4203974	GO:0004674, GO:0005524, GO:0006468, GO:0004672, GO:0004713	PF00069.20
MDP0000704184	LRR	4487753	4488703	GO:0005515	PF13855.1
MDP0000124998	Pkinase	4647145	4649326	GO:0004674, GO:0005524, GO:0006468, GO:0004672	PF00069.20
MDP0000316456	Pkinase	5153338	5155732	GO:0004674, GO:0005524, GO:0016020, GO:0016021, GO:0006468, GO:0004672, GO:0004713	PF00069.20
MDP0000776336	Pkinase	5318521	5318955	GO:0004674, GO:0005524, GO:0005529, GO:0006468, GO:0004672	PF00069.20
MDP0000635687	Pkinase	5616270	5618691	GO:0004674, GO:0005524, GO:0005529, GO:0006468, GO:0004672, GO:0004713	PF00069.20
MDP0000877654	Pkinase	5624955	5626019	GO:0004674, GO:0005524, GO:0005529, GO:0006468, GO:0048544	PF00069.20
MDP0000205199	Pkinase	5788366	5790962	GO:0004674, GO:0005524, GO:0005529, GO:0006468, GO:0004672, GO:0004713	PF00069.20

Table 3-8 Disease resistant University of Minnesota advanced selections screened with SSR markers at *Rvi19* and *Rvi20* for R gene pyramiding. A '?' indicates not enough information to determine the identity by descent of the haplotype.

Selection	Parent	Parent	LG1	LG15
MNX63	Liberty	Minnewashta	*Rvi6*	
MNX64	Freedom	Wildung	*Rvi6*	
MNX71	1789	Honeycrisp	*Rvi19*	*Rvi20*
MNX80	Honeycrisp	Liberty	*Rvi6, Rvi19*	*Rvi20?*
MNX88	Honeycrisp	Freedom	*Rvi6*	*?*
MNX95	Sawa	Honeycrisp	*Rvi6*	*?*
MNY06	Sawa	Honeycrisp	*Rvi6*	*Rvi20*
MNY18	Sawa	Honeycrisp		*Rvi20?*
MNY21	Honeycrisp	GoldRush	*Rvi6*	*Rvi20*
MNY27	NY65707	Minnewashta	*Rvi6*	
MNY31	Honeycrisp	NY61345		*Rvi20*
MNY32	MN1505	Witos	*Rvi6*	
MNY35	Discovery	Williams Pride	*Rvi17?*	
MNY36	Honeycrisp	Liberty		*Likely*
MNY48	Honeycrisp	Jonafree		*Rvi20*
MNY49	Honeycrisp	Sawa		*Rvi20*
MNY60	MN1802	Wililams Pride	*Rvi17? Rvi6*	
MNY61	NY65707	Honeycrisp	*Rvi19, Rvi6*	*Rvi20?*

Figures

Figure 3-1 Pedigree of population AE1022 ('Honeycrisp' × 'Twin Bee Gala') that was was utilized for mapping apple scab resistance gene loci on LG1 and LG15.

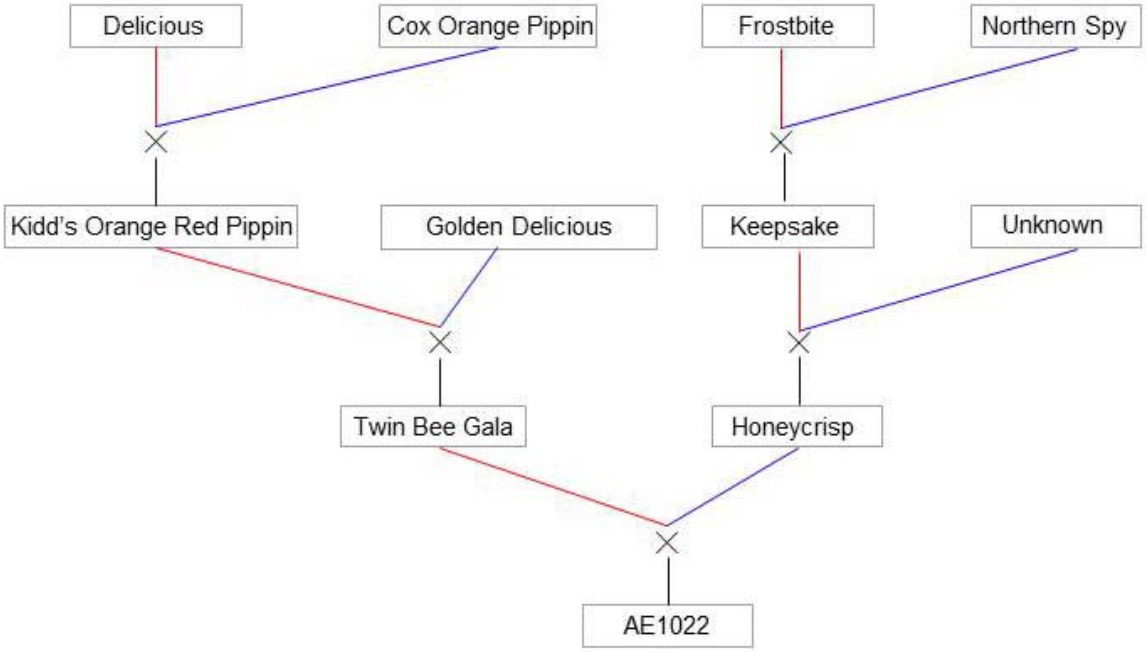

Figure 3-2 Genetic maps and QTL histograms for linkage group 1. Highly significant LOD-3 threshold is shown as a dashed vertical line. Marker ss475882286 (red font, underlined) is the SNP marker most strongly associated with the resistance locus during linkage mapping and in Kruskal-Wallis mapping. (A) Consensus SNP 'Honeycrisp' with QTL position (box) and 2-LOD interval (whiskers) when screened with isolate 1914D (green) during interval mapping. No significant QTL were detected with isolate GR19142b (red dashed line). (B) Remapping of QTL in 'Honeycrisp' × 'Twin Bee Gala' linkage map with SNP and SSR markers (blue font).

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Figure 3-2

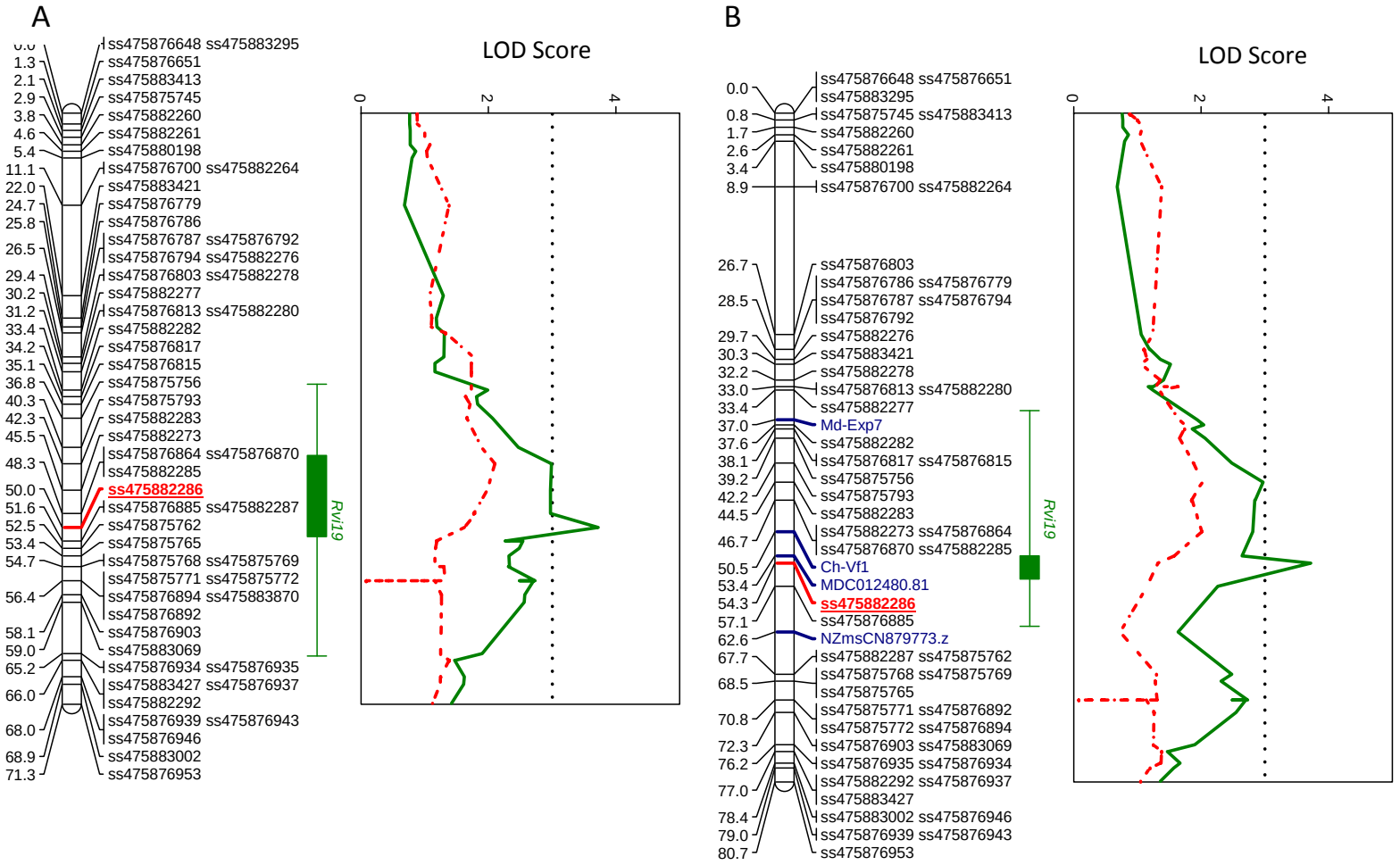
A
LOD Score
0
2
4
1.3 ss475876651
2.1 ss475883413
2.9 ss475875745
3.8 ss475882260
4.6 ss475882261
5.4 ss475880198
11.1 ss475876700 ss475882264
22.0 ss475883421
24.7 ss475876779
25.8 ss475876786
26.5 ss475876787 ss475876792
ss475876794 ss475882276
29.4 ss475876803 ss475882278
30.2 ss475882277
31.2 ss475876813 ss475882280
33.4 ss475882282
34.2 ss475876817
35.1 ss475876815
36.8 ss475875756
40.3 ss475875793
42.3 ss475882283
45.5 ss475882273
48.3 ss475876864 ss475876870
ss475882285
50.0 ss475882286
51.6 ss475876885 ss475882287
52.5 ss475875762
53.4 ss475875765
54.7 ss475875768 ss475875769
56.4 ss475875771 ss475875772
ss475876894 ss475883870
ss475876892
58.1 ss475876903
59.0 ss475883069
65.2 ss475876934 ss475876935
66.0 ss475883427 ss475876937
ss475882292
68.0 ss475876939 ss475876943
ss475876946
68.9 ss475883002
71.3 ss475876953
Rvi19
B
LOD Score
0
2
4
0.0 ss475876648 ss475876651
0.8 ss475883295
1.7 ss475875745 ss475883413
2.6 ss475882260
3.4 ss475882261
8.9 ss475880198
ss475876700 ss475882264
26.7 ss475876803
28.5 ss475876786 ss475876779
ss475876787 ss475876794
ss475876792
29.7 ss475882276
30.3 ss475883421
32.2 ss475882278
33.0 ss475876813 ss475882280
33.4 ss475882277
37.0 Md-Exp7
37.6 ss475882282
38.1 ss475876817 ss475876815
39.2 ss475875756
42.2 ss475875793
44.5 ss475882283
46.7 ss475882273 ss475876864
ss475876870 ss475882285
50.5 Ch-Vf1
53.4 MDC012480.81
54.3 ss475882286
57.1 ss475876885
62.6 NZmsCN879773.z
67.7 ss475882287 ss475875762
68.5 ss475875768 ss475875769
ss475875765
70.8 ss475875771 ss475876892
ss475875772 ss475876894
72.3 ss475876903 ss475883069
76.2 ss475876935 ss475876934
77.0 ss475882292 ss475876937
ss475883427
78.4 ss475883002 ss475876946
79.0 ss475876939 ss475876943
80.7 ss475876953
Rvi19

Figure 3-3 Genetic maps and interval mapping QTL histograms for linkage group 15. Highly significant LOD-3 threshold is shown as a dashed vertical line. Marker ss475883045 and ss475882502 are the SNP marker most strongly associated with the resistance locus during linkage mapping and in Kruskal-Wallis mapping (red font, underlined). (A) Consensus SNP 'Honeycrisp' map with QTL position (box) and 2-LOD interval (whiskers) when screened with isolate 1914D (green) during interval mapping. No significant QTL were detected with isolate GR19142b (red dashed line). (B) Remapping of QTL in 'Honeycrisp' × 'Twin Bee Gala' linkage map with SNP and SSR markers (blue font). No QTL detected with isolate GR19142b (red dashed line).

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Figure 3-3

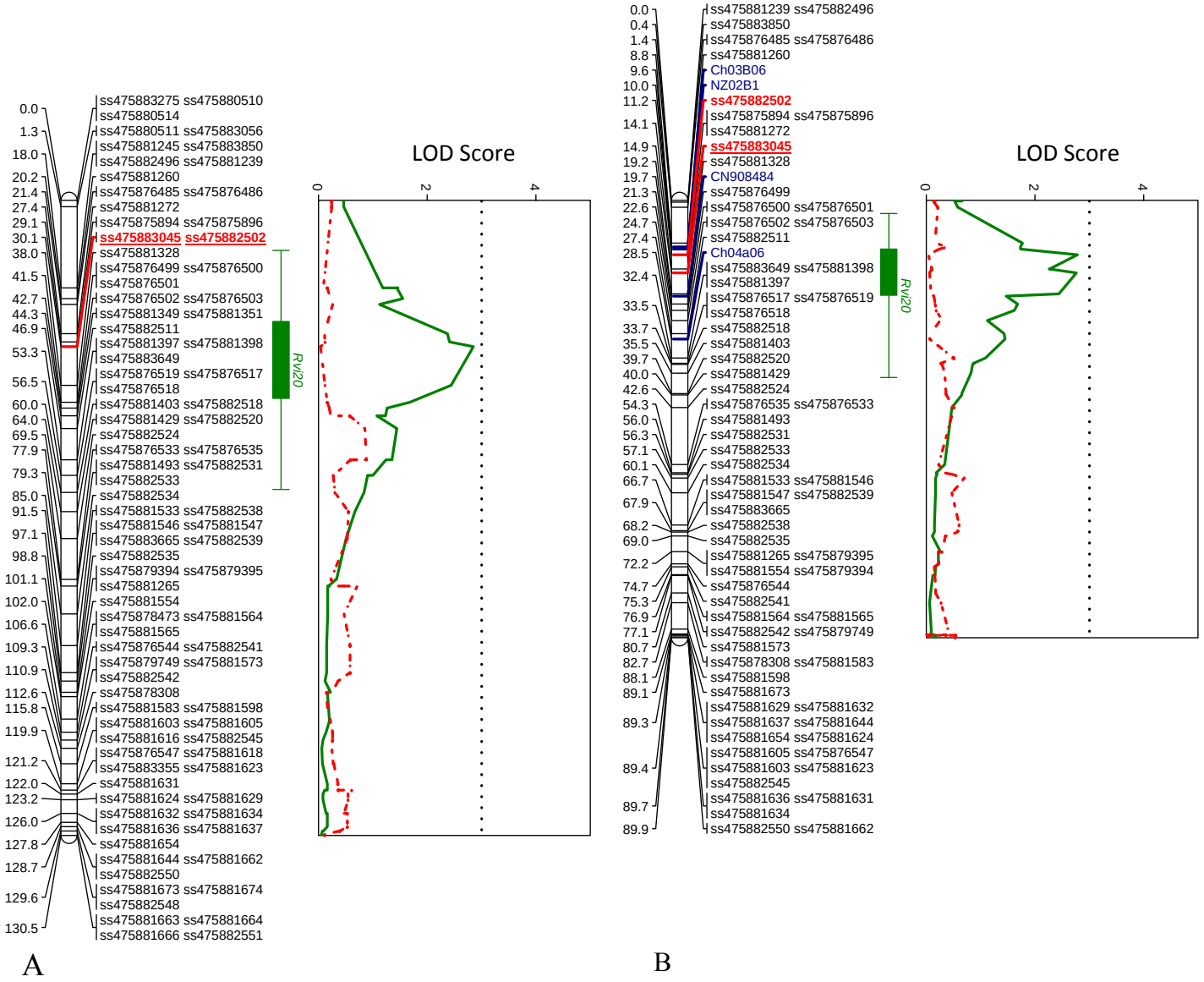
0.0 ss475883275 ss475880510
ss475880514
1.3 ss475880511 ss475883056
18.0 ss475881245 ss475883850
ss475882496 ss475881239
20.2 ss475881260
21.4 ss475876485 ss475876486
27.4 ss475881272
29.1 ss475875894 ss475875896
30.1 ss475883045 ss475882502
38.0 ss475881328
41.5 ss475876499 ss475876500
ss475876501
42.7 ss475876502 ss475876503
44.3 ss475881349 ss475881351
46.9 ss475882511
53.3 ss475881397 ss475881398
ss475883649
56.5 ss475876519 ss475876517
ss475876518
60.0 ss475881403 ss475882518
64.0 ss475881429 ss475882520
69.5 ss475882524
77.9 ss475876533 ss475876535
79.3 ss475881493 ss475882531
ss475882533
85.0 ss475882534
91.5 ss475881533 ss475882538
97.1 ss475881546 ss475881547
ss475883665 ss475882539
98.8 ss475882535
101.1 ss475879394 ss475879395
ss475881265
102.0 ss475881554
106.6 ss475878473 ss475881564
ss475881565
109.3 ss475876544 ss475882541
110.9 ss475879749 ss475881573
ss475882542
112.6 ss475878308
115.8 ss475881583 ss475881598
119.9 ss475881603 ss475881605
ss475881616 ss475882545
121.2 ss475876547 ss475881618
ss475883355 ss475881623
122.0 ss475881631
123.2 ss475881624 ss475881629
126.0 ss475881632 ss475881634
ss475881636 ss475881637
127.8 ss475881654
128.7 ss475881644 ss475881662
ss475882550
129.6 ss475881673 ss475881674
ss475882548
130.5 ss475881663 ss475881664
ss475881666 ss475882551
Rvi20
LOD Score
0
2
4
A
0.0 ss475881239 ss475882496
0.4 ss475883850
1.4 ss475876485 ss475876486
8.8 ss475881260
9.6 Ch03B06
10.0 NZ02B1
11.2 ss475882502
14.1 ss475875894 ss475875896
ss475881272
14.9 ss475883045
19.2 ss475881328
19.7 CN908484
21.3 ss475876499
22.6 ss475876500 ss475876501
24.7 ss475876502 ss475876503
27.4 ss475882511
28.5 Ch04a06
32.4 ss475883649 ss475881398
ss475881397
33.5 ss475876517 ss475876519
ss475876518
33.7 ss475882518
35.5 ss475881403
39.7 ss475882520
40.0 ss475881429
42.6 ss475882524
54.3 ss475876535 ss475876533
56.0 ss475881493
56.3 ss475882531
57.1 ss475882533
60.1 ss475882534
66.7 ss475881533 ss475881546
67.9 ss475881547 ss475882539
ss475883665
68.2 ss475882538
69.0 ss475882535
72.2 ss475881265 ss475879395
ss475881554 ss475879394
74.7 ss475876544
75.3 ss475882541
76.9 ss475881564 ss475881565
77.1 ss475882542 ss475879749
80.7 ss475881573
82.7 ss475878308 ss475881583
88.1 ss475881598
89.1 ss475881673
89.3 ss475881629 ss475881632
ss475881637 ss475881644
ss475881654 ss475881624
ss475881605 ss475876547
89.4 ss475881603 ss475881623
ss475882545
89.7 ss475881636 ss475881631
ss475881634
89.9 ss475882550 ss475881662
Rvi20
LOD Score
0
2
4
B

Figure 3-4 SSR and SNP haplotypes spanning *Rvi19* l on LG1 demonstrating resistance inherited from 'Frostbite', through 'Keepsake', 'Honeycrisp', and into resistant progeny (gray) of the 'Honeycrisp' × 'Twin Bee Gala' mapping population.

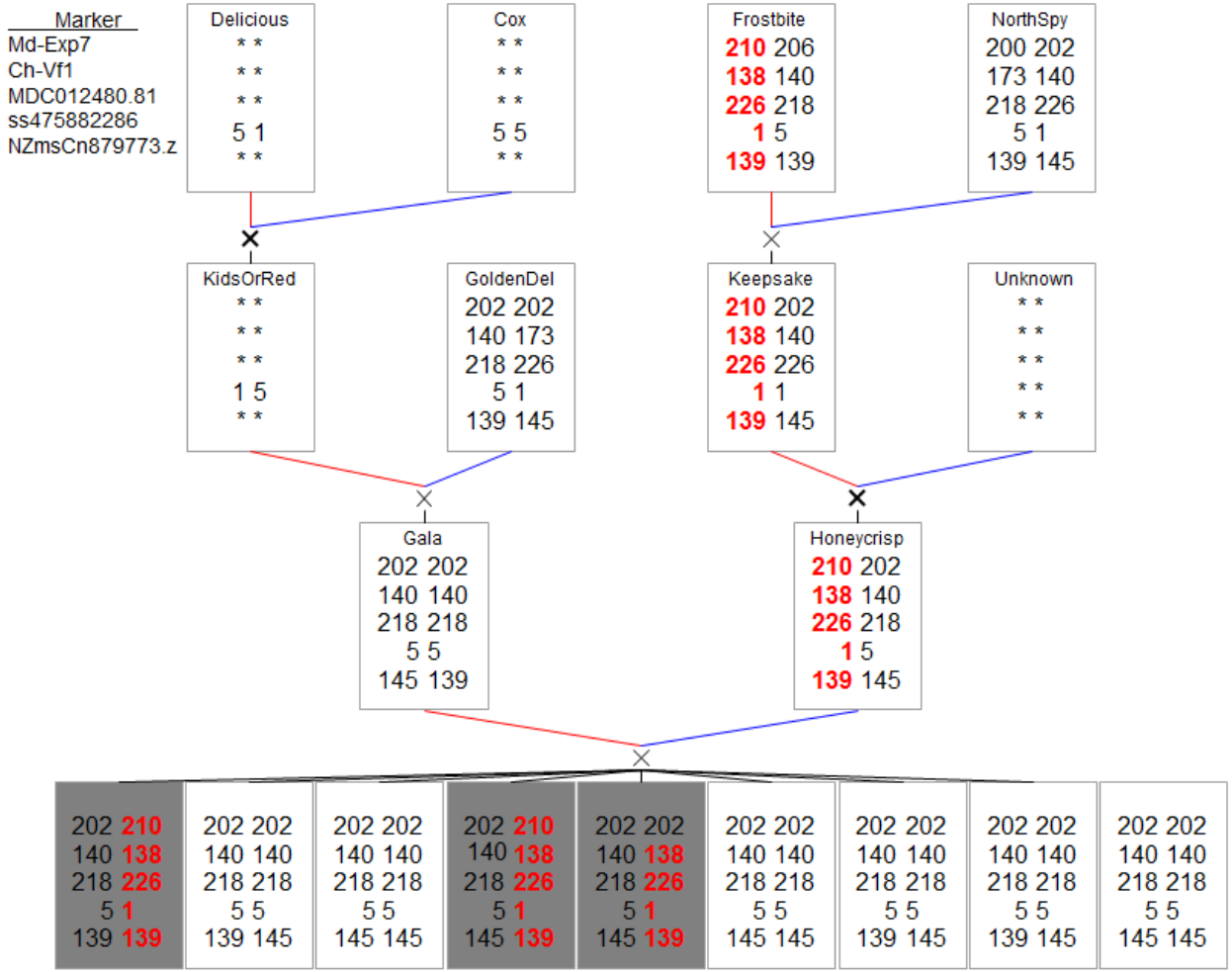

Figure 3-5 SNP and SSR haplotype spanning *Rvi20* on LG15 demonstrating the inheritance of the resistant haplotype (red) from the unknown parent of 'Honeycrisp' in the resistant 'Honeycrisp' × 'Twin Bee Gala' progeny (gray boxes).

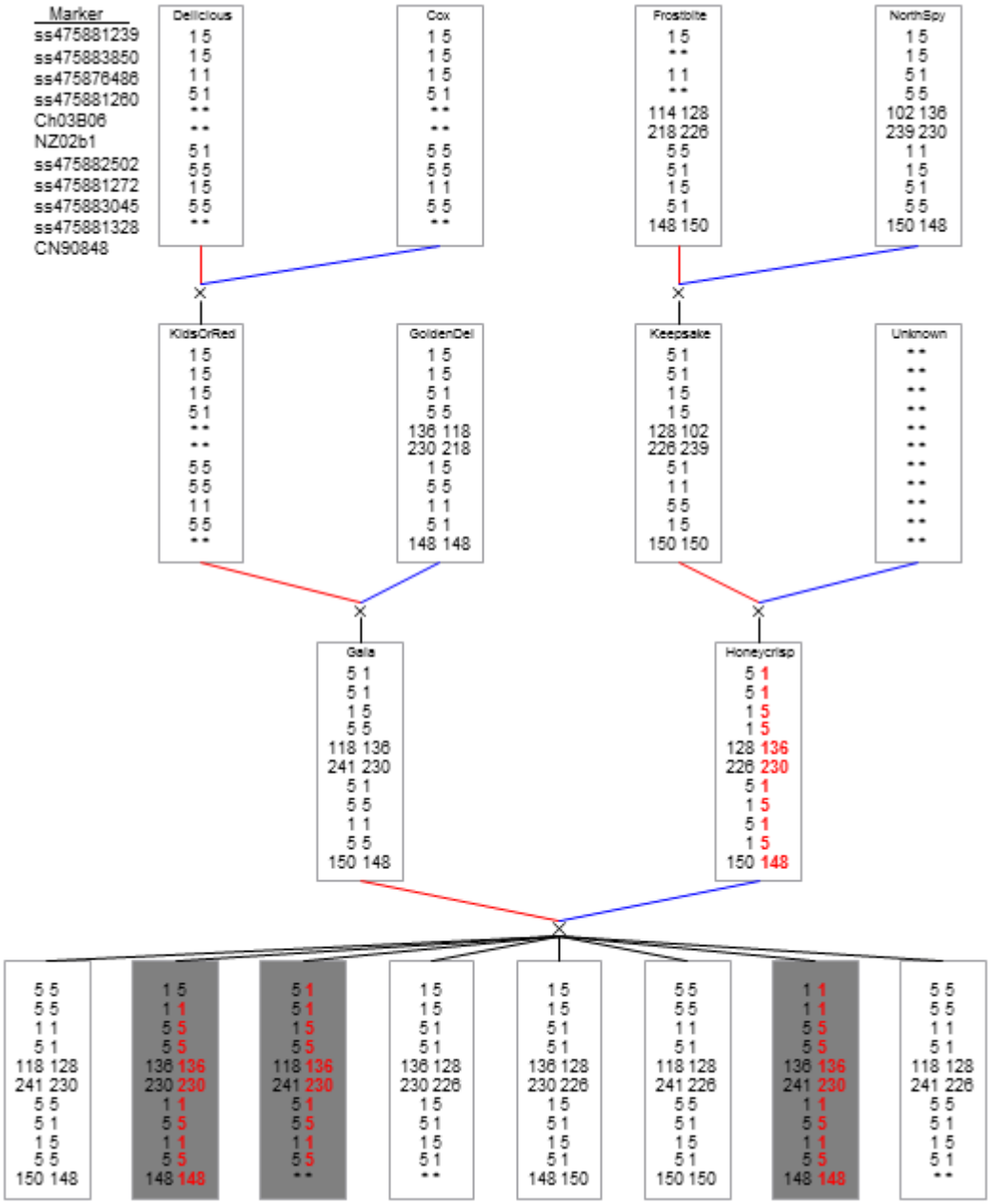

Figure 3-6 Alignment of the *Rvi19* SNP and SSR haplotype with the physical position of the markers on the apple genome sequence for chromsome 1. Candidate resistance genes are positioned on the physical map based on their start site (Table 3-6a). The motif(s) the candidate gene encodes for is displayed [leucine reach repeat (LRR), leucine rich repeat N-terminal domain (LRRNT), nucelotide binding (NB-arc, NBS), protein kinase, and Toll-Interleukin-1 Receptor (TIR)]. Not shown are the 239 other predicted genes in the region. The red and underlined SNP marker is most strongly associated with the resistance at this locus during mapping experiments.

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Figure 3-6

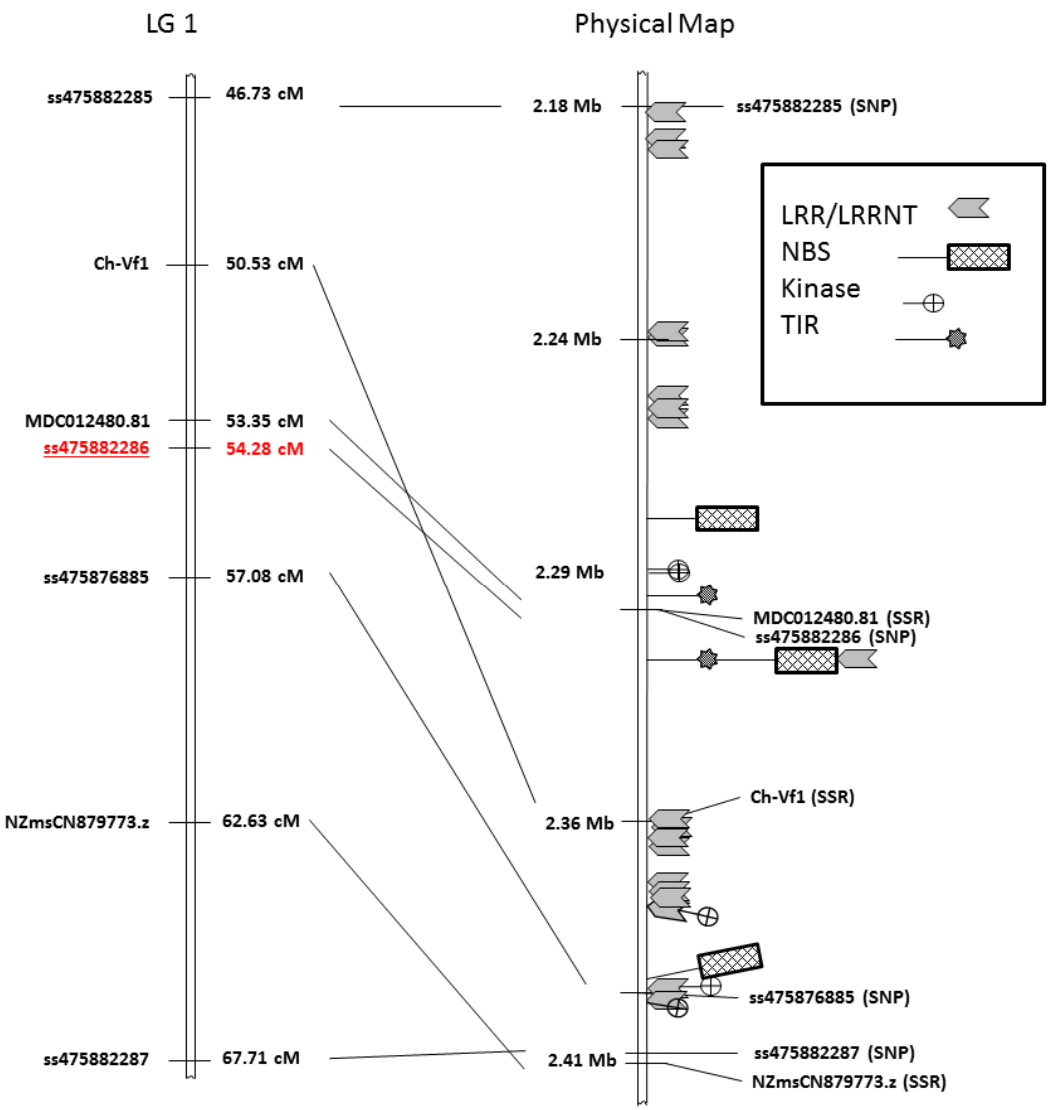
LG 1
Physical Map
ss475882285
46.73 cM
Ch-Vf1
50.53 cM
MDC012480.81
53.35 cM
ss475882286
54.28 cM
ss475876885
57.08 cM
NZmsCN879773.z
62.63 cM
ss475882287
67.71 cM
2.18 Mb
2.24 Mb
2.29 Mb
2.36 Mb
2.41 Mb
ss475882285 (SNP)
LRR/LRRNT
NBS
Kinase
TIR
MDC012480.81 (SSR)
ss475882286 (SNP)
Ch-Vf1 (SSR)
ss475876885 (SNP)
ss475882287 (SNP)
NZmsCN879773.z (SSR)

Figure 3-7 Alignment of the *Rvi20* SNP and SSR haplotype with the physical position of the markers on the apple genome sequence for chromsome 1. Candidate resistance genes are positioned on the physical map based on their start site (Table 3-6b). The motif(s) the candidate gene encodes for is displayed [leucine reach repeat (LRR), leucine rich repeat N-terminal domain (LRRNT), nucelotide binding (NB-arc, NBS), protein kinase, and Toll-Interleukin-1 Receptor (TIR)]. Not shown are the other 437 other predicted genes in the region. The red and underlined SNP markers are most strongly associated with the resistance at this locus during mapping experiments.

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Figure 3-7

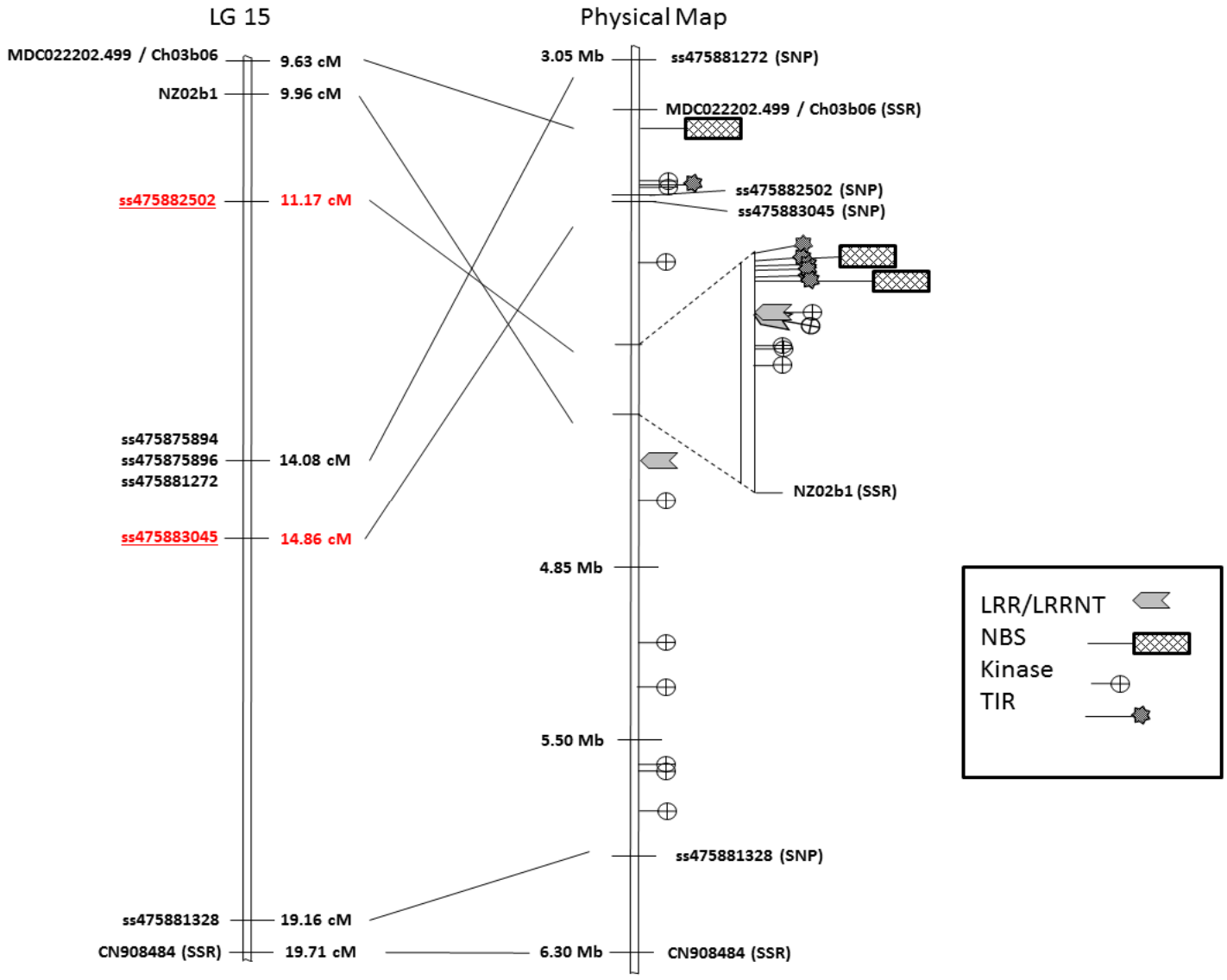
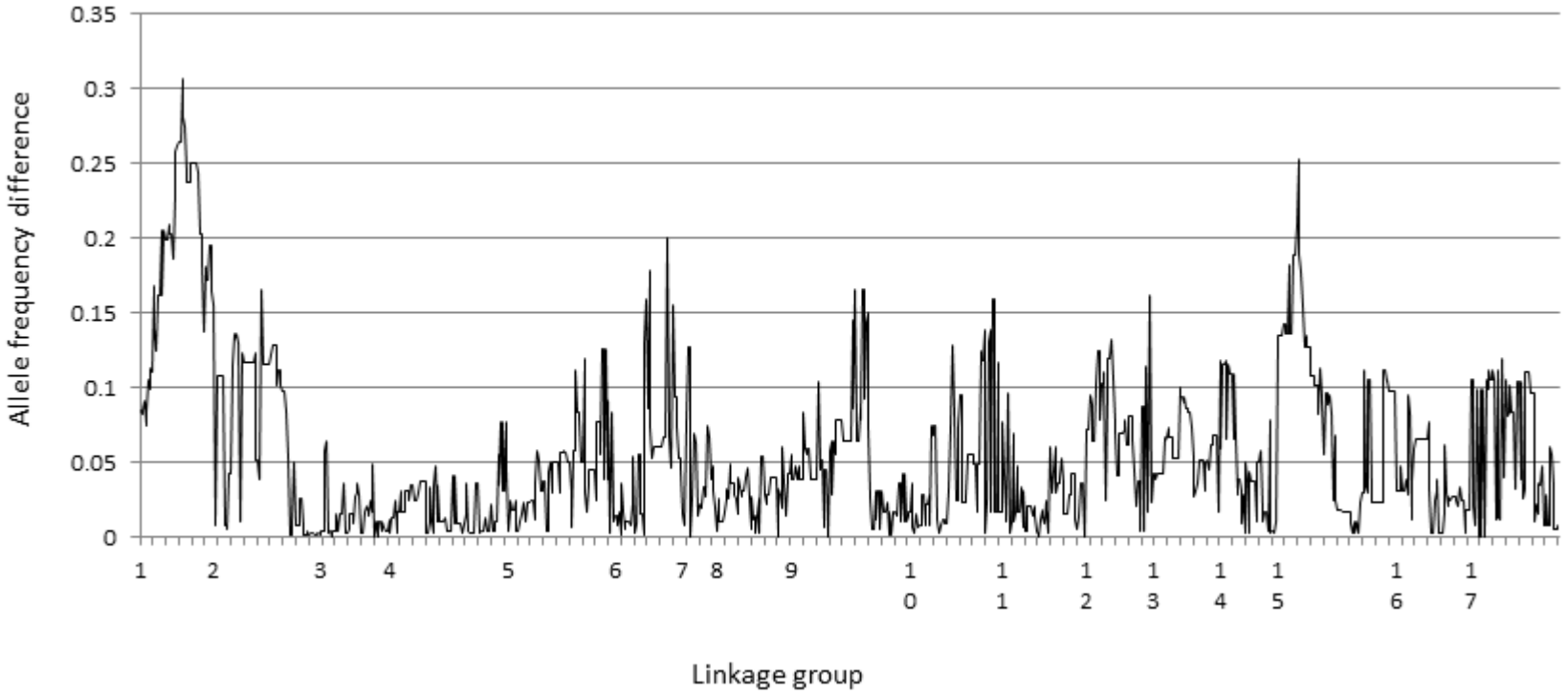

LG 15
Physical Map
MDC022202.499 / Ch03b06
9.63 cM
NZ02b1
9.96 cM
ss475882502
11.17 cM
ss475875894
ss475875896
14.08 cM
ss475881272
ss475883045
14.86 cM
ss475881328
19.16 cM
CN908484 (SSR)
19.71 cM
3.05 Mb
ss475881272 (SNP)
MDC022202.499 / Ch03b06 (SSR)
ss475882502 (SNP)
ss475883045 (SNP)
NZ02b1 (SSR)
4.85 Mb
5.50 Mb
ss475881328 (SNP)
6.30 Mb
CN908484 (SSR)
LRR/LRRNT
NBS
Kinase
TIR

Figure 3-8 Population AE1022 was screened for resistance with single spore isolate1914D. Allele frequency differences between pooled resistant and susceptible seedlings were plotted against SNPs along the 'Honeycrisp' consensus linkage map. This non-parametric approach detected peaks on LG1 and 15 which correspond to SNP markers detected by linkage mapping (JoinMap) and QTL mapping (MapQTL; Kruskal-Wallis and interval mapping) approaches for the two resistance loci identified in the 'Honeycrisp' progeny.



Chapter 4

Population genetic structure of Minnesota isolates of *Venturia inaequalis* collected from diverse *Malus* species and cultivars

Evaluating the genetic diversity in populations of plant pests is an important step in disease monitoring, and is critical in developing and testing disease resistant cultivars. Apple scab, incited by the fungus *Venturia inaequalis*, is the most important disease in apple (*Malus x domestica*) in Minnesota and worldwide. This is due to the large number of chemical inputs required to manage the disease, and the consequence of fruit loss due to unsightly lesions, poor storage, and overall reduced plant health. Over eighty monoconidial isolates of *V. inaequalis* were collected and maintained in culture from leaf and apple fruit lesions collected from six locations in Minnesota. The collection locations included a crab apple collection, residential settings, and germplasm evaluation locations for the University of Minnesota plant breeding project. Twelve simple sequence repeat (SSR) markers were utilized for assessing the population structure and genetic diversity of the fungal isolates, however only eleven markers were polymorphic. Variation within populations accounted for 90% and 96% of the total variation when using a Haploid or Haploid-SSR distance matrix, respectively. The ϕ and R_{st} values were 0.095 and 0.04 for these models, indicating gene flow among populations. Nei's pairwise distance matrix separated population 6 (from Grand Rapids, MN) from a cluster of populations 1, 2, and 3. The neighbor-joining and unweighted pair group method with arithmetic mean clustering algorithms showed no relationship between the geographic collection locations and genetic clustering. This lack of population stratification was

supported by Structure analysis. The Mantel tests detected no spatial autocorrelation between the geographic and genetic distance matrices.

Introduction

Apple scab, caused by the fungal pathogen *Venturia inaequalis* (Cke.) Wint., is the major disease affecting apples grown in temperate climates around the world. This disease can have a significant economic impact as diseased fruit are not marketable. Complete crop loss is possible if steps are not taken in the orchard to reduce infection. Regular fungicide applications are the most effective way to limit disease. Fungicides increase production costs and also lead to concerns of environmental pollution, health concerns for growers, and pesticide residues in foods for consumers (Ellis et al., 1998; Broggini et al., 2010). Organic management approaches aim to control the disease and include sanitation practices such as shredding leaves on the orchard floor in the fall or early spring to limit disease pressure and thus the number of fungicide applications (Sutton et al., 2000). The deployment of scab resistant cultivars also offers growers sustainable options for reducing infection in an orchard.

Venturia inaequalis is a hemi-biotrophic ascomycete that produces both sexual spores (ascospores) and asexual spores (conidia) which are capable of causing infection on apple trees. The infection begins each spring with the release of ascospores from pseudothecia (fruiting bodies) which have over-wintered in leaf tissue on the ground or on other plant tissue (e.g., budscales) from the previous season (MacHardy, 1996). The ascospores are the result of sexual reproduction between compatible mating types that occurs on leaves on the orchard floor. Air currents move the ascospores to the host plant where the primary infection occurs on young leaves and sepals. The scab lesions that

develop from the primary inoculum produce conidia which are able to cause secondary infections. The conidia are dispersed in wet weather by wind and splashing rain, leading to infection on leaves, stems, and fruit that can spread throughout the orchard. The polycyclic, secondary infection can persist throughout the growing season and into leaf senescence. In Israel, no sexual cycle has been observed because of the warm winter conditions leading to populations of clonal lineages (Boehm et al., 2003).

A major objective of apple breeding programs is the development of cultivars with durable disease resistance and high quality fruit traits. A number of scab resistant cultivars have been developed, but these are not widely grown due to consumer preference for higher quality fruit varieties. The development of scab resistant cultivars faces both genetic (linkage drag) and marketing challenges. Any new apple cultivar must be an outstanding alternative or replacement to an existing, consumer-recognized cultivar. The deployment of scab resistant cultivars can place evolutionary pressure on scab populations, leading to new races able to overcome host resistance in less than ten years (Parisi et al., 1993).

Flor (1956) described the classic gene-for-gene model for plant-pathogen interactions in the flax (*Linum usitatissimum*)-flax rust (*Melampsora lini*) pathosystem. The gene-for-gene relationship was soon after described in the *Malus*-*Venturia inaequalis* pathosystem (Williams and Shay, 1957; Boone, 1971). When a spore lands and initiates an infection site on the plant, the presence of the pathogen Avr protein is recognized (directly or indirectly) by the corresponding plant R protein, triggering a defense response (Dangl and Jones, 2001). From a population standpoint, pathogen *Avr* genes can become virulent against the host through mutational selection pressures that result in

the Avr protein avoiding recognition by the R proteins (Ellis et al., 1999; Abramovitch et al., 2006). The deployment of cultivars with single R genes has led to identification of new apple scab races (Races 6 and 7) which can overcome the R cognate gene (Parisi et al., 1993; Bénaouf and Parisi, 2000).

In the compatible reaction, the symptoms include circular lesions that are green, necrotic, or chlorotic on the leaf surface. The margin may or may not be defined, and the lesion may change color as the older portion in the middle of the lesion and supporting leaf tissue die. Symptoms may also include the presence of dark mycelial growth on either adaxial or abaxial leaf surface or fungal growth along the lateral and mid veins. There are eight physiologic races of *V. inaequalis* found around the world, six of which are known to be present in the United States as Race 7 was recently detected (Dayton and Williams, 1968; Beckerman et al., 2009). Race 6 (Germany) (Parisi et al., 1993) was detected on 'Prima', a cultivar carrying the *Vf* scab resistance gene, and race 7 (Great Britain) was detected on *M. floribunda* 821 (Bénaouf and Parisi, 2000). In New Zealand, race 8 was discovered while describing a new resistance gene interaction in progeny of *M. sieversii* (Bus et al., 2005a).

Natural gene flow in the pathogen can be very slow, as splash dispersal is the main mechanism of movement of conidia. Infected leaf litter is spatially restricted within an orchard and the development and/or movement of races to other orchards may be slow or non-existent. Local adaptation of strains and cultivars develops because of limited movement of the pathogen within and among orchards. The planting of mixed cultivar orchards would impart differential resistance, thus influencing the adaptation of local *V. inaequalis* strains, slowing down the spread of highly virulent strains (Sierotzki et al.,

1994). Knowing the races present in an orchard or growing region through continual monitoring can give insight as to how R-genes are defeated or how resistant cultivars and management practices should be implemented (www.vinquest.ch; (Patocchi et al., 2009)).

The movement of pathotypes (Races 6 and 7) that are virulent to a resistant gene could affect the durability of *Rvi6* (*Vf)* resistant cultivars (Tenzer and Gessler, 1997). Though natural gene flow is highly restricted, human activity has been an important factor in distributing races around the globe. Spores could potentially be moved on infected fruit from orchard to orchard and even further down the international supply chain. The movement of infected budwood, rootstock, and nursery trees also plays a role. The pathogen is not restricted to the orchard industry. Apples that are planted throughout the landscape as ornamentals (i.e. crabapples of a number of *Malus* species), for fruit, or in wild habitat can be sources of inoculum (Lê Van et al., 2011).

The population structure and/or the genetic variation of *V. inaequalis* of several geographic regions has been the focus of several studies in Czech Republic (Melounova et al., 2004), Spain (Martinez-Bilbao and Murillo, 2005), Sweden (Sandskär and Liljeroth, 2005), Brazil (Schenato et al., 2008), and India (Padder et al., 2011). The population structure was compared between Asian (China and India) and UK isolates, with increased diversity observed in the European population (Xu et al., 2008). The increased knowledge at the interface of plant breeding and pathogen population structure can lead to improved control measures for the disease (Beckerman et al., 2009).

Studies in population structure and genetic diversity can be conducted by evaluating physiological race structure by inoculating a set of differential apple genotypes with monosporic isolates in the greenhouse, and/or genetic diversity analyses

using molecular markers to detect polymorphism between isolates (Tenzer and Gessler, 1997; Tenzer et al., 1999; Martinez-Bilbao and Murillo, 2005; Schenato et al., 2008). Other studies have planted the differential set into an orchard or utilized portable screening orchards (potted differential set) to monitor and detect apple scab races (Sandskär and Liljeroth, 2005). Difficulties arise with quantifying the amount of each race present and virulence characteristics of the races. To date, assessing incidence (a survey of populations present) and genetic variation are important tools for monitoring the pathogen.

Molecular markers can be useful in assessing genetic variation in a population, but may not be able to identify specific races. Tenzer et al. (1999) observed significant variation within 11 *V. inaequalis* populations, but little variation between different populations. The markers utilized in assessing genetic diversity have not been shown to be correlated with *Avr* genes. One cannot determine if rare alleles that are shared between populations are identical by decent and the result of genetic drift, or if they have independently evolved (Tenzer et al., 1999). Restriction fragment length polymorphism (RFLP) markers have been used to demonstrate significant diversity within populations which have been attributed to the movement of diseased plants and fruits (Tenzer and Gessler, 1999). Polymorphic microsatellite simple sequence repeat (SSR) markers are an affordable and repeatable marker platform which can be multiplexed in PCR and fragment analysis. A number of SSR markers have been developed for *V. inaequalis* for the development of genetic linkage maps and for studies of genetic diversity (Tenzer et al., 1999; Guérin et al., 2004).

A collection of single spore isolates in Minnesota was curated in an effort to characterize the genetic diversity in mixed plantings of *Malus* species and cultivars. Isolates were derived from fruit and leaf tissues on managed and residential plantings. The isolates were collected for utilization in screening apple seedling populations as part of the University of Minnesota apple breeding program. Genetic diversity of the isolates was evaluated using SSR markers (Table 4-1). It is hypothesized that these genetic indices should provide support in identifying unique isolates for screening of resistance genes. It is also hypothesized that the geographic distance between sampling locations will result in allele frequency differences such that they can be defined as separate *V.inaequalis* populations.

Materials and Methods

Monoconidial Isolate Collection

Scab lesions were collected from University of Minnesota locations at Minnesota Landscape Arboretum (Chaska, MN), the Horticultural Research Center (HRC; Excelsior, MN), the University of Minnesota-Twin Cities campus (St. Paul, MN), the North Central Research and Outreach Center (Grand Rapids, MN), and from residential trees in Minneapolis, MN (Table 4-2). Lesions were collected on both leaves and infected fruit from both known and unknown cultivars, including ornamental crab apples of diverse *Malus* species (Table 4-3). Lesions were collected at different times during the growing season across years.

Infected leaves were collected and placed into individual paper bags or coin envelopes and stored over night at room temperature (~20º C) to dry. The air-dried lesions were manually cut from the leaf tissue, placed into 1.5 ml microcentrifuge tubes,

and stored at -20 ºC or immediately used for spore isolation (Barbara et al., 2008). Some conidia were isolated from air dried lesions that were stored at room temperature for 3-6 months. To isolate individual spores, sterile, distilled water (0.2 - 0.5 mL) was added to a microcentrifuge tube with a portion of scab lesion. The tube was agitated for ~30 seconds by vortexing to wash the conidia from the lesion. The concentration of each conidial suspension was determined with a hemocytometer and adjusted to ~ 5 x 10^3 conidia mL^{-1}. The outside bottom of a 3% agar petri plate (100 mm) was marked with a permanent marker with ~10 evenly spaced 1 cm^2 circles. The spore suspension was pipetted in 2-3 µL aliquots onto the agar at each of the pre-marked regions to aid in locating germinating spores (Ho and Ko, 1997). Plates were placed in the dark and incubated for 24 h at 20ºC (Sierotzki et al., 1994). Using a dissecting microscope under sterile conditions (laminar flow hood), individual germinated spores were removed with a scalpel or glass needle by gently touching the germ tube or conidia and lifting it off the plate (Goh, 1999). Alternatively, agar blocks containing individual spores were cut and replated separately. Individual spores were placed onto potato dextrose agar (PDA) amended with the antibiotic Rifamycin (50 mg L^{-1}) (MP Biomedicals, LLC, Solon, OH, USA) added after autoclaving (Parker et al., 1995; Barbara et al., 2008). Single spore isolates were sealed in Parafilm, incubated at 20ºC under black lights or under ambient lighting. Cultures were allowed to grow for up to 4 weeks or until the diameter of the colony reached ~ 1 cm.

Colonies were maintained through sub-culturing on PDA or were stored at -20º C as described here. For long-term storage, several milliliters of sterile water were added to the surface of the agar and a sterile wire loop was scraped gently across the surface to

loosen conidia/mycelia. Whatman #1 filter papers (GE Healthcare Life Sciences, Piscataway, NJ) were cut into ~1 cm^2 squares and double autoclaved in a glass petri dish wrapped in foil. Approximately 10 squares were placed on the same plate to soak up the fungal suspension. Additional sterile water is added as needed to moisten the paper. The petri plate was resealed with Parafilm and incubated for an additional 10 d to allow mycelium to grow onto the filter paper. The filter papers were then removed and placed in a single layer inside double-autoclaved coin envelopes and allowed to dry overnight. The entire envelope including the filter paper squares were placed into sterile vials and stored at -20ºC (Bus, personal communication, 2010). Individual cultures were reinitiated by placing a filter paper square onto PDA using sterile techniques and incubating as described above.

DNA Extraction and SSR Genotyping

Agar plugs (3 mm^3) were cut from growing colonies using sterile techniques and placed into 1.2 µL microcentrifuge tubes with 0.20g silica desiccant beads (PillowPak, Dessicare, Inc., Reno, NV). After drying overnight, mycelia were homogenized for 3-5 minutes in a Retsch MM301 Mixer Mill (Retch, Haan, Germany). DNA was extracted using a silica bead extraction protocol modified for 1.2 µL microcentrifuge tubes instead of a 96-well format (Edge-Garza et al., submitted). Samples with limited tissue disruption were ground by hand using disposable pestles after the addition of heated 250 µL extraction buffer. Following hand grinding the remaining 250µL was added to the microcentrifuge tube and the extraction protocol was followed

Table 4-1 lists the 12 microsatellite markers, including primers, utilized in this study for genotyping the Minnesota *V. inaequalis* isolates. All forward primers included

an 18-bp M13 (TGTAAAACGACGGCCAGT) sequence tag to allow for incorporation of an M13-tagged fluorescent dye (Hex, Ned, 6-Fam) used in capillary electrophoresis (Schuelke, 2000). Primers were multiplexed with the same dye when PCR products ranges were not expected to overlap based on known reported allele sizes. PCRs were carried out in PCR plates in 13µL reaction volumes consisting of 1uL DNA template, 6µL GoTaq® Colorless Master Mix (M714; Promega Corporation, Madison, WI, USA), 0.6µL volume of each primer (10mM), and 0.6µL fluorescent label (10 mM). DNA was amplified using the GeneAMP® 2700 thermal cycler [Applied Biosystems (Life Technologies), Foster City, CA] or the MWG-Biotech Primus96 Plus thermal cycler [MWG-Biotech (Eurofins MWG Operon) Huntsville, AL]. The thermal cycler conditions were: 1 cycle of 3 min at 94°C, 30 cycles of 94°C for 45s, 58°C for 45s, 72°C for 45s; 9 cycles of 94°C for 40s, 53°C for 45s, 72°C for 45s; a final extension at 72°C for 30 min; and products held at 4°C.

For each PCR plate, a subsample of amplicons was separated on a 3% agarose gel (0.5x sodium borate buffer, 250V, 30 m) to confirm the presence of amplicons of each primer pair within a multiplex. The plates were sealed with aluminum foil seals and wrapped in foil to prevent degradation from ambient light. The PCR products were submitted to the University of Minnesota Genomics Center (St. Paul, MN) for fragment analysis using the Applied Biosystems 3730xl Genetic Analyzer and the internal size standard ROX. The electropherograms were analyzed using the PeakScaner ™ v1.0 software (Applied Biosystems, Foster City, CA) and manually binned using Microsoft Excel 2010 (Microsoft, Redmond, WA).

Data Analysis

The isolates were assigned to six populations based on collection location (Table 4-2). The data were corrected to remove clones (same alleles at all loci) at a collection location using GenAlEx 6.5 (Peakall and Smouse, 2012). This was necessary as asexual spores (conidia) were collected from lesions during the asexual stages of the pathogen life cycle, often times from separate lesions on the same host leaf. Different lesions within or among trees may have been incited by the same isolate. Indices of marker and population diversity were calculated including *h* (haploid genetic diversity), H_s (heterozygosity within populations), H_t (total heterozygosity), G_{st} (fixation index), G'_{st} [Nei's corrected fixation index for small populations], and G''_{st} (standardized fixation index corrected for small populations) using the software programs Genodive (Meirmans and Van Tienderen, 2004) and GenAlEx 6.5 (Peakall and Smouse, 2012). The number of alleles and the effective number of alleles for each population and across all populations were calculated.

The SSR genotypes were analyzed to create a genetic distance matrix in GenAlEx 6.5 (Peakall and Smouse, 2012). Since SSR markers were utilized, genetic distances were calculated for haploids following two algorithms available in GenAlEx. The first model, which we refer to as 'Haploid', calculates distance by scoring any pair of alleles that are equivalent as 0 and different as 1, and sums the distances over loci to calculate the genetic distance between two isolates. The second model (Haploid-SSR) assumes a step-wise rate of mutation and considers the squared difference in allele size $[(S1-S2)^2]$ at each pairwise calculation, then sums the value across all loci. GenAlEx was also utilized to perform the analysis of molecular variance (AMOVA) between the six populations.

For haploid datasets, the AMOVA output Φ_{PT} is analogous to F_{ST} for the Haploid model and analogous to R_{ST} when using the Haploid-SSR model (Peakall et al., 1995; Peakall and Smouse, 2012). Nei's genetic distance and genetic identity were calculated between pairs of populations from the original data set in GenAlEx6.5 following Nei (1972, 1978). Principal coordinates analyses (PCoA) were conducted to visualize relatedness of populations based on Nei's genetic distance matrix. Phylip 3.695 was utilized for calculating phylogenetic trees using unweighted pair group method (UPGMA) with randomization and the neighbor joining method. Trees were visualized and annotated in EvolView software (Zhang et al., 2012). Mantel tests were conducted in GenAlEx between geographic distance matrices and each genetic distance matrices, and between the genetic distance matrices.

The assignment of isolates into clusters was further completed using a Bayesian analysis method in STRUCTURE 2.3.3 (Pritchard et al., 2000). The model did not take into account the *a priori* populations except for in estimating the number of clusters to explore in the model (K = 2 to 10). Additional parameters included admixture mode, Monte Carlo Markov Chain burn-in with 100,000 iterations and a run of 500,000 iterations. Three repetitions were run for each model to check for convergence in determining the likelihood value of K [L(K)]. The likely value of K, LnP(D), derived from the natural logarithm of the posterior probability (P) of data (D), and ΔK, and empirical statistic based on the second-order rate of change and the variance of LnP(D) were calculated in Structure Harvester (Evanno et al., 2005; Earl and von Holdt, 2011).

An AMOVA based K-means clustering (Meirmans, 2012) was conducted to consider population structure using different parameters in GenoDive. Missing data were

filled in with random values within GenoDive, as recommended in the software manual. Population assignment was calculated using likelihood ratio thresholds to place isolates into new groups using resampling (Monte Carlo test, $\alpha = 0.002$, 100 replications, 7,652 resampled individuals, clustering from k = 2 to k = 10).

Results

From 3 to 31 isolates were sampled to represent each of the six collection locations. The number of alleles for describing the genetic diversity of each population ranged from 1.667 to 4.167 and effective number of alleles from 1.394 to 2.451 (Table 4-1). Table 4-4 provides measurements of the genetic diversity of the 12 SSR markers utilized in screening the isolates within and averaged among all populations. The SSR marker 1aac4f was not polymorphic in any of the populations. Marker Victc1/2 had the most alleles (16) and an effective number of alleles of 3.66. The mean number of alleles for all loci was 5.7, with an effective number of 1.70 alleles. The mean *H* value across populations ranged from 0.250 (Pop6) to 0.384 (Pop1). The average number of alleles varied greatly between Pop1 (24.333) and Pop6 (2.750). The mean number of alleles across all loci and populations was 11.472. The average genetic differentiation at a single locus (G_{st}) was 0.089, with ranges from -0.005 (Victc1/2) to 0.399 (Vigt10/ε). Nei's fixation index (G'_{st}) and the correction index (G''_{st}) adjust the G-statistic for small populations to 0.105 and 0.175, respectively.

Pairwise Φ_{PT} or R_{ST} values between the 6 populations were significant for a number of pairwise combinations (Table 4-5). The Haploid genetic distance matrix differentiated the populations more than the Haploid-SSR analysis. The Haploid pairwise Φ differentiated Population 6 being significantly ($p < 0.05$) different from all other

populations. Population 2 and Population 3 were not significantly different. The Haploid-SSR distance matrix separated Population 2 from Population 1, Population 4, and Population 5. Consensus between the two matrices demonstrated significant ($p < 0.05$) differences for the following comparisons: Population 1:Population 2, Population 2:Population 4, and Population 2:Population 5. Principal coordinates 1 and 2 (axes 1 and 2) are plotted from the PCoA of each genetic distance matrix which demonstrates graphically the differences between genetic distance matrices(Figure 4-1). For the Haploid genetic distance matrix the first two axes accounted for 49.1 and 32.07% of the variation. For Haploid-SSR, the first two axes accounted for 100% of the variation, with 87.74% attributed to axis 1.

Analysis of molecular variance (AMOVA) partitioned the variance components for both genetic distance matrices. For distance matrix Haploid-SSR, the percentage of variance attributed to within populations was 96% ($\Phi_{PT} = 0.040$, $p = 0.108$) and the 4% attributed to between population variation. The distance matrix Haploid partitioned the variation to 90% within population and 10% between populations ($R_{ST} = 0.095$, $p = 0.003$). The Φ_{PT} statistic (and similarly R_{ST} for Haploid-SSR) is equivalent to Wright's F-statistic, with p values based on 999 permutations.

Nei's pairwise genetic distance matrix for the 6 populations (Table 4-7) provides a third model for distinguishing distance between populations and is spatially represented based on coordinates 1 and 2 of PCoA which account for 46.88 and 26.80% of the variation, respectively (Figure 4-2). The largest difference (0.307) was between Population 3 and Population 6.

The three-way comparison of genetic matrices using the Mantel test showed significant R^2 values for the matrices Haploid and Haploid-SSR ($R^2 = 0.2936$, $p = 0.010$). The Mantel test between the genetic distance matrices and the geographic distance matrix each produced R^2 values considerably < 0.01 and did not show any relationship between these matrices (Table 4-8).

The clustering method utilized in Structure did not reveal strong support for any K value for the posterior probability LnP(D) (Figure 4-3a). K = 2 had the lowest value, but large variances around other K values made it impossible to support any population structure with this metric. Figure 4-3b shows the ΔK values which support a population with K = 8 sub populations, and a second peak at K = 5 indicating potential further population structure. Visualization of the clusters as bar plots within Structure suggests no population structure, i.e. all individuals had similar profiles with equal membership in each cluster (not shown).

The Meirman's method for K-means clustering of populations (AMOVA, simulated annealing with 50,000 steps, missing data) provided two posterior models for clustering based on Bayesian information criterion (k = 6) or the Calinski and Harabasz pseudo-F statistic (k = 2). The variance components comprising the Bayesian information criterion (BIC) show 69.5% of the variance within clusters, and 30.5% of the variance attributed to among clusters (*rho* = 0.305) when BIC = 428.457. The *a priori* model using the assigned population of k = 6 has BIC score of 412.706, *rho* = 0.036.

The cluster dendrograms created using UPGMA and neighbor joining algorithms resulted in unique topologies for each methodology and distance matrix utilized in its

construction. For the sake of brevity only the neighbor-joining dendrogram for the Haploid-SSR matrix is shown (Figure 4-4).

Discussion

Single spore isolates of the apple scab causal agent *V. inaequalis* were collected and curated as part of the University of Minnesota apple breeding program for screening *Malus* germplasm for disease resistance. These were resolved into 81 unique isolates using 12 microsatellite (SSR) markers. Two isolates were identical based on their SSR genotypes and presumed to be clones so only one was retained for use in studying genetic diversity. One SSR (1aac4f) was not polymorphic in these populations. The six sample locations were selected to represent different growing areas, management conditions, and to capture the diversity of *Malus* germplasm assembled in Minnesota. Samples were requested from organic and traditional growers for this study, however no samples were submitted. Population 1 was sampled from a mixed planting of *Malus* species accessions including interspecific hybrids used primarily as flowering ornamental (i.e. crab apple) trees and curated as part of an arboretum display collection. Management practices were similar between Populations 2 and 3; except that Population 3 contains both fungicide treated and untreated orchard blocks for apple scab. The isolates were collected on hosts that included unknown crabapple trees, pollinator trees (e.g. 'Manchurian' and 'Snowdrift'), resistant dessert apples (e.g. 'Honeycrisp'), and susceptible cultivars (e.g. McIntosh, Minneiska, Minnewashta). Populations 2, 3, and 6 consisted of isolates collected from apple seedlings grown and evaluated as part of the University of Minnesota apple breeding program. Crabapples were the primary source of isolates samples in populations 4 and 5, grown as unmanaged landscape trees planted in

Minneapolis, MN, and St. Paul, MN, respectively. The North Central Research and Outreach Center at Grand Rapids, MN, is the most northern sample location in in the study and is a planting of advanced seedlings and cultivars. Population 6 was a small sample of isolates which included many trees previously grown at the location of population 2 and other regions. Population 6 is most the physically isolated from apples in landscape plantings or orchard plantings.

The anonymous SSR markers chosen for this study varied in their polymorphisms within and among populations. Additional SSR markers not reported above (Vitcca7/P, Vitg11/70, 1tc1g, M42, Vigt8/146, Viga3/Z; Tenzer et al., 1999; Guerin et al., 2004) were tested in these isolates, but were not included as they did not produce reliable amplicons (missing data points, multiple peaks, etc.). The data reported included some missing data points (null alleles) which may have been due to amplification conditions during PCR. Missing data should not affect most calculations; however, K-means clustering in GenoDive was tested with missing data and filled-in data, with both yielding very similar results.

The genetic diversity (H_s) within the populations was similar (0.30 to 0.45) and relatively high which is consistent with other studies of the pathogen (Tenzer et al., 1999). As discussed by Tenzer et al. (1999), rare alleles shared between populations are less likely to have evolved independently, but are identical by decent through gene flow among those populations. The majority of the variance observed using either distance matrix was attributed to variation within the populations. However, this was only significant for the Haploid-SSR distance matrix which had a moderate fixation index (R_{ST} = 0.095). The low (Φ_{PT} = 0.04) value in the Haploid model indicates that the populations

are sharing (or have recently shared) genetic diversity. The AMOVA output and pairwise F-statistics between populations are not congruent.

Following the suggestions of Peakall and Smouse (2012) two models for genetic distance were used in calculating the distance matrices utilized in exploring population differences. The Haploid model differentiated the populations in more pairwise combinations than the Haploid-SSR matrix. The latter model considers the step-wise mutation behavior of SSRs, but produced several warnings in the analysis output. This may explain some differences in the AMOVA output. A number of pairwise R_{ST} values were negative, but converted to 0.000 to demonstrate no difference between populations. The PCoA (Fig 4-1) reflects the disparity between the matrices. Nei's pairwise matrix is more similar to the Haploid model, and provides additional support that populations 4, 5, and 6 were unique from populations 1, 2, and 3.

Mantel tests are a useful tool for comparing matrices to determine correlations that may exist inherently in sampling procedures and to account for genetic similarity that is a function of geographic distance. The current study showed a moderate correlation between the two genetic distance matrices which provides support for using both models in interpreting the population structure. The Mantel tests between the genetic and geographic distance matrices had no linear relationship, which demonstrates no spatial autocorrelation amongst all sites, but does not consider the possibility of autocorrelation between subsets of the study. Geographic distance may account for the similarity between Populations 1, 2, and 3, but the spatial autocorrelation is not supported by Mantel tests.

Although Structure analyses suggested a population structure with K = 8 populations, visualization of the bar plot (not shown) indicated equal membership representation of each cluster across all isolates. The ΔK value often is the real value of K in the sampled population (Evanno et al., 2005), but is not confidently supported in the current data set. The lack of informative K clustering in Structure provides additional support that there is gene flow between populations. It is possible that the limited number of markers used in characterizing the isolates may not be sufficient for characterizing population structure.

Other clustering algorithms suggest population structures with k = 2 (pseudo-F) or k = 6 (BIC) clusters, including the strongest support for the *a priori* 6 populations assigned based on geographic sampling locations. The BIC model is optimal for random mating populations, whereas pseudo-F statistic is more appropriate for non-random mating (Meirmans, 2012). Random mating may be limited in *V. inaequalis* as sexual reproduction occurs when isolates of different mating types infect the same susceptible host (leaf). Host-specific interactions may lead to population differentiation when host ranges do not overlap (Guérin et al., 2007; Leroy et al., 2013). Apple trees with genetic resistance may impart specialization and thus population structure for *V. inaequalis* populations capable of infecting those resistant hosts. The most widely introgressed R gene in apple scab resistant cultivars, *Rvi6* (*Vf*), has been shown to impact population substructure by splitting the population (K = 2) in a mixed orchard of *Rvi6* and *rvi6* hosts (Leroy et al., 2013).

Ascospore dispersal in the early spring most likely accounts for the longest dispersal range for the pathogen locally on air currents. Holb et al. (2004)showed that

ascospore dispersal was effectively limited at 33 m. Modeling and simulation studies show that spores can move up to 5 km (Aylor, 1998), which could explain some gene flow between populations 1, 2, and 3. Human intervention is more likely to have had a role by moving infected plant materials. Trees transferred between locations 2, 3, and 6 may have contributed to pathogen gene flow. The pathogen reproduces asexually only after the primary infection, where conidia are generally distributed by splash dispersal throughout the orchard. High host specificity and low cross-variety disease transmission may lead to genetic differentiation and maintenance of population, although this does not seem likely in the populations studied (Gladieux et al., 2011). Reproductive isolation and long distance dispersal can lead to new virulent strains. New strains may not be randomly mating due to host specificity, or they remain undetected due to founder effects (Guérin and Le Cam, 2004; Guérin et al., 2007).

The dendrograms produced by UPGMA and neighbor joining clustering using the Haploid and Haploid-SSR distance matrices produced dramatically different results. The general trend among dendrograms was a limited geographic basis for population assignment. Membership within any cluster was not restricted to the original population, and many clusters had isolates from several of the sampling locations.

The evidence presented here indicates that despite a range of genetic diversity in the isolates sampled across Minnesota, there is no clear delineation of population structure. The differences within populations can be attributed to the random mating of the pathogen and life-histories of the orchards from which they were sampled. Each collection location was comprised of diverse *Malus* germplasm with individual trees that may impart selection pressure on the pathogen differently. This diverse host base may

account for the introduction of diverse *V. inaequalis* alleles that are now panmictic within and possibly across collection locations. There may not have been sufficient time or selection pressures to accumulate unique mutations due to the heterogeneous host structure and relatively young age of the host plantings (Gladieux et al., 2010).

A limitation of the current study is the number of SSR markers utilized. Increased markers across the genome would provide additional information especially related to marker linkage and mutations. Two genetic distance models were examined to explore inherent biases in the step-wise mutation models associated with SSR markers. The different results suggest that both models should be utilized. As molecular genotyping becomes more affordable, and genomic resources become available for *V. inaequalis*, other technologies such as genotyping by sequencing could provide information on (possibly functional) sequence variants (single nucleotide polymorphisms, microsatellite repeats, indels, etc.) that could differentiate isolates and population structure. Sequence information would be especially useful if avirulence genes were identified that were able to overcome the major scab resistance genes deployed in apple cultivars. A next step in studying these isolates would be screening against the differential set of *Malus* genotypes to determine the avirulence gene structure for each isolate. Knowledge of the avirulence genes would be important for the apple breeding program in appropriately selecting isolates for screening parents and segregating seedling populations for disease resistance genes.

The newly identified scab resistance loci *Rvi19* and *Rvi20* have been widely distributed with the planting of the cultivar Honeycrisp. 'Honeycrisp' has been planted extensively across Minnesota and in apple growing regions in the United States and

Canada. Although it is generally planted in mixed orchards and managed with fungicides for scab control, this cultivar will undoubtedly impact *V. inaequalis* evolution and population structure. This study identified isolates capable of infecting the 'Honeycrisp' host, and a thorough investigation of the *Avr* alleles in the pathogen can provide additional information about the races present in Minnesota. By documenting the genetic diversity of *V. inaequalis* isolates in Minnesota, including those capable of infecting 'Honeycrisp', we provide a snapshot in time of the population structure which could be compared to future samples to explore the effect of 'Honeycrisp' on the agricultural landscape.

Tables

Table 4-1 Microsatellite primer sequences for SSR markers utilized in screening *Venturia inaequalis* isolates. Observed allele fragment size ranges given for the single spore isolates evaluated.

Marker	Forward Primer	Reverse Primer	Allele Size	SOURCE	GenBank Accession
1tc1a	TCGAGATCCTCAACTTCCTT	TTTTAACTGTGCGGCCTG	117-120	Tenzer et al., 1999	
1tc1b	CGATTGGGGATATGAAGACTT	TTAGTAATCAAATCGCACCCA	180-248	“”	
1aac3b	AGCGCTAGGTCGTGAAATC	TTTCTGAAGTGTGTGGGACAT	119-166	“”	
Vicacg8/42	TGTCAGCCACGCTAGAAG	CACCGGACGAATCATGC	168-220	“”	AY491486
1aac4b	GGTGAGGAGGGAGAGACGAG	CATCACGCCCCTATCAAAC	177-180	“”	
1aac4f	CTTGACAGACCACAGCGAC	CTGACTGAGAGTGGCATCG	101	“”	
1aac4h	TCGTTCATCGTTCGTTTTTTCG	AATAGTGCGTACCCATATATCCA	202-213	“”	
Vigt10/ε	GCAGTGCAGGAATAGTAAGG	GCTGTGATACCAGAGAACGA	167-173	Guerin et al., 2004	AY491495
Victc1/2	CTTACCTCTCACTTGCTAAC	GTTCTGACAAGACTGTGTTG	181-233	“”	AY491476
Vitg9/129	CTAATTCAACTCGCTGCGTC	TTTCAGCCAGCTAACCTAGG	278-286	“”	AY502077
Vict1/130	GATTGGTGACGCATGTGT	GCTGGAGATTGCGTAGAC	148-152	“”	AY491478
Vica9/x	TCGCGCATCACTATCTACAC	AGACAGGAATGTGGTGGAAG	226-236	“”	AY491492

Table 4-2 Population, collection location, centroid geographic coordinates (decimal), number of individuals sampled (N), number of alleles (Num), effective number of alleles (Eff No.), and genetic diversity measurement H_s.

		Centroid Coordinates					
Pop	Location	North	West	N	Num	Eff No.	H_s
1	University of Minnesota Landscape Arboretum, Chaska, MN	44.85999	-93.62312	31	4.167	2.451	0.41
2	Horticultural Research Center Farm 1, Excelsior, MN	44.86743	-93.62967	15	3.167	2.003	0.44
3	Horticultural Research Center Farm 2, Excelsior, MN	44.86571	-93.60033	5	1.417	1.394	0.30
4	Kingfield Neighborhood, Minneapolis, MN	44.94483	-93.25253	7	2.083	1.711	0.32
5	University of Minnesota, Twin-Cities, St. Paul, MN	44.98618	-93.35580	20	2.833	2.068	0.45
6	North Central Research and Outreach Center, Grand Rapids, MN	47.24293	-93.50123	3	1.667	1.583	0.44

Table 4-3 *Venturia inaequalis* isolates collected from 6 locations in Minnesota. Isolate name, *Malus* host genotype, lesion tissue type, GPS coordinates (decimal), and population assignment are listed.

Isolate	Host Genotype	Tissue	North	West	Pop
1914	*M. x domestica 'Minneiska'*	fruit	44.86925	-93.6331	1
ArbHopa	*M. 'Hopa'*	Leaf	44.85837	-93.6226	1
Arnold	*M. arnoldiana*	leaf	44.85765	-93.6229	1
Brevipes	*M. brevipes*	Leaf	44.85845	-93.6221	1
Brier	*M.* 'Brier'	Leaf	44.85842	-93.6229	1
DavidA	*M.* 'David'	Leaf	44.85805	-93.6224	1
EchtA	*M.* 'Echtermeyer'	Leaf	44.85793	-93.6213	1
EchtB	*M.* 'Echtermeyer'	Leaf	44.85793	-93.6213	1
GlenMills	*M. xhumi* 'Glen Mills'	Leaf	44.85772	-93.6227	1
IllinoisA	*M. baccata* 'Illinois'	Leaf	44.86117	-93.6029	1
IllinoisB	*M. baccatta* 'Illinois'	Leaf	44.86117	-93.6029	1
Ioensis	*M. ioensis*	Leaf	44.85827	-93.6231	1
JohnDownie	*M.* 'John Downie'	Leaf	44.85727	-93.6235	1
KerrA	*M. baccataxdomestica x M. domestica* (Dolgo x Haralson)	Leaf	44.87833	-93.6400	1
KerrB	*M. baccataxdomestica x M. domestica* (Dolgo x Haralson)	leaf	44.87833	-93.6400	1
LemoniiA	*M. purpurea 'Lemonei'*	Leaf	44.85797	-93.6223	1
LemoniiB	*M. purpurea* 'Lemonei'	Leaf	44.85797	-93.6223	1
MarthaDolgoA	*M. 'Martha Dolgo'*	Leaf	44.85838	-93.6227	1
MBaccataD	*M. baccata*	Leaf	44.85727	-93.6235	1
PcrabA	*M. ioensis* 'Prairie Rose'	Leaf	44.85825	-93.6231	1
PCrabC	*M. ioensis* 'Prairie Rose'	Leaf	44.85825	-93.6231	1
PcrabC	*M. ioensis* 'Prairie Rose'	Leaf	44.85825	-93.6231	1
Pixie	*M.* 'Pixie'	Leaf	44.85805	-93.6228	1
SnowMagic	*M.* 'Snow Magic'	Leaf	44.85753	-93.6228	1
SoulardiiA	*M. soulardii*	Leaf	44.85828	-93.6235	1
SoulardiiB	*M. soulardii*	Leaf	44.85828	-93.6235	1
SoulardiiC	M. soulardii	Leaf	44.85828	-93.6235	1
Thunder	*M.* 'Thunderchild'	Leaf	44.86003	-93.6223	1
TuresA	*M. 'Tures'*	Leaf	44.85823	-93.6249	1

Table 4-3 continued (2 of 3)

TuresB	*M.* 'Tures'	Leaf	44.85823	-93.6249	1
Turesc	*M.* 'Tures'	Leaf	44.85823	-93.6249	1
Applehouse	unknown	Leaf	44.86843	-93.6358	2
Bball	unknown	Leaf	44.87733	-93.6364	2
HC12A	*M. x domestica* 'Honeycrisp'	fruit	44.86927	-93.6415	2
HC12B	*M. x domestica* 'Honeycrisp'	fruit	44.86927	-93.6415	2
HC12C	*M. x domestica* 'Honeycrisp'	fruit	44.86927	-93.6415	2
HCEarly	*M. x domestica* 'Honeycrisp'	fruit	44.86927	-93.6415	2
HoneyGold	*M. x domestica* 'Honeygold	Leaf	44.86905	-93.6435	2
Mac	*M. x. domestica* 'McIntosh'	Leaf	44.86948	-93.6363	2
Manchurian	*M. baccata mandschurica*	leaf	44.86478	-93.6333	2
MN1914c	*M. x domestica* 'Minneiska'	Leaf	44.86925	-93.6331	2
MN1914D	*M. x domestica* 'Minneiska'	Leaf	44.86925	-93.6331	2
Snowdrift	*M.* 'Snowdrift'	Leaf	44.86478	-93.6333	2
ZestarA	*M. x domestica* 'Minnewashta'	Leaf	44.86068	-93.5981	2
ZestarB	*M. x domestica 'Minnewashta'*	Leaf	44.86068	-93.5981	2
ZestarFruit	*M. x domestica* 'Minnewashta'	fruit	44.86068	-93.5981	2
4060254A	*M. x domestica* 'Honeycrisp' seedling	fruit	44.8668	-93.603	3
4060254B	*M. x domestica* 'Honeycrisp' seedling	fruit	44.86681	-93.6025	3
80wa	unknown	fruit	44.86498	-93.5989	3
80wb	unknown	fruit	44.86498	-93.5989	3
80wc	unknown	fruit	44.86498	-93.5989	3
1331A	unknown	Leaf	44.98127	-93.1856	4
1331B	unknown	Leaf	44.98127	-93.1856	4
CrabD	unknown	Leaf	44.93037	-93.2778	4
Mplscrabf0a	unknown	Leaf	44.93037	-93.2778	4
MplsCrabf0c	unknown	Leaf	44.93037	-93.2778	4
UK2	unknown	Leaf	44.93008	-93.2816	4
UK2_2	unknown	Leaf	44.93008	-93.2816	4
AnimalA	unknown	Leaf	44.98215	-93.1978	5
AnimalB	unknown	Leaf	44.98215	-93.1978	5
BioA	unknown	Leaf	44.99567	-93.1820	5
BioSciB	unknown	Leaf	44.99567	-93.1820	5

Table 4-3 continued (3 of 3)

BioSciC	*unknown*	Leaf	44.99567	-93.1820	5
Cereal	unknown	Leaf	44.98812	-93.1795	5
CowsA	unknown	Leaf	44.98221	-93.1860	5
CowsB	unknown	Leaf	44.98221	-93.1860	5
Cowsc	unknown	Leaf	44.98221	-93.1860	5
FieldNorthA	unknown	Leaf	44.98327	-94.8707	5
FieldNorthB	unknown	Leaf	44.98327	-94.8707	5
FitchA	unknown	Leaf	44.98253	-93.1855	5
FitchB	unknown	Leaf	44.98253	-93.1855	5
FitchC	unknown	Leaf	44.98253	-93.1855	5
ForestryA	unknown	Leaf	44.98633	-93.1933	5
ForestryB	unknown	Leaf	44.98633	-93.1933	5
StPaulcampus	unknown	Leaf	44.98838	-93.1826	5
SugarTymeA	*M.*'Sutyzam' Sugar Tyme® PP7062	Leaf	44.98743	-93.1900	5
SugarTymeB	*M.*'Sutyzam' Sugar Tyme® PP7062	Leaf	44.98743	-93.1900	5
SugarTymeC	*M.*'Sutyzam' Sugar Tyme® PP7062	Leaf	44.98743	-93.1900	5
1731	*M. x domestica*	Leaf	47.24645	-93.5004	6
GR1914A	*M. x domestica* 'Minneiska'	fruit	47.24117	-93.5017	6
GR1914b	*M. x domestica* 'Minneiska'	fruit	47.24117	-93.5017	6

Table 4-4 Microsatellite genetic diversity measures for 6 populations of *V. inaequalis* isolates including number of alleles (N), effective number of alleles (EffNo), haploid genetic diversity (*h*), within population (H_s), total (H_t), and fixation indices (G_{st}, G'_{st} (Nei's), and corrected G''_{st}). Probability (*P)* based on G'_{st}. Mean values for each population are given. The average number of alleles within each population and the grand mean number of alleles among all populations are shown.

			h^z											
Locus	N^y	$EffNo^y$	Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	H_s^y	H_t^y	G_{st}^y	$G'^{y,x}_{st}$	$G''^{y,w}_{st}$	$P^{y,v}$
1tc1a	2	1.34	0.278	0.480	0.480	0.000	0.000	0.000	0.292	0.319	0.084	0.099	0.140	0.115
1tc1b	9	1.55	0.542	0.667	0.000	0.612	0.499	0.500	0.413	0.510	0.191	0.220	0.375	0.014
1aac3b	9	2.09	0.349	0.000	0.000	0.245	0.715	0.000	0.607	0.632	0.040	0.047	0.120	0.184
Vicacg8/42	4	1.71	0.311	0.124	0.480	0.408	0.547	0.444	0.496	0.465	-0.067	-0.082	-0.163	0.863
1aac4b	2	1.03	0.121	0.000	0.000	0.000	0.000	0.000	0.036	0.033	-0.086	-0.105	-0.109	0.801
1aac4f	1	1.00	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	---	---	---
1aac4h	9	1.48	0.393	0.198	0.000	0.278	0.357	0.500	0.408	0.533	0.236	0.270	0.456	0.047
Vigt10/ε	4	1.33	0.386	0.292	0.560	0.500	0.379	0.000	0.320	0.532	0.399	0.443	0.652	0.007
Victc1/2	16	3.66	0.835	0.796	0.625	0.375	0.844	0.667	0.869	0.864	-0.005	-0.006	-0.048	0.500
Vitg9/129	5	1.61	0.654	0.561	0.625	0.375	0.379	0.444	0.492	0.508	0.031	0.037	0.072	0.339
Vict1/130	3	1.12	0.091	0.231	0.000	0.320	0.215	0.000	0.123	0.113	-0.094	-0.115	-0.131	0.801
Vica9/x	4	2.44	0.653	0.631	0.625	0.560	0.581	0.444	0.701	0.715	0.019	0.023	0.078	0.319
Mean	5.7	1.70	0.384	0.332	0.283	0.306	0.376	0.250	0.396	0.435	0.089	0.105	0.175	
Average no .of alleles			24.333	14.083	4.583	6.000	17.083	2.750						
Grand mean			11.472											

[z] Mean haploid genetic diversity calculated as: $h = 1 - \Sigma pi^2$. Where *pi* is the frequency of the *i*th allele. Calculated in GenAlEx 6.5.
[y] Measures of diversity calculated with GenoDive.
[x] Nei's (1987) G-statistic corrected for small populations.
[w] Corrected standardized fixation index.

Table 4-5 Pairwise population Φ_{PT} (Phi_{PT}) and R_{ST} values for two measures of genetic distance calculated in GenAlEx6.5: Haploid above diagonal, Haploid-SSR below, bold = $p < 0.05$.

	Pop1	Pop2	Pop3	Pop4	Pop5	Pop6
Pop1	…	**0.077**	0.051	0.050	**0.065**	**0.167**
Pop2	**0.144**	…	0.067	**0.162**	**0.155**	**0.237**
Pop3	0.000	0.000	…	**0.126**	**0.153**	**0.206**
Pop4	0.000	**0.199**	0.000	…	0.040	**0.200**
Pop5	0.017	**0.065**	0.000	0.000	…	**0.113**
Pop6	0.106	0.004	0.000	0.094	0.009	…

Table 4-6 Analysis of molecular variance (AMOVA) using two measurements of genetic distance (Haploid, Haploid-SSR) calculated in GenAlEx6.5 using 80 unique isolates and 12 microsatellite markers. Results for among and within population variation, degrees of freedom (df), sums of squares (SS), mean squares (MS), genetic variance (Est. Var), and percent variation attributed to different levels of the hierarchy.

Method	Source	df	SS	MS	Est. Var.	% Var.	Φ_{PT}[z]	P[y]
Haploid	Among Pops	5	33.223	6.645	0.308	10%	0.095	0.003
	Within Pops	75	219.271	2.924	2.924	90%		
	Total	80	252.494		3.232	100%		
							(R_{ST})	
Haploid-SSR	Among Pops	5	463201	92640.23	2551.27	4%	0.04	0.108
	Within Pops	75	4636749	61823.33	61823.33	96%		
	Total	80	5099951		64374.60	100%		

[z] Φ_{PT} Phi statistic is analogous to Wright's F-statistics (F_{ST}), R_{ST} is the equivalent in Haploid-SSR
[y] p values are based on 999 permutations

Table 4-7 Nei's pairwise genetic distance matrix for 6 populations of *V. inaequalis* isolates collected in Minnesota and screened with 12 microsatellite markers.

	Pop1	Pop2	Pop3	Pop4	Pop5	Pop6
Pop1	…					
Pop2	0.050	...				
Pop3	0.118	0.127	…			
Pop4	0.181	0.191	0.285	…		
Pop5	0.129	0.181	0.268	0.155	…	
Pop6	0.307	0.370	0.397	0.342	0.213	…

Table 4-8 Results of Mantel tests calculated between three matrices: genetic distance Haploid, genetic distance Haploid-SSR, and geographic distance. Mantel correlation (R^2) and *P* value are shown for each comparison.

Matrices	*P*	R^2
Haploid: Haploid-SSR	0.010	0.29
Geographic:Haploid	0.300	< 0.01
Geographic:Haploid-SSR	0.435	< 0.01

Figures

Figure 4-1 Principal Component Analysis (PCoA) of pairwise *Φ* (*Phi*) values for Haploid (blue triangle) and *Rst* values for Haploid-SSR (red circle) distance matrices plotted using coordinates (axes) 1 and 2 for each pairwise population matrix (Table 4-5).

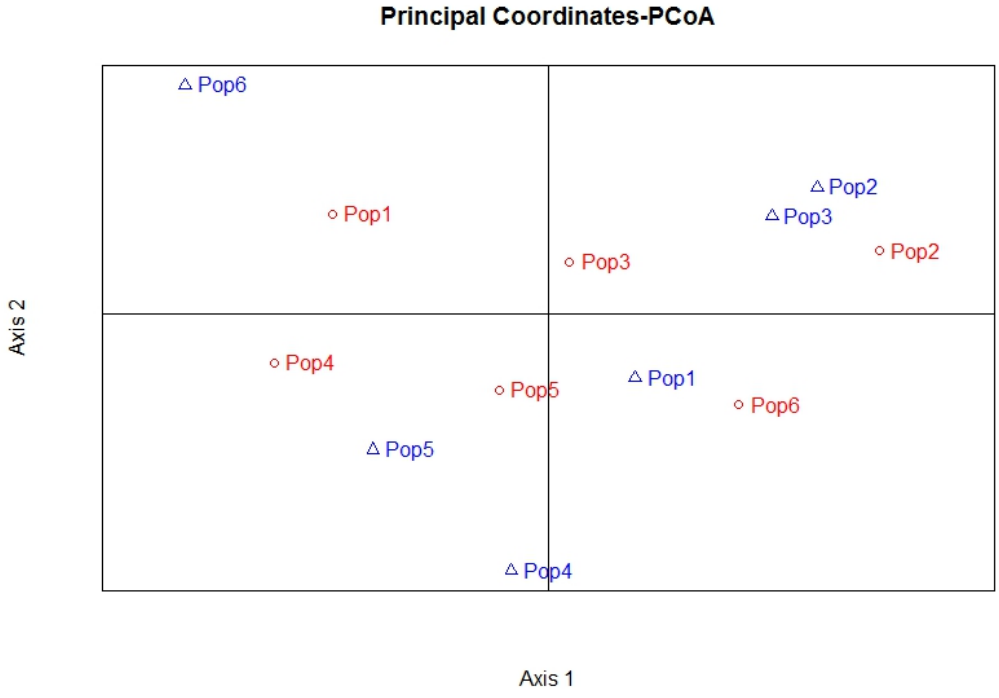


Figure 4-2 Principal Component Analysis of Nei's pairwise distance matrix (Table 4-7) for 6 *V. inaequalis* isolates plotted on coordinates (axes) 1 and 2.

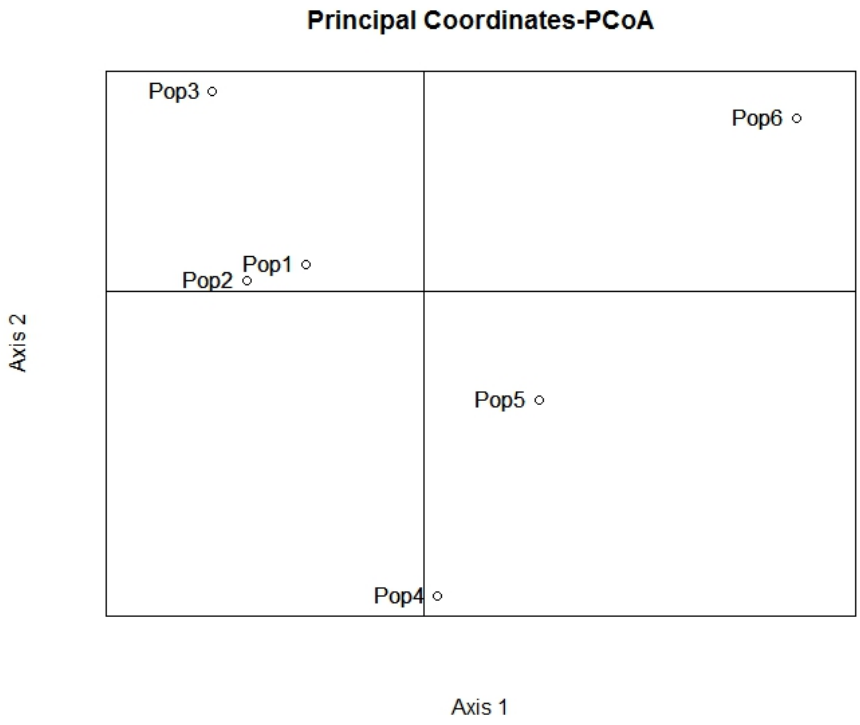
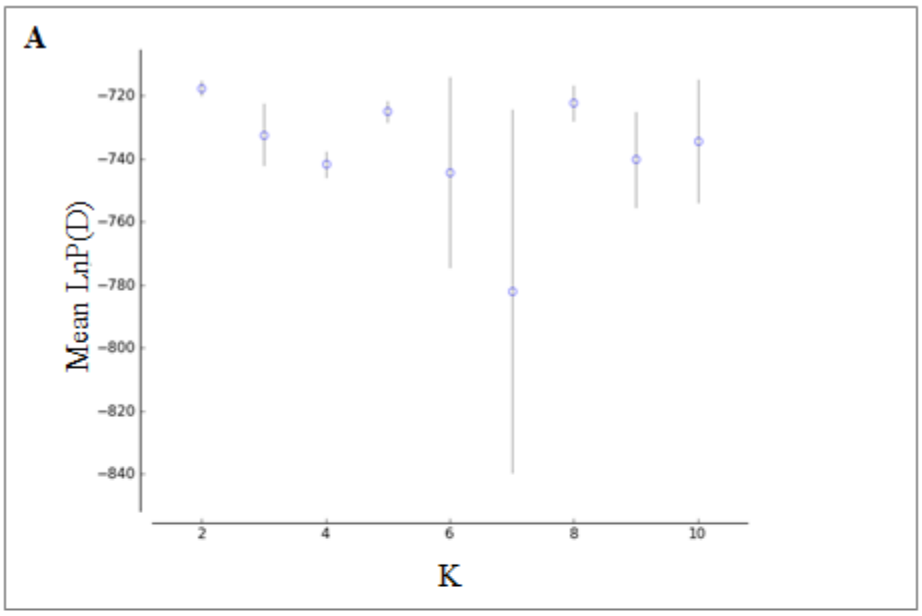


Figure 4-3 Structure Harvester plots for the posterior probability for the most likely value of K. (A) Mean LnP(D) value and standard deviation at each value of K as produced in Structure. (B) ΔK value for each level of K populations.



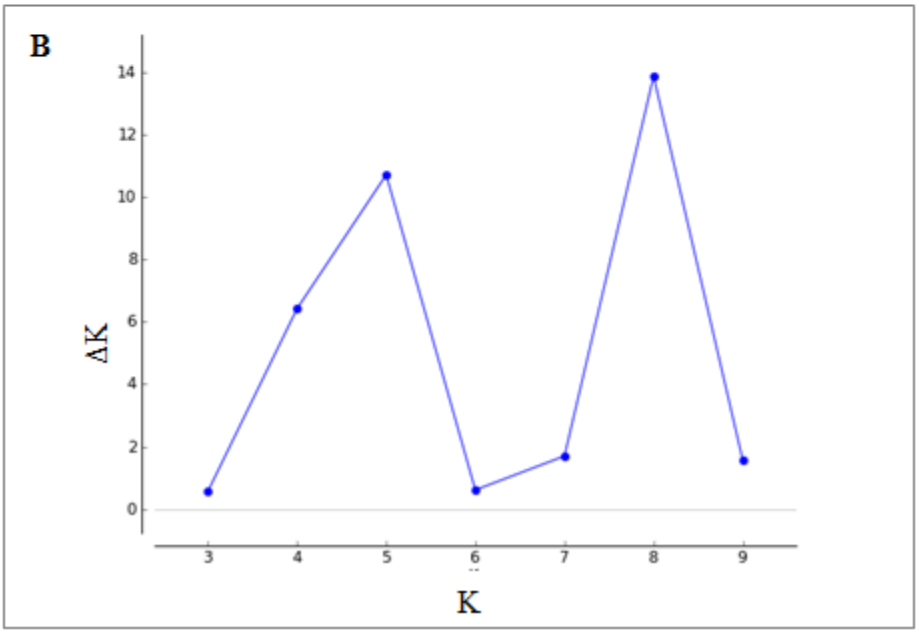
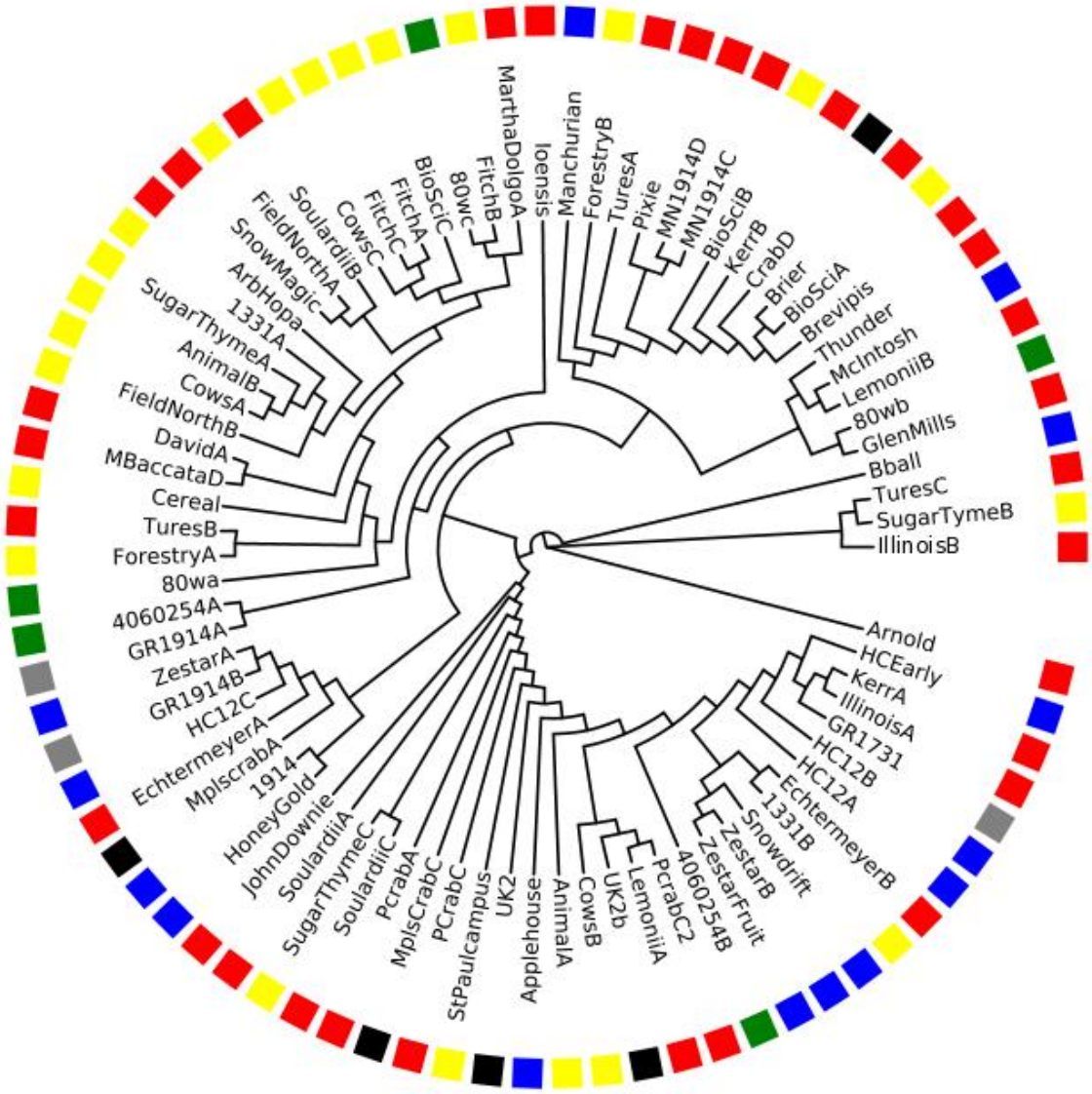


Figure 4-4 Neighbor-joining tree based on the Haploid-SSR genetic distance data matrix. Colors indicate collection location (Population1-red, Population 2-blue, Population 3-green, Population 4-black, Population 5-yellow, Population 6-gray).



Conclusions

The apple cultivar Honeycrisp was released due to its explosively crisp, fresh eating qualities that have resulted in its prominence in breeding programs around the globe. At the time of release, its pedigree was known only from breeding records. Molecular marker fingerprinting has allowed a putative pedigree to be built which represents two generations of one parental line (Figure 3-1; Cabe et al., 2005). A resistant ancestor for 'Honeycrisp' was not expected and the known pedigree provides little support for a known major R gene for apple scab. To date, most major scab resistance genes have been introduced into the cultivated apple from related *Malus* species (MacHardy, 1996). Therefore, 'Honeycrisp' was not evaluated for scab resistance in controlled studies prior to release. Grower reports, breeder field evaluations, and a study of an organic orchard system (Berkett et al., 2008) provided evidence for resistance in 'Honeycrisp' to *V. inaequalis*.

In order to investigate the genetic control of scab resistance in 'Honeycrisp', several experiments were conducted. A high-density, consensus SNP linkage map was developed from three 'Honeycrisp' full-sib families for positioning of the resistance loci as well as fruit texture traits (Schmitz, 2013). To screen the host seedling populations, a collection of *V. inaequalis* isolates was developed and curated from six collection locations in Minnesota. These single spore isolates served as the basis for screening individuals in the 'Honeycrisp' pedigree, including segregating progeny populations, for R gene and QTL mapping.

The consensus 'Honeycrisp' map (Ch. 1) is one of the first reports of a high-density SNP map in the cultivated apple using the IRSC 8K SNP array. The map used for the grouping assignment was developed from a rootstock progeny population (M432; Ananaviciute et al., 2012), which may not adequately reflect the local genomic positions of markers in scion cultivars. QTL mapping in RosBREED using FlexQTL has focused on the physical positions of the markers, which does not account for potential problems in the construction of the genetic map and may not accurately reflect linkage between markers in study populations. The RosBREED approach does not consider problems inherent in the physical map contig alignments or the possibility of paralogous SNPs mapping to multiple genomic locations. The map reported in Chapter 1 identified a number of SNPs (> 10%) which mapped to positions in the linkage map other than their mapped position onto the 'Golden Delicious' pseudo-chromosomes. The 'Honeycrisp' map offers the advantage of having very high quality marker calls due to the stringency applied at several steps in the process. Furthermore, because 'Honeycrisp' is heterozygous at every locus, SNP haplotypes for this cultivar are potentially informative at every SNP in the Excel Haplotyping (pers. communication Cameron Peace). Additionally the consensus map and the contributing parental maps were developed from the populations utilized in the current study.

Greenhouse experiments were conducted to confirm that 'Honeycrisp' was in fact demonstrating resistance to infection by *V. inaequalis*. Macro- and microscopic observation showed that as early as 7 dpi, 'Honeycrisp' and all of its known ancestors exhibited a defense response. This included necrotic/chlorotic lesions (class 2) through

class 3b (lesions with sporulation). The grandparent, 'Northern Spy', showed stellate necrosis. Because mixed isolates were used, susceptible reactions were in some of the reactions. The presence of auto-fluorescence in cleared leaf tissues provided strong evidence for a genetic response to the invasion of a pathogen as part of programmed cell death or HR (Bus et al., 2005b). The results of Chapter 2 support the hypothesis that at least one gene, and possibly two, is present in 'Honeycrisp' through the known pedigree inherited through 'Keepsake'.

To test the hypothesis that at least one resistance gene was present in 'Honeycrisp', seedling populations were developed by crossing 'Honeycrisp' with susceptible parents. In the USA, three populations were inoculated in the greenhouse with single spore isolates. The seedlings exhibited class 0, 2, 3a, 3b, stellate necrosis, and 4 symptoms. The segregation ratios were evaluated with the Chi-square test to determine if 1:1 or 3:1 segregation models were appropriate. Considering intermediate class 3b as resistant resulted in supporting a 3 resistant : 1 susceptible segregation ratio that is expected when two independent genes are involved in resistance. A third population (2009-077) from New Zealand was screened in the Netherlands with four single spore isolates. Segregation ratios supported 1, 2, and 3 resistance gene models, depending on the isolate used (Table 3-2). Population 2009-007 is hypothesized to have a third R gene from its ancestor Russian apple R12740-7A.

A single segregating population (AE1022; n=125) was SNP genotyped with the IRSC 8k array (Chagné et al., 2012) for mapping of the scab resistance loci. Different mapping approaches were utilized due to the binary and quantitative nature of the

greenhouse disease screening. Linkage analysis, using the resistance reaction as a marker, mapped scab resistance on LGs 1 and 15. These same regions were identified with QTL mapping approaches in MapQTL (Van Ooijen, 2004) for interval mapping and Kruskal-Wallis analyses (Figures 3-2 and 3-3). These resistance loci were named *Rvi19* and *Rvi20*. The SNP regions were saturated with SSR markers so that linked markers could be identified for routine screening of progeny and potential parents in marker assisted breeding. Novel SSR were developed from contigs spanning the QTL when necessary. SSRs spanning the QTL were mapped to their physical locations on the 'Golden Delicious' genome sequence at GDR. The SSRs were also utilized for identifying scab resistant advanced selections in the University of Minnesota breeding program that had at least one known scab resistant parent.

Rvi19 is transmitted from 'Frostbite' to 'Keepsake' to 'Honeycrisp', and into the resistant progeny of 'Honeycrisp'. The Ch-Vf1 SSR haplotype for *Rvi19* is identical by state with *Rvi17*, from 'Antonovka'. The Ch-Vf1 allele (138 bp) is shared in common between these loci and will be useful in MAB for distinguishing *Rvi1/Rvi19* resistance from *Rvi6* (158 bp) at this locus. Additional research will be needed to determine if *Rvi17* and *Rvi19* are allelic. *Rvi20* cannot be traced through the known 'Honeycrisp' progeny, and appears to be transmitted through the unknown parent. The resistant SNP haplotype is common in apple germplasm and is shared by 'Golden Delicious' and 'Gala', both susceptible cultivars at this locus.

The physical and genetic positions of the SNP and SSR loci spanning the *Rvi19* and *Rvi20* haplotypes were compared. The predicted genes in these regions were

downloaded from the genome browser available on GDR (Jung et al.2008; Velasco et al., 2010). The peptide sequences were evaluated for those encoding common R gene motifs. From these, a number of candidate genes (Table 3-7 and Table 3-8) were identified at each haplotype that can serve as starting points for identifying the genes conferring functional resistance.

A field experiment was also conducted to map the resistance QTL in four 'Honeycrisp' full-sib families grown at the University of Minnesota Horticultural Research Center in Victoria, MN (Appendix). The resistance locus *Rvi19* was detected in some of the FlexQTL models. Because the inoculum in this study included natural sources, as well as from heavily infected leaves from the University of Minnesota Landscape Arboretum crabapple collection (Chaska, MN), this locus appears to have more broad spectrum resistance. Additional QTL were detected on LG15 and LG17, although *Rvi20* was not specifically detected. The pathogen race structure in the field may limit the detection of *Rvi20*. Other resistance QTL may be population specific and should be further explored.

The collection of 80+ Minnesota *V. inaequalis* isolates was genotyped with 12 SSR markers to explore the genetic diversity of the germplasm assembled. Within population variation accounted for the majority of the variation observed. No consistent population structure was detected, as was no relationship between the geographic collection location and genetic distance matrices. The diversity of the isolates may be an artifact of the obligate sexual cycle that occurs before the start of each infection season in the spring. Furthermore, gene flow between populations is possible due to the movement

of spores naturally (in wind) or the movement of infected plant materials (among and within sites). The collection includes isolates capable of inciting infection on the resistant cultivar 'Honeycrisp'. Additional research should screen the collection against a differential set of apple scab resistant selections for establishing the race of each isolate (Bus et al., 2012; Patocchi et al., 2009). This will allow for the curation of a reduced set of isolates representative of the races present in in Minnesota for screening seedling populations. The current collection represents a diverse set of germplasm that provides a baseline for monitoring the pathogen on the landscape.

Literature Cited

Abramovitch, R.B., J.C. Anderson, and G.B. Martin. 2006. Bacterial elicitation and evasion of plant innate immunity. Nat Rev Mol Cell Biol 7(8): 601–611.

Aderhold, R. 1899. Auf welche weise konnen wir dem immer weitteren umsichgreifen des dem Pilz gegenuber am widerstandsfahigsten gezeigt? Pomol. Monatshefte XLV: 266–272.

Aldwinckle, H.S., H.L. Gustafson, and R.C. Lamb. 1976. Early determination of genotypes for apple scab resistance by forced flowering of test cross progenies. Euphytica 25(1): 185–191.

Aldwinckle, H.S., and M. Malnoy. 2009. Plant regeneration and transformation in the Rosaceae. Transgenic Plant J. 3: 1–39.

Alves, S.A.M., and R.M. Beresford. 2013. Evaluation of three models for predicting *Venturia inaequalis* ascospore release in Southern Brazil. N.Z. Plant Prot. 66(303-307).

Ameson, P.A. 1998. Apple Scab. Available at http://jpkc.jluhp.edu.cn/zwkx/zwbl/improve/ref/ppathw3/Pa2200t0.htm#references (verified 9 Dec. 2013).

Antanaviciute, L., F. Fernández-Fernández, J. Jansen, E. Banchi, K.M. Evans, R. Viola, R. Velasco, J.M. Dunwell, M. Troggio, and D.J. Sargent. 2012. Development of a dense SNP-based linkage map of an apple rootstock progeny using the Malus Infinium whole genome genotyping array. BMC Genomics 13(1): 203.

Aylor, D.E. 1998. The Aerobiology of Apple Scab. Plant Dis. 82(8): 838–849.

Baker, G.A. 1999. Consumer preferences for food safety attributes in fresh apples: Market segments, consumer characteristics, and marketing opportunities. J. Agric. Resour. Econ. 24(1): 80–97.

Baldi, P., A. Patocchi, E. Zini, C. Toller, R. Velasco, and M. Komjanc. 2004. Cloning and linkage mapping of resistance gene homologues in apple. TAG Theor. Appl. Genet. 109(1): 231–239.

Barbara, D.J., A.L. Roberts, and X.-M. Xu. 2008. Virulence characteristics of apple scab (*Venturia inaequalis*) isolates from monoculture and mixed orchards. Plant Pathol. 57(3): 552–561.

Barnes, E.H., and E.B. Williams. 1960. A biochemical response of apple tissues to fungus infection. Phytopathology 50: 844–6.

Beckerman, J., J. Chatfield, and E. Draper. 2009. A 33-year evaluation of resistance and pathogenicity in apple scab--crabapples pathosystem. HortScience 44(3): 599–608.

Belfanti, E., E. Silfverberg-Dilworth, S. Tartarini, A. Patocchi, M. Barbieri, J. Zhu, B.A. Vinatzer, L. Bolland, C. Gessler, and S. Sansavini. 2004. The *HcrVf2* gene from a wild apple confers scab resistance to a transgenic cultivated variety. Proc. Natl. Acad. Sci. USA. 101(3): 886 –890.

Bénaouf, G., and L. Parisi. 2000. Genetics of host-pathogen relationships between *Venturia inaequalis* Races 6 and 7 and *Malus* species. Phytopathology 90(3): 236–242.

Bennett, M., M. Gallagher, J. Fagg, C. Bestwick, T. Paul, M. Beale, J. Mansfield. 1996. The hypersensitive reaction, membrane damange and accumulation of autofluorescent phenolics in lettuce cells challenged by *Bremia lactucae*. Plant J. 9(6): 851-865.

Berkett, L., M. Garcia, R. Moran, H. Darby, R. Parsons, J. Hayden, T. Bradshaw, S. Kingsley-Richards, and M. Cromwell. 2008. The OrganicA Project: Organic disease management in orchards with "newer" cultivars. Int. Organ. Biol. Integr. Control Bull.: 436-440.

Bink, M.C. a. M., M.P. Boer, C.J.F. ter Braak, J. Jansen, R.E. Voorrips, and W.E. van de Weg. 2008. Bayesian analysis of complex traits in pedigreed plant populations. Euphytica 161(1-2): 85–96.

Boehm, E.W.A., S. Freeman, E. Shabi, and T.J. Michailides. 2003. Microsatellite primers indicate the presence of asexual populations of *Venturia inaequalis* in coastal Israeli apple orchards. Phytoparasitica 31(3): 236–251.

Bolar, J.P., J.L. Norelli, K.-W. Wong, C.K. Hayes, G.E. Harman, and H.S. Aldwinckle. 2000. Expression of endochitinase from *Trichoderma harzianum* in transgenic apple increases resistance to apple scab and reduces vigor. Phytopathology 90(1): 72–77.

Boone, D.M. 1971. Genetics of *Venturia inaequalis*. Annu. Rev. Phytopathol. 9(1): 297–318.

Boudichevskaia, A., H. Flachowsky, and F. Dunemann. 2009. Identification and molecular analysis of candidate genes homologous to *HcrVf* genes for scab resistance in apple. Plant Breed. 128(1): 84–91.

Bowen, J.K., C.H. Mesarich, V.G.M. Bus, R.M. Beresford, K.M. Plummer, and M.D. Templeton. 2011. *Venturia inaequalis*: the causal agent of apple scab. Mol. Plant Pathol. 12(2): 105–122.

Broggini, G.A.L., V.G.M. Bus, G. Parravicni, S. Kumar, R. Groenwold, and C. Gessler. 2010. Genetic mapping of 14 avirulence genes in an EU-B04 × 1639 progeny of *Venturia inaequalis*. Fungal Genet. Biol. In Press.

Bus, V.G.M., P.A. Alspach, M.E. Hofstee, and L.R. Brewer. 2002. Genetic variability and preliminary heritability estimates of resistance to scab (*Venturia inaequalis*) in an apple genetics population. N.Z. J. Crop Hortic. Sci. 30(2): 83.

Bus, V.G.M., H.C.M. Bassett, D. Bowatte, D. Chagné, C.A. Ranatunga, D. Ulluwishewa, C. Wiedow, and S.E. Gardiner. 2010. Genome mapping of an apple scab, a powdery mildew and a woolly apple aphid resistance gene from open-pollinated Mildew Immune Selection. Tree Genet. Genomes 6(3): 477–487.

Bus, V.G.M., F.N.D. Laurens, W.E. Van De Weg, R.L. Rusholme, E.H.A. Rikkerink, S.E. Gardiner, H.C.M. Bassett, L.P. Kodde, and K.M. Plummer. 2005a. The *Vh8* locus of a new gene-for-gene interaction between *Venturia inaequalis* and the wild apple *Malus sieversii* is closely linked to the *Vh2* locus in *Malus pumila* R12740-7A. New Phytol. 166(3): 1035–1049.

Bus, V.G.M., E.H.A. Rikkerink, V. Caffier, C.E. Durel, S.E. Gardiner, C. Gessler, R. Groenwold, F. Laurens, B. Le Cam, J. Luby, B. Meule, M. Kellerhals, L. Parisi, A. Patocchi, K.M. Plummer, H.J. Schouten, S. Tartarini, and W.E. van de Weg. 2009. A proposal for the nomenclature of *Venturia inaequalis* races. Acta Hort 814: 739–746.

Bus, V.G.M., E.H.A. Rikkerink, W.E. van de Weg, R.L. Rusholme, S.E. Gardiner, H.C.M. Bassett, L.P. Kodde, L. Parisi, F.N.D. Laurens, E.J. Meulenbroek, and K.M. Plummer. 2005b. The *Vh2* and *Vh4* scab resistance genes in two differential hosts derived from Russian apple R12740-7A map to the same linkage group of apple. Mol. Breeding 15(1): 103-116.

Bus, V.G.M., E.H.A. Rikkerink, V. Caffier, C.-E. Durel, and K.M. Plummer. 2011. Revision of the nomenclature of the differential host-pathogen interactions of *Venturia inaequalis* and *Malus*. Annu. Rev. Phytopathol. 49(1): 391–413.

Bus, V.G.M., W.E. van de Weg, A. Peil, F. Dunemann, E. Zini, F.N.D. Laurens, J. Blažek, V. Hanke, and P.L. Forsline. 2012. The role of Schmidt 'Antonovka' in apple scab resistance breeding. Tree Genet. Genomes 8(4): 627–642.

Cabe, P.R., A. Baumgarten, K. Onan, J.J. Luby, and D.S. Bedford. 2005. Using microsatellite analysis to verify breeding records: A study of `Honeycrisp' and other cold-hardy apple cultivars. HortScience 40(1): 15 –17.

Calenge, F., A. Faure, M. Goerre, C. Gebhardt, W.E. Van de Weg, L. Parisi, and C.-E. Durel. 2004. Quantitative trait loci (QTL) analysis reveals both broad-spectrum and isolate-specific QTL for scab resistance in an apple progeny challenged with eight isolates of *Venturia inaequalis*. Phytopathology 94(4): 370–379.

Celton, J.-M., D.S. Tustin, D. Chagné, and S.E. Gardiner. 2008. Construction of a dense genetic linkage map for apple rootstocks using SSRs developed from *Malus* ESTs and *Pyrus* genomic sequences. Tree Genet. Genomes 5(1): 93–107.

Chagné, D., R.N. Crowhurst, M. Troggio, M.W. Davey, B. Gilmore, C. Lawley, S. Vanderzande, R.P. Hellens, S. Kumar, A. Cestaro, R. Velasco, D. Main, J.D. Rees, A. Iezzoni, T. Mockler, L. Wilhelm, E. Van de Weg, S.E. Gardiner, N. Bassil, and C. Peace. 2012. Genome-wide SNP detection, validation, and development of an 8K SNP array for apple. PLoS ONE 7(2): e31745.

Cheng, F.S., N.F. Weeden, S.K. Brown, H.S. Aldwinckle, S.E. Gardiner, and V.G.M. Bus. 1998. Development of a DNA marker for *Vm*, a gene conferring resistance to apple scab. Genome 41(2): 208–214.

Chevalier, M., Y. Lespinasse, and S. Renaudin. 1991. A microscopic study of the different classes of symptoms coded by the *Vf* gene in apple for resistance to scab (*Venturia inaequalis*). Plant Pathol. 40(2): 249–256.

Clark, M.D., V.G.M. Bus, J.J. Luby, and J.M. Bradeen. submitted. Characterization of the defense response to *Venturia inaequalis* in 'Honeycrisp' apple, its ancestors, and progeny. Eur. J. Plant Pathol.

Clark, M.D., C.A. Schmitz, U.R. Rosyara, J.J. Luby, and J.M. Bradeen. Accepted with revisions. A consensus 'Honeycrisp' apple (*Malus* × *domestica*) genetic linkage map from three full-sib progeny populations. Tree Genet. Genomes.

Clark, M.D., Schmitz, C. A., U.R. Rosyara, J.J. Luby, and J.M. Bradeen. 2013. A high resolution SNP linkage map of a 'Honeycrisp' apple (*Malus* x *domestica*) progeny. Plant and Animal Genome Conf. XXI. San Diego, CA. 14 January 2013. https://pag.confex.com/pag/xxi/webprogram/Paper7625.html (Accessed 31 Dec. 2013).

Cmelik, Z., P. Zadravec, S. Tojnko, B. Schlauer, A. Vogrin, and T. Unuk. 2007. Sensory evaluation of fruit of some organically grown scab-resistant apple cultivars. Acta Hortic. 737: 53–58.

Costa, F., W.E. Weg, S. Stella, L. Dondini, D. Pratesi, S. Musacchi, and S. Sansavini. 2008. Map position and functional allelic diversity of *Md-Exp7*, a new putative expansin gene associated with fruit softening in apple (*Malus* × *domestica* Borkh.) and pear (*Pyrus communis*). Tree Genet. Genomes 4(3): 575–586.

Crandall, C.S. 1926. Apple breeding at the University of Illinois. University of Illinois Agricultural Experiment Station, Urbana, Ill.

Crosby, J.A., J. Janick, P.C. Pecknold, S.S. Korban, P.A. O'Connor, S.M. Ries, J. Goffreda, and A. Voordeckers. 1992. Breeding apples for scab resistance: 1945-1990. Acta Hortic. 317: 43–55.

Croxall, H.E., D.C. Gwynne, and J.E.E. Jenkins. 1952a. The rapid assessment of apple scab on fruit. Plant Pathol. 1: 89–92.

Croxall, H.E., D.C. Gwynne, and J.E.E. Jenkins. 1952b. The rapid assessment of apple scab on leaves. Plant Pathol. 1: 39–41.

Dai, G.H., M. Nicole, C. Andary, C. Martinez, E. Bresson, B. Boher, J.F. Daniel, and J.P. Geiger. 1996. Flavonoids accumulate in cell walls, middle lamellae and callose-rich papillae during an incompatible interaction between *Xanthomonas campestris* pv. *malvacearum* and cotton. Physiol. Mol. Plant Pathol. 49(5): 285–306.

Dangl, J.L., and J.D.G. Jones. 2001. Plant pathogens and integrated defence responses to infection. Nature 411(6839): 826–833.

Davidson, R.S. 1996. Application of fluorescence microscopy to a study of chemical problems. Chem. Soc. Rev. 25(4): 241–253.

Dayton, D.F., and E.B. Williams. 1968. Independent genes in *Malus* for resistance to *Venturia inaequalis*. Proc. Am. Soc. Hort. Sci. 92: 89–94.

Dunemann, F., and J. Egerer. 2010. A major resistance gene from Russian apple 'Antonovka' conferring field immunity against apple scab is closely linked to the *Vf* locus. Tree Genet. Genomes 6(5): 627–633.

Durel, C.E., F. Calenge, L. Parisi, W.E. Weg, L.P. Kodde, R. Crassweller, C. Gessler, M. Thiermann, F. Dunemann, F. Gennari, S. Tartarini, and Y. Lespinasse. 2004. An overview of the position and robustness of scab resistance QTLs and major genes by aligning of genetic maps in five apple progenies. Acta Hortic. 663: 135–140.

Earl, D.A., and B.M. von Holdt. 2011. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv. Genet. Resour. 4(2): 359–361.

Edge-Garza, D.A, T.V. Rowland, Jr., S. Haendiges, and C. Peace. A high-throughput and cost-efficient DNA extraction protocol for the tree fruit crops apple, sweet cherry, and peach relying on silica beads during tissue sampling. submitted to Molecular Breeding (12/31/2013).

Ellis, J., P. Dodds, and T. Pryor. 2000. Structure, function and evolution of plant disease resistance genes. Curr. Opin. Plant Biol. 3(4): 278–284.

Ellis, M.A., D.C. Ferree, R.C. Funt, and L.V. Madden. 1998. Effects of an apple scab-resistant cultivar on use pattern of inorganic and organic fungicides and economics of disease control. Plant Dis. 82(2): 428–433.

Ellis, J.G., G.J. Lawrence, J.E. Luck, and P.N. Dodds. 1999. Identification of regions in alleles of the flax rust resistance gene *L* that determine differences in gene-for-gene specificity. Plant Cell Online 11(3): 495 –506.

Erdin, N., S. Tartarini, G.A.L. Broggini, F. Gennari, S. Sansavini, C. Gessler, and A. Patocchi. 2006. Mapping of the apple scab-resistance gene *Vb*. Genome 49(10): 1238–1245.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol. 14(8): 2611–2620.

Evans, K., Y. Guan, J. Luby, M. Clark, C. Schmitz, S. Brown, B. Orcheski, C. Peace, E. Van De Weg, and A. Iezzoni. 2012. Large-scale standardized phenotyping of apple in RosBREED. Acta Hortic. 945: 233–238.

Faircloth, B.C. 2008. MSATCOMMANDER: detection of microsatellite repeat arrays and automated, locus-specific primer design. Mol. Ecol. Resour. 8(1): 92–94.

FAOSTAT. 2013. FAOSTAT. Available at faostat3.fao.org (verified 1 October 2013).

Fernández-Fernández, F., L. Antanaviciute, M.M. van Dyk, K.R. Tobutt, K.M. Evans, D.J.G. Rees, J.M. Dunwell, and D.J. Sargent. 2012. A genetic linkage map of an apple rootstock progeny anchored to the *Malus* genome sequence. Tree Genet. Genomes 8(5): 991–1002.

Fischer, M., C. Fischer, and W. Dierend. 2005. Evaluation of the stability of scab resistance in apple: a co-operation between gene bank curator, breeder and fruit grower. Plant Genet. Resour. Newsl. 142: 36–42.

Flor, H.H. 1956. The complementary genic system in flax and flax rust. Adv. Genet. 8: 29–54.

Galli, P., G.A.L. Broggini, C. Gessler, and A. Patocchi. 2010. Phenotypic Characterization of the *Rvi15* (*Vr2*) apple scab resistance. J. Plant Pathol. 92(1): 219–226.

Gardiner, S.E., H.C.M. Bassett, D.A.M. Noiton, V.G.M. Bus, M.E. Hofstee, A.G. White, R.D. Ball, R.L.S. Forster, and E.H.A. Rikkerink. 1996. A detailed linkage map around an apple scab resistance gene demonstrates that two disease resistance classes both carry the *Vf* gene. Theor. Appl. Genet. 93(4): 485–493.

Gasic, K., Y. Han, S. Kertbundit, V. Shulaev, A.F. Iezzoni, E.W. Stover, R.L. Bell, M.E. Wisniewski, and S.S. Korban. 2009. Characteristics and transferability of new apple EST-derived SSRs to other Rosaceae species. Mol. Breed. 23(3): 397–411.

Gau, A.E., M. Koutb, M. Piotrowski, and K. Kloppstech. 2004. Accumulation of pathogenesis-related proteins in the apoplast of a susceptible cultivar of apple (*Malus domestica* cv. Elstar) after infection by *Venturia inaequalis* and constitutive expression of PR genes in the resistant cultivar Remo. Eur. J. Plant Pathol. 110(7): 703–711.

Gessler, C., A. Patocchi, S. Sansavini, S. Tartarini, and L. Gianfranceschi. 2006. *Venturia inaequalis* resistance in apple. Crit. Rev. Plant Sci. 25(6): 473–503.

Gessler, C., and D. Stumm. 1984. Infection and stroma formation by *Venturia inaequalis* on apple leaves with different degrees of susceptibility to scab. J. Phytopathol. 110(2): 119–126.

Gianfranceschi, L., B. Koller, N. Seglias, M. Kellerhals, and C. Gessler. 1996. Molecular selection in apple for resistance to scab caused by *Venturia inaequalis*. Theor. Appl. Genet. 93(1): 199–204.

Gilmore, B., K. Hummer, and N. Bassil. 2011. DNA extraction protocols from dormant buds of twelve woody plant genera. J. Amer. Pom. Soc. 65(4): 201–207.

Gladieux, P., X.G. Zhang, I. Róldan-Ruiz, V. Caffier, T. Leroy, M. Devaux, S. van Glabeke, E. coart, B. Le Cam. Evolution of the population structure of *Venturia inaequalis*, the apple scab fungus, associated with the domestication of its host. Mol. Ecol. 19(4): 658-674.

Gladieux, P., F. Guérin, T. Giraud, V. Caffier, C. Lemaire, L. Parisi, F. Didelot, and B. Le Cam. 2011. Emergence of novel fungal pathogens by ecological speciation: importance of the reduced viability of immigrants. Mol. Ecol. 20(21): 4521–4532.

Glazebrook, J. 2001. Genes controlling expression of defense responses in Arabidopsis — 2001 status. Curr. Opin. Plant Biol. 4(4): 301–308.

Glazebrook, J., W. Chen, B. Estes, H.-S. Chang, C. Nawrath, J.-P. Métraux, T. Zhu, and F. Katagiri. 2003. Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. Plant J. 34(2): 217–228.

Goh, T.K. 1999. Single-spore isolation using a hand-made glass needle. Fungal Divers. 2: 47–63.

Goland, C., and S. Bauer. 2007. When the apple falls close to the tree: Local food systems and the preservation of diversity. Renew. Agric. Food Syst. 19(04): 228–236.

Goodman, R.N., and A.J. Novacky. 1994. The hypersensative reaction in plants to pathogens: A resistance phenomenon. APS Press, St. Paul, MN.

Grattapaglia, D., and R. Sederoff. 1994. Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross: mapping strategy and RAPD markers. Genetics 137(4): 1121–1137.

Guan, Y. 2013. QTL identification and DNA marker evelopment for individual sugars in apple. PhD Dissertation Washington State University, Pullman, WA.

Guérin, F., and B. Le Cam. 2004. Breakdown of the scab resistance gene *Vf* in apple leads to a founder effect in populations of the fungal pathogen *Venturia inaequalis*. Phytopathology 94(4): 364–369.

Guérin, F., P. Franck, A. Loiseau, M. Devaux, and B. Le Cam. 2004. Isolation of 21 new polymorphic microsatellite loci in the phytopathogenic fungus *Venturia inaequalis*. Mol. Ecol. Notes 4(2): 268–270.

Guérin, F., P. Gladieux, and B.L. Cam. 2007. Origin and colonization history of newly virulent strains of the phytopathogenic fungus *Venturia inaequalis*. Fungal Genet. Biol. 44(4): 284–292.

Guilford, P., S. Prakash, J.M. Zhu, E. Rikkerink, S. Gardiner, H. Bassett, and R. Forster. 1997. Microsatellites in *Malus* × *domestica* (apple): abundance, polymorphism and cultivar identification. Theor. Appl. Genet. 94(2): 249–254.

Gusberti, M., A. Patocchi, C. Gessler, and G.A.L. Broggini. 2012. Quantification of *Venturia inaequalis* growth in *Malus* × *domestica* with quantitative real-time polymerase chain reaction. Plant Dis. 96(12): 1791–1797.

Gygax, M., L. Gianfranceschi, R. Liebhard, M. Kellerhals, C. Gessler, and A. Patocchi. 2004. Molecular markers linked to the apple scab resistance gene *Vbj* derived from *Malus baccata jackii*. Theor. Appl. Genet. 109(8): 1702–1709.

Hammond-Kosack, K.E., and J.D.G. Jones. 1997. Plant disease resistance genes. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48(1): 575–607.

Hancock, J.F., J.J. Luby, S.K. Brown, and G.A. Lobos. 2008. Apples. p. 1–38. *In* Temperate Fruit Crop Breeding. J.F.Hancock (ed.) Springer Netherlands.

Harker, F.R., F.A. Gunson, and S.R. Jaeger. 2003. The case for fruit quality: an interpretive review of consumer attitudes, and preferences for apples. Postharvest Biol. Technol. 28(3): 333–347.

Heath, M.C. 1998. Involvement of reactive oxygen species in the response of resistant (hypersensitive) or susceptible cowpeas to the cowpea rust fungus. New Phytol. 138(2): 251–263.

Heath, M.C. 2000. Hypersensitive response-related death. Plant Mol. Biol. 44(3): 321–334.

Heath, M.C., Z.L. Nimichuk, and H. Xu. 1997. Plant nuclear migrations as indicators of critical interactions between resistant or susceptible cowpea epidermal cells and invasion hyphae of the cowpea rust fungus. New Phytol. 135(4): 689–700.

Hemmat, M., S.K. Brown, N.F. Weeden, H.S. Aldwinckle, and S.A. Mehlenbacher. 2003. Identification and mapping of markers for resistance to apple scab from 'Antonovka' and 'Hansen"s baccata# 2'. Acta Hortic. 622: 153–162.

Ho, W.C., and W.H. Ko. 1997. A simple method for obtaining single-spore isolates of fungi. Bot. Bull. Acad. Sinica. 38.

Holb, I.J., B. Heijne, J.C.M. Withagen, and M.J. Jeger. 2004. Dispersal of *Venturia inaequalis* ascospores and disease gradients from a defined inoculum source. J. Phytopathol. 152(11-12): 639–646.

Holzapfel, C., B. Meisel, F. Thümmler, C. Leser, and D. Treutter. 2012. Differential gene expression in leaves of a scab susceptible and a resistant apple cultivar upon *Venturia inaequalis* inoculation. Trees 26(1): 121–129.

Hoover, E., D. Bedford, and D. Foulk. 2000. Apples for Minnesota and their culinary uses. University of Minnesota, St Paul, MN.

Hulbert, S.H., C.A. Webb, S.M. Smith, and Q. Sun. 2001. Resistance gene complexes: Evolution and utilization. Annu. Rev. Phytopathol. 39: 285–312.

Hyten, D.L., J.R. Smith, R.D. Frederick, M.L. Tucker, Q. Song, and P.B. Cregan. 2009. Bulked segregant analysis using the GoldenGate Assay to locate the locus that confers resistance to soybean rust in soybean. Crop Sci. 49(1): 265.

Ignatov, A., and A. Bodishevskaya. 2011. Malus. p. 45–64. *In* Wild crop relatives: Genomic and breeding resources. Kole, C. (ed.),Springer Berlin, Heidelberg.

Illumina (2006) Illumina SNP genoptype: Infinium II assay workflow. Illumina, Inc., Towne Centre Drive, San Diego, CA. http://www.illumina.com/Documents/products/workflows/workflow_infinium_ii.pdf Accessed 10 April 2013.

Illumina Inc. 2010a. GenomeStudio Genotyping module v1.0, User Guide. Illumina, Inc. Towne Centre Drive, San Diego, CA.

Illumina Inc. 2010b. InfiniumH, genotyping data analysis: A guide for analyzing InfiniumH genotyping data using the IlluminaH GenomeStudio Genotyping Module. Illumina Inc., Towne Centre Drive, San Diego, CA.

Illumina Inc. 2010c. Improved cluster generation with Gentrain2, Tech Note. Illumina Inc., Town Centre Drive, San Diego, CA.

Janick, J. 2006. The PRI apple breeding program. HortScience 41(1): 8–10.

Janick, J., J.N. Cummins, S.K. Brown, and M. Hemmat. 1999. Fruit Breeding, Vol. I. Tree and Tropical Fruits. Wiley, New York.

Jeun, Y.-C., and K.-H. Lee. 2005. Observations of infection structures after inoculation with *Colletotrichum orbiculareon* the leaves of cucumber plants pre-inoculated with two bacterial strains *Pseudomonas putida* or *Micrococcus luteus*. Mycobiology 33(3): 131.

Jha, G., K. Thakur, and P. Thakur. 2009. The *Venturia* apple pathosystem: pathogenicity mechanisms and plant defense responses. J. Biomed. Biotechnol. 10 pp. doi:10.1155/2009/680160

Joshi, S.G., J.M. Soriano, A. Kortstee, J.G. Schaart, F.A. Krens, E. Jacobsen, and H.J. Schouten. 2008. Development of cisgenic apples with durable resistance to apple scab. Acta Hortic. 839:403–406.

Jung, S., T. Lee, A. Blenda, R. Svancara, A. Abbott, and D. Main. 2008. GDR (Genome Database for Rosaceae): integrated web-database for Rosaceae genomics and genetics data. Nucleic Acids Res. 36 (D1034-D1040).

Kellerhals, M., and B. Furrer. 1994. Approaches for breeding apples with durable disease resistance. Euphytica 77(1-2): 31–35.

Kelley, K., J. Hyde, J. Travis, and R. Crassweller. 2010. Accessing consumer preferences of scab-resistant apples: A sensory evaluation. HortTechnology 20(5): 885–891.

Khan, M.A., Y. Han, Y.F. Zhao, M. Troggio, and S.S. Korban. 2012. A multi-population consensus genetic map reveals inconsistent marker order among maps likely attributed to structural variations in the apple genome. PLoS ONE 7(11): e47864.

King, G.J., F.H. Alston, L.M. Brown, E. Chevreau, K.M. Evans, F. Dunemann, J. Janse, F. Laurens, J.R. Lynn, C. Maliepaard, and others. 1998. Multiple field and glasshouse assessments increase the reliability of linkage mapping of the *Vf* source of scab resistance in apple. Theor. Appl. Genet. 96(5): 699–708.

King, G.J., C. Maliepaard, J.R. Lynn, F.H. Alston, C.E. Durel, K.M. Evans, B. Griffon, F. Laurens, A.G. Manganaris, E. Schrevens, S. Tartarini, and J. Verhaegh. 2000. Quantitative genetic analysis and comparison of physical and sensory descriptors relating to fruit flesh firmness in apple (*Malus pumila* Mill.). Theor. Appl. Genet. 100(7): 1074–1084.

King, G.J., S. Tartarini, L. Brown, F. Gennari, and S. Sansavini. 1999. Introgression of the *Vf* source of scab resistance and distribution of linked marker alleles within the *Malus* gene pool. Theor. Appl. Genet. 99(6): 1039–1046.

Kollar, A. 1998. Characterization of an endopolygalacturonase produced by the apple scab fungus, *Venturia inaequalis*. Mycol. Res. 102(03): 313–319.

Komjanc, M., S. Festi, L. Rizzotti, L. Cattivelli, F. Cervone, and G. De Lorenzo. 1999. A leucine-rich repeat receptor-like protein kinase (LRPKm1) gene is induced in *Malus* × *domestica* by *Venturia inaequalis* infection and salicylic acid treatment. Plant Mol. Biol. 40(6): 945–957.

Kuhn, B.F., and A.K. Thybo. 2001. Senory quality of scab-resistant apple cultivars. Postharvest Biol. Technol. 23: 41–50.

Lamb, R.C., and J.M. Hamilton. 1969. Environmental and genetic factors influencing the expression of resistance to scab (*Venturia inaequalis* Cke. Wint.) in apple progenies. J. Amer. Soc. Hort. Sci. 94: 554–557.

Lateur, M., and C. Populer. 1994. Screening fruit tree genetic resources in Belgium for disease resistance and other desirable characters. Euphytica 77(1): 147–153.

Lawson, D.M., M. Hemmat, and N.F. Weeden. 1995. The use of molecular markers to analyze the inheritance of morphological and developmental traits in apple. J. Am. Soc. Hort. Sci. 120(30): 532–537.

Lefrancq, B., M. Lateur, and A. Rondia. 2003. Screening method for polygenic scab resistance within an apple breeding programme: Relationship between early greenhouse screening test on young seedlings and their scab susceptibility in natural field conditions. Acta Hortic. 663: 793–798.

Leroy, T., C. Lemaire, F. Dunemann, and B.L. Cam. 2013. The genetic structure of a *Venturia inaequalis* population in a heterogeneous host population composed of different *Malus* species. BMC Evol. Biol. 13(1): 64.

Lespinasse, Y., C. Pinet, F. Laurens, C.E. Durel, and L. Parisi. 2002. European Research for Durable Resistance to scab on apple: the DARE Project. Acata Hortic. 595: 17–22.

Lê Van, A., C.E. Durel, B. Le Cam, and V. Caffier. 2011. The threat of wild habitat to scab resistant apple cultivars. Plant Pathol. 60(4): 621–630.

Liebhard, R., M. Kellerhals, W. Pfammatter, M. Jertmini, and C. Gessler. 2003a. Mapping quantitative physiological traits in apple (*Malus* x *domestica* Borkh.). Plant Mol. Biol. 52(3): 511–526.

Liebhard, R., B. Koller, L. Gianfranceschi, and C. Gessler. 2003b. Creating a saturated reference map for the apple (*Malus* × *domestica* Borkh.) genome. Theor. Appl. Genet. 106(8): 1497–1508.

Liebhard, R., B. Koller, A. Patocchi, M. Kellerhals, W. Pfammatter, M. Jermini, and C. Gessler. 2003c. Mapping quantitative field resistance against apple scab in a 'Fiesta' × 'Discovery' progeny. Phytopathology 93(4): 493–501.

Luby, J.J., P.A. Alspach, V.G.M. Bus, and N.C. Oraguzie. 2002. Field resistance to fire blight in a diverse apple (*Malus* sp.) germplasm collection. J. Am. Soc. Hort. Sci. 127: 245–253.

Luby, J.J., and D. Bedford. 1992. Honeycrisp apple. University of Minnesota, St. Paul, MN.

Luby, J., P. Forsline, H.S. Aldwinckle, V.G.M. Bus, and M. Geibel. 2001. Silk road apples--collection, evaluation, and utilization of *Malus sieversii* from Central Asia. Hortscience 36: 225–231.

Luby, J.J., and D.V. Shaw. 2001. Does marker-assisted selection make dollars and sense in a fruit breeding program? HortScience 35: 872–879.

MacHardy, W.E. 1996. Apple Scab: Biology, epidemiology, and management. APS Press, St. Paul.

Maliepaard, C., F.H. Alston, G. Van Arkel, L.M. Brown, E. Chevreau, F. Dunemann, K.M. Evans, S.E. Gardiner, P. Guilford, A.W. Van Heusden, and others. 1998. Aligning male and female linkage maps of apple (*Malus pumila* Mill.) using multi-allelic markers. Theor. Appl. Genet. 97(1): 60–73.

Maliepaard, C., J. Jansen, and J.W. Van Ooijen. 1997. Linkage analysis in a full-sib family of an outbreeding plant species: Overview and consequences for applications. Genet. Res. 70(03): 237–250.

Malnoy, M., M. Xu, E. Borejsza-Wysocka, S.S. Korban, and H.S. Aldwinckle. 2008. Two receptor-like genes *Vfa1* and *Vfa2*, confer resistance to the fungal pathogen *Venturia inaequalis* inciting apple scab disease. Mol. Plant. Microbe Interact. 21: 448–458.

Martinez-Bilbao, A., and J. Murillo. 2005. Six races of *Venturia inaequalis* are found causing apple scab in Spain. Plant Dis. 89(8): 908.

McDowell, J.M., and S.A. Simon. 2006. Recent insights into *R* gene evolution. Mol. Plant Pathol. 7(5): 437–448.

McKay, S.J., J.M. Bradeen, and J.J. Luby. 2011. Prediction of genotypic values for apple fruit texture traits in a breeding population derived from 'Honeycrisp'. J. Am. Soc. Hortic. Sci. 136(6): 408–414.

Meirmans, P.G. 2012. AMOVA-based clustering of population genetic data. J. Hered. 103(5): 744–750.

Meirmans, P.G., and P.H. Van Tienderen. 2004. Genotype and genodive: two programs for the analysis of genetic diversity of asexual organisms. Mol. Ecol. Notes 4(4): 792–794.

Melounova, M., P. Vejl, P. Sedlak, A. Reznerova, M. Tesarova, J. Blazek, and J. Zoufala. 2004. The variability of *Venturia inaequalis* Cke. races in the Czech Republic and the accumulation of resistance genes in apple germplasm. Plant Soil Environ. 50(9): 416–423.

Meyers, B.C., A. Kozik, A. Griego, H. Kuang, and R.W. Michelmore. 2003. Genome-wide analysis of NBS-LRR-encoding genes in Arabidopsis. Plant Cell 15(4): 809–834.

Micheletti, D., M. Troggio, A. Zharkikh, F. Costa, M. Malnoy, R. Velasco, and S. Salvi. 2011. Genetic diversity of the genus *Malus* and implications for linkage mapping with SNPs. Tree Genet. Genomes 7(4): 857–868.

Michelmore, R. 1995. Molecular approaches to manipulation of disease resistance genes. Annu. Rev. Phytopathol. 33(1): 393–427.

Michelmore, R.W., and B.C. Meyers. 1998. Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. Genome Res. 8(11): 1113 – 1130.

Michelmore, R.W., I. Paran, and R.V. Kesseli. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. U.S.A. 88(21): 9828.

Mills, W.D. 1944. Efficient use of sulfur dusts and sprays during rain to control apple scab. Cornell Ext. Bull. 630: 4.

Nei, M. 1972. Genetic distance between populations. Am. Nat. 106(949): 283–292.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89(3): 583–590.

Nicholson, R.L. 1977. Host-pathogen interactions preceding the hypersensitive reaction of *Malus* sp. to *Venturia inaequalis*. Phytopathology 77(1): 108.

Nicholson, R.L., and R. Hammerschmidt. 1992. Phenolic compounds and their role in disease resistance. Annu. Rev. Phytopathol. 30(1): 369–389.

Nicholson, R.L., S. Van Scoyoc, and E.B. Williams. 1973. Response of detached apple leaves to *Venturia inaequalis*. Phytopathology 63: 649–650.

Nusbaum, C.J., and G.W. Keitt. 1938. A cytological study of host-parasite relations of *Venturia inaequalis* on apple leaves. J. Agric. Res. 56: 595–618.

Ognjanov, V., K. Gasic, and D. Vujanic-Varga. 1999. Mildew and scab resistance of apple cultivars, selections and progenies. Acta Hortic. 484: 455–462.

Padder, B.A., M.D. Shah, M. Ahmad, T.A. Sofi, F.A. Ahanger, and A. Hamid. 2011. Genetic differentiation among populations of *Venturia inaequalis* in Kashmir: A north-western state of India. Asian J. Plant Pathol. 5: 75–83.

Paris, R., V. Cova, G. Pagliarani, S. Tartarini, M. Komjanc, and S. Sansavini. 2009. Expression profiling in *HcrVf2*-transformed apple plants in response to *Venturia inaequalis*. Tree Genet. Genomes 5(1): 81–91.

Parisi, L., Y. Lespinasse, J. Guillaumes, and J. Krüger. 1993. A new race of *Venturia inaequalis* virulent to apples with resistance due to the *Vf* gene. Phytopathology 83(5): 533–537.

Parker, D.M., U.W. Hilber, M. Bodmer, F.D. Smith, C. Yao, and W. K\öller. 1995. Production and transformation of conidia of *Venturia inaequalis*. Phytopathology 85(1): 87–91.

Patocchi, A., A. Frei, J.E. Frey, and M. Kellerhals. 2009. Towards improvement of marker assisted selection of apple scab resistant cultivars: *Venturia inaequalis* virulence surveys and standardization of molecular marker alleles associated with resistance genes. Mol. Breed. 24: 337–347.

Patocchi, A., L. Gianfranceschi, and C. Gessler. 1999. Towards the map-based cloning of *Vf*: fine and physical mapping of the *Vf* region. Theor. Appl. Genet. 99(6): 1012–1017.

Patocchi, A., M. Walser, S. Tartarini, G.A.L. Broggini, F. Gennari, S. Sansavini, and C. Gessler. 2005. Identification by genome scanning approach (GSA) of a microsatellite tightly associated with the apple scab resistance gene *Vm*. Genome 48(4): 630–636.

Peakall, R., and P.E. Smouse. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. Bioinformatics 28(19): 2537–2539.

Peakall, R., P.E. Smouse, and D.R. Huff. 1995. Evolutionary implications of allozyme and RAPD variation in diploid populations of dioecious buffalograss *Buchloë dactyloides*. Mol. Ecol. 4(2): 135–148.

Pre-Aymard, C., E. Fallik, A. Weksler, and S. Lurie. 2005. Sensory analysis and instrumental measurements of `Anna' apples treated with 1-methylcyclopropene. Postharvest Biol. Technol. 36(2): 135–142.

Pritchard, J.K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155(2): 945–959.

Punta, M., P.C. Coggill, R.Y. Eberhardt, J. Mistry, J. Tate, C. Boursnell, N. Pang, K. Forslund, G. Ceric, J. Clements, A. Heger, L. Holm, E.L.L. Sonnhammer, S.R. Eddy, A. Bateman, and R.D. Finn. 2012. The Pfam protein families database. Nucleic Acids Res. 40(Database Issue): D290–D301.

R Core Team. 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Rees, D.J.G., J.-M. Celton, A. Cristoffels, D. Sargent, and X. Xu. 2010. Whole-genome SNP sequencing of selected progeny individuals allows anchoring of draft genome sequence to a linkage map for the apple scab pathogen *Venturia inaequalis*. Acta Hortic. 859: 295–300.

Richberg, M.H., D.H. Aviv, and J.L. Dangl. 1998. Dead cells do tell tales. Curr. Opin. Plant Biol. 1(6): 480–485.

Rosyara, U.R., M.C.A.M. Bink, E. van de Weg, G. Zhang, D. Wang, A. Sebolt, E. Dirlewanger, J. Quero-Garcia, M. Schuster, and A.F. Iezzoni. 2013. Fruit size QTL identification and the prediction of parental QTL genotypes and breeding values in multiple pedigreed populations of sweet cherry. Mol. Breed. 32(4): 875–887.

Rozen, S., and H. Skaletsky. 1999. Primer3 on the WWW for general users and for biologist programmers. p. 365–386. *In* Misener, S., Krawetz, S.A. (eds.), Bioinformatics Methods and Protocols. Methods in Molecular Biology™. Humana Press.

Sandskär, B., and E. Liljeroth. 2005. Incidence of races of the apple scab pathogen (*Venturia inaequalis*) in apple growing districts in Sweden. Acta Agric. Scand. Sect. B-Plant Soil Sci. 55(2): 143–150.

Schatz, M.C., A.L. Delcher, and S.L. Salzberg. 2010. Assembly of large genomes using second-generation sequencing. Genome Res. 20(9): 1165–1173.

Schenato, P.G., R.M. Valdebenito-Sanhueza, and V. Duarte. 2008. Determinaçao da presença da raça 1 de *Venturia inaequalis* no Sul do Brasil. Trop Plant Pathol: 281–287.

Schmidt, M. 1938. *Venturia inaequalis* (Cooke) Aderhold. Zücht. 10(9-11): 280–291.

Schmitz, C.A. 2013. Enabling marker-assisted breeding for fruit texture traits in progeny of the apple cultivar Honeycrisp. MSc Thesis. University of Minnesota, St. Paul, MN.

Schneider, C.A., W.S. Rasband, and K.W. Eliceiri. 2012. NIH IMage to ImageJ: 25 years of image analysis. Nat. Methods 9: 671–675.

Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. Nat. Biotechnol. 18(2): 233–234.

Shay, J.R., and L.F. Hough. 1952. Evaluation of apple scab resistance in selections of *Malus*. Am. J. Bot. 39(4): 288–297.

Sierotzki, H., M. Eggenschwiler, O. Boillat, J.M. McDermott, and C. Gessler. 1994. Detection of variation in virulence toward susceptible apple cultivars in natural populations of *Venturia inaequalis*. Phytopathology 84: 1005–1009.

Sierotzki, H., and C. Gessler. 1998. Genetic analysis of a cross of two *Venturia inaequalis* strains that differ in virulence. J. Phytopathol. 146(10): 515–519.

Smereka, K.J., W.E. Machardy, and A.P. Kausch. 1987. Cellular differentiation in *Venturia inaequalis* ascospores during germination and penetration of apple leaves. Botany 65(12): 2549–2561.

Smith, S.M., and S.H. Hulbert. 2005. Recombination events generating a novel *Rp1* race specificity. Mol. Plant. Microbe Interact. 18: 220–228.

Soriano, J.M., S.G. Joshi, M. Van Kaauwen, Y. Noordijk, R. Groenwold, B. Henken, W.E. Van de Weg, and H.J. Schouten. 2009. Identification and mapping of the novel apple scab resistance gene *Vd3*. Tree Genet. Genomes 5(3): 475–482.

Soufflet-Freslon, V., L. Gianfranceschi, A. Patocchi, and C.E. Durel. 2008. Inheritance studies of apple scab resistance and identification of *Rvi14*, a new major gene that acts together with other broad-spectrum QTL. Genome 51(8): 657–667.

Sutton, D.K., W.E. MacHardy, and W.G. Lord. 2000. Effects of shredding or treating apple leaf litter with urea on ascospore dose of *Venturia inaequalis* and disease buildup. Plant Dis. 84(12): 1319–1326.

Tartarini, S. 1996. RAPD markers linked to the *Vf* gene for scab resistance in apple. Theor. Appl. Genet. 92(7): 803–810.

Tartarini, S., F. Gennari, D. Pratesi, C. Palazzetti, S. Sansavini, L. Parisi, A. Fouillet, V. Fouillet, and C.E. Durel. 2004. Characterisation and genetic mapping of a major scab resistance gene from the old Italian apple cultivar 'Durello di Forlì'. Acta Hortic. 663: 129–133.

Tartarini, S., L. Gianfranceschi, S. Sansavini, and C. Gessler. 1999. Development of reliable PCR markers for the selection of the *Vf* gene conferring scab resistance in apple. Plant Breed. 118(2): 183–186.

Tartarini, S., and S. Sansavini. 2003. The use of molecular markers in pome fruit breeding. Acta Hortic. 622: 129–140.

Tenzer, I., and C. Gessler. 1997. Subdivision and genetic structure of four populations of *Venturia inaequalis* in Switzerland. Eur. J. Plant Pathol. 103(6): 565–571.

Tenzer, I., and C. Gessler. 1999. Genetic diversity of *Venturia inaequalis* across Europe. Eur. J. Plant Pathol. 105(6): 545–552.

Tenzer, I., S. degli Ivanissevich, M. Morgante, and C. Gessler. 1999. Identification of microsatellite markers and their application to population genetics of *Venturia inaequalis*. Phytopathology 89(9): 748–753.

Tomerlin, J.R., and A.L. Jones. 1983. Development of apple scab on fruit in the orchard and during cold storage. Plant Dis. 67(2): 147–150.

Tong, C., D. Krueger, Z. Vickers, D. Bedford, J. Luby, A. El-Shiekh, K. Shackel, and H. Ahmadi. 1999. Comparison of softening-related changes during storage of `Honeycrisp' apple, Its parents, and `Delicious'. J Amer Soc Hort Sci 124(4): 407–415.

Valsangiacomo, C., and C. Gessler. 1988. Role of the cuticular membrane in ontogenic and *Vf*-resistance of apple leaves against *Venturia inaequalis*. Phytopathology 78: 1066–1068.

Vanblaere, T., I. Szankowski, J. Schaart, H. Schouten, H. Flachowsky, G.A.L. Broggini, and C. Gessler. 2011. The development of a cisgenic apple plant. J. Biotechnol. 154(4): 304–311.

Vanderplank, J.E. 1978. Genetic and molecular basis of plant pathogenesis. 6: 167 pp.

Vanderplank, J.E. 1982. Host-pathogen interactions in plant disease. Academic Press, New York, NY.

van Ooijen, J.W. 2004. MapQTL v5, Software for the mapping of quantitiatve trait loci in experimental populations. Kyazma B.V., Wageningen, Netherlands..

van Ooijen, J.W. 2006. JoinMap4, Software for the calculation of genetic linkage maps in experimental populations. Kyazma B.V., Wageningen, the Netherlands.

van Ooijen, J.W. 2011. Multipoint maximum likelihood mapping in a full-sib family of an outbreeding species. Genet. Res. 93(05): 343–349.

Velasco, R., A. Zharkikh, J. Affourtit, A. Dhingra, A. Cestaro, A. Kalyanaraman, P. Fontana, S.K. Bhatnagar, M. Troggio, D. Pruss, S. Salvi, M. Pindo, P. Baldi, S. Castelletti, M. Cavaiuolo, G. Coppola, F. Costa, V. Cova, A. Dal Ri, V. Goremykin, M. Komjanc and others. 2010. The genome of the domesticated apple (*Malus* × *domestica* Borkh.). Nat Genet 42(10): 833–839.

Verma, S., C. Schmitz, M. Clark, Y. Guan, B. Orcheski, J. Harsman, K. Evans, S.K. Brown, J.R. McFerson, N. Bassil, U.R. Rosyara, M. Bink, J. Luby, E.V.D. Weg, A. Iezzoni, and C. Peace. 2013. Geneome-wide QTL analysis uncovers SNP marker-based functional alleles for apple "Fresh Sensation" traits. ASHS, Palm Dessert, CA.

Vinatzer, B.A., A. Patocchi, L. Gianfranceschi, S. Tartarini, H.B. Zhang, C. Gessler, and S. Sansavini. 2001. Apple contains receptor-like genes homologous to the *Cladosporium fulvum* resistance gene family of tomato with a cluster of genes

cosegregating with *Vf* apple scab resistance. Mol. Plant. Microbe Interact. 14(4): 508–515.

Voorrips, R.E. 2002. MapChart: Software for the graphical presentation of linkage maps and QTLs. J. Hered. 93(1): 77–78.

Voorrips, R.E., M.C.A.M. Bink, and W.E. van de Weg. 2012. Pedimap: Software for the visualization of genetic and phenotypic data in Pedigrees. J. Hered. 103(6) 903-907.

Wang, A., H.S. Aldwinckle, P. Forsline, D. Main, G. Fazio, S. Brown, and K. Xu. 2011. EST contig-based SSR linkage maps for *Malus* × *domestica* cv Royal Gala and an apple scab resistant accession of *M. sieversii*, the progenitor species of domestic apple. Mol. Breed. 29(2): 379-397.

Wayne, M.L., and L.M. McIntyre. 2002. Combining mapping and arraying: An approach to candidate gene identification. Proc. Natl. Acad. Sci. 99(23): 14903–14906.

Wilcox, W. 2001. Apple Scab. New York State Agricultural Experiment Station.

Williams, E.B., and J. Kuc. 1969. Resistance in Malus to Venturia Inaequalis. Annu. Rev. Phytopathol. 7(1): 223–246.

Williams, E.B., and J.R. Shay. 1957. The relationship of genes for pathogenicity and certain other characters in *Venturia inaequalis* (Cke.) Wint. Genetics 42(6): 704–711.

Win, J., D.R. Greenwood, and K.M. Plummer. 2003. Characterisation of a protein from *Venturia inaequalis* that induces necrosis in *Malus* carrying the *Vm* resistance gene. Physiol. Mol. Plant Pathol. 62(4): 193–202.

Xu, M.L., and S.S. Korban. 2000. Saturation mapping of the apple scab resistance gene *Vf* using AFLP markers. Theor. Appl. Genet. 101(5): 844–851.

Xu, M., and S.S. Korban. 2002. A cluster of four receptor-like genes resides in the *Vf* locus that confers resistance to apple scab disease. Genetics 162(4): 1995.

Xu, M., and S.S. Korban. 2004. Somatic variation plays a key role in the evolution of the Vf gene family residing in the *Vf* locus that confers resistance to apple scab disease. Mol. Phylogenet. Evol. 32(1): 57–65.

Xu, X., T. Roberts, D. Barbara, N. Harvey, L. Gao, and D. Sargent. 2009. A genetic linkage map of *Venturia inaequalis*, the causal agent of apple scab. BMC Res. Notes 2(1): 163.

Xu, M.L., J. Song, Z. Cheng, J. Jiang, and S.S. Korban. 2001. A bacterial artificial chromosome (BAC) library of *Malus floribunda* 821 and contig construction for positional cloning of the apple scab resistance gene *Vf*. Genome 44(6): 1104–1113.

Xu, X., J. Yang, V. Thakur, A. Roberts, and D.J. Barbara. 2008. Population variation of apple scab (*Venturia inaequalis*) isolates from Asia and Europe. Plant Dis. 92(2): 247–252.

Yang, H.Y., S.S. Korban, J. Kr\üger, and H. Schmidt. 1997. The use of a modified bulk segregant analysis to identify a molecular marker linked to a scab resistance gene in apple. Euphytica 94(2): 175–182.

Yepes, L.M., and H.S. Aldwinckle. 1993. Selection of resistance to *Venturia inaequalis* using detached leaves from in vitro-grown apple shoots. Plant Sci. 93(1-2): 211–216.

Young, N.D. 1996. QTL mapping and quantitative disease resistance in plants. Annu. Rev. Phytopathol. 34(1): 479–501.

Young, N.D. 2000. The genetic architecture of resistance. Curr. Opin. Plant Biol. 3(4): 285–290.

Zhang, H., S. Gao, M.J. Lercher, S. Hu, and W.-H. Chen. 2012. EvolView, an online tool for visualizing, annotating and managing phylogenetic trees. Nucleic Acids Res. 40(W1): W569–W572.

Appendix
Field screening of four 'Honeycrisp' full-sib families identifies QTL for apple scab resistance using a pedigree based mapping approach

Materials and Methods

Plant material

Four full-sib families that share 'Honeycrisp' as common parent were evaluated in this study and were grown at the University of Minnesota Horticultural Research Center in Excelsior, MN (McKay et al., 2011). The alternate parents include the presumed susceptible cultivars Pitmastin Pineapple and Monark, and the advanced breeding lines MN1702 and MN1764. These populations are part of a replicated, field planting that comprises six full-sib families and representative parents. Seedlings and parents were budded onto B-9 dwarfing rootstock and planted on ~2 foot spacing in a double row with 10ft spacing between rows. The majority of the trees were in the fourth leaf; however some individuals were re-budded due to graft failure since the time of establishment in 2008. Many of these trees were in their first or second year of fruit production at this location. These trees were maintained as part of a limited integrated pest management program that did not include any fungicide applications. A subset of each population was genotyped for QTL mapping.

Inoculum

Preliminary observations in spring and summer 2011 indicated that disease pressure in this planting block was not sufficient to cause significant scab infection for evaluation. In order to increase inoculum levels, two approaches were utilized to introduce both primary (ascospore) and secondary (conidia) inocula.

Scab infected leaves were collected fall 2011 from a mixed planting of crabapple species at the University of Minnesota Landscape Arboretum (Chanhassen, MN) and over wintered in plastic mesh bags until late winter in an unheated storage building. In February, the bags were placed on a gravel bed and secured to the ground for development of pseudothecia and ascospores. Before bud break, leaves were removed from the mesh bags and scattered at 10 foot intervals within the double row of trees on the orchard floor.

In spring 2012, leaves were collected from the same crab apple collection for inoculation with a conidial suspension. Leaves were collected in cotton bags from the ground and also from heavily infected trees. The leaves were allowed to dry in the bags until conditions were favorable for field inoculation.

Spore suspensions were created by agitating leaves in a pipet wash reservoir filled partway with water and using the basket as a plunger to agitate the leaves. The leaf particles were filtered with 2 layers of cheesecloth and the suspensions were stored in 4L glass jugs at 4°C until taken to the orchard were they were held on ice. Conidia concentrations were calculated with a hemocytometer and adjusted to final concentration (10,000 conidia ml^{-1}) in the field when mixed with additional water in the sprayer.

On 12 June 2012, trees were sprayed with the spore suspension using a hand-pump back-pack sprayer. The operator specifically targeted each tree in the row and focused on areas of new growth to increase the potential of infection/resistance reaction. New leaves were sprayed until visibly wet.

Leaf wetness was maintained in the orchard through the use of an irrigation system using MP-3000 sprinkler heads (Hunter Industries, San Marcos, CA The sprinkler heads were on ~ 3 foot risers and were spaced between alternate double rows of trees where coverage reached the entire canopy (Figure A1). Water was applied for ~10-15 min every hour for ~ 8h until night fall (2200h), when conditions were cool and dew was expected. Intermittent water application was resumed at 0700h the next day for 3 hours.

Scab Assessment

Field scoring was conducted on three occasions utilizing the 6 point scale developed by Chevalier (1991). Leaves were observed on both adaxial and abaxial sides. No attempt was made to quantify resistance, but only to detect resistance reaction(s) or susceptibility indicated by sporulation. The first screening was on 30 May 2012 prior to inoculation with the conidial suspension. If lesions were observed on fruit, the tree was scored as 4 (susceptible). Two additional evaluations were conducted on 26 June 2012 and 7 September 2012. A composite score for each individual was assigned based on the replicate planting and all three observation dates. The most susceptible score was used if there was variation in scoring. For QTL analysis, scores were converted to a quantitative scale [0; 1; 2; 3 (3a); 3.25 (3a-3b); 3.50 (3b), 3.75 (3b-4); 4].

Segregation of the resistance classes for each family was tested to assess the hypothesis that one or two scab resistance genes are present in 'Honeycrisp' and inherited in the progeny. A 1:1 segregation ratio supports one dominant R gene, whereas a 3:1 segregation supports two genes. Segregation ratios are also utilized to test the hypothesis that the parent crossed to 'Honeycrisp' is in fact susceptible and not contributing a resistant allele in the seedling population.

DNA Extraction and SNP Genotyping

A subset of each 'Honeycrisp' full-sib population was selected for SNP genotyping. DNA extraction was conducted following the procedures outlined in Clark et al. (accepted; Chapter 1) for seedlings, parents, and other ancestors. SNP marker calls were made in GenomeStudio.

FlexQTL Analysis

QTL mapping was conducted with a consensus 'Honeycrisp' genetic map with 1091 SNP markers and an average distance of 1.36 cM between consecutive markers. A genome-wide, pedigree-based mapping approach using FlexQTL(v.0.099103 and v.0.099105) was performed using the field scab resistance data of the pedigreed populations (Bink et al., 2008; Rosyara et al., 2013). Five separate analyses were conducted on the dataset to test models with different assumptions (Table A2). The first three models explored distinct subsets of the 17 linkage groups. After QTL were detected in models 1, 2, and 3, additional analysis (Model 4) explored those linkage groups with support for a QTL in Models 1, 2 or 3. Model 5 was based on the *a priori* knowledge of scab resistance QTL on LG 1 and 15 detected in the 'Honeycrisp' × 'Twin

Bee Gala' greenhouse-screened mapping population. All analyses used 200k sweeps with thinning every 100 iterations in the Markov Chain Monte Carlo (MCMC) simulation. The number of QTL explored in each model was set for the *a priori* value of 2, and either a max of 4 (Models 2,3, and 5) or 6 (Models 1 and 4) QTL.

QTL positions were visualized in MapChart (Voorrips, 2002) or within VisualFlexQTL. Convergence (effective chain size and trace plots) of the MCMC model was examined to ensure that the results of the model would be supported prior to assessing significance levels of the QTL analysis. Bayes factor (BF) thresholds provided in the software set limits for detecting QTL at a linkage group. BF scores that support a QTL at a locus include: 2 to 5 (positive), 5 to 10 (strong), and >10 (decisive). Heritability, in the narrow sense, was calculated from FlexQTL output using the formula: $h^2 = V_{QTL}/V_p$, where V_{QTL} is the additive variance of a QTL, and V_P the total phenotypic variance.

Results

In the field, the majority of susceptible reactions were observed on the abaxial leaf surface. Scab resistance reactions, when visible, were generally limited to the adaxial surface. The majority of individuals scored as resistant were classified as 0, no resistance reaction. Observations of the disease incidence in the orchard block for each of the families in 2012 supported a 1:1 segregation ratio (combined $p = 0.62$; Table A1) when 3b was considered as resistant. This supports a single gene in the resistant parent, 'Honeycrisp'.

Models 1, 2, and 3 explored different segments of the genome using the same parameters for detecting QTL. Convergence as indicated by effective chain size was met for each model (data not shown). Table A2 reports the BF values for each model. Table A3 shows the genomic position of the QTL on the consensus 'Honeycrisp' linkage map and posterior probability for a QTL within the model. Model 1 supported at QTL on LG XXXX. Model 2 supported a QTL on LG 9 at ~ 68 cM which is flanked by the SNP markers ss475879535 and ss457879540. Model 3 detected two QTL; LG 15 (~54 cM; ss475883649 and ss475876519) and LG 17 (~75 to 82 cM; ss475881264 to ss475876632). The trace plot and probability plot for Model 3 is shown in Figure A2. Model 4 explored the QTL detected in the previous models and supported only a QTL on LG 1 (50.51 cM; (ss475882286). The trace plot and probability plot for Model 4 is shown in Figure A3. Model 5 explored LGs 1 and 15, and detected the same QTL as in models 1 and 4 (trace plot and posterior probability plot shown in Figure A4).

Discussion

The QTL models tested for field scab resistance in FlexQTL analysis provided consistent support for a QTL (or a major gene) flanking SNP ss475882286 on LG 1, the same locus previously detected and named *Rvi19* (Chapter 3). The QTL detected in model 3 on LG15, maps ~24 cM below the previously detected *Rvi20* resistance locus. Replicate analyses with different MCMC parameters (varying priors and seeds, different combinations of linkage groups) would be need to be tested to determine the accuracy, repeatability, as well as the precision of the placement of the QTL in the study population.

The other QTL detected may represent resistance in the alternate parent and will require additional exploration of the data to determine the parental haplotypes contributing to resistance. Field-based studies for scab resistance often require multiple years of assessment for accurate phenotyping. Disease pressures, even in untreated orchard blocks, may not be sufficiently high to screen segregating populations. Spore dispersal is limited and environmental conditions varied. By bringing in mixed inoculum and overhead watering for maintaining leaf moisture, the incidence of disease was increased sufficiently to detect QTL in 2012. Approximately 50% of the genotypes in the orchard block were resistant (1:1 segregation ratio).

From this analysis, it appears that *Rvi19* provides a broader spectrum of resistance than *Rvi20*. The defense response observed in the 'Honeycrisp' families could be attributed to (i.e. detected as) *Rvi19*, despite the mixed inoculum. This research supports the need for the development of durable cultivars, combining several R genes into a single genotype to protect against different races of the pathogen. *Rvi20* would not be suitable alone for resistance, at least when grown in locations with the unknown races present in the current study.

Table A1. Four full-sib populations designated by the parent crossed to 'Honeycrisp' (resistant parent) and screened in the field with mixed inoculum for apple scab resistance. The number of individuals in the population, and resistance class on the Chevalier et al. (1991) scale are given. No class 1 or 2 individuals were observed. Several individuals remained unscored. The segregation R (resistant) : S (susceptible) counts for each population where classes 0-3b were considered resistant and classes 3b-4 and 4 were considered susceptible.

		Resistance Class									
Population	N	0	3a	3a-b	3b	3b-4	4	unscored	R : S	X^2	*p*
MN1702	66	27	1	--	--	1	35	2	1:1	1.00	0.31
MN1764	170	92	--	--	2	3	67	6	1:1	3.51	0.06
Monark	105	45	--	1	2	2	53	2	1:1	0.48	0.49
Pitmastin Pineapple	81	40	--	1	--	3	37	--	1:1	0.01	0.91
Total	422	204	1	2	4	9	192	10	1:1	0.24	0.62

Table A2 Five different models explored in FlexQTL to test for field resistance to apple scab. Markov Chain Monte Carlo (MCMC) sweeps and thinning parameters for each model, and the number of QTL explored (*a priori* and maximum) are given. The linkage groups tested, and corresponding Bayes Factor for a single QTL on that linkage group are given. **Bold** values represent a decisive QTL on that linkage group in the model. In all models there was no support for more than one QTL on any given linkage group.

Model	MCMC		*a priori*/Max	Linkage group and Bayes Factor																
	Sweeps	Thinning		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	200K	100	2/6	**30.7**	-5.4	-6.2	-5.4	-6.0	-5.5	-5.8	-5.8	-	-	-	-	-	-	-	-	-
2	200K	100	2/4	-	-	-	-	-	-1.4	-2.5	-2.1	**29.4**	-1.5	-	-	-	-	-	-	-
3	200K	100	2/4	-	-	-	-	-	-	-	-	-	-	-6.6	-6.0	-6.8	-6.3	**29.4**	-5.6	**30.3**
4	200K	100	2/6	**29.4**	-	-	-	-	-	-	-	-5.7	-	-	-	-	-	-6.4	-	-5.8
5	200K	100	2/4	**28.3**	-	-	-	-	-	-	-	-	-	-	-	-	-	-6.0	-	-

Table A3 Genetic position on the consensus 'Honeycrisp' linkage map [linkage group (LG) and position (cM)], and posterior probabilities for QTL identified in each model.

Model	LG	Position (cM)	Posterior Probability
1	1	50.84—51.18	0.983
2	9	68.51 – 68.69	0.891
3	15	54.50 – 55.05	0.981
	17	75.50 – 82.49	0.991
4	1	50.51 – 50.76*	0.981
5	1	50.51 – 50.77*	0.967

*QTL maps to the same resistance locus *Rvi19* as in 'Honeycrisp' × 'Twin Bee Gala' greenhouse population (Chapter 3)

Figure A1. Irrigation system for maintaining leaf wetness following application of a conidial suspension of *V. inaequalis* at the University of Minnesota Horticultural Research Center (Victoria, MN).

Figure A2: Model 3: Trace plots and posterior probability of quantitative trait loci for field resistance to apple scab as calculated in FlexQTL. Parameters are given. Bayes factor (BF) is indicated for linkage groups showing a significant QTL. Linkage groups 11 to 17, MCMC sweeps 200K, thinning 100. Explored QTL *a priori* 2 to max 4. LG15 BF = 29.4; LG17 BF=30.3.

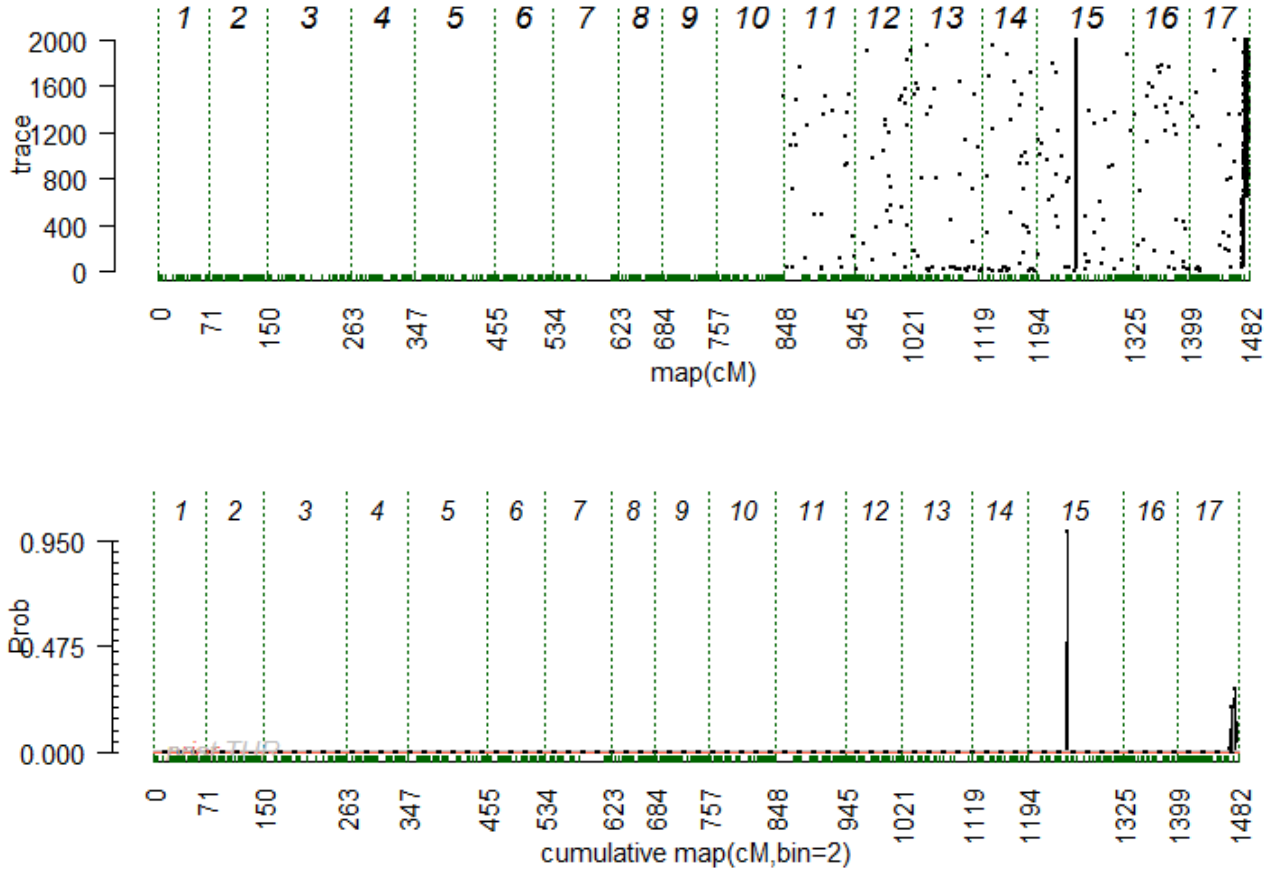


Figure A3: Model 4: Trace plots and posterior probability of quantitative trait loci for field resistance to apple scab as calculated in FlexQTL. Parameters are given. Bayes factor (BF) is indicated for linkage groups showing a significant QTL. Linkage groups 1,9,15,and 17. MCMC sweeps 200K, thinning 100. Explored QTL *a priori* 2 to max 6. LG1 BF = 29.4.

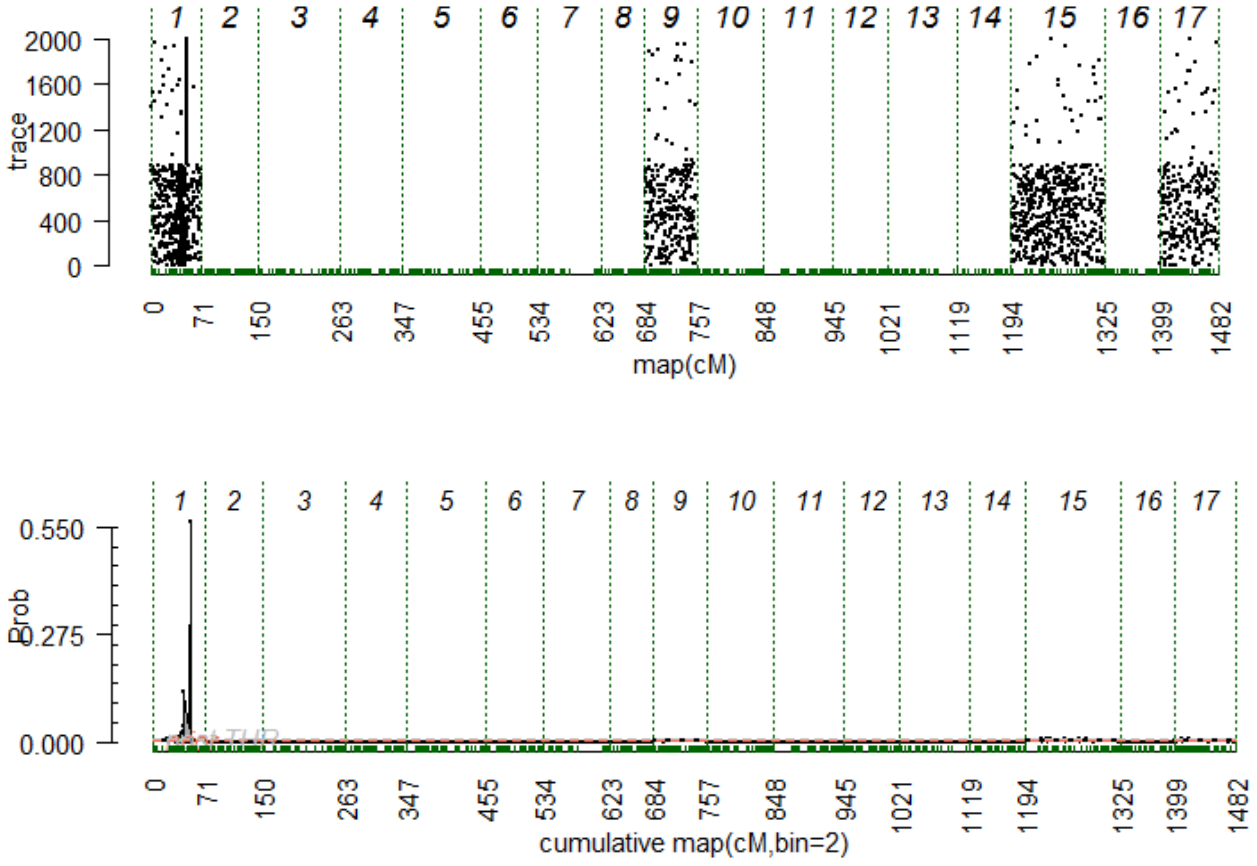


Figure A4: Model 5. Trace plots and posterior probability of quantitative trait loci for field resistance to apple scab as calculated in FlexQTL. Parameters are given. Bayes factor (BF) is indicated for linkage groups showing a significant QTL. Linkage groups 1 and 15, MCMC sweeps 200K, thinning 100. Explored QTL *a priori* 2 to max 4. LG1 BF = 28.3.