MAPPING QUANTITATIVE TRAIT LOCI ASSOCIATED WITH RESISTANCE TO
BACTERIAL SPOT (*XANTHOMONAS ARBORICOLA* PV. *PRUNI*) IN PEACH

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ABSTRACT


Bacterial spot, caused by *Xanthomonas arboricola* pv. *pruni (Xap)*, is a serious disease that can affect peach fruit quality and production worldwide. This disease causes severe defoliation and blemishing of fruit, particularly in areas with high rainfall, strong winds, high humidity and sandy soil. The molecular basis of its tolerance and susceptibility in peach is yet to be understood. To study the genetics of the peach response to *Xap*, an $F_2$ segregating population between two peach cultivars, 'Clayton', a resistant phenotype, and 'O'Henry', which is very susceptible to *Xap*, was created. Phenotypic data for leaf and fruit response to *Xap* infection were collected over three years at two locations: the Sandhills Research Station, Jackson Springs, North Carolina (NC) and the Sandhill Research and Education Center, Pontiac, South Carolina (SC).

Phenotypic data for leaf and fruit organs were collected with 26 data points in total. Our phenotypic data suggest that *Xap* resistance in peach is a quantitative trait, and leaf and fruit resistance is regulated by separate genetic factors. In addition, relative humidity higher than 80% from petal fall to shucks off (gererally from March 15th to April 15th) plays a significant role on the occurrence of *Xap* disease incidence and severity.

A genetic map was initially developed using SSR markers, however, only thirteen SSR markers were put on the linkage map. Therefore, sixty three individuals exhibiting high tolerance/resistance to *Xap* were genotyped with an IPSC 9K peach SNP array v1.

Out of 8,144 SNPs 1,341 were used to construct a high-density genetic linkage map. This map covers a genetic distance of 421.4 cM with an average spacing of 1.6 cM and is used for mapping QTLs responsible for *Xap* in peach. 95% of the mapped SNP markers on the linkage map showed consistency with the marker order on the peach genome v1.0 assembly. A QTL analysis revealed 14 QTLs involved in *Xap* resistance: 3 on linkage group (LG) 1; two on each LG2, 3, 4 and 8; and one on each LG5, 6, and 7. One major *Xap.Pp.CO-4.1* on LG4 was associated with *Xap* resistance in leaf, and one major QTL *Xap.Pp.CO-5.1* on LG5, was associated with *Xap* resistance on both leaf and fruit and two major QTLs. While *Xap.Pp.CO-1.2* and *Xap.Pp.CO-6.1* on LG1 and 6, was associated with *Xap* resistance in fruit.

# DEDICATION

I dedicate this dissertation to my wonderful family. My mother, Zhuanxian Ji, always encouraged me to conquer the problems from the research in the past three years. When I was in a hard time, my father, Shuanzhao Yang, always stand out to provide good suggestions to deal with those difficulties. Since I was not at home for a long time I owed a small debt to my younger brother, Xifan Yang, he took many responsibilities of mine in our family.

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CHAPTER 1

LITERATURE REVIEW

**Introduction**

Peach [*Prunus persica* (L.) Batsch] is native to China, and belongs to the subfamily Prunoideae of the Rosaceae. Prunoideae, species that produce hard and lignified seed buried in an edible and juicy mesocarp, include: *P. domestica* L. (European or prune plum), *P. salicina* Lindl. (Japanese plum), *P. cerasus* L. (sour cherry), *P. avium* L. (sweet cherry), *P. armeniaca* L. (apricot), *P. amygdalus* L. (almond), and *P. persica* L. (peach). Peach is a temperate fruit, generally distributed between latitudes $30^o$ and $45^o$ N and S. In the U.S., peach is one of the most important economic fruits with 1194 metric ton in 2009, contributing to 7% of the peach production in the world (USDA, 2009). South Carolina and Georgia rank second and third, respectively, in US peach production, accounting for an average of 226 million lbs annually, with an average value of $63 million dollars (NASS, 2004).

The main objective in many fruit breeding programs, whether it's fresh consumption or canning is developing peach cultivars to satisfy commercial requirements/preferences. Early breeding programs focused on the improvement of physiological and quality characteristics of peach, including fruit color, firmness, attractiveness, taste, ripening time, cold hardiness, and adaptation to various environmental conditions. Disease and pest resistance have also been one of the major

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goals in many breeding programs. Many of the most globally spread diseases and pests, such as powdery mildew, brown rot, bacterial canker, bacterial spot, nematodes, *plum pox virus* (PPV, sharka disease), leaf curl, peach tree borers, and green aphids, show variable levels of economic impact on peach production (Scorza and Sherman, 1996; Abbott et al., 2007; Hancock et al., 2008).

However, breeding disease resistant cultivars is not an easy task. Peach breeding in general is time consuming due to long breeding cycles, large plant size and growing space requirements and difficulty of selecting important traits. A breeder has to wait at least 3 years for trees to bear fruits for evaluation. Most disease resistance traits are polygenic in nature and controlled by many genes residing at so called quantitative trait loci (QTLs) (Young, 1996), e.g. powdery mildew (Foulongne et al., 2003), bacterial spot (Yang et al., 2010), peach tree short life (Blenda et al., 2006, 2007), PPV (Decroocq et al., 2005), and leaf curl (Virul et al., 1998). In addition, sources of resistance are usually found in wild relatives or cultivars with lower agronomical value, so introgression of resistance characters into commercial peach cultivars usually requires several generations of backcrossing to reinstate the favorable genotype. Some diseases manifest only under certain environmental conditions and show erratic occurrence (Ferri et al., 2002), making it harder to evaluate fruits on trees in the field. Molecular-assisted breeding (MAB), however, allow the pre-selection of traits long before they are expressed. Furthermore, if tightly linked markers with traits of interest were known, desirable individuals could be selected from progeny, thus facilitating the process of disease resistance breeding in peach (Stockinger et al., 1996).

# DNA marker application in *Prunus* species

Molecular marker developed from plant DNA sequences have been routinely employed in analysis of various aspects of the *Prunus* genome including genetic variability, genome fingerprinting, genome mapping, gene location, plant breeding, etc. At the beginning, morphological, cytogenetic, and isozomic markers were used to construct linkage maps. However, those markers were limited in numbers and insufficient to build comprehensive linkage maps, resulting in inadequacy to perform genetic studies, such as interactions between gene and environment, and gene epistasis. Various types of molecular markers are utilized to evaluate DNA polymorphism and are generally classified as hybridization-based markers, polymerase chain reaction (PCR)-based markers, and sequencing-based markers, including Restriction Fragment Length Polymorphisms (RFLPs), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSRs), Sequence Related Amplified Polymorphisms (SRAPs), and Single Nucleotide Polymorphisms (SNPs). In recent years, due to their high throughput nature, PCR and sequencing-based markers are preferred in genetic studies.

RAPD is a PCR-based genetic assay that uses short and single primers of arbitrary nucleotide sequences to detect sequence polymorphisms in DNA. However, the stoichastic nature of DNA amplification with arbitrary random sequence primers causes low reproducibility such as faint or fuzzy products, and difficulty in band scoring (Joshi et al., 1999). However, since the dominant nature of RAPD markers allows the detection of many loci at the same time it was widely used to saturate the linkage map in *Prunus* L.

(Sonsinski et al., 1998; Dettori et al., 2001; Joobeur et al., 2000; Verde et al., 2005). Furthermore, if RAPD markers happen to be linked to agronomically important traits, they can easily be converted into PCR-based, co-dominant, Cleaved Amplified Polymorphic Sequence (SCARs) markers, which reflect the allelic variations at a single locus.

AFLP is a robust and reliable molecular marker assay where molecular markers are generated by a combination of restriction digestion and PCR amplification, therefore detecting much more polymorphism per reaction (Vos et al., 1995). Most AFLP fragments are unique in the genome and thus can be exploited as anchor sites in linkage map development in *Prunus* L. (Ehrlich et al., 1991). Therefore, AFLP are also widely used in *Prunus* L. to construct linkage maps (Dirlewanger et al., 1998 and 1999; Sonsinski et al., 1998; Verde et al., 2005; Blenda et al., 2007; Fan, 2010). Similar to RAPD fragments, AFLP fragments of interest can also be converted into SCARs.

Simple Sequence Repeat markers (SSRs) are also PCR-based markers that can be used to detect sequence polymorphisms in DNA. The discovery that 30-90% of the genome of virtually all species is composed of randomly distributed repetitive DNA, resulted in the development of microsatellite markers which are highly polymorphic in nature (Moore et al., 1991). SSR markers consist of one to six bp long monomer sequences that are repeated several times. Specifically designed primers flanking the tandem repeats, are used to amplify unique fragments that are usually genomically unique and also have a potential to detect multiple alleles. Due to their abundance, high

polymorphism, co-dominance, reproducibility, and transferability to related species, SSRs are emerging as a marker of choice for linkage and comparative mapping, genotype identification, QTL tagging, and marker-assisted selection (MAS) (Cipriani et al., 1999; Aranzana et al., 2002, 2003a, 2003b; Dirlewanger et al., 2004b; Liu et al., 2007; Mnejja et al., 2010). SSR markers are species specific and estimated to detect above 20% of polymorphism in peach (Dirlewanger et al., 2006; Blenda et al., 2007; Ogundiwin et al., 2009; Fan, 2010; Cao et al., 2011).

SSR markers were used to develop over twenty genetic linkage maps in *Prunus*, including three maps between two hybrid *Prunus* species, one almond map, four maps in apricot, one map in cherry, and five linkage maps in peach (www.rosaceae.org). SSR markers have also been used to saturate an almond x peach (T x E) linkage map (Joobeur et al., 1998) that has served as the reference map for the genus (Aranzana et al., 2003b; Dirlewanger et al., 2004b; Howad et al., 2005). This reference map has facilitated location of different major genes and QTLs in a unique map, the search for markers to saturate specific genome regions, and/or the establishment of map comparisons with other *Prunus* species (Dirlewanger et al., 2004b). The reference map and peach physical map (Aranzana et al., 2003b; Horn et al., 2005) have fostered development of a *Prunus* resistance map with 90 SSR markers which was used to locate the loci associated with resistance (Lalli et al., 2005).

The completion of genome sequencing of many organism genomes allowed the development of a new marker system, SRAP, for DNA fingerprinting (Li and Quiros,

2001).The SRAP markers target the amplification of coding regions in the genome. Arbitrary forward and reverse primers can be designed and combined for PCR reaction, first under a low annealing temperature for unspecific amplification and then increasing the annealing temperature for specific amplification. This method is efficient to create sufficient polymorphism for linkage map construction. A number of plant resistance genes were isolated and characterized to share similar sequence information (Bent, 1996; Jones, 1996; Hammond-Kosack and Jones, 1997). In addition, many resistance genes tend to cluster in regions of the plant genome (Hulbert et al., 2001). Markers derived from the putative resistance genes therefore can saturate regions of resistance 'hot spots' to facilitate location of candidate genes or even isolation of genes of interest. This approach has been applied in grape (Donald et al., 2002), apple (Baldi et al., 2004), peach (Lalli et al., 2005), chestnut rose (Xu et al., 2005), and raspberry (Samuelian et al., 2008).

SNPs are characterized as co-dominant and bi-allelic markers caused by single nucleotide changes (i.e. transition, transversion, deletion or insertion) in the genomic sequences (Vignal et al., 2002). SNP frequency in Rosaceae was found to be 1/100 for non-coding sequences and 1/225 for exonic sequences (Sargent et al., 2009; Illa et al., 2010). As a result, SNP markers are far more abundant than any other marker system per unit of genome sequence. A large number of SNP markers covering the entire genome are desirable to facilitate molecular breeding efforts such as genome wide association studies, fine mapping, genomic selection and marker-assisted selection in peach. Therefore, several efforts to perform genome-scale single nucleotide polymorphism discovery in peach using next generation sequencing platforms have recently been

revealed (Ahmad et al., 2011; Verde et al., 2012). However, considering recombination events, SNP makers are less informative than SSR markers in a given number of individuals (Slate, 2008; Ball et al., 2010).

## **Bacterial spot disease (*Xanthomonas arboricola* pv. *pruni*)**

Bacterial spot, caused by *Xanthomonas arboricola* pv. *pruni* (*Xap*), is a serious disease that can affect all cultivated *Prunus* species and their hybrids (EPPO, 1997). It was first described on plum in the United States by Smith (1903), and soon after *Xap* was identified on peach and other stone fruits (Rolfs, 1915). The most severe infections, over 50% infections, were reported on Japanese plum (*P. salicina*), Korean cherry (*P. japonica*) and hybrids, as well as on peach and nectarines (*P. persica*) and their hybrids (Ritchie, 1995). The rapid spread of bacterial spot disease across different countries had been recently noticed. It was reported that the disease is present and widespread in China, South Africa and Uruguay, whereas local outbreaks were also reported in several other countries such as Bulgaria, Romania, Moldova, Russia, Ukraine, India, Pakistan, Japan, Korea, Canada, USA, Mexico, Argentina, Brazil, and Australia (EPPO, 2006).

Bacterial spot disease is normally characterized as various sized spots on leaves, stems, blossoms, and the most obvious symptoms are found on the fruit. The disease is favored by warm temperature and high humidity. When the disease develops rapidly in the population, it is referred to as blights. After high inoculation by the pathogen, most of plant leaves will appear blighted or with a tattered surface, which can damage the whole plant. Diseased leaves of dicotyledonous plants exhibit angular spots, because the

pathogen is restricted by veins; whereas in monocotyledon leaves show streaks or stripes (Agrios, 2005). Lesions on fruit surface can cause skin cracking when extending deep into the fruit flesh, gum may exude from the injured areas (Agrios, 2005).

Bacterial spot pressure varies between seasons, sites and production areas. Generally the eastern region of the United States shows higher infection pressure than the arid regions of the western United States, such as California. It is estimated that 25% of the bearing acreage in Georgia and South Carolina require some level of bacterial spot control (NSSA, 2004). Traditional control of bacterial spot involves spraying bactericides such as copper-based compound and the antibiotic oxytetracycline (Ritchie, 1995). However, with the environmentally conscious public, chemical control of bacterial spot through bactericides is coming under close scrutiny. Thus interest in developing resistant peach cultivars has moved to the forefront of breeding programs. Many cultivars from breeding programs in eastern US have medium to high level of resistance, i.e. 'Candor', 'Clayton', 'Contender', 'Encore', 'Juneprince', and 'Redrose' (Okie, 1998). Unfortunately, some of the best wholesale market peaches are highly susceptible, such as 'O'Henry', which are still planted or widely used as parents in the development of new cultivars, because of their competitive advantage in the market. Due to the quantitative nature of bacterial spot disease resistance, molecular markers tagging with the resistance traits can be used as an efficient tool to speed up the breeding process. Bacterial spot incidence in peach was evaluated by planting seedlings and selections in the field (Werner et al., 1986). Several alternative methods have been proposed, including greenhouse inoculations, detached leaf tests, and others (Daines and Hough, 1951;

Civerolo and Keil, 1976; Randhawa and Civerolo, 1985; Hammerschlag, 1988), but breeders have not used them because they are not sufficiently reliable or worth the effort.

To date, the molecular mechanism of resistance or susceptibility to bacterial spot is not clear. Sherman and Layne (1981) suggested that dominant genes are involved in the resistance. Later, the resistance of leaf and fruit in peach was suggested to be controlled by separate genetic factors (Keil nad Fogle, 1974; Simeone, 1985; Werner et al., 1986). The first study of genetic factors involved in the resistance to *Xap* was reported in Yang et al. (2010), and suggested the polygenic characteristics of *Xap* resistance. Interestingly, one putative QTL region was found on Linkage Group (LG) 4 (Yang et al., 2011). Only partial linkage groups were constructed in the study, restricting the QTL analysis. Furthermore, no QTL with major effects was indicated. Socquet-Juglard et al. (2011) using low density SSR linkage map (Dondini et al., 2007), identified four genomic regions related to *Xap* resistance in apricot and reported a single QTL on LG5 being of interest for MAS. However, to date no tightly linked markers or isolation of genes associated with *Xap* resistance were reported.

## Mapping disease resistance traits in *Prunus*

Peach, is the best characterized of the *Prunus* species and is the genetic study model for Rosaseae (Abbott et al., 2002). In comparison to other Rosaceae, peach has a short juvenile phase (2-3 years), is a diploid species (2n=16) with a relatively small genome, only ~220 Mbp, twice that of Arabidopsis (Baird et al. 1994; Sosinski et al., 2009). More than twenty genetic maps have been constructed with peach and other

*Prunus* species (Abbott et al., 2008). The *Prunus* reference map was constructed from an inter-specific almond cv. 'Texas' x peach cv. 'Earlygold' (abbr. T x E) $F_2$ mapping population (Joobeur et al. 1998; Aranzana et al. 2003b). Considering all markers bin-mapped or mapped with the whole T x E population (Dirlewanger et al. 2004b), the reference map consists of at least 1,803 sequence-based markers, of which 264 are SSR and 796 are SNP markers, corresponding to a density of 0.29 cM/marker (Illa et al., 2010). Given that all these markers are transferable to other *Prunus*, as they can be associated to a specific DNA sequence, they are invaluable for map construction in other populations and useful anchors for comparison between the whole genome sequence of peach and its linkage map (Illa et al., 2010). High quality peach genome sequence v1 has been recently released, and several genomic databases housing Rosaceae genomic resources are also available, including the Genomic Database for Rosaceae (http://www.rosaceae.org/), ESTree (http://www.itb.cnr.it/estree/), GenBank (http://www.ncbi.nlm.nih.gov/) , etc.

Genetic mapping is widely used to determine the location of genes or QTLs and characterize agronomically important traits. To date, over 14 genetic maps have been constructed to facilitate discovery of resistance genes for several key plant diseases: leaf curl, nematode, PPV, powdery mildew, and peach tree short life. Resistance to leaf curl disease and to nematodes are more likely to be controlled by several predominant genes, which were mapped on LG3 and LG6 (Viruel et al., 1998), and on LG2 and LG7, respectively (Abbott et al., 1998; Lu et al., 1998; Jauregui, 1998; Yamamoto and Hayashi, 2002; Bliss et al., 2002; Claverie et al., 2004a; Dirlewanger et al., 2004a; Gillen

and Bliss, 2005). QTLs for resistance to PPV were found on all linkage groups except LG3 (Decroocq et al., 2005; Soriano et al., 2008; Marandel et al., 2009; Rubio et al., 2009, 2010; Dondini et al., 2010), and three QTLs (*PPV.RD-1.1*, *PPV.RD-5.1*, and *PPV.RD-7.1*) co-localized in several studies. A major QTL of resistance to powdery mildew was found on LG6 (Dirlewanger et al., 1996; Quarta et al., 2000; Verde et al., 2002; Foulongne et al., 2003), and was later genetically linked to leaf color (Pascal et al., 2010). QTLs were also discovered responsible for the resistance/tolerance to peach tree short life (Blenda et al., 2006, 2007). One QTL region on the upper part of LG2 was involved in resistance/tolerance to peach tree short life (Liu, 2009). Using candidate gene approach and probe hybridization analysis, a total of 42 regions of resistance were mapped on all linkage groups except on LG3, in a *Prunus* resistance map (Lalli et al., 2005). Upper parts of LG1, LG2 and LG7 are considered resistance 'hot spots' where disease resistance genes reside.

Linkage mapping using small populations in plants generally can locate the genes or QTLs to only 10 to 20 cM, due to the limited recombination events. To narrow down a genomic region of interest the population size must be increased. Recently a map-based cloning method was successfully used to clone an R gene *Ma/TNL 1* in plum, which confers a complete-spectrum resistance to root-knot nematodes (Claverie et al., 2011). In this study, over 3000 individuals were used to map *Ma/TNL 1* gene.

Association mapping or linkage disequilibrium mapping has been employed as another tool to map some complex traits in major crop species, i.e. maize, wheat, barley,

rice, and etc. (Zhu et al., 2008). While linkage analysis searches for association within populations developed from bi-parental crosses, association mapping exploits recombination events in history or natural genetic diversity. Association mapping is based on the principle that over multiple generations of recombination, correlations only with markers tightly linked to the trait of interest will remain. Two strategies are used in association study: the candidate-gene approach, utilizing polymorphism of candidate genes to relate the trait when there is evidence to support involvement of those genes, and the genome-wide scan approach, scanning the whole genome to search for the signals associated with the trait (Zhu et al., 2008). Recently, candidate-gene association mapping was used to map the chilling requirement in peach (Fan, 2010). The candidate gene *DAM6* associated with bud break was verified using association mapping with 65 different peach germplasm accessions. Conversely, genome-wide association mapping uses a high amount of polymorphic markers such as SNP and a next generation sequencing platforms to set up a high resolution genetic map. Currently, no research based on this strategy is reported yet for *Prunus* L.

## **Markers assisted selection/breeding in *Prunus***

The identification of markers or "tags" tightly linked to genes of interest makes it possible to select for desired alleles indirectly. MAS appear to have promise in the development of disease-resistant cultivars. Suggested uses of molecular tags in fruit and nut breeding include following resistance alleles in several crosses over several generations, identifying seedlings likely to be resistant in the presence of the pathogen, constructing pyramids of resistance genes without the need for progeny testing, and more

rapidly eliminating the donor parent genome in a modified backcross program (Mehlenbacher, 1991).

Some traits that show a continuous distribution in a segregating population may actually be controlled by a small number of loci (Paterson et al., 1991), and genetic analysis using molecular markers in conjunction with a linkage map can allow identification of the number and location of these loci. To develop new peach cultivars with improved traits, MAB using markers tightly linked to gene(s) of interest can be used to follow introgression of desired traits into elite commercial lines. There are several examples of discovery of tightly linked markers associated with disease and/ or pathogen resistance in peach. For example, nematode resistance loci *Mi* (*Meloidogyne incognita*), and *Mij* (*Meloidogyne javanica*) in 'Nemared' were found to be tightly linked with one SSR marker, pchgms1, and one Sequence-Tag Sites (STS) marker on LG2 (Lu et al., 1999; Sosinski et al., 2000). Later, five additional STS markers tightly linked to *Mia* (*Meloidogyne arenaria*) and *Mja* loci were discovered (Yamamoto and Hayashi, 2002). In addition, two SSR markers on LG7 tightly linked to Mja resistance gene, susceptibility allele (CPPCT022), and resistance allele (CPSCT026) were also reported (Claverie et al., 2004b; Van Ghelder et al., 2010). SSR marker PaCITA5 showed a strong correlation with a PPV resistance gene on LG1 (Lambert et al., 2007; Sicard et al., 2008; Soriano et al., 2008; Lalli et al., 2008; Rubio et al., 2010). However, low resolution of genetic maps hampers the discovery of markers linked to traits of interest. Abbott et al. (2009) estimated that 1 cM of genomic regions on a linkage map could correspond to as little as 100 kb of genome sequence, which might contain approximately 30 genes (Georgi et al.,

2003). Average spacing between the markers larger than 1 cM or more than 10 cM is observed in genetic maps mentioned above, thus raising doubt in the reliability and confidence of detected molecular markers tightly linked to the disease of interest.

Functional markers require various allele sequences of functionally characterized genes. They are derived from the polymorphic sites of the genes whose effects were identified and associated with the plant phenotype (Andersen and Lübberstedt, 2003). The application of functional markers associated with disease resistance in *Prunus* L. is not available yet.

Application of a functional marker, *endoPG*, which is associated with peach fruit texture and adherence facilitates parental and seedling MAS for desirable fruit characteristic suitable for final utilization of peach, canning or fresh consumption. The gene *endoPG* encodes the cell wall pectin-cleaving enzyme known as endopolygalacturonase controlling fruit softening (melting/non-melting and freestone/clingstone) in peach, apricot, and plum (http://www.rosaceae.org/node/176). Over 12 alleles were discovered in the *Freestone-Melting flesh* locus, allowing functional markers derived from *endoPG* alleles to establish association profiles with different peach cultivars (Peace et al., 2007).

With the discovery of more disease resistance genes, more functional markers should become available for disease resistance MAB. For example, an R gene *Ma/TNL 1* in plum was reported, conferring nematode resistance (Claverie et al., 2011), and cloning

of *Rm2* gene resistance to green peach aphid is also under the way (Lambert and Pascal, 2011).

MAS/MAB will supplement but not replace traditional breeding methods, and will likely be most useful for traits that are controlled by few loci and that are either expensive or difficult to evaluate by classical methods (Lande, 1992). For some pathogens, it may be difficult to provide conditions that provide uniform infection for precise screening—but such conditions must be provided when identifying marker loci. Theoretically, MAS is superior to conventional methods if the fraction of the additive variance explained by the markers exceeds the narrow sense heritability of the trait (Dudley, 1993). However, most traits in fruit and nut crops are highly heritable (Hansche, 1983). Therefore, linkage maps facilitate the identification and localization of genes controlling important traits, subsequently allowing marker-assisted selection and positional cloning of genes (Staub et al., 1996; La Rosa et al., 2003).

## Project objectives

The overall objective of this project was to develop a genetic linkage map based on 'Clayton' x 'O'Henry' segregating population to facilitate mapping of quantitative trait loci associated with bacterial spot resistance in peach with the ultimate goal of enabling MAS for leaf and fruit bacterial spot resistance in peach. The specific objectives are:

1) Development of a genetic linkage map using an $F_2$ population segregating for bacterial spot resistance;

2) Development of a phenotyping protocol and collection of field data for leaf and fruit response to bacterial spot;

3) Using a genetic linkage map and phenotypic data to detect QTL(s) associated with bacterial spot resistance in leaf and fruit;

4) Perform comparative analyses using detected putative QTL region(s) and available peach genomic resources to discover tightly linked DNA markers and /or candidate resistance genes associated with bacterial spot resistance in peach.

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CHAPTER 2


BACTERIAL SPOT (*XANTHOMONAS ARBORICOLA* PV. *PRUNI*) RESISTANCE IN

CLAYTON X O'HENRY PEACH POPULATION


**<u>Introduction</u>**

Bacterial spot, caused by *Xanthomonas arboricola* pv. *pruni (Xap)*, is a severe disease of *Prunus* spp. across the world. Particularly, the fruit crops almond, peach, cherry, plum, and apricot are the main targets of *Xap* (EPPO, 1997). Bacterial spot was first described in 1902 on plums in North America (Smith, 1903), and it is referred to as bacterial leaf spot, shot-hole, and black spot. Different disease symptoms were observed on leaves, twigs, and fruits, weakening the vigor of the tree year by year, and decreasing the fruit quality and production severely (Ritchie, 1995).

In peach, the source of *Xap* primarily resides in the intercellular spaces of the cortex, phloem, and xylem parenchyma towards the tips of twigs over the winter.  In the spring, this source of *Xap* starts multiplying from the intercellular spaces, initiating the primary inoculum from the twigs as a spring canker (EPPO, 1997). Inoculum of these cankers is dispersed by rain, wind, or wounding to infect the new growth leaves. Then lesions developing from these infected leaves exude the multiplied *Xap* to initiate secondary infections. A season with high temperatures and frequent rains accompanied by fairly heavy winds and heavy dews always favors severe infection (Ritchie, 1995).

Infection of a peach leaf can give a small, pale-green to yellow, circular or irregular water-soaked lesions (Ritchie, 1995). Most obvious symptoms are yellow, chlorotic leaves with grayish colored and angular spot lesions, formed when water droplets aggregate at the leaf tip, mid-rib, and/or along leaf margin. Lesions might enlarge and coalesce into larger shot holes, causing tattered and dark brown leaf appearance (Ritchie, 1995). Later in the season, mechanical or pesticide spray damages on foliage might mimic the disease symptoms (Ritchie, 1995).

Fruit infection happens after petal fall usually starting as a small circular brown spot. The margins of lesions are frequently water-soaked, often with light-green haloes (EPPO, 1997). When the fruit is growing, small lesions on the fruit surface may merge to form large surface cracks or sunken deep pitting into the fruit flesh (Ritchie, 1995). Light yellow gum flow, particularly after rain, may occur from bacterial wounds; which may be confused with insect damage that has clear gum color (Agrios, 2005).

Control of bacterial spot disease currently relies on pesticides, such as copper-containing compounds, and antibiotics, such as oxytetracycline. However, inappropriate application of copper compounds can cause foliage damage in peach, resulting in grayish discoloration, shot holes, and premature leaf drops (Ritchie, 1995). If coupled with the environmental and economic concern of oxytetracycline use, chemical protection can be limited in the orchard. Generally, once bacterial spot is established in the orchard, control of the disease is very difficult, especially for highly susceptible cultivars when favorable environmental conditions remain (Ritchie, 1995). Therefore, planting resistant peach

cultivars is a better choice. Many resistant cultivars have been developed in public breeding programs, the most resistant of which were 'Candor' and 'Clayton' from the University of North Carolina (Okie, 1998). However, none of them have excellent fruit quality required by producers and desired by consumers.

The agronomic importance of incorporating durable resistance with high fruit quality in newly developed cultivars resulted in substantial research in elucidating genetic control of disease resistance. Disease resistance in plants is mostly quantitative in nature and is associated with many genes of various effects (Young, 1996). Therefore, mapping quantitative trait loci (QTL) for disease resistance in plants became focus of many studies (Scorza and Sherman, 1996; Abbott et al., 2007; Hancock et al., 2008), and is one of the main objectives in breeding programs. For elucidation of genetic control and detection of genes or QTLs associated with trait of interest, beside genetic linkage map phenotypic data are of utmost importance. Obtaining informative and reliable phenotypic data for disease response in field conditions is not an easy task. Visual estimation of disease incidence or severity is the main method that has been used since 19th century (Cobb, 1892). Results drawn from the subjective method could be affected by different factors, including difference in the experience level of person who is collecting phenotypic data (O'Brien et al., 1992; Nutter et al., 1993; Nita et al., 2003; Bock et al., 2008). In addition, in a host plant population the disease incidence or severity of each individual is also interfered by the environmental factors such as temperature, humidity, and distribution of pathogen inoculum.

Several types of rating scales comprising continuous or discrete variables (Sheskin, 1997) are used to measure disease severity: nominal or descriptive scales, category scales, and ordinal rating scale. In nominal or descriptive scales, disease is graded into two or three classes with descriptive terms such as "susceptibility", "tolerance", or "resistance". For some plant diseases, symptoms are observed with percent area, which is generally rated using category scales. For example, this method was applied to define disease severity of *Plum pox virus* on *Prunus davidiana* (Decroocq et al., 2005; Marandel et al., 2009; Rubio et al., 2009). Plants were assigned a severity rating based on 5-category scale from 0-4, where 0 = healthy, 1 =slight resistant, 2 = moderate resistant, 3 = moderate susceptible, and 4 = susceptible. Diease severity was also assessed for leaf symptoms. An ordinal scales grades the disease severity into arbitrary classes that represent the increasing severity of symptoms. This method is quite widely used for diseases, such as those caused by viruses that are not easy to quantify (Madden et al., 2007). It allows staging the disease development as the symptoms become increasingly severe, and also provides a rapid way for evaluators to assess a large number of plants in a breeding program (Bock et al., 2010).

The objective of this study was to assess *Xap* response in parents and $F_2$ progeny segregating for *Xap* resistance to facilitate discovery of QTLs associated with *Xap* resistance in peach.

## Materials and methods

### Plant material

Phenotyping evaluation for *Xap* was performed on a $F_2$ segregating population of 188 hybrids originating from a 'Clayton' x 'O'Henry' cross (hereafter referring to C x O population), where 'Clayton' is highly resistant to *Xap* for botht leaf and fruit, and 'O'Henry' is highly susceptible to *Xap* for leaf and fruit. Self rooted cuttings were used to establish the plantings in three replicates at two locations: Clemson University, Sandhill Research and Education Center, Pontiac, SC and North Carolina State University, Sandhills Research Station, Jackson Springs, NC. One replicate was kept as a backup at the ARS-USDA Southeastern Fruit and Tree Nut Laboratory at Byron, GA. All the 188 individual accessions were planted in two rows, each representing a replicate, with 3ft spacing between the trees and 12ft between the rows and standard horticultural practices were applied.

### Phenotypic evaluation

Bacterial inoculum was prepared by growing *Xanthomonas arboricola* pv. *pruni* (*Xap*) on the agar medium (sucrose peptone, PDA, nutrient agar, and 1% glucose or sucrose) for 36-48 hours. The cultures were washed off from the media with sterile water and bacterial suspension with the optical density of 1.0-1.5 or greater (600 nm) was prepared. The bacterial spot (*Xap*) suspension was prepared and applied during first year in each location, 2008 for NC and 2009 for SC, on the top of each young tree (approx. two year old) in early spring from late petal fall to shuck split to ensure presence of inoculum in each tree. Field response to *Xap* infection on leaf and fruits was assessed as

explained in Yang et al. (2011) (Table 2.1; Figures 2.1 and 2.2). In detail, leaf symptoms were evaluated once a month from May to July during two seasons in NC (2008 and 2009) and SC (2009 and 2011).

Fruit symptoms were evaluated once in June, and severity of infection was recorded (Figure 2.2). No data was collected on leaves and fruit at the GA locations in this period (2008 to 2011). Phenotypic data were organized in datasets as explained in Rubio et al. (2010). The number of individuals in each data point is summarized in Appendix I

**Table 2.1.** Phenotypic scoring used to assess bacterial spot (*Xap)* infection on peach leaf and fruit.

| Class | *Symptoms | |
|---|---|---|
| | **Leaf** | **Fruit** |
| 0 | No leaves with symptoms | No fruits with symptoms |
| 1 | 1-5% diseased leaves or observed defoliation | 1-5% fruit surface with spot lesions |
| 2 | 6-10% diseased leaves or observed defoliation | 6-10% fruit surface with spot lesions |
| 3 | 11-25% diseased leaves or observed defoliation | 11-25% fruit surface with spot lesions |
| 4 | 25-50% diseased leaves or observed defoliation | 25-50% fruit surface with spot lesions |
| 5 | > 50% diseased leaves or observed defoliation | > 50% fruit surface with spot lesions |

*Note: For the purpose of genetic study of *Xap* resistance in peach, the phenotypic scoring for leaf was based on all leaves symptoms on each individual tree. The phenotypic scoring for fruit was based on the most severe individual fruit symptom on each individual tree.

**Figure 2.1.** Bacterial spot (*Xap*) affecting peach tree - leaf symptoms. 0, highly resistant peach tree; 5, highly susceptible peach tree.

. Statistical analysis

Descriptive statistics, mean and standard deviation of leaf and fruit phenotypic data were calculated using IBM® SPSS® Statistics (19.0.0, 2010). The ratings (i.e., 0-5) were averaged across disease evaluation cycles, replications, years, and locations. ANOVA (P< *0.05*) was used to compare the mean scores of all individual accessions.

Weather conditions, including temperature and humidity data for 2008, 2009, 2010, and 2011 for SC (Zip code 29045) and NC (Zip code 27218) were collected from the local weather stations.

**Figure 2.2.** Bacterial spot (*Xap*) symptom severity on peach leaf and fruit. A. Leaf symptoms are shown in six different severity categories based on 0-5 scale, with 0 – no symptoms; 1 –water soaked lesions; 2 – tattered patterns on the leaf tip and leaf rib; 3 – coalesced water-lesion and shot holes; 4 – yellow leaf and 5 – premature leaf drop; b. 0-5 scale applied for fruit evaluation.

## Results

### Phenotypic data evaluation on bacterial spot resistance

Phenotypic evaluation of the bacterial spot incidence in the peach C x O segregating population and parents showed variability between years and locations. Leaf disease incidence data collected in SC were more severe in 2011 than 2009 considering overall performance of the progeny (Figure 2.3). More than half of the progeny were considered highly resistant/tolerant (0 and 1) after the first year of assessing the disease incidence in SC. The initial 60% (i.e., 96) of individuals considered resistant or highly tolerant (class 0 and 1) in 2009 decreased to 2% (3) in 2011, while the number of individuals in highly susceptible classes 4 and 5 increased from 8 (5%) in 2009 to 94 (61%) in 2011. A similar situation was observed between years in the NC phenotypic data, where leaf response for *Xap* incidence during 2008 was slightly more severe than in 2009 (Figure 2.4). During these two years, the percentage of resistant individuals (clasees 0 and 1) increased from 14% (i.e., 16) in 2008 to 18% (i.e., 19) in 2009, while the number of individuals clustered in classes 4 and 5 decreased from 8 (7%) in 2008 to 2 (2%) in 2009. On the other hand, leaf symptoms in NC were recorded only twice in 2009 due to inability to differentiate between bacterial spot and other damage on leaf tissue caused by abiotic and biotic factors, i.e. mechanical damages, nutrition deficiencies, or other disease symptoms. There were ten more individuals clustered in resistant/highly tolerant classes (0 and 1) in 2009 than in 2008, but the average number of individuals decreased from 11 to 3 in 2008 and 2009, respectively. In addition, highly significant

differences (P< *0.001*) between two years of leaf *Xap* incidence were observed in both

SC and in NC data (see Appendix II).
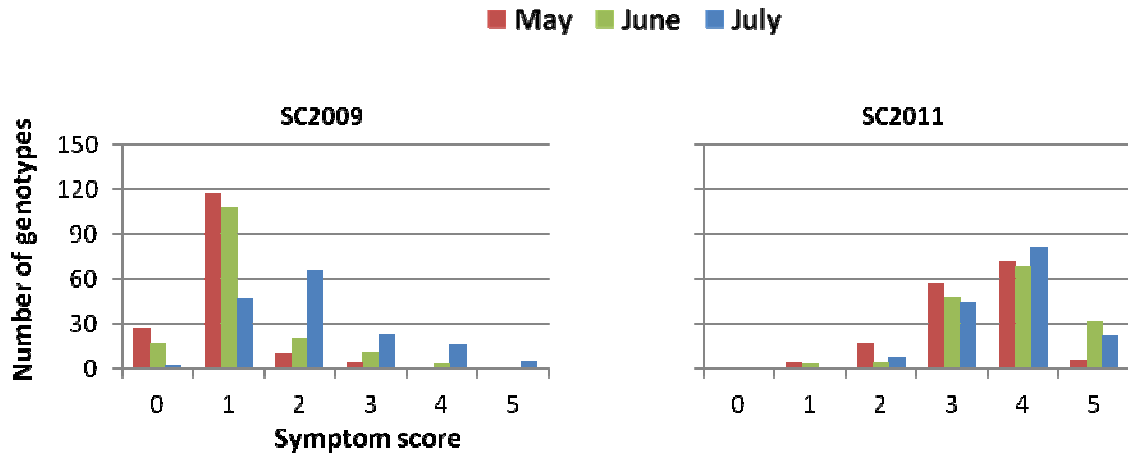


**Figure 2.3.** Distribution of progeny from C x O segregating population in different disease severity classes based on leaf response to *Xap* in South Carolina in two years, 2009 and 2011. Scores of each class and the number of genotypes are represented at the *x* and *y* axes, respectively. Each data point represents an average of two replicates of disease score for leaf recorded in May, June, and July. SC, South Carolina

Leaf response to *Xap* infection for C x O population evaluated in SC became more severe as the season progressed in both evaluation years (2009 and 2011). During 2009, the average number of individuals recorded as resistant/highly tolerant, classes 0 and 1, gradually decreased from 145 (91%) in the first cycle, to 125 (79%) and 50 (31%) in the second and third cycle, respectively. At the same time, the average number of progeny in highly susceptible classes (4 and 5) increased from 1 in the first cycle, to 3 in the second, and 19 in the third cycle. During the second evaluation year, 2011, very few progeny (2%) exhibited resistance/high tolerance to leaf *Xap* incidence (Figure 2.3).

Moreover, more than 50% of progeny were classified as moderately susceptible (classes 2 and 3) to highly susceptible (classes 4 and 5) at the beginning of the season, or the first evaluation cycle completed in May.
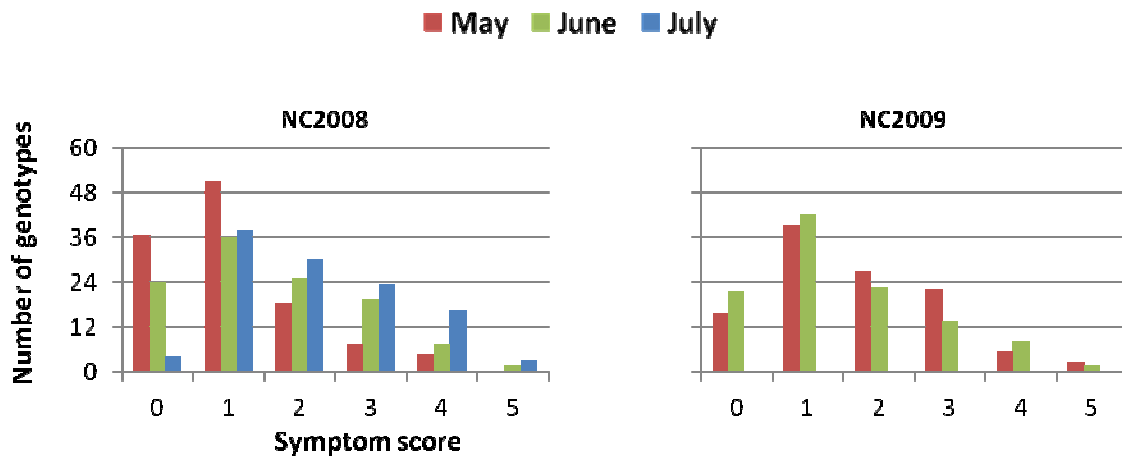


**Figure 2.4.** Distribution of progeny from C x O segregating population in different disease severity classes based on response to *Xap* in North Carolina in two years, 2008 and 2009. Scores of each class and the number of genotypes are represented at the *x* and *y* axes, respectively. Each data point represents an average of two replicates of disease score for leaf recorded in May, June, and July. NC, North Carolina.

Leaf response to *Xap* infection throughout the season in NC showed similar a trend to that observed in SC (Figure 2.4). Statistically significant differences (P< *0.05*) were detected among each cycle in the SC A replicate in both 2009 and 2011; B replicate in 2009, and in NC C replicate in 2008, and D replicate in both 2008 and 2009 (See Appendix II).
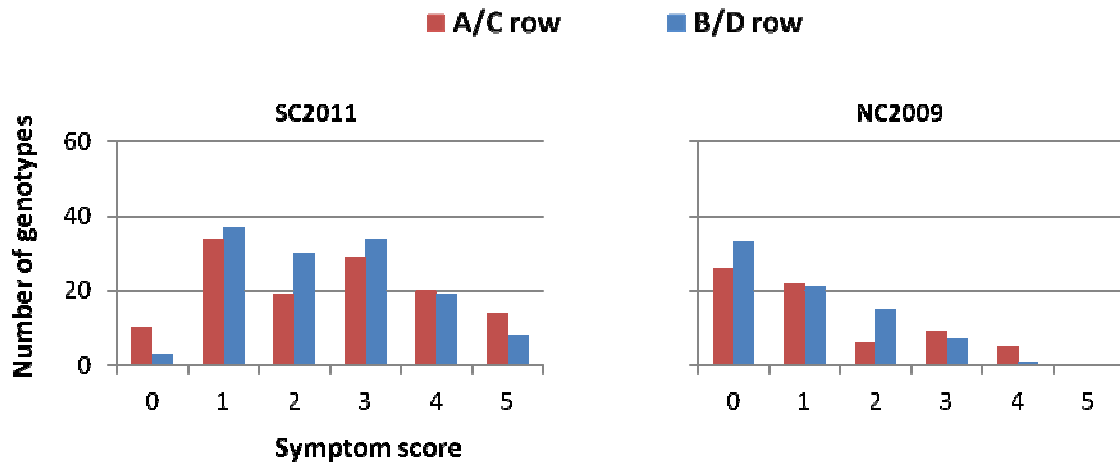
**Figure 2.5.** Distribution of progeny from C x O segregating population in different disease severity classes based on fruit response to *Xap* at SC and NC in 2011 and 2009, respectively. Scores of each class and the number of genotypes are represented at the *x* and *y* axes, respectively. Each data point represents one cycle of disease evaluation on fruit from A, B, C, and D rows, repectively. A and B rows are from SC and C and D rows are from NC. Each row represents one replicate. SC, South Carolina; NC, North Carolina.

Effects of location on leaf response to *Xap* infection were obvious between SC and NC in 2009, as the data points revealed highly significant difference (P< *0.001*) (See Appendix II). None of the accessions were highly leaf resistant/ tolerant (classes 0 and 1) after 2 years of evaluations in SC. However, 15 accessions had consistently exhibited high susceptibility (classes 4 and 5) to *Xap*. Highly resistant/tolerant fruit (classes 0 and 1) were observed in eighteen accessions, while eleven trees exhibited high susceptibility (classes 4 and 5).

In NC, after two years of evaluation, 16 trees with consistently high resistance/tolerance (classes 0 and 1) and 8 trees with consistently high susceptibility

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(classes 4 and 5) were observed. The number of individual trees with consistently high resistant/tolerant and high susceptibility was 19 and 2, respectively. Overall the percentage of highly resistant individuals and highly susceptible individuals varied in different disease evaluation cycles, replicates, years, and locations (Figure 2.4 and 2.5). The proportion of C x O population classified in the extreme classes, highly resistant and highly susceptible, for leaf data varied from 0.6% to 20% and from 0.6% to 49%, respectively. However, proportion of C x O population with highly resistant fruit was 5% in SC (2011), and 40% in NC (2009). On the other hand, the proportion of the C x O population having highly susceptible fruit ranged from 6% in A to 10% in B replicate in SC in 2011. However, no progeny with highly susceptible fruit to *Xap* infection was observed in NC in 2009.

A few individual accessions performed consistently across different years and locations. The accession 076 showed consistently moderate resistance across seasons and locations. Ten individual accessions exhibited low *Xap* incidence on fruit in both SC and NC, but only one, accession 031, was scored "0" in both locations. The symptom on the leaf of the highly resistant parent 'Clayton' varied from "1" to "3" in SC (2011) and NC (2009), nevertheless no symptom on the fruit were observed (data not shown). At the same time, the highly susceptible parent, 'O'Henry', exhibited high leaf and fruit susceptibility to *Xap* in both locations and all seasons (score ≥3).
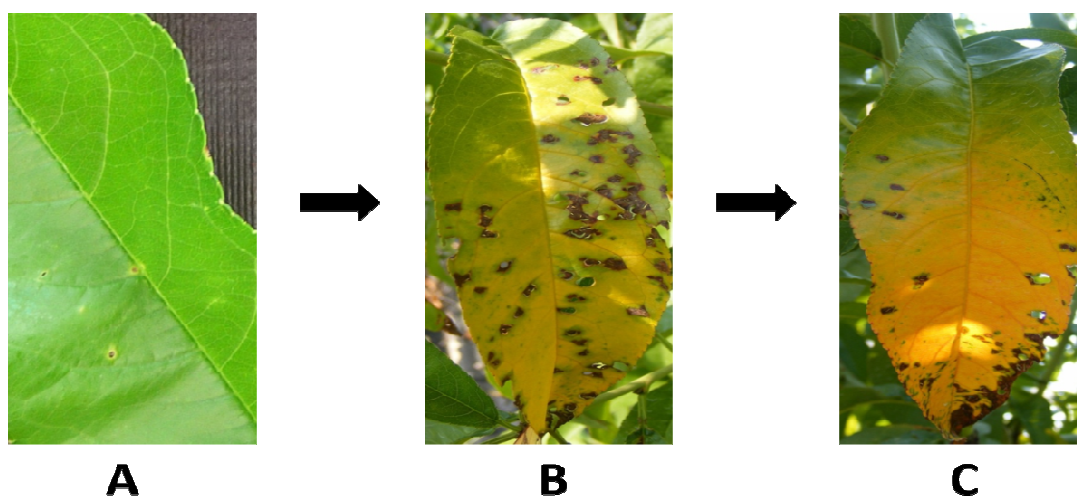
**Figure 2.6.** Leaf symptoms caused by bacterial spot in different development stage. A. Water-soaked foliar lesions are formed on the new emerged leaves; B. As the lesion continues, the lesion centers may become dark or purple in color and necrotic, or form a shot-hole appearance; C. In the final stage, infected leaves become chlorotic and yellow and prematurely drop.

The level of *Xap* disease severity varied in different plant growth stage. Primary infection spread on newly growing leaves in late spring or early summer, producing water-soaked lesions on the leaf blades (Figure 2.6A). After the bacteria were transmitted by wind, rain, or insects, those lesions formed in the early stage, coalesced and enlarged to form shot holes and the tattered leaf surface (Figure 2.6B). Chlorotic and yellow leaves were observed at the end of July, and were generally accompanied with the premature leaf drop (Figure 2.6C). This was consistent with the phenotypic rating results, in which values of the rating scores increased from the first to the third evaluation cycle (Figure 2.3 and 2.4).

Leaf and fruit symptoms on the same tree did not necessarily exhibit the same disease severity level. In some cases similar severity level of *Xap* incidence was observed on both leaf and fruit on the same accession, for example 031 and 192. *Xap* incidence on different fruits on the same tree did not always reach the same severity level (Figure 2.7A) and different response to *Xap* infection and disease development were observed on leaf and fruit for some accessions (Figure 2.7B), such as 98, 111, 112, 116, and 180, where fruit appeared to be resistant to *Xap* but leaves were not (see Appendix I).



**Figure 2.7.** Leaf and fruit symptoms caused by *Xap*. A. Variability in *Xap* inoculum distribution results in different symptom severity on two peach individual fruits; B. Different response to *Xap* observed on leaf (3) and fruit (0).

## Weather conditions anlaysis

Temperature and relative humidity along with the presence of bacterial inoculum are important factors for disease establishment and development. The average temperature recorded during March – May period did not show much variation and had

shown similar fluctuation in the evaluation years at the two locations (Figure 2.8 and 2.9).
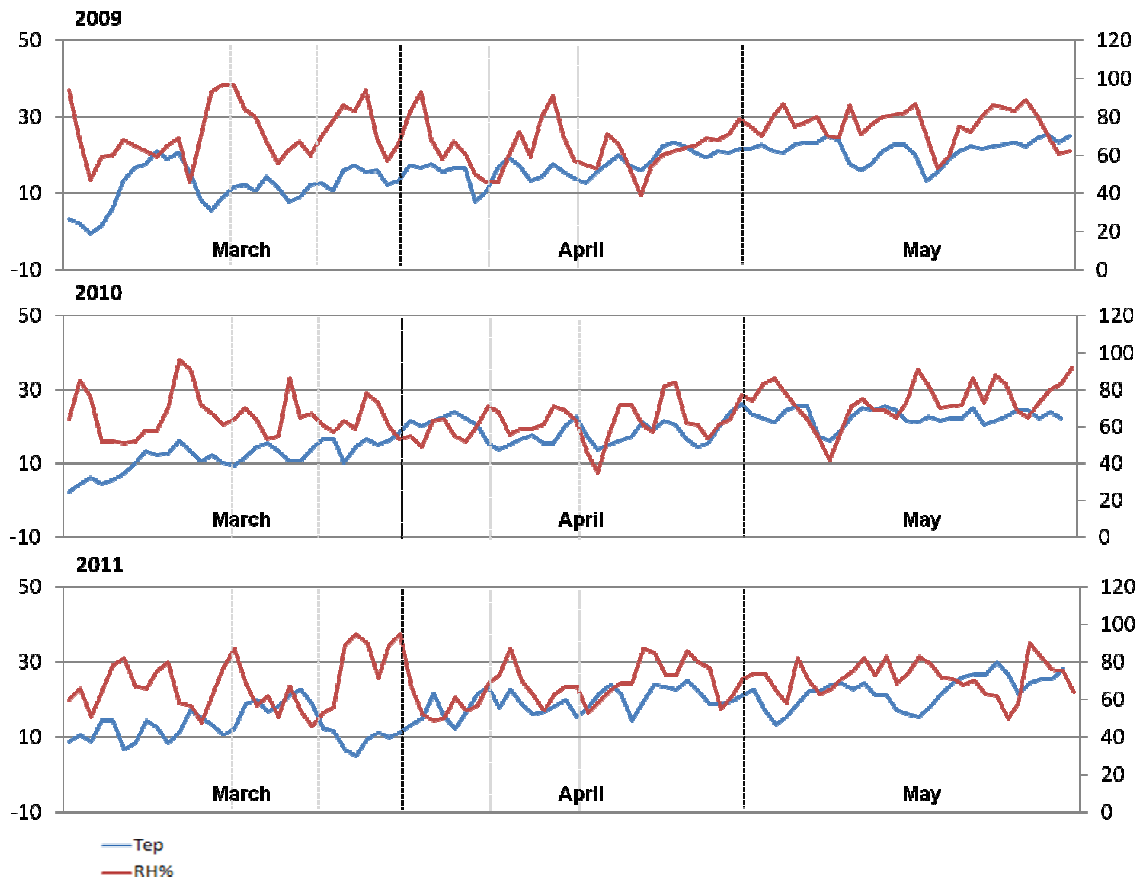


**Figure 2.8.** Average temperature and relative humidity variation from March to May (2009-2011) in SC. The blue line represents the average temperature (T), and the red line represents the average relative humidity (RH%).

The average relative humidity however was variable during the March – May period in evaluation years at research locations in both SC and NC. During the first two weeks of March, relative humidity above 80% was observed in all three years in NC (2008, 2009, and 2010), and SC (2009, 2010, and 2011) (Figure 2.8 and 2.9). Relative

humidity above 80% in the period from March 15th to April 15th was recorded in NC in 2008 and 2009 and in SC in 2009 and 2011. In the same period during 2010 at both NC and SC locations, the recorded average relative humidity was less than 60%.
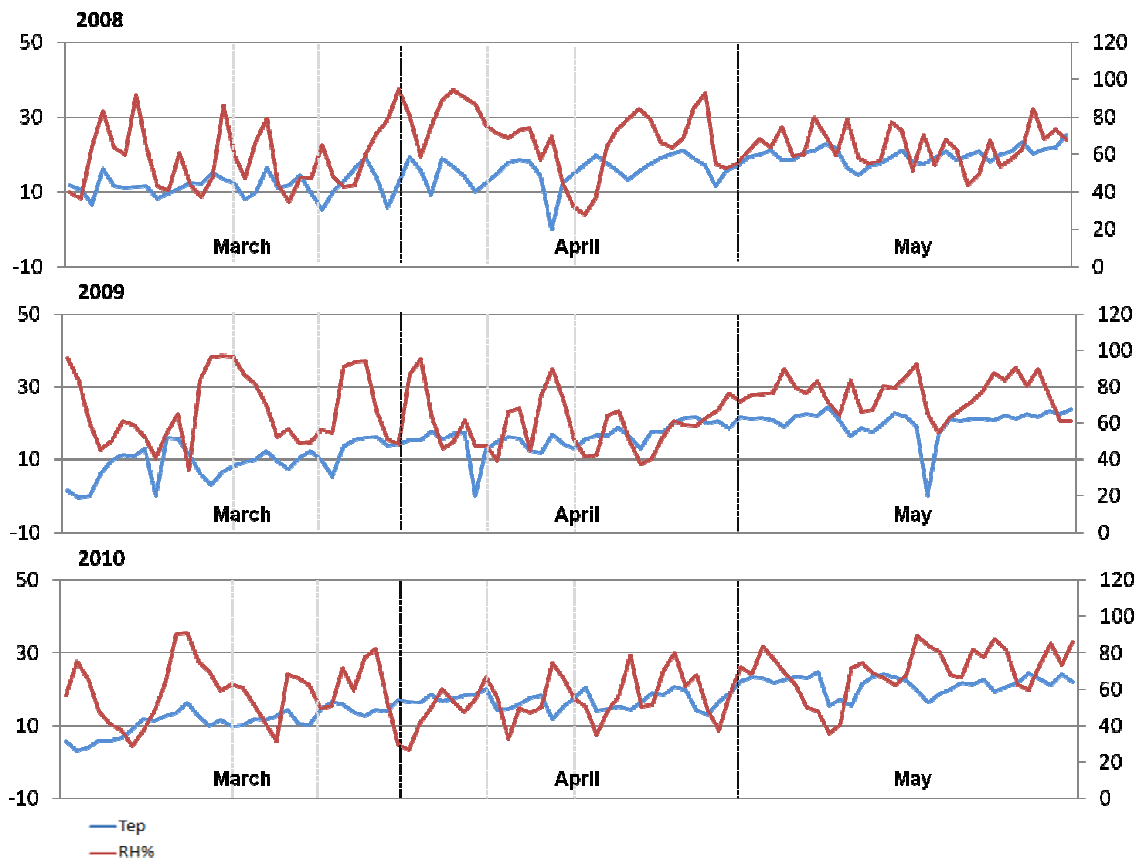


**Figure 2.9.** Average temperature and relative humidity variation from March to May (2008-2010) in NC. The blue line represents the average temperature (Tep), and the red line represents the average relative humidity (RH%).

However, from April 16th to May 31st, relative humidity was generally above 80% in both SC and NC (Figure 2.8 and 2.9). In an average year in the period from the second week of March to second week of April, a peach tree undergoes phenological

phases from petal fall to shuck split. *Xap* infection and symptom development have been observed in NC in 2008 and 2009, and in SC in 2009 and 2011 seasons, while no disease symptoms were observed in 2010 at both locations.

## Discussion

Assessing *Xap* resistance in peach was based on visual observation of symptoms on the leaf and fruit. Disease establishment and development is dependent on environmental conditions, such as humidity, wind, temperature, abiotic stress, and presence of inoculum in the period from petal fall to shuck split developmental stages of peach (Ritchie, 1995). Temperature and relative humidity were considered as two main factors in each season. Temperature does not seem to be very important in the early stages of infection as much as it is later for disease spread. In our study *Xap* disease symptoms were observed in all experimental years except 2010 in both SC and NC. That probably was due to higher than 80% relative humidity was present from March 15th to April 15th in 2008 and 2009 (NC), and 2009 and 2011 (SC). In addition, among the leaf data points, there were more individuals categorized in classes 4 and 5 in 2011 than 2009 in SC (Figure 2.3). This trend was also observed in NC, where more individuals clustered in classes 4 and 5 in 2008 than 2009 (Figure 2.4). Taking all together, this indicated the four weeks from March to April could affected the disease severity. The parameter, higher than 80% relative humidity from March 15th to April 15th could be useful to predict *Xap* disease incidence and severity in NC and SC. Therefore, disease incidence observed during our experiment confirmed that relative humidity above 80% was of utmost importance for disease infection. Moreover, since the rainfall frequency is considered as another important factor accounting for the occurrence of disease (Dr. Ritchie, pers communication), more investigation needs to be evaluated on the precipitation from March to May in the future.

We propose that *Xap* resistance in peach is expressed as a quantitative trait and leaf and fruit resistances are regulated by different genetic factors. 'O'Henry' is a cultivar highly susceptible to *Xap* infection in both leaf and fruit, and 'Clayton' is highly resistant to *Xap* in both leaf and fruit (Okie, 1998). Therefore, the resistant and susceptible characters should follow the Mendelian inheritance in their progeny. As expected, all the 24 data points collected from the $F_2$ progeny, showed continuous distributions from 0-5 scale, suggesting that several genetic factors might be involved in *Xap* resistance. In addition, since the percentage of highly susceptible individuals varied from 0.6% to 49% in leaf data points, whereas the percentage varied from 6% to 10% in fruit data points, suggesting that three to seven genes are estimated to be involved in leaf resistance, and three to five genes in fruit resistance. We only found few individuals that showed consistent resistance or susceptibility for both the leaf and fruit on a same individual accession to *Xap* infection. It is probably because leaf and fruit resistance or susceptibility is regulated by separate gene (s). Such an assumption is in agreement with Werner et al. (1986).

Unreliable disease assessments might lead to incorrect conclusions of QTLs being drawn from the phenotypic data, which in turn mislead to the wrong actions being taken in molecular assisted breeding (Poland and Nelson, 2011). Rating method therefore is critical for QTL mapping, and a strong attention was given this point to make sure the scoring was careful to obtain precision and accuracy of the disease level. Visual observation of symptoms is suggested to be more accurate (Bardsley and Ngugi, 2010), since it has been practiced and understood in evaluation of disease severity over 100

years (Cobb, 1892). However, inconsistent evaluation of leaf disease incidence was observed between replicates and locations. Leaf data points showed significant difference (P< *0.05*) between replicates in SC (2009) and NC (2008), and as well as the fruit data points in SC (2011) (Appendix II). Such discrepancy might due to that the disease incidence was evaluated by two different raters in SC (2009), or the possible environmental effects on the replicates for the evaluation of leaf disease incidence in NC (2008) and of fruit disease incidence in SC (2011). Such discrfepancy was also noticed in several other studies (Bock et al., 2008; Nita et al., 2003; Nutter et al., 1993; O'Brien et al., 1992). However, Poland and Nelson (2011) indicated that such a variation might differentiate the later estimated QTL effects, but will not affect the accurate detection of QTLs positions.

Observed decreasing average number of individuals in highly resistant/tolerant classes (0 and 1) and the increasing average number in highly susceptible classes (4 and 5) from the first to the third cycle in both SC and NC suggests that the *Xap* disease severity is cumulative which is in agreement with the fact that disease becomes more severe at the end of the peach growing season (Ritchie, 1995). Therefore, the ordinal scale method was adequate for our phenotyping, as evidenced from the reports by Madden et al. (2007) and Bock et al. (2010). A recently reported PCR method to quantify *Xap* incidence from naturally infected symptomatic or asymptomatic peach materials (Pagani, 2004; Palacio-Bielsa et al., 2010) would be a valuable addition to visual observation data.

## Conclusions

An ordinal scale of 0-5 was applied in this study to collect the phenotypic data for leaf and fruit response to *Xap*. Continuous distribution of level of susceptibility in individuals was observed for all 26 leaf and fruit data points. In addition, only a few trees show consistent resistance or susceptibility for both leaf and fruit to *Xap* infection in different years and locations. Overall, the conclusion is that *Xap* resistance in peach is a quantitative trait, and separate genetic factors control the resistance of leaf and fruit to *Xap*. We estimate that three to seven genes might be associated with leaf resistance, and three to five genes for fruit resistance to *Xap* in peach. In addition, relative humidity higher than 80% from petal fall to shucks off (generally from March 15th to April 15th) is suggested to predict *Xap* disease incidence and severity.

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CHAPTER 3

CONSTRUCTION OF A PEACH GENETIC LINKAGE MAP USING SIMPLE

SEQUENCE REPEAT (SSR), RESISTANCE GENE ANALOGS (RGAS) AND

SINGLE NUCLEOTIDE POLYMORPHISM (SNP) MARKERS

## **Introduction**

Peach [*Prunus persica* (L.) Batsch] is the second economically most important

fruit crop in US, and is the most important in the genus *Prunus*. Peach is a diploid (2n =

16), self-compatible autogamous species, with a small haploid genome ~220 Mbp, almost

twice the size of Arabidopsis (Baird et al., 1994; Sosinski et al., 2009). It is genetically

the best-characterized species in the genus *Prunus* and Rosaceae family. Peach breeding

is time consuming and labor-intensive, due to juvenility and space required for growing

trees. Therefore, marker-assisted selection (MAS) of parents and seedlings would be

advantageous for peach breeding, allowing an early and efficient selection of traits long

before they are expressed. If prior knowledge of a linkage relationship between marker

loci and traits of interest were known undesirable individuals could be eliminated from

progeny and more resources could be devoted to the promising genotypes (Stockinger et

al., 1996).

Several genetic maps were developed in peach using molecular markers

(Chaparro et al., 1994; Rajapakse et al., 1995; Abbott et al., 1998; Dirlewanger et al.,

1998; Lu et al., 1998; Yamamoto et al., 2001; Blenda et al., 2007; Ogundiwin et al.,

2009; Fan et al., 2010). Linkage maps were also developed from interspecific progenies: almond x peach (Foolad et al., 1995; Joobeur et al., 1998; Jauregui et al., 2001; Aranzana et al., 2002; Bliss et al., 2002), peach x *Prunus ferganensis* (Quarta et al., 1998, 2000; Dettori et al., 2001; Verde et al., 2005), and *Prunus davidiana* x peach (Foulongne et al., 2003). The Texas (almond) x Earlygold (peach) linkage map (T x E) has become a reference map for *Prunus* (Joobeur et al., 1998; Aranzana et al., 2003; Dirlewanger et al., 2004; Howad et al., 2005; Illa et al., 2010). Although, the position of 21 major genes and 28 QTLs on the *Prunus* reference map are known (Abbott et al., 2008), many important agronomic traits are still not mapped and markers for routine MAS are lacking.

Markers of choice for developing genetic linkage maps are simple sequence repeats (SSRs) and single nucleotide polymorphisms (SNPs). SSR markers are co-dominant, abundant, highly polymorphic and transferable among Rosaceae and especially *Prunus* L. (Gasic et al., 2008; Mnejja et al., 2010; Illa et al., 2011). In addition, even low coverage maps developed with few SSR markers, that are already mapped in other maps are sufficient to serve as framework map (Slate et al., 2008; Ball et al., 2010). SNP markers are the most abundant markers per unit of genome sequence, with estimated frequency of 1/100 and 1/225 in non-coding (intronic) and coding (exonic) sequences in Rosaceae, respectively (Sargent et al., 2009; Illa et al., 2010). Candidate gene approaches have proven useful for finding association between genes involved in relevant metabolic pathways and major genes or QTLs in fruit trees (Dirlewanger et al., 2004). Several resistance gene analogs (RGAs) have been mapped in *Prunus* L. (Bliss et al., 2002) and are placed in similar genomic positions as genes or QTLs that determine disease

resistance such as sharka (Decroocq et al., 2005) and root-knot nematode resistance (Cao et al., 2011). Many resistant genes tend to be clustered in the plant genome (Hulbert et al., 2001) and RGA markers can be used to saturate regions of resistance, so called 'hot spots', and facilitate detection of the resistance gene of interest. The objective of this research was to construct a linkage map in a peach population segregating for several agronomical traits including bacterial spot resistance, and use it to detect genes responsible for the disease resistance and other traits of interest. Existence of such a map would further facilitate development of markers for marker-assisted breeding (MAB) in peach and other *Prunus* L.

## Materials and methods

### Plant material

The $F_2$ mapping population (n = 188) was obtained from selfing a single individual derived from controlled pollination of 'Clayton' x 'O'Henry' (Figure 3.1). Clayton is yellow, melting, freestone peach selected from a 'Pekin' x 'Candor' cross from the North Carolina peach breeding program (Figure 3.1) and is resistant to bacterial spot (*Xanthomonas arboricola* pv. *pruni*). 'O'Henry' is a high quality, yellow, melting and freestone peach that originated in Red Bluff, California in 1968 from Merrill Bonanza O.P. (Okie 1998), and it is highly susceptible to bacterial spot (*Xanthomonas arboricola* pv. *pruni*).

The 'Clayton' x 'O'Henry' population (hereafter referred to as C x O) also segregates for flower type (Sh/sh) and skin pubescence (G/g). 'Clayton' has non-showy flowers, and 'O'Henry' has showy flowers and is heterozygous for skin pubescence. These two phenotypic traits are controlled by a single gene, with non-showy flower (Sh) and pubescent skin (G) being dominant and showy flower (sh) and glabrous skin (g) being recessive (Blake, 1932; Bailey and French, 1949). The mapping population has been maintained in two replicates at two locations: Clemson University Sandhill Research and Education Center, Pontiac, SC, and North Carolina State University, Sandhills Research Station, Jackson Springs, NC; and in one replicate at the ARS-USDA Southeastern Fruit and Tree Nut Laboratory, Byron, GA.
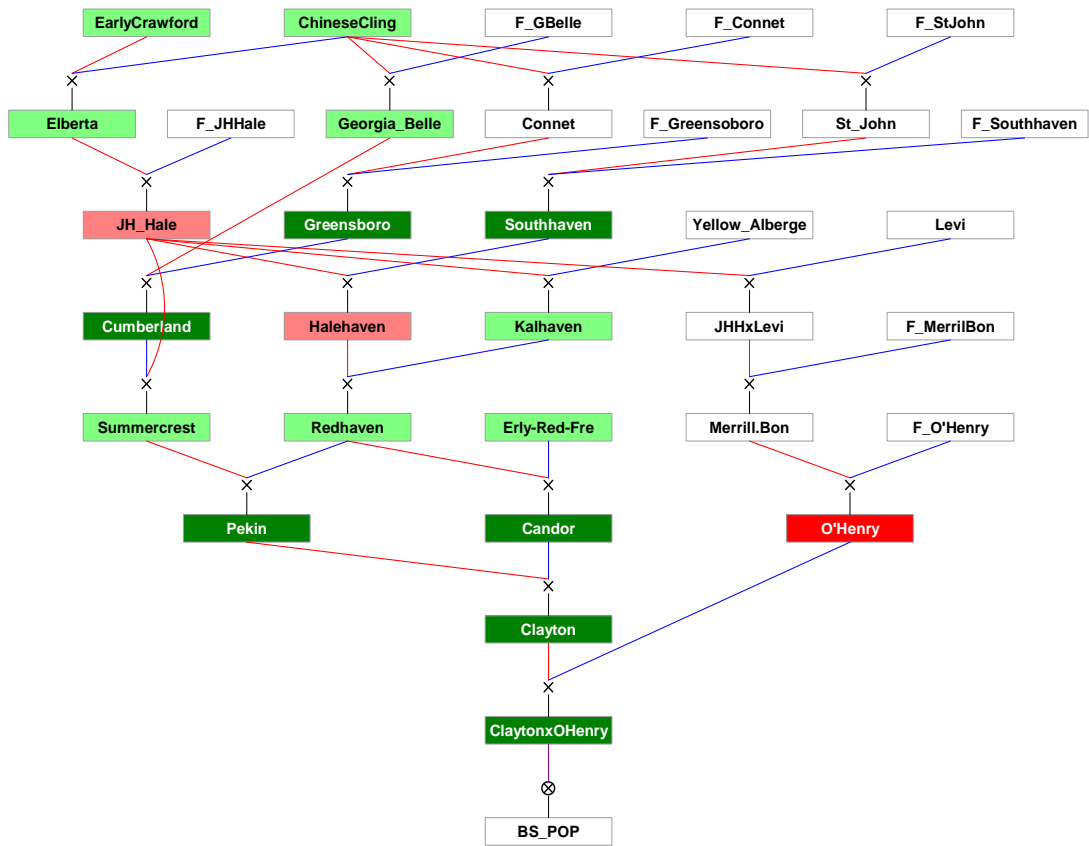
**Figure 3.1.** Pedigree analysis of mapping population showing bacterial spot resistance/susceptibility. Green - resistant; Red – susceptible based on Okie (1998). The greener, the more resistant; the redder, the more susceptible; and white no data available. The map was created by Pedimap (Voorrips, 2007).

**Table 3.1.** Summary of SSR markers used.

| Species | SSR name | Origin | Reference | PCR[1] T | S | M | N | C |
|---|---|---|---|---|---|---|---|---|
| *P. persica* | EPPB | cDNA library | Dirlewanger pers. comm. | 20 | 0 | 15 | 1 | 4 |
| | EPPCU | | GDR[2] | 69 | 3 | 45 | 8 | 13 |
| | EPPISF | | Vendramin *et al.*, 2007 | 16 | 1 | 12 | 0 | 3 |
| | M | | Yamamoto *et al.*, 2000 | 15 | 0 | 8 | 5 | 2 |
| | pchcms | | Sosinski *et al.*, 2000 | 5 | 0 | 2 | 2 | 1 |
| | BPPCT | Genomic library | Dirlewanger *et al.*, 2002 | 41 | 5 | 20 | 11 | 5 |
| | CPPCT | | Aranzana *et al.*, 2002 | 35 | 3 | 19 | 10 | 3 |
| | MA | | Yamamoto *et al.*, 2002; Yamamoto *et al.*, 2005 | 44 | 5 | 31 | 0 | 8 |
| | pchgms | | Sosinski *et al.*, 2000 | 35 | 0 | 22 | 4 | 9 |
| | UDP | | Cipriani *et al.*, 1999; Testolin *et al.*, 2000; Testolin, pers. comm. | 22 | 5 | 10 | 3 | 4 |
| | MD | Gene sequences | Yamamoto *et al.*, 2005 | 7 | 0 | 6 | 0 | 1 |
| *P. armeniaca* | AMPA | cDNA library | Hagen *et al.*, 2004 | 15 | 1 | 12 | 0 | 2 |
| | Pac | | Decroocq *et al.*, 2003 | 10 | 0 | 8 | 1 | 1 |
| | AMPA | Genomic library | Hagen et al., 2004 | 13 | 2 | 8 | 2 | 1 |
| | aprigms | | Lalli *et al.*, 2008 | 9 | 0 | 5 | 2 | 2 |
| | ssrPaCITA | | Lopes *et al.*, 2002 | 22 | 2 | 14 | 3 | 3 |
| | UDAp | | Messina *et al.*, 2004 | 45 | 3 | 26 | 8 | 8 |
| *P. dulcis* | EPDCU | cDNA library | GDR | 12 | 2 | 7 | 2 | 1 |
| | CPDCT | Genomic library | Mnejja *et al.*, 2005 | 20 | 3 | 10 | 6 | 1 |
| | UDA | | Testolin *et al.*, 2004 | 41 | 1 | 26 | 8 | 6 |
| *P. avium* | EMPA | Genomic library | Clarke and Tobutt, 2003 | 21 | 0 | 8 | 3 | 10 |
| | EMPaS | | Vaughan and Russell, 2004 | 14 | 0 | 6 | 1 | 7 |
| | PS | | Joobeur *et al.*, 2000; Cantini *et al.*,2001 | 2 | 0 | 0 | 0 | 2 |
| | UCD-CH | | Struss *et al.*, 2003 | 6 | 0 | 4 | 0 | 2 |
| *P. cerasus* | PceGA | | Downey and Lezzoni, 2000 | 1 | 0 | 1 | 0 | 0 |
| *P. salicina* | CPSCT | Genomic library | Mnejja *et al.*, 2004 | 34 | 2 | 20 | 11 | 1 |
| Total | | | | 574 | 38 | 345 | 91 | 100 |

[1]Results of PCR amplification; Tested – number of tested markers*; S* number of segregating markers; number of monomorphic markers; N, No product, C complex. CPSCT022 and M20a are synonymous of BPPCT014

[2]GDR, Genome Database for Rosaceae

## DNA extraction

Fresh young leaves were harvested, refrigerated during transportation and stored at −80°C until needed. Frozen tissue was grounded in liquid nitrogen using mortar and pestle, and DNA extraction was performed according to Kobayashi et al. (1998). In addition, DNA was treated with RNase A at 37 °C for 30 min. RNA-treated samples were precipitated with isoproponal, and dissolved in 200 µl AE (10 mM TrisCl; 0.5 mM EDTA, pH 8.0) buffer. DNA quantity and quality were measured using a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and confirmed by electrophoresis on 1% TBE (1 M Tris, 0.9 M Boric Acid, and 0.01 M EDTA) agarose gel. Final dilutions of 10 ng/µl were created for PCR reaction.

## Microsatellite and Resistance gene analog markers

A set of SSR markers developed in *Prunus* were tested for their polymorphism between the parents and informativeness in the progeny (Table 3.1). Nucleotide-binding site–leucine-rich repeat (NBS-LRR) resistance gene analog (RGA) sequences from the gene bank (NCBI) were used to develop an additional 48 markers (see Appendix III), and NBS1 – NBS32 markers were acquired from Cao et al. (2011).

## SSR and RGA marker analysis

PCR amplifications were run on two platforms with different conditions for each. For fragment separation on 3% high resolution MetaPhor® (Cambrex Charles City Inc, IA) agarose – 1X TBE gels PCR amplifications were performed in a total volume of 15µl with final concentrations of 50 ng of DNA, 0.2 µM of both primers, 200 µM of each

dNTP (New England Biolabs, Ipswich, MA), and 0.5 U of New England Biolabs Taq DNA polymerase in 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$ and 50 mM KCl. For fragment detection using the ABI 3130 (Applied Biosystems, Foster City, CA), PCR conditions were the same as above with the exception of 0.02 µM of M-13–tagged forward primer, 0.2 µM of reverse primer and 0.2 µM of M-13–tagged dye (6'-FAM, VIC, NED, or PET) (ABI). Thermo Scientific MBS Satellite Thermal Cyclers (Thermo Fisher Scientific, Waltham, MA) thermocyclers were used. The conditions used were 3 min of initial denaturation at 94°C, 1 min at 94°C, 1 min at annealing temperature (Ta), and 1 min at 72°C for 35 cycles, then a final extension step of 5 min at 72°C for all primer combinations. When performing PCR reaction for multifluorophore fragment analysis, the above conditions were followed except for primer pairs with Ta significantly lower than 58ºC (Ta for M-13 forward primer), when additional 4 cycles are performed at the annealing temperature of the SSR marker followed by 35 cycles at the annealing temperature of the M-13, as described above. PCR amplicons were visualized on either 3% MetaPhor® - 1X TBE agarose gels along New England Biolabs low molecular weight DNA marker with ethidum bromide under UV light, or pooled together (4 different fluorophore), cleaned up with ExoSAP-IT (USA Scientific or USB) according manufacturer protocol and run on ABI 3130 with GeneScan™ 600 LIZ® (Applied Biosystems) internal size standard. Polymerase chain reaction products separated on agarose gel were analyzed visually and for those separated on the ABI 3130, Gene Mapper V.4.0 (Applied Biosystem) was used for genotype scoring.

## DNA isolation and SNP genotyping

Isolation of genomic DNA and subsequent Infinium assay was performed as explained in Verde et al. (2012). In short, genomic DNA was isolated from fresh young leaves of 63 C x O progeny using the E-Z 96 Tissue DNA Kit (Omega Bio-Tek, Inc., Norcross, GA, USA), and quantitated with the Quant-iT™ PicoGreen® Assay (Life Technologies, Grand Island, NY, USA), using the Victor multiplate reader (Perkin Elmer Inc., San Jose, CA, USA). Concentrations were adjusted to a minimum of 50 ng/µl in 5 µl aliquots and submitted to the Research Technology Support Facility at Michigan State University (East Lansing, MI, USA) where the Infinuium assay was performed following the manufacturer's protocol (Illumina Inc.). After amplification, PCR products were hybridized to VeraCode microbeads via the address sequence for detection on a VeraCode BeadXpress Reader. SNP genotypes were scored with the Genotyping Module of GenomeStudio Data Analysis software (Illumina Inc.). A GenTrain score of >0.4 and a GenCall 10% of >0.2 were applied to remove most SNPs that did not cluster (homozygous) or had ambiguous clustering. SNPs homozygous for alternate allele in two parents as well as SNPs homozygous in one and heterozygous in other parent were considered for mapping. $F_2$ population type codes were applied (Van Ooijen et al., 2006).

## Linkage map construction using SSR and RGA markers

Genetic linkage map of the 'Clayton' x 'O'Henry' segregating population was generated using Mapmaker/exp 3.0 software (Lincoln et al., 1992) as explained in Yang et al. (2011). Kosambi mapping function was used for markers linkage analysis. Chi-square test was applied to calculate the segregation distortion of individual marker ($P <$

*0.05*). Linkage groups were established using default parameters and a recombination fraction of 0.30. Finally, this map was compared to T x E reference map (Dirlewanger et al., 2004).

Linkage map construction using SSR and SNP markers

Linkage analyses were performed using JoinMap 4.1 (Van Ooijen et al., 2006) and R/QTL package (Broman, 2003). The deviations from Mendelian ratio were tested using chi-square-goodness-of-fit test (P< *0.05*) available in JoinMap 4.1. Polymorphic SNP markers and 35 SSR markers from previous work (Yang et al., 2011) were initially grouped by JoinMap. Each group was then compared to the peach genome v1.0 (GDR, www.rosaceae.org) sequence and edited for the SNP position, and separately re-created by R/QTL, using minimum 6.0 log of odds (LOD) and 0.35 maximum recombination frequency. The plotting of marker order in each group was accomplished by 'plot.rf'. The final linkage map was constructed using 'ripple' and 'mapthis' functions (*P<0.005*). Marker orders that conflicted with the physical map were adjusted and recalculated based on LOD scores using 'switchorder' function in R/QTL. The map distances were calculated using Kosambi (1944) mapping function. Accuracy of the linkage map was iteratively checked and confirmed by calculating pairwise recombination fractions across genome, and comparing marker order to the physical location on the peach genome v1.0.

Comparison of the physical and genetic map

The set of SNPs mapped in each linkage group were aligned with their position on the peach genome using MapChart2.2 (Voorrips, 2002) and co-linearity among the linkage and physical map was evaluated.

## Results

### SSR genotyping

The SSR markers used in the study have been developed from several different *Prunus* species. The SSR markers were derived from peach (309), almond (73), apricot (114), sweet cherry (43), sour cherry (1), and plum (34) (Table 3.1). Overall, 84% of SSRs successfully amplified in all samples. Lowest amplification, 68%, was achieved with SSRs originating from plum and highest, 91%, with those originating from cherry (Table 3.1). Out of the 169 EST-SSR markers, 7 markers (4%) were segregating in the progeny. Whereas 31 (7%) out of 405 SSR markers derived from genomic sequences were polymorphic in the progeny. Consequently, only 7% (38) of successfully amplified SSRs were polymorphic between the parents and could be used for map development (Figure 3.2 and 3.3).
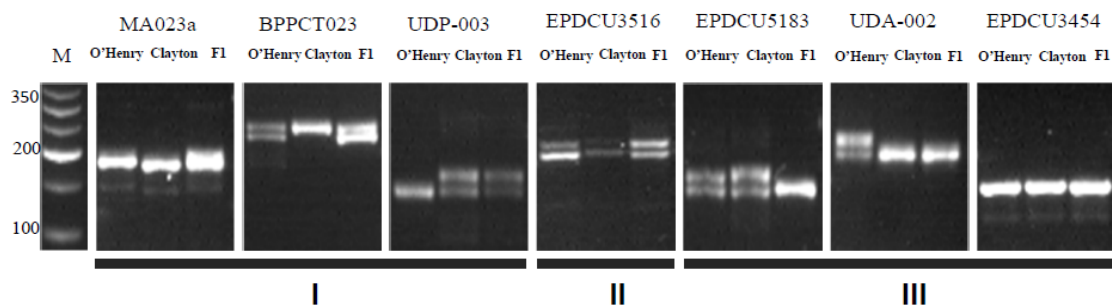


**Figure 3.2.** Screening of SSR markers using gel electrophoresis. I polymorphic markers; II putative polymorphic marker – depending on the segregation in the population; III monomorphic markers.

Out of 574 SSR and 48 NBS markers tested, 41 were polymorphic and informative in the C x O mapping population. Of those, 23 (4%) SSRs and 3 (6%) NBS markers were scored using high resolution agarose gels (Figure 3.4) and 12 (2%) SSRs using multifluorophore fragment analysis (Figure 3.3).
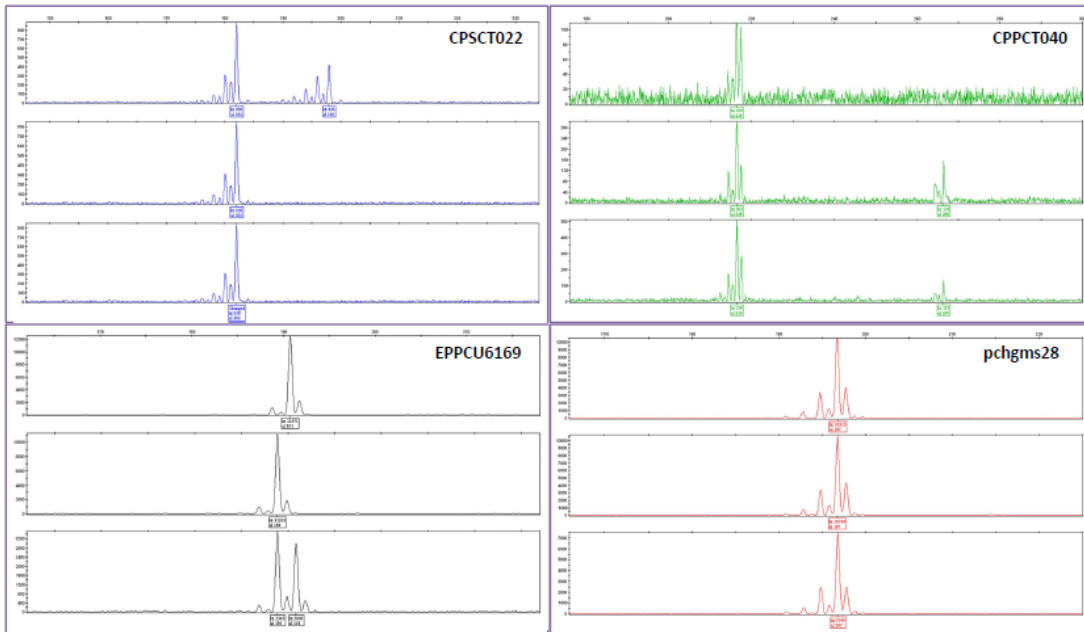


**Figure 3.3.** Screening of SSR markers using multifluorophore fragment analysis. Four SSR markers, two polymorphic and two monomorphic, each labeled with different fluorophore. Top to bottom panels in each quadrant: O'Henry, Clayton, and $F_1$.

The limited number of segregating markers was only sufficient to construct a partial linkage map composed of thirteen markers in three linkage groups (Figure 3.5.). Linkage group 3 was comprised of 7 SSR markers and covered distance of 97 cM. The partial map covered 164 cM distance with average spacing of 12.6 cM between the markers.

To increase map density, additional 48 NBS markers from *Prunus* were tested. Three NBS markers, NBS28, NBS30, and NBS35, were found polymorphic, but could not be mapped in the C x O population.
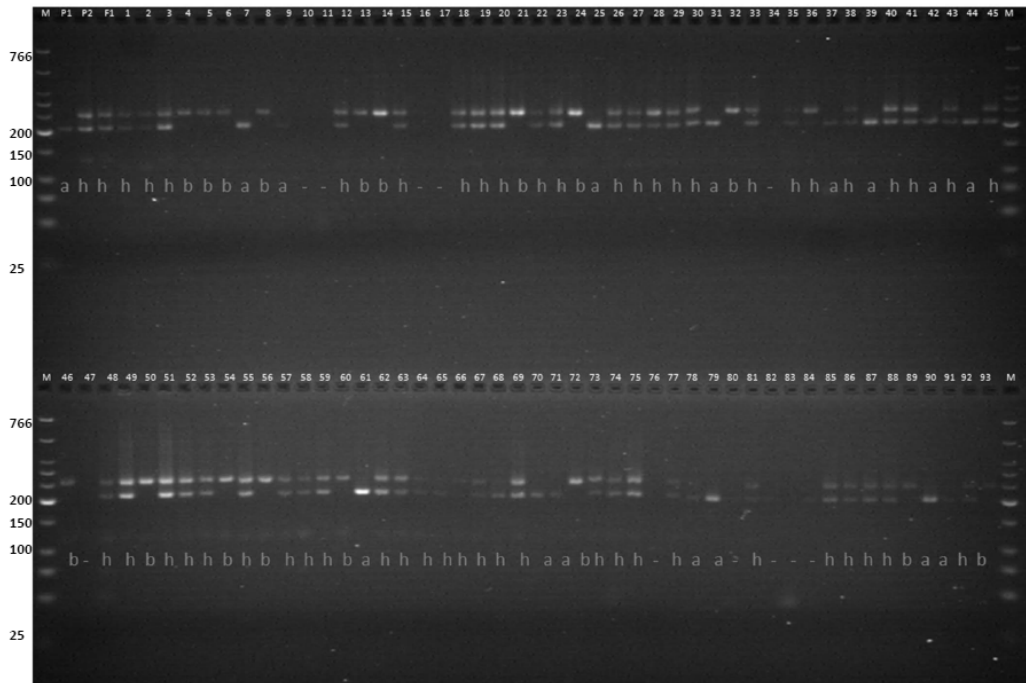


**Figure 3.4.** Genotyping of C x O population with SSR marker MA064a using 3% MetaPhor agarose gel. a: haploid as the pollen parent; b: haploid as the mother parent; h: heterozygote as the $F_1$.

## SNP Genotyping

Out of 8,144 SNP markers on International Peach Sequence Consortium (IPSC) peach 9K SNP array v1, 5,317 (65%) had GT>0.6 and were considered for linkage analysis. Although polymorphism between 'Clayton' and 'O'Henry' was observed in 65% of SNPs, only 33% (1,764) of the polymorphic SNPs were informative in progeny

and could be used in linkage analysis. The number of polymorphic/informative SNPs was

further reduced to 1,341 (25%) by removing SNPs with more than 20% missing data.
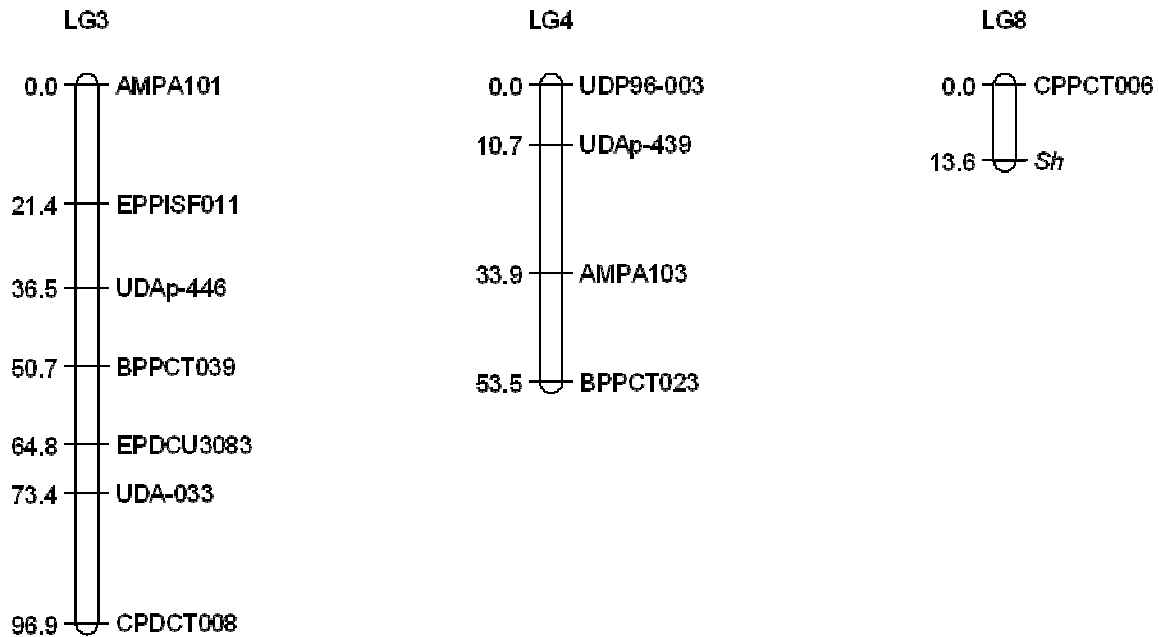


**Figure 3.5.** Partial linkage map derived for the C x O progeny using SSR markers. Linkage groups have been labeled according to the Texas x Earlygold reference map (T x E). The names of the markers are listed on the right. Mapping distances are listed on the left and given in centiMorgans. One morphological marker Sh was mapped and it is italicized.

Map construction

All polymorphic markers, SSRs and SNPs, were used to construct the final

linkage map. The 1,167 (87%) SNPs and two SSR markers, ssrPaCITA16 and

CPPCT006, were successfully mapped in 8 linkage groups. Two hundred and sixty-three

SNP markers could not be mapped in the C x O population and were removed from

further analysis. Approximately 78% of the mapped SNPs shared the same map positions, due to the absence of recombination caused by the small number of accessions genotyped (Table 3.2). For the clarity of figures, a single SNP marker was selected for each unique position and map figures produced (Figure 3.8).

**Table 3.2.** Summary of SNP and SSR markers used in the development of C x O linkage map.

| Group | Mapped markers | Unlinked markers | Mapped to the same position | Sum |
|-------|----------------|------------------|------------------------------|-----|
| LG1 | 63 | 31 | 148 | 242 |
| LG2 | 20 | 36 | 156 | 212 |
| LG3 | 32 | 15 | 99 | 146 |
| LG4 | 40 | 23 | 226 | 289 |
| LG5 | 15 | 15 | 14 | 44 |
| LG6 | 15 | 4 | 44 | 63 |
| LG7 | 41 | 41 | 139 | 221 |
| LG8 | 32 | 9 | 85 | 126 |
| Sum | 258 | 174 | 911 | 1343 |

Accuracy of the linkage map was iteratively checked and confirmed by two methods. First, pairwise recombination fractions across the genome were calculated with the R/QTL software, and the marker order was confirmed through running the 'jittermap' function. The red diagonal present in the plot of pairwise recombination fractions suggests that the order of grouped markers on each group is accurate (Figure 3.6). The presence of a well-defined red diagonal showed that consecutive markers have the smallest recombination fraction with highest LOD ratio. Further, no genotype errors were found using 'jittermap' function (Figure 3.7). Second, marker order was also confirmed

against their position on peach genome v 1.0 sequence (www.rosaceae.org). Six regions identified on LG1, LG2, LG3, LG7, and LG8, respectively, revealed inverted marker positions relative to the peach genome assembly v1.0. Finally, an order of 5% of the mapped markers was adjusted using R/QTL. A map with good order was obtained in spite of a highly distorted telomeric region on G7 between SNP_IGA_763311 and SNP_IGA_792619.
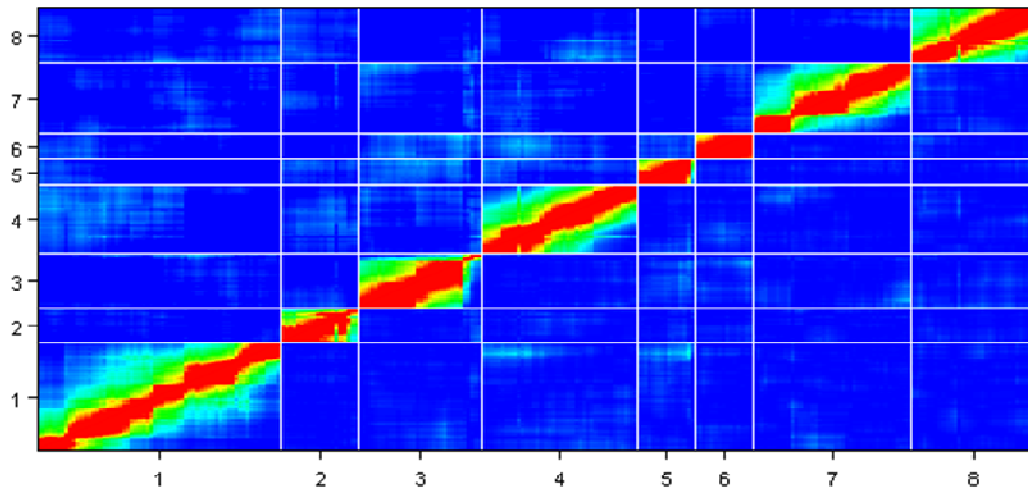


**Figure 3.6.** Pairwise recombination fractions and LOD scores on eight linkage groups. A well-defined red diagonal represents that consecutive markers have the smallest recombination fraction with highest LOD ratio to reflect the accuracy of the grouped markers and the order of markers on each group.
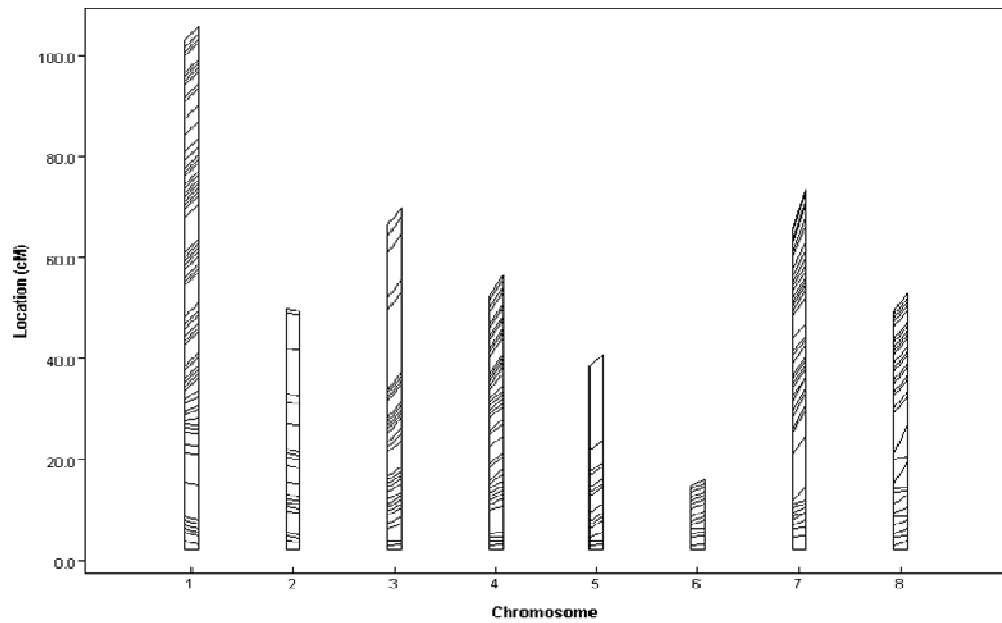
**Figure 3.7.** Jitter map created from R/QTL software. All the markers were shuffled to create the jitter linkage map to reflect the accuracy of the previously constructed map. Short solid lines in those bars represent different markers. The distance between different markers is marked by the white space.
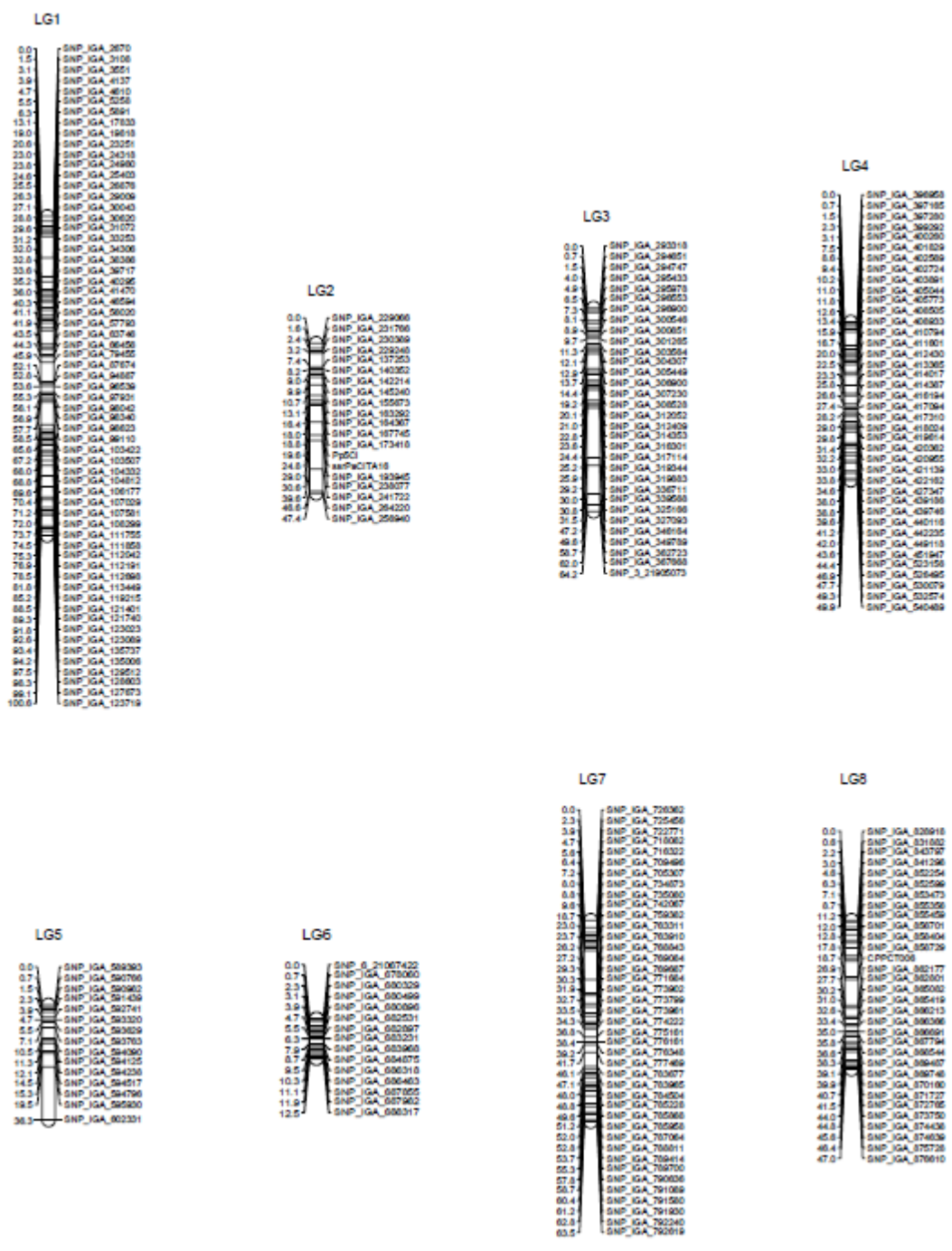
**Figure 3.8.** Linkage map derived from the C x O progeny using two SSR and 256 SNP markers. Linkage groups have been labeled LG1 to LG7 according to the 'Texas' x 'Earlygold' (T x E) *Prunus* reference map. The names of the markers are listed on the right. Mapping distances are listed on the left and given in centiMorgans.

The average marker density considering 258 markers was 1.63 cM/marker. Among mapped SNP markers, 31 deviated significantly from the chi-square expectations; 24 (13.9%) and 12 (4.7%) at the 5% and 1% threshold, respectively. The number of unique map positions, mapped on each linkage group, ranged from 15 on LG5 and LG6, to 63 in LG1, with an average of 27 markers per LG (Table 3.2). The average marker density ranged from 0.8 cM / marker in LG6 to 2.4 cM / marker in LG2 and 5. The length of LGs was variable, with LG1 being the largest, 100.6 cM, and LG6 covering the shortest distance 12.5 cM (Table 3.3). Larger gaps were observed on LG3 with 15.7 cM and on LG5 with 16.8 cM.

**Table 3.3.** Comparison of the C x O linkage map with the peach physical map. Only 256 SNP markers that were used to represent the map positions were considered for the calculation.

| Group | C x O linkage map | | | Coverage (%) | Marker Density | | Average coverage (kb/cM) |
| | Marker No. | Physical length (Mb) | Genetic distance (cM) | | kb | cM | |
|---|---|---|---|---|---|---|---|
| G1 | 63 | 45 | 100.6 | 96 | 700 | 1.6 | 447 |
| G2 | 20 | 15 | 47.4 | 56 | 800 | 2.4 | 316 |
| G3 | 32 | 21 | 64.2 | 95 | 700 | 2 | 327 |
| G4 | 40 | 24 | 49.9 | 80 | 600 | 1.2 | 481 |
| G5 | 15 | 6 | 36.3 | 33 | 400 | 2.4 | 165 |
| G6 | 15 | 3 | 12.5 | 14 | 200 | 0.8 | 240 |
| G7 | 41 | 17 | 63.5 | 77 | 400 | 1.5 | 268 |
| G8 | 32 | 12 | 47 | 57 | 400 | 1.5 | 255 |
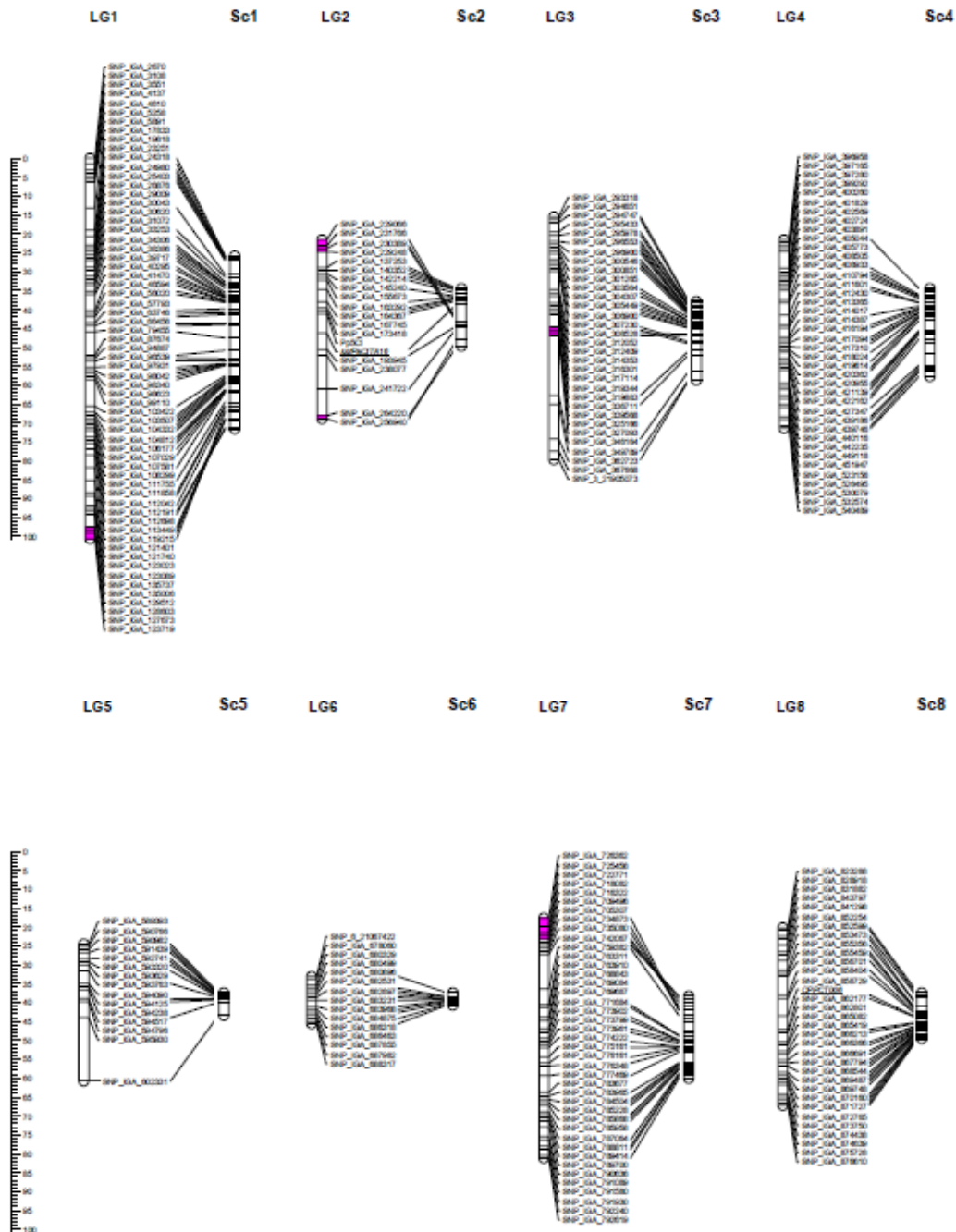
**Figure 3.9.** Comparison of linkage map derived from the C x O population with the peach genome v1.0. SSR markers are underlined. A fixed ruler is placed on the left. One unit of C x O genetic map represents one centimorgan, while one unit of the physical map represents one megabase pair. The shaded areas in the linkage groups represent the inverted regions in comparison to the physical map. Sc = scaffold.

Comparison of the physical and genetic map

Linkage positions of the 95% of SNP markers in the C x O linkage map were in agreement with their positions on the pseudomolecules/scaffolds of peach genome v 1.0. Six regions in the C x O map, involving six markers on LG1, four on LG2, four on LG3, seven markers on LG7, and two markers on LG8, appeared inverted relative to the physical map (Figure 3.9). Linkage groups 4, 5 and 6 exhibit high homology with the 'dhLovell' physical map. The physical length of the C x O linkage map was estimated to cover 63% of the pseudomolecules of peach genome v 1.0. The largest coverage of 96% was achieved between LG1 and pseudomolecule one and the lowest between LG6 and pseudomolecule six (14%). In addition, the estimated average coverage per marker on the pseudomolecules ranged from 1/800 kb on LG2 to 1/200 kb on LG6 (Table 3.3).

**<u>Discussion</u>**

Low variability and narrow genetic base among peach cultivars (Scorza 1985; Scorza et al., 1988) is often a major obstacle for developing linkage maps and elucidating genes responsible for traits of interest. Genetic linkage maps, both intra- and inter-specific, were used to generate consensus reference map for the *Prunus* genome (Aranzana et al., 2003) and location of genes/traits mapped in various crosses (Dirlewanger et al., 2004). Markers such as SSRs are often sufficiently informative to construct linkage maps with the decent coverage in a diverse background. The estimated percentage of informative SSR markers for development of peach linkage maps among any given peach cultivar was around 20% (Dirlewanger et al., 2006; Blenda et al., 2007; Ogundiwin et al., 2009; Fan, 2010; Cao et al., 2011). However, when segregating populations are created between closely related cultivars to reveal inheritance of a specific trait that number is even lower, only 7% in case of the C x O population. Pedigree analysis revealed a highly similar genetic background between the C x O parents 'Clayton' and 'O'Henry', which share the same grandparent 'J.H. Hale', therefore highly reducing the variability detectable with screened SSR markers (Figure 3.1).

One of the intended uses of the C x O map was to facilitate mapping of genes responsible for resistance to bacterial spot (*Xanthomonas arboricola* pv. *pruni*) in peach. Therefore resistance gene analogs (RGAs) were surveyed for their informativeness in C x O progeny and potential for inclusion in the C x O linkage map. Number of isolated plant resistant genes share similar sequence information (Bent, 1996; Jones, 1996; Hammond-

Kosack and Jones, 1997). NBS-LRR genes are the most abundant RGAs distributed in the plant genome, i.e. 149 in Arabidopsis, 317 in *Populus*, 480 in rice (Kohler et al., 2008), and approximately 420 in peach (see Appendix V). In addition, 68 out of 97 functionally characterized resistance genes belong to NBS-LRR or NBS-LRR-like genes (Ingvarden et al., 2008). Therefore, linkage maps constructed with RGA markers were successfully obtained in grape (Donald et al., 2002), apple (Baldi, et al. 2004; Calenge et al., 2005), peach (Lalli et al., 2005), chestnut rose (Xu et al., 2005), and raspberry (Samuelian et al., 2008).

Since limited number of polymorphic SSRs could be utilized for C x O genetic map development, to achieve better resolution SNP markers were used. Recently, several reports have been published on generating SNP resources in peach (Ahmad et al., 2011; Verde et al., 2012). The SNPs on the IPSC peach 9K SNP array v1 (Verde et al., 2012), used in our study, cover most of the peach genome with markers well distributed over all chromosomes. The average gap size across the genome achieved was 26.7 kb that increases to 31.5 kb when considering only polymorphic SNPs. The average ratio of genetic to physical distance in peach is about 440 kb/cM (Dirlewanger et al., 2004; Verde et al., 2012), which gives an average of 13.3 polymorphic SNPs per cM for the array (Verde et al., 2012). In our genetic map, the SNP marker density was estimated from 165 kb/cM to 447 kb/cM (Table 3.3), although two gaps were observed on LG3 (15.7 cM) and LG5 (16.8 cM). Such high marker density is almost equivalent to the *Prunus* reference map with an average of 0.92 cM per marker (www.rosaceae.org/), and is higher than the marker density achieved in other peach genetic maps, 3.3 cM in J x F

(Dirlewanger et al., 2006), 4.7 cM in 'Guardian®' x 'Nemaguard' (Blenda et al., 2007), 4.2 cM 'Contender' x 'Fla.92-2C', (Fan et al., 2009), and 4.0 cM in 'Dr. Davis' x 'Georgia Belle' map (Ogundiwin et al., 2009).

Approximately 78% of the mapped SNPs shared an unique map position, due to the absence of recombination caused by the small number of accessions genotyped. Inclusion of genotyping data from additional progeny from the C x O population is needed to improve map resolution and distinguish betweem SNPs mapped at the same location. Nonetheless, 95% of the 256 mapped SNP positions on the C x O linkage map were in agreement with the order on the peach genome assembly v1 (GDR, www.rosaceae.org). Six inverted regions on LG1, LG2, LG3, LG7 and LG8 were observed, possibly due to the wrong marker order assignment (Dirlewanger et al., 2004) or chromosome translocation (Yammamoto et al., 2001). Selective genotyping strategy allows QTL detection using superior progeny that contains alleles of interest (Navabi et al., 2009). It is reverse approach from bin mapping applied in peach (Howad et al., 2005) where phenotypic data is used to select progeny for genotyping. Selective genotyping of a subset of progeny chosen for their phenotypic performance proved to be cost effective method of achieving high density linkage map suitable for mapping QTLs associated with traits of interest in our case disease resistance.

## Conclusions

Testing of the 574 SSR markers developed from five different *Prunus* species resulted in 38 polymorphic markers for linkage map development. Consequently, three

partial linkage groups were obtained with only 13 SSR markers. Three polymorphic RGA markers were also developed; unfortunately, they could not be put on the linkage map. Finally, SNP markers were also used to develop a fine resolution of linkage map. This map contains 1167 SNP markers and two SSR markers, covering 421.4 cM on eight linkage groups. Since the length of our linkage map is shorter than the reference map with 591 cM, more progeny will be genotyped with SNP markers to get a complete linkage map in the future.

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CHAPTER 4


MAPPING QTLS ASSOCIATED WITH RESISTANCE TO BACTERIAL SPOT

(*XANTHOMONAS ARBORICOLA* PV. *PRUNI)* IN PEACH


**<u>Introduction</u>**

Bacterial spot, caused by *Xanthomonas arboricola* pv. *pruni* (*Xap*), is a serious disease that can affect nearly all cultivated *Prunus* species and their hybrids (EPPO, 1997). It was first described on plum in the United States by Smith (1903). *Xap* was also identified on peach and other stone fruits (Rolfs, 1915; Dunegan, 1932). The most severe infection was reported on Japanese plum (*P. salicina*), Korean cherry (*P. japonica*) and plum hybrids, and on peach and nectarines (*P. persica*) and their hybrids, with over 50% infection (Ritchie, 1995). Nowadays, the pathogen is present and widespread in China, South Africa and Uruguay, whereas local outbreaks have been reported in many other countries such as Bulgaria, Romania, Moldova, Russia, Ukraine, India, Pakistan, Japan, Korea, Canada, USA, Mexico, Argentina, Brazil and Australia (EPPO, 2006). *Xap* can affect leaves, twigs and fruits, and severe infection results in premature leaf defoliation, tree weakening, reduced fruit quality and yield (Ritchie, 1995), making them often not marketable. Traditionally, spraying bactericides, such as antibiotic oxytetracycline or copper-based compounds, is the method to control the disease in the peach orchards (Ritchie, 1995). However, with the environmentally conscious public, chemical control of *Xap* is coming under close scrutiny. Thus interest in developing resistant peach cultivars has moved to the forefront in breeding programs.

Peach cultivars vary greatly in susceptibility to *Xap* and the most effective control is through the use of host plant resistance (Werner et al., 1986). Unfortunately, many resistant cultivars lack specific desirable fruit and marketing characteristics (Okie, 1998). The breeding program in North Carolina was successful in developing series of *Xap*-resistant cultivars, the most resistant of which were 'Clayton' and 'Candor' (Okie et al., 2008), through introgressing resistance characters from the cultivar 'Elberta' into the popular commercial cultivar 'J.H. Hale' (Okie, 1998). However, considerable variation was noticed in disease incidence from year to year, and under favorable conditions for infection all cultivars show at least some symptoms, although highly resistant cultivars have been identified (Keil and Fogle, 1974; Simeone, 1985; Werner et al., 1986). Integration of a genomics approach and traditional breeding facilitates more efficient introgression of *Xap* resistance in newly developed peach cultivars. A molecular breeding approach via the application of DNA markers tagging the resistance loci of interest offers pre-selection of resistant individuals, therefore, can accelerate the breeding process. The application of marker-assisted breeding (MAB) requires well developed genetic resources. Peach is one of the best characterized fruit tree species and serves as a model for genetics studies in Rosaceae and other tree species (Dirlewanger et al., 2004; Shulaev et al., 2008). The available *Prunus* reference map (Dirlewanger et al., 2004) along with release of peach genome sequence v1 (Sosinski et al., 2009) and recently developed Infinium SNP genotyping resources (Gasic et al., 2012; Verde et al., 2012) offer vast resources for marker detection and MAB application.

The high number of resistant cultivars released in many eastern US breeding programs suggested that dominant genes were involved in *Xap* resistance (Sherman and Layne, 1981). Later, the inconsistent performance of susceptibility on leaf and fruit in peach indicated that separate genetic factors might regulate the leaf and fruit resistance (Werner et al., 1986). However, the molecular mechanism of resistance/susceptibility to *Xap* is not yet clear. Recently there were several attempts to understand molecular basis of *Xap* resistance in *Prunus* (Yang et al., 2010, 2011; Socquet-Juglard et al., 2011). Yang et al. (2010) suggested polygenic nature of *Xap* resistance in peach. One putative QTL region was detected on linkage group 4, but the low density linkage map restricted the QTL analysis and discovery of other QTLs with major effects (Yang et al., 2011). Additionally, Socquet-Juglard et al. (2011) using a low density SSR linkage map (Dondini et al., 2007), identified four genomic regions related to *Xap* resistance in apricot and reported a single QTL on linkage group 5 being of interest for marker assisted selection. However, to date no tightly linked markers or isolation of genes associated with *Xap* resistance were reported.

The aim of the present study was to use previously developed *Xap* linkage map (Yang et al., 2011 and chapter III) to map QTLs responsible for *Xap* resistance in peach. The overall goal was to determine the mode of inheritance of leaf and fruit *Xap* resistance in peach and develop reliable markers linked to the resistance locus for MAB and introgression of *Xap* resistance into commercial peach cultivars.

## Materials and methods

### Plant material

*Xap* QTLs were mapped in the 'Clayton' x 'O'Henry' (referred to as C x O) (Yang et al., 2011) mapping set, which consisted of 63 plants, with the highest *Xap* resistance, and two parents. 'Clayton' is yellow, melting, freestone peach selected from a 'Pekin' x 'Candor' cross in the North Carolina peach breeding program; and is resistant to bacterial spot (*Xanthomonas arboricola* pv. *pruni*). 'O'Henry' is high quality yellow, melting and freestone peach that originated in Red Bluff, California in 1968 from Merrill Bonanza O.P. (Okie, 1998); and is highly susceptible to *Xap*. The C x O population also segregates for flower type (Sh/sh) and skin pubescence (G/g). 'Clayton' has non-showy flowers, and 'O'Henry' has showy flowers and is heterozygous for skin pubescence. These two phenotypic traits are controlled by a single gene, with non-showy flower (Sh) and pubescent skin (G) being dominant and showy flower (sh) and glabrous skin (g) being recessive (Blake, 1932; Bailey and French, 1949). The mapping population was maintained in two replicates at three locations: Sandhill Research and Education Center, Pontiac, SC; Sandhills Research Station, Jackson Springs, NC, and ARS-USDA Southeastern Fruit and Tree Nut Laboratory, Byron, GA.

### Assessment of *Xap* incidence

*Xap* bacterial suspension, developed from mixture of isolates, was applied on two-year old trees in early spring of 2008 in NC and 2009 in SC from late petal fall to shuck split to ensure the presence of inoculum in each tree. Field response to *Xap*

infection on leaf and fruits was assessed as explained in Yang et al. (2011) and Chapter II (Table 2.1). In short, leaf symptoms were evaluated once a month from May to July during two seasons at two locations, NC (2008 and 2009) and SC (2009 and 2011). Fruit symptoms were evaluated once in June, and severity of infection was recorded. Phenotypic data were organized in datasets as explained in Rubio et al. (2010).

## DNA isolation and SNP genotyping

Isolation of genomic DNA and subsequent Infinium assay was performed as explained in Verde et al. (2012). In short, genomic DNA was isolated from fresh young leaves of 63 C x O progeny using the E-Z 96 Tissue DNA Kit (Omega Bio-Tek, Inc., Norcross, GA, USA), and quantitated with the Quant-iT™ PicoGreen® Assay (Life Technologies, Grand Island, NY, USA), using the Victor multiplate reader (Perkin Elmer Inc., San Jose, CA, USA). Concentrations were adjusted to a minimum of 50 ng/µl in 5 µl aliquots and submitted to the Research Technology Support Facility at Michigan State University (East Lansing, MI, USA) where the Infinuium assay was performed following the manufacturer's protocol (Illumina Inc.). After amplification, PCR products were hybridized to VeraCode microbeads via the address sequence for detection on a VeraCode BeadXpress Reader. SNP genotypes were scored with the Genotyping Module of GenomeStudio Data Analysis software (Illumina Inc.). A GenTrain score of >0.4 and a GenCall 10% of >0.2 were applied to remove most SNPs that did not cluster (homozygous) or had ambiguous clustering. SNPs homozygous for alternate allele in two parents as well as SNPs homozygous in one and heterozygous in other parent were considered for mapping. $F_2$ population type codes were applied (Van Ooijen et al., 2006).

Linkage map construction

Linkage analyses were performed using JoinMap 4.1 (Van Ooijen et al., 2006) and R/QTL package (Broman, 2003). The deviations from a Mendelian ratio were tested using a Chi-square-goodness-of-fit test (P<*0.05*) available in JoinMap 4.1. Polymorphic SNP markers and 35 SSR markers from previous work (Yang et al., 2011) were initially grouped by JoinMap. Each group was then compared to the peach genome v1.0 (GDR, www.rosaceae.org) sequence and edited for the SNP position. Then, each group was separately re-created by R/QTL, using minimum 6.0 log of odds (LOD) and 0.35 maximum recombination frequency. The plotting of marker order in each group was accomplished by 'plot.rf'. The final linkage map was constructed using 'ripple' and 'mapthis' functions (P< *0.005*). Marker orders that conflicted with the physical map were adjusted and recalculated based on LOD scores using 'switchorder' function in R/QTL. The map distances were calculated using Kosambi's mapping function (Kosambi, 1944). Accuracy of the linkage map was iteratively checked and confirmed by calculating pairwise recombination fractions across genome, and comparing marker order to the physical location on the peach genome v1.0.

Comparison of the position of the SNPs in the physical and genetic map

The set of SNPs mapped in each linkage group were aligned with their position on the peach genome using MapChart2.2 (Voorrips, 2002) and co-linearity among the linkage and physical map was evaluated.

Statistical analysis

Mean and standard deviation were calculated, and the *Xap* resistance scores were tested for normality. Broad-sense heritability ($H^2$) of genotypic mean values was estimated using the formula $H^2 = \sigma_g^2 / (\sigma_g^2 + \sigma_e^2)$, where $\sigma_g^2$ is the genotypic variance and $\sigma_e^2$ the environmental variance as described in Rubio et al. (2010).

QTL analysis and mapping of *Xap* resistance

*Xap* incidence data, collected for leaf and fruit, were organized in datasets, according to Rubio et al. (2010). In detail, three data points, collected for bacterial spot incidence on leaf for each accession replicate, in each season, for both locations, and maximum values for each data point, location and year were organized in 36 leaf datasets. The bacterial spot incidence on peach fruit for each accession replicate was documented once at each location and most severe symptoms have been used as the performance. In addition, the maximal score was extracted and six fruit datasets obtained. Uneven number of individuals was noticed during the different scoring seasons or locations due to the tree death. Therefore, each dataset was not comprised of the scores from all 63 individuals. Totally, 42 datasets were constructed and used for QTL analysis.

Phenotypic data were tested for the normality of distribution using Windows-QTL-Cartographer V2.5 (Wang et al., 2007; http://statgen.ncsu.edu/qtlcart/WQTLCart.htm). Detection of putative QTLs was performed using composite interval mapping (CIM), with a 1,000-permutation test, as described by Rubio et al. (2010). Nonparametric test based on the Kruskal-Wallis (KW) (Kruglyak and Lander, 1995) and multiple regression (MR) with the threshold of 0.5%

were conducted using the MapQTL 6.0 software (Van Ooijen et al., 2009) for data sets that departed from normality. In addition, a less stringent threshold of 5% was applied in case no putative QTLs were detected by CIM, MR and/or KW. MR analysis was used to estimate the percentage of phenotypic variation ($R^2$) explained for each individual QTL and for all QTLs ($R^2$t).

## Results

### Phenotypic evaluation of resistance to *Xap*

Phenotypic evaluation of *Xap* incidence was obtained over 4 seasons, from 2008-2011, at two locations, SC and NC (Figure 4.1; Table 4.1). *Xap* incidence on leaves was evaluated in both locations only during 2009, and no significant difference between average symptom score was observed (Table 4.1). The *Xap* incidence scores in most leaf and all fruit datasets were close to normal distribution, for the 36/6 leaf/fruit datasets, since only 12 leaf datasets were rejected at 5% level (Table 4.1), among which four involved maximal scores (Table 4.1 and Figure 4.1). Data obtained from SC showed a higher average value of *Xap* incidence in 2011 (4.41) than in 2009 (1.83). A similar trend was observed for NC data where *Xap* incidence was higher in 2008 (2.19) than 2009 (1.93) (Table 4.1). *Xap* incidence on fruit was recorded once in 2008 and 2011 in NC and SC, respectively. Seven individual accessions showed low *Xap* incidence on fruit in both SC and NC, but only one, 031, was scored "0" for both locations. Leaf symptoms on the highly resistant parent 'Clayton' varied from "1" to "3" in different years and locations; however, no symptoms on the fruit were detected. At the same time, the highly susceptible parent, 'O'Henry', exhibited high leaf and fruit susceptibility to *Xap* in both locations and all seasons (score ≥3).

The mean values were generally lower in early evaluation stages, with lowest for A1-SC09LEA (0.73) and C1-NC09LEA (0.55) (Table 4.1). As expected, the highest mean values were scored in datasets representing the maximal disease symptoms with the highest in MaxA-SC11LEA (4.27) (Table 4.1). The range of symptom scores was wide in

both locations and all years, with the narrower scores observed in SC (0-1) and NC (0-2) in 2009 and the widest in SC (1-5) and NC (0-5) in 2011 and 2008, respectively. Effects of environmental factors were evaluated with broad-sense heritability, which ranged from 0.15 (B1-SC09LEA) to 0.84 (MaxD-NC08LEA) in 36 leaf datasets, suggesting the important environmental factors involved in leaf resistance to *Xap* (Table 4.1). Higher heritability (over 0.8) for the six fruit datasets, however, suggested the minor environmental effects on fruit resistance to *Xap* infection.

## SNP Genotyping

SNP genotyping was performed on a subset of 63 progeny from C x O population exhibiting the highest leaf *Xap* resistance (classes 0 and 1) and where the disease response was in agreement between the two locations. The individual sample call rate was ≥ 99% for 63 individual sample and the two parents, except for #134 for which genotyping was successful for 74.1% of available SNPs on the IPSC peach 9K SNP v1 array. Out of 8,144 SNP markers on an array, 5,317 (65%) had GT>0.6 and were considered for linkage analysis. Although polymorphism between 'Clayton' and 'O'Henry' was observed in 64% of SNPs, only 33% (1,764) of the polymorphic SNPs were informative in progeny and could be used in the linkage analysis. The number of polymorphic/informative SNPs was further reduced to 1341 (25%) by removing SNPs with more than 20% missing data.

**Table 4.1.** Summary of the statistics computed with the phenotypic data of leaves and fruit obtained from C x O progeny.

| Datasets | Population size | Mean (SD) | Range | Skewness | Kurtosis | S test value | Heritability ($H^2$) |
|---|---|---|---|---|---|---|---|
| A1-SC09LEA | 51 | 0.73 (0.60) | 0-3 | 0.17 | 0.77 | 22.37 | 0.31 |
| A2-SC09LEA | 51 | 1.00 (0.20) | 0-2 | 0.00 | 0.05 | 1348.2 | - |
| A3-SC09LEA | 51 | 0.96 (0.20) | 0-1 | -0.04 | 0.04 | 1329.8 | - |
| B1-SC09LEA | 58 | 0.83 (0.38) | 0-1 | -0.10 | 0.09 | 35.24 | 0.15 |
| B2-SC09LEA | 58 | 1.31 (1.14) | 0-4 | 1.33 | 5.26 | 7.66 | 0.81 |
| B3-SC09LEA | 58 | 0.65 (0.81) | 0-4 | 0.71 | 2.08 | 26.57 | 0.62 |
| A1-SC11LEA | 51 | 3.63 (0.72) | 1-5 | -0.48 | 1.61 | 33.66 | 0.52 |
| **A2-SC11LEA** | **52** | **3.96 (0.74)** | **2-5** | **-0.10** | **0.86** | **0.54** | **0.54** |
| **A3-SC11LEA** | **51** | **3.86 (0.53)** | **2-5** | **-0.02** | **0.29** | **1.12** | **0.28** |
| **B1-SC11LEA** | **59** | **3.25 (0.82)** | **1-5** | **-0.28** | **1.75** | **4.29** | **0.63** |
| **B2-SC11LEA** | **58** | **3.53 (1.08)** | **1-5** | **-0.88** | **4.60** | **5.07** | **0.79** |
| **B3-SC11LEA** | **59** | **3.73 (0.87)** | **2-5** | **-0.06** | **1.39** | **0.80** | **0.67** |
| **C1-NC08LEA** | **44** | **0.84 (0.83)** | **0-3** | **0.33** | **1.26** | **2.63** | **0.64** |
| **C2-NC08LEA** | **42** | **1.31 (1.02)** | **0-3** | **0.20** | **2.38** | **1.47** | **0.76** |
| **C3-NC08LEA** | **43** | **1.84 (0.87)** | **1-4** | **0.37** | **1.36** | **2.95** | **0.67** |
| D1-NC08LEA | 38 | 0.87 (1.09) | 0-4 | 0.73 | 9.57 | 42.64 | 0.79 |
| D2-NC08LEA | 37 | 1.19 (1.17) | 0-5 | 1.84 | 9.22 | 13.15 | 0.82 |
| **D3-NC08LEA** | **37** | **1.62 (1.09)** | **0-4** | **0.90** | **4.62** | **3.08** | **0.79** |
| **C1-NC09LEA** | **42** | **0.55 (0.67)** | **0-2** | **0.60** | **0.58** | **4.97** | **0.44** |
| **C2-NC09LEA** | **42** | **1.86 (1.03)** | **0-4** | **0.88** | **2.46** | **1.07** | **0.76** |
| **D1-NC09LEA** | **36** | **0.69 (0.82)** | **0-3** | **0.69** | **1.60** | **5.96** | **0.63** |
| **D2-NC09LEA** | **36** | **1.42 (1.05)** | **0-5** | **0.36** | **2.70** | **1.53** | **0.77** |
| MaxA-SC09LEA | 51 | 1.06 (0.37) | 0-3 | 0.16 | 0.38 | 747.25 | 0.14 |
| **MaxB-SC09LEA** | **58** | **1.83 (1.05)** | **0-4** | **0.74** | **2.74** | **5.20** | **0.77** |
| **MaxA-SC11LEA** | **52** | **4.27 (0.50)** | **3-5** | **0.42** | **0.16** | **2.71** | **0.24** |
| **MaxB-SC11LEA** | **59** | **4.08 (0.75)** | **2-5** | **-0.17** | **0.89** | **1.60** | **0.55** |
| **Max-NCC08LEA** | **44** | **1.95 (0.83)** | **1-4** | **0.20** | **1.15** | **1.55** | **0.64** |
| **MaxD-NC08LEA** | 38 | **1.66 (1.26)** | **0-5** | **1.90** | **9.30** | **6.58** | **0.84** |
| **MaxC-NC09LEA** | **42** | **1.88 (1.02)** | **0-4** | **-0.05** | **2.44** | **0.91** | **0.76** |
| **MaxD-NC09LEA** | **36** | **1.42 (1.05)** | **0-3** | **0.36** | **2.70** | **1.53** | **0.77** |
| Max-SC09LEA | 60 | 1.83 (1.01) | 1-4 | 0.78 | 2.43 | 6.82 | 0.76 |
| Max-SC11LEA | 63 | 4.41 (0.59) | 2-5 | -0.18 | 0.68 | 28.47 | 0.27 |
| Max-SC LEA | 63 | 4.41 (0.59) | 2-5 | -0.18 | 0.68 | 28.47 | 0.27 |
| **Max-NC08 LEA** | **54** | **2.19 (1.05)** | **0-5** | **0.50** | **3.66** | **1.69** | **0.77** |
| **Max-NC09 LEA** | **54** | **1.93 (1.01)** | **0-4** | **-0.08** | **2.09** | **2.14** | **0.75** |
| **Max-NCLEA** | **55** | **2.53 (0.96)** | **0-5** | **-0.19** | **2.94** | **0.92** | **0.73** |
| **A1-SC11FRU** | **43** | **1.77 (1.43)** | **0-5** | **2.31** | **11.59** | **4.57** | **0.88** |
| **B1-SC11FRU** | **42** | **2.10 (1.14)** | **0-5** | **0.78** | **4.84** | **1.98** | **0.81** |
| **Max-SC11FRU** | **50** | **2.22 (1.39)** | **0-5** | **0.81** | **8.56** | **1.57** | **0.87** |
| **C1-NC09FRU** | **20** | **1.50 (1.36)** | **0-4** | **1.58** | **8.98** | **1.43** | **0.86** |
| **D1-NC09FRU** | **28** | **0.93 (1.15)** | **0-3** | **1.18** | **4.23** | **3.22** | **0.81** |
| **Max-NC09FRU** | **40** | **1.40 (1.26)** | **0-4** | **0.96** | **5.71** | **2.40** | **0.84** |

Each dataset name reflects replication (A, B, C, D), evaluation (1, 2, 3), location (SC – South Carolina; NC – North Carolina), year (2008; 2009; 2011), and plant organ (LEA – leaf; FRU – fruit). Those datasets that show normal distribution are bolded. The critical values for the rejection of normality are 5.99 and 9.21 at the 5% and 1% levels, respectively.
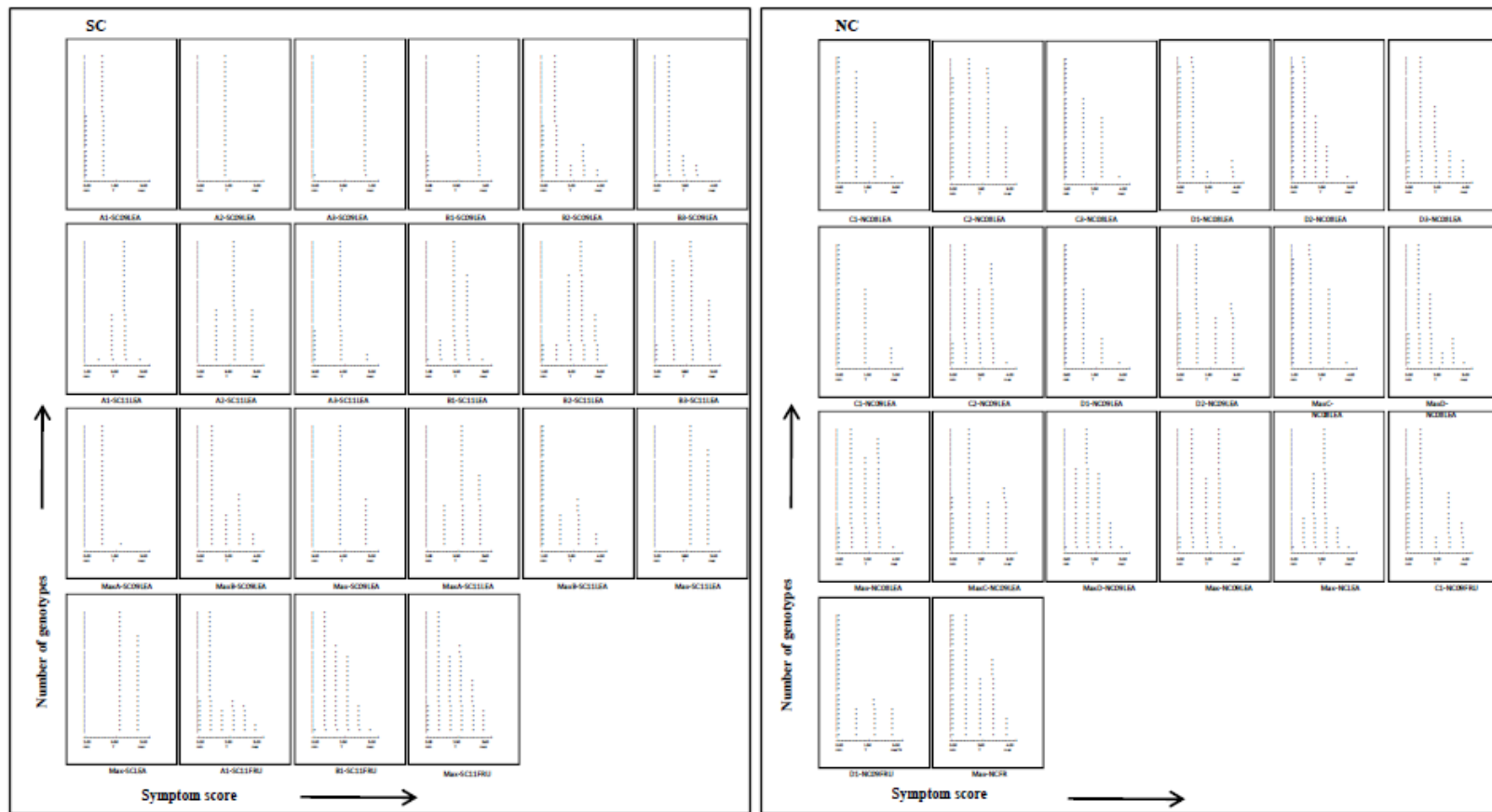
**Figure 4.1.** Seedling distributions in the different symptom classes according to the data set analyzed. Scores of each class and the number of genotypes are represented on the *x* and *y* axes, respectively. The figure summarizes 42 datasets collected from two replicates of SC (2009 and 2011) and NC (2008 and 2009) for both leaf and fruit. Each dataset name reflects replication (A, B, C, D); evaluation (1, 2, 3); location (SC – South Carolina; NC – North Carolina); year (2008; 2009; 2011); and plant organ (LEA – leaf; FRU – fruit). Graphs are created by QTL Cartographer.

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## Map construction

A genetic linkage map was constructed using a subset of 63 progeny. The 1,167 (87%) SNPs were successfully mapped on 256 map positions in 8 linkage groups (Figure 4.2). Two hundred and sixty-three SNP markers could not be mapped in the C x O population and were removed from further analysis. Approximately 78% of the mapped SNPs shared same map positions, due to the absence of recombination caused by the small number of accessions genotyped. For the clarity of figures, a single SNP marker was selected for each unique position and map figures produced (Figure 4.2). In addition, two SSR markers, ssrPaCITA16 and CPPCT006, were also mapped in linkage group (LG) 2 and 8, respectively. The average marker density considering 258 markers was 1.63 cM/marker. Among mapped SNP markers, 31 deviated significantly from the Chi-square expectations; 24 (13.9%) and 12 (4.7%) at the 5% and 1% threshold, respectively. The number of unique map positions, mapped on each linkage group, ranged from 15 in LG5 and LG6 to 63 in LG1, with a mean of 27. The average marker density ranged from 0.8 cM/marker in LG6 to 2.4 cM/marker in LG2 and LG5. The LGs length was variable, with LG1 being the largest, 100.6 cM, and LG6 covering the shortest distance 12.5 cM. Two gaps larger than 15 cM were observed in LG3 and LG5.
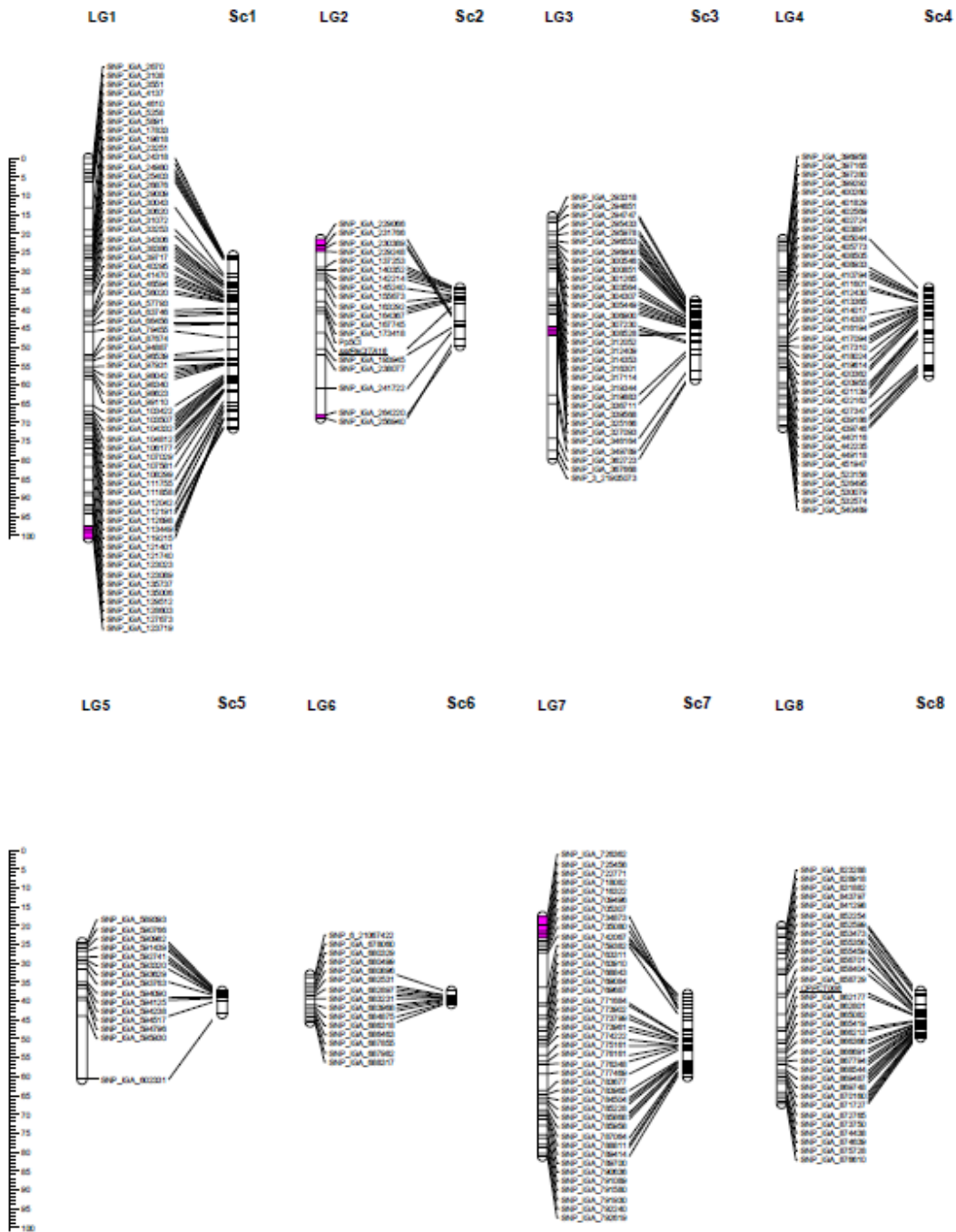
**Figure 4.2.** Linkage map derived for the C x O population and its comparison with the peach genome v1.0. SSR markers placed in *Prunus* reference (T x E) map are underlined. A fixed ruler is placed on the left, one unit of C x O genetic map represents one cM, while one unit of the physical map represents one Mbp. The shaded areas in the linkage groups represent the inverted regions in comparison to the physical map. Sc = scaffold.

Comparison of the physical and genetic map

Linkage positions of the 95% of SNP markers in the C x O linkage map were in agreement with their positions on the pseudomolecules/scaffolds of peach genome v 1.0. Six regions in the C x O map, involving six markers on LG1, six on LG2, four on LG3, seven markers on LG7, and two markers on LG8, appeared inverted relative to the physical map (Figure 4.3).

Linkage groups 4, 5 and 6 exhibit high homology with the 'dhLovell' physical map. From Chapter III, the physical length of the C x O linkage map was estimated to cover 63% of the pseudomolecules/scaffolds of peach genome v 1.0. The physical length was estimated with the largest coverage on scaffold one (96%), and lowest on scaffold six (14%). In addition, the estimated average coverage per marker on the pseudomolecules/scaffolds ranged from 1/200 kb (LG6) to 1/800 kb (LG2).

QTL analysis

QTL analysis was performed for each of the 36 leaf and 6 fruit datasets. A total of fourteen regions associated with *Xap* resistance in C x O map were detected with at least two independent analyses (KW, MR, CIM) and the less stringent threshold (5%) for KW or MR. These QTLs were designated as *Xap.Pp.CO-1.1*, *Xap.Pp.CO-1.2*, *Xap.Pp.CO-1.3*, *Xap.Pp.CO-2.1*, *Xap.Pp.CO-2.2*, *Xap.Pp.CO-3.1*, *Xap.Pp.CO-3.2*, *Xap.Pp.CO-4.1*, *Xap.Pp.CO-4.2*, *Xap.Pp.CO-5.1*, *Xap.Pp.CO-6.1*, *Xap.Pp.CO-7.1*, *Xap.Pp.CO-8.1*, and *Xap.Pp.CO-8.2*, according to pathogen, species, population, linkage group, and position from the top of the LG (Table 4.2). The locations and effects of detected QTLs are summarized in Table 4.2 and their locations in the linkage groups in Figure 4.3.
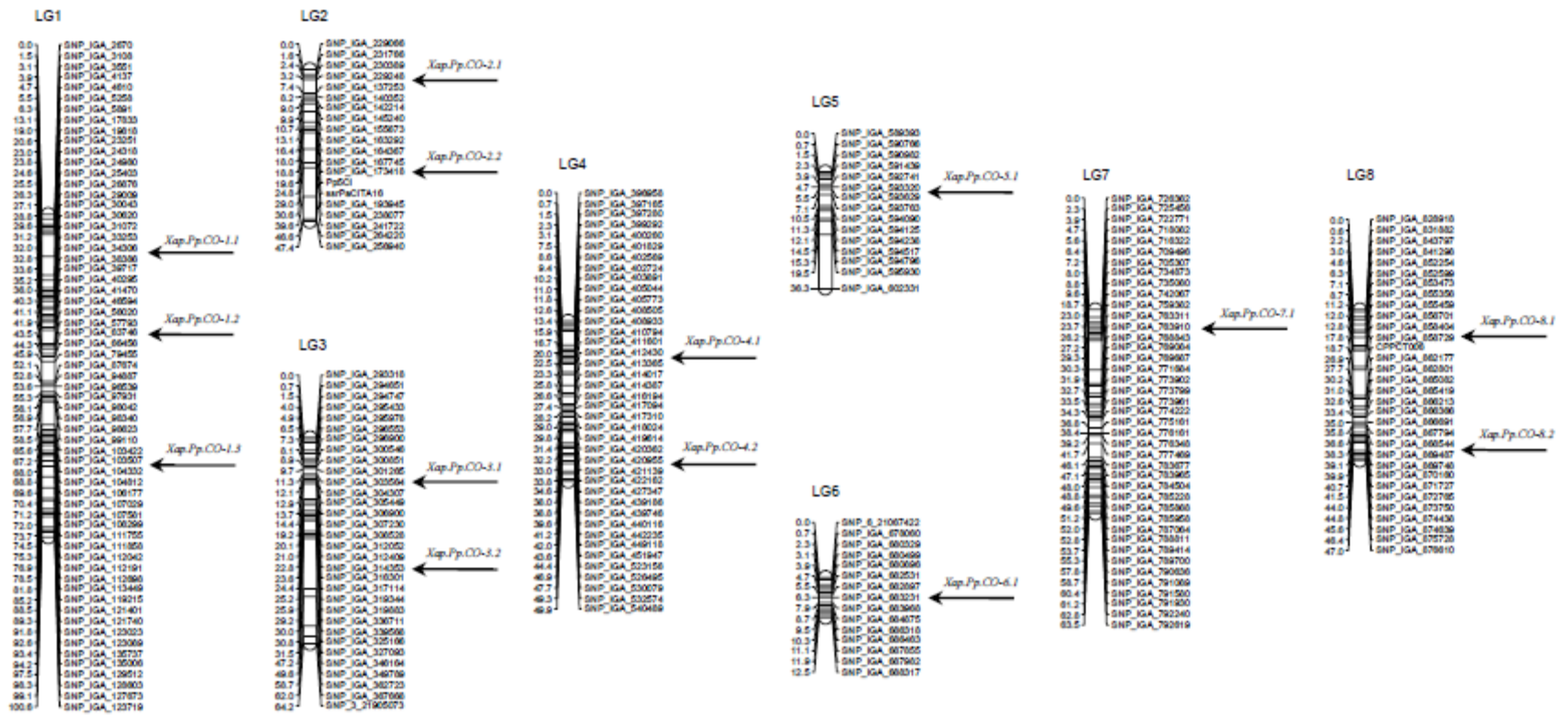
**Figure 4.3.** QTLs mapped on the C x O linkage map. QTLs are figured with an arrow on the right of the linkage groups. The QTL name reflects pathogen (*Xap*); species (*Prunus persica* – Pp); population (CO); the linkage group (LG) on which QTLs were identified; and a position from the top of the LG.

**Table 4.2.** Summary of the QTLs detected for each scoring dataset by Kruskal-Wallis test (KW), multiple regression (MR), and composite interval mapping (CIM). The QTL name reflects pathogen (*Xap*); species (*Prunus persica* – Pp); population (CO); the linkage group (LG) on which QTLs were identified; and a position from the top of the LG.

| Datasets[1] | QTL | LG | Closest marker[2] | KW P value[2] | MR Posit. | P value[2] | CIM Posit. | LOD[3] | LOD$_t$[4] | Add.[5] | R$^2$[6] | R$^2_t$[7] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A3-SC09LEA | *Xap.Pp.CO-4.2* | 4 | SNP_IGA_439186 | <0.005 | 38 | 0.001 | - | - | - | -0.14 | - | 25.7 |
| B1-SC09LEA | *Xap.Pp.CO-3.2* | 3 | SNP_IGA_339568 | <0.05 | 30 | <0.001 | - | - | - | -0.08 | 10.6 | 34.5 |
| | *Xap.Pp.CO-4.2* | 4 | SNP_IGA_451947 | <0.005 | 43.6 | 0.005 | - | - | - | -0.19 | 21.2 | |
| B2-SC09LEA | *Xap.Pp.CO-3.1* | 3 | SNP_IGA_295433 | <0.05 | 4 | 0.001 | - | - | - | 0.16 | - | 15.4 |
| A1-SC11LEA | *Xap.Pp.CO-4.1* | 4 | SNP_IGA_408505 | <0.01 | 12.6 | 0.005 | - | - | - | 0.23 | 46.3 | 56.4 |
| | *Xap.Pp.CO-8.2* | 8 | SNP_IGA_867794 | <0.0001 | 35.8 | <0.001 | - | - | - | 0.68 | 21.6 | |
| **A2-SC11LEA** | ***Xap.Pp.CO-1.1*** | **1** | **SNP_IGA_5891** | **<0.01** | **6.3** | **0.003** | **6.3** | **5** | **3.5** | **0.4** | **12.8** | **31.4** |
| | *Xap.Pp.CO-3.1* | 3 | SNP_IGA_303564 | <0.05 | 11.3 | 0.017 | 11.3 | 3.6 | 3.5 | 0.34 | 19.7 | |
| A3-SC11LEA | *Xap.Pp.CO-1.1* | 1 | SNP_IGA_17833 | <0.01 | 13.1 | 0.005 | - | - | - | 0.09 | - | 18.8 |
| B1-SC11LEA | *Xap.Pp.CO-4.2* | 4 | SNP_IGA_440116 | <0.01 | 39.6 | 0.005 | - | - | - | 0.54 | - | 16.7 |
| B2-SC11LEA | *Xap.Pp.CO-7.1* | 7 | SNP_IGA_742067 | <0.005 | 9.6 | 0.001 | - | - | - | -0.78 | - | 21.4 |
| **B3-SC11LEA** | ***Xap.Pp.CO-8.2*** | **8** | **SNP_IGA_871727** | **<0.005** | **40.7** | **<0.001** | **40.7** | **7.1** | **3.6** | **0.62** | **-** | **27.2** |
| C2-NC09LEA | *Xap.Pp.CO-3.2* | 3 | SNP_IGA_325166 | <0.001 | 30.8 | <0.001 | - | - | - | -0.72 | - | 37.3 |
| D1-NC09LEA | *Xap.Pp.CO-4.2* | 4 | SNP_IGA_421139 | <0.05 | 33 | 0.003 | - | - | - | 0.57 | 24.4 | 45.7 |
| | *Xap.Pp.CO-5.1* | 5 | SNP_IGA_591439 | <0.05 | 2.3 | 0.001 | 2.3 | 4.1 | 3.9 | 0.34 | 19.4 | |
| D2-NC09LEA | *Xap.Pp.CO-1.3* | 1 | SNP_IGA_103422 | <0.05 | 65.6 | 0.003 | - | - | - | 0.6 | 36.6 | 54.5 |
| | *Xap.Pp.CO-4.1* | 4 | SNP_IGA_411601 | <0.005 | 16.7 | 0.039 | 16.7 | 4.3 | 3.7 | 0.78 | 45.5 | |
| | *Xap.Pp.CO-4.2* | 4 | SNP_IGA_420955 | <0.01 | 32.2 | 0.032 | - | - | - | 0.86 | 44.9 | |
| MaxB-SC09LEA | *Xap.Pp.CO-3.1* | 3 | SNP_IGA_295433 | <0.01 | 4 | 0.003 | - | - | - | 0.28 | - | 18.1 |

| Datasets[1] | QTL | LG | Closest marker[2] | KW P value[2] | MR Posit. | P value[2] | CIM Posit. | LOD[3] | LODt[4] | Add.[5] | R²[6] | R²t[7] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MaxA-SC11LEA | *Xap.Pp.CO-1.1* | 1 | SNP_IGA_5891 | <0.005 | 6.3 | 0.001 | 6.3 | 3.9 | 3.4 | 0.33 | - | 23.3 |
| MaxB-SC11LEA | *Xap.Pp.CO-8.1* | 8 | SNP_IGA_841298 | <0.005 | 3 | 0.001 | - | - | - | 0.23 | - | 20.2 |
| MaxC-NC08LEA | *Xap.Pp.CO-1.3* | 1 | SNP_IGA_112042 | <0.05 | 75.3 | 0.003 | - | - | - | 0.3 | 14.5 | 34.1 |
| | *Xap.Pp.CO-4.2* | 4 | SNP_IGA_440116 | <0.05 | 39.6 | 0.007 | - | - | - | -0.22 | 17.3 | |
| MaxC-NC09LEA | *Xap.Pp.CO-2.1* | 2 | SNP_IGA_137253 | <0.05 | 7.4 | 0.004 | - | - | - | 0.45 | 32.6 | 47.9 |
| | *Xap.Pp.CO-3.1* | 3 | SNP_IGA_304307 | <0.005 | 12.1 | <0.001 | - | - | - | -0.58 | 17.3 | |
| MaxD-NC09LEA | *Xap.Pp.CO-1.3* | 1 | SNP_IGA_111755 | <0.05 | 73.7 | 0.01 | - | - | - | 0.59 | 36.6 | 50.8 |
| | *Xap.Pp.CO-4.1* | 4 | SNP_IGA_411601 | <0.005 | 16.7 | - | 16.7 | 4.3 | 3.7 | 0.78 | 46.1 | |
| | *Xap.Pp.CO-4.2* | 4 | SNP_IGA_420955 | <0.01 | 32.2 | 0.046 | - | - | - | 0.86 | 41.6 | |
| Max-NC08LEA | *Xap.Pp.CO-1.3* | 1 | SNP_IGA_107029 | <0.05 | 70.4 | 0.008 | - | - | - | 0.54 | - | 16.5 |
| Max-NC09LEA | *Xap.Pp.CO-2.1* | 2 | SNP_IGA_140352 | <0.005 | 8.2 | 0.002 | 8.3 | 3.8 | 3.5 | 0.53 | - | 20.6 |
| **A1-SC11FRU** | ***Xap.Pp.CO-1.2*** | **1** | **SNP_IGA_34306** | **<0.05** | **23** | **0.012** | **31.2** | **5.6** | **3.6** | **0.85** | **30.6** | **43.6** |
| | *Xap.Pp.CO-6.1* | 6 | SNP_IGA_682531 | <0.01 | 4.7 | <0.001 | 4.7 | 3.9 | 3.6 | 1.17 | 18 | |
| B1-SC11FRU | *Xap.Pp.CO-1.2* | 1 | SNP_IGA_39717 | <0.01 | 33.6 | 0.001 | - | - | - | 0.69 | 21.9 | 44.1 |
| | *Xap.Pp.CO-3.1* | 3 | SNP_IGA_300851 | <0.01 | 8.9 | 0.001 | - | - | - | 0.45 | 23.2 | |
| Max-SC11FRU | *Xap.Pp.CO-1.2* | 1 | SNP_IGA_40295 | <0.05 | 35.2 | 0.012 | 32.3 | 4.3 | 3.4 | 0.8 | 20.7 | 33 |
| | *Xap.Pp.CO-6.1* | 6 | SNP_IGA_682531 | <0.01 | 4.7 | 0.003 | - | - | - | 0.92 | 15.6 | |
| **D1-NC09FRU** | *Xap.Pp.CO-1.2* | 1 | SNP_IGA_63746 | <0.05 | 43.5 | <0.001 | 43.6 | 4.1 | 4 | 0.85 | 17.3 | 60.7 |
| | ***Xap.Pp.CO-3.2*** | **3** | **SNP_IGA_325166** | **-** | **30.8** | **0.008** | **30.8** | **5.4** | **4** | **0.23** | **24.6** | |
| Max-NC09FRU | *Xap.Pp.CO-2.2* | 2 | SNP_IGA_238077 | <0.01 | 30.6 | 0.004 | - | - | - | 0.37 | 35.4 | 51.3 |
| | *Xap.Pp.CO-5.1* | 5 | SNP_IGA_594090 | <0.005 | 10.5 | <0.001 | 11.3 | 4.1 | 3.7 | 0.94 | 26.2 | |

[1] Each dataset name reflects replication (A, B, C, D), evaluation (1, 2, 3), location (SC – South Carolina; NC – North Carolina), year (2008; 2009; 2011), and plant organ (LEA – leaf; FRU – fruit).

[2] Closest marker is given by the Kruskal-Wallis test. P value is the significance of the association between the marker and the QTL. Threshold was set above 0.05.

[3] Logarithm of odds score under composite interval mapping, those QTLs between LOD1 and LOD2 confidence interval are bolded.

[4] LOD threshold under composite interval mapping.

[5] Additive effects.

[6] Individual contribution to the variance accounted for by the QTL (%).

[7] Total variance explained by the model (%)

The phenotypic variation explained by the MR analysis models fitting all the QTLs varied from 15.4% to 56.4% in leaf datasets, and ranged from 33% to 60.7% in fruit datasets (Table 4.2). The phenotypic variance of QTLs, associated only with leaf resistance to *Xap*, ranged from 16.7% to 54.5% (Table 4.2). *Xap.Pp.CO-4.1* with the strongest effect (> 45%) was detected via KW, CIM and MR analysis methods by one dataset from SC (2011) and two datasets from NC (2009). *Xap.Pp.CO-4.2* was detected by seven datasets spanning all years and both locations, via KW and MR analysis methods (P< *0.05*), with phenotypic variance variying from 16.7% to 44.9%. *Xap.Pp.CO-1.1* and *Xap.Pp.CO-8.2* were detected only by two datasets from SC (2011) with phenotypic variance varying from 18.8% to 31.4%, and *Xap.Pp.CO-1.3* was detected by four datasets from NC (2008 and 2009) with phenotypic variance from 16.5% to 54.5%.

*Xap.Pp.CO-3.1*, *Xap.Pp.CO-3.2*, and *Xap.Pp.CO-5.1* are all involved in both leaf and fruit resistance to *Xap* with phenotypic variance ranging from 15.4% 18.1%. Out of those, *Xap.Pp.CO-3.1* was detected by three leaf datasets from SC (2011), one leaf dataset from NC (2009), and one fruit dataset from NC (2009), with 15.4% to 18.1% phenotypic variance. The phenotypic variance of QTLs associated only with fruit resistance to *Xap* ranged from 33% to 60.7% (Table 4.2). Only *Xap.Pp.CO-1.2* was detected by three datasets from both SC (2011) and one dataset from NC (2009), with phenotypic variance ranging from 33% to 60.7%. Although *Xap.Pp.CO-6.1* was detected only by two datasets from SC (2011) using KW and MR analysis, it was detected by all

six fruit datasets using CIM analysis with the LOD threshold lowered at 2.0 (data not shown).

Additive effects were also calculated to speculate the origins of resistance alleles (Table 4.2). Additive effects of seven QTLs, *Xap.Pp.CO-1.1*, *Xap.Pp.CO-1.2, Xap.Pp.CO-1.3*, *Xap.Pp.CO-2.1*, *Xap.Pp.CO-4.1*, *Xap.Pp.CO-6.1*, and *Xap.Pp.CO-8.2* varied from 0.09 to 1.17. The positive values suggest that the resistance alleles originate from the resistant parent 'Clayton'. While *Xap.Pp.CO-7.1* showed a negative additive value (-0.78), indicating the possible contribution of resistance alleles from susceptible parent 'O'Henry'. However, the remaining six QTLs showed both positive and negative additive effects, and require further investigation to determine the origins of resistant alleles.

## Discussion

### C x O genetic map

Development of SNPs genetic linkage map in peach has not yet been reported although several reports of development of SNP marker resources for peach have recently been published (Ahmad et al., 2011; Gasic et al., 2012; Verde et al., 2012). Estimated SNP frequency of 1/100 in non-coding / intronic and 1/225 in coding / exonic genome regions have been reported (Sargent et al., 2009; Illa et al., 2010). The IPSC peach 9K SNP v1 array contains 8,144 high quality SNPs covering all eight peach chromosomes with an average spacing of 26.7 kb between SNPs, which were all detected in exonic regions of peach genome (Verde et al., 2012). In our genetic map, the estimated SNP marker density was ranging from 0.8 cM to 2.4 cM, or 1/165 kb to 1/447 kb. The accuracy of the high resolution C x O genetic map was confirmed through pairwise recombination fractions analysis and comparison with peach genome assembly v1 (GDR, www.rosaceae.org). Several inversions of SNP marker order (<10 cM) were observed in LG1, LG2, LG3, LG7, and LG8 (Figure 4.2). When comparing the positions of anchor markers between T x E and 13 other *Prunus* maps, Drilewanger et al, (2004) observed occasional divergences between maps of different species and attributed it to the mapping of different duplicates of markers (RFLPs or SSRs) that have more than one copy in different regions of the *Prunus* genome. Moreover, order inversions affected almost always pairs of loci that are close together in the T x E map (~10 cM), suggesting that they were rather caused by errors in the assignment of marker order than to inversion of chromosome fragments. Only one major chromosomal rearrangement has been

documented in peach, a reciprocal translocation between G6 and G8 that was demonstrated in the $F_2$ progeny of almond (cv. Garfi) x peach (cv. Nemared) (Jauregui et al., 2001) and in the peach $F_2$ cv. Akame x cv. Juseitou (Yamamoto et al., 2001). The C x O map also has one inverted region larger than 15 cM on the upper part of LG2 (Figure 4.2) that might be due to the translocation of chromosome fragments. Genotyping of more progeny from this population is necessary to support a hypothesis of the chromosome fragment translocation.

## Genetics basis of quantitative resistance to *Xap*

Our study indicates that *Xap* resistance in peach is a quantitative trait controlled by polygenic factors, which is supported by the evidence in literature where cultivars reported resistant have quite diverse pedigrees (Okie, 1998). Thus, the rating method applied in our research was critical for QTL mapping, and a strong attempt was made to ensure accuracy and precision of the applied score. Visual observation of symptoms was carried out twice on each genotype to assess the whole tree performance in each cycle of evaluation using the ordinal scale method, which is deemed more reliable and accurate (Bardsley and Ngugi, 2010). Recently, a PCR method for detection of a specific ABC transporter gene of *Xap* was developed to facilitate detection of disease in the field (Pagani, 2004; Palacio-Bielsa et al., 2010). Symptoms of *Xap* are cumulative in each season cycle; therefore maximal score was used to detect the potential QTLs. Maximal score was deemed as a good parameter in revealing potential for disease severity development and genetic basis for it in each genotype (Rubio et al., 2010). Datasets

acquired from each cycle were also used to capture additional QTLs, in order to elucidate genetic control of *Xap* resistance in natural environment (Rubio et al., 2010).

Our findings suggest that the leaf and fruit resistance to *Xap* in peach are regulated by different QTLs, which is in agreement with the reports of Werner et al. (1986). In our study, we detected total of 14 QTLs involved in *Xap* resistance. This is higher than the number of genes associated with bacterial spot resistance reported in pepper (6) and tomato (5) (Stall et al., 2009), but similar to rice (19) (Nino-Liu et al., 2006). The QTL *Xap.Pp.CO-4.1*, with the major effects of $R^2 > 45\%$, was associated only with leaf resistance to *Xap* and co-localized with the QTL region on LG4 detected in our previous study (Yang et al., 2011). Furthermore, this QTL region includes marker AG8A on LG4 of *Prunus* resistance map, which is associated with powdery mildew resistance (Lalli et al., 2005). Another putative *Xap.Pp.CO-4.2* co-localizes with the SSR marker BPPCT036 on LG4 of the *Prunus* resistance map and is also associated with powdery mildew resistance (Lalli et al., 2005). These findings suggest pleiotropic effect indicating that this region of peach genome harbors resistance genes associated with resistance to both bacterial spot and powdery mildew in peach. In addition, Grube et al. (2000) suggested that highly similar R genes may confer resistance to different pathogen types, while highly similar pathogen races may employ different R genes. On the other hand, *Xap.Pp.CO-3.1* and *Xap.Pp.CO-5.1* were detected by both leaf and fruit datasets, and seem to co-localize with the QTLs on LG3 and LG5 also reported in apricot (Socquet-Juglard et al., 2011). Higher resolution maps and a set of shared markers between the C x O and apricot genetic maps are necessary to confirm if it is indeed the same region in

both species responsible for *Xap* resistance in *Prunus*. Additionally, two QTLs, *Xap.Pp.CO-1.2* and *Xap.Pp.CO-6.1*, associated only with fruit datasets were also detected in C x O population. All these findings reveal the complexity of *Xap* resistance in peach and suggest existence of different genes involved in leaf and fruit resistance as well as those more general resistance genes that elicit resistant response to both leaf and fruit *Xap* infection in peach.

For seven of the putative QTLs identified in this study, favorable alleles conferring high resistance were inherited from the resistant parent 'Clayton' as expected. However, one QTL, *Xap.Pp.CO-7.1* with a favorable allele for resistance seems to originate from the susceptible parent 'O'Henry'. It is possible that 'O'Henry' contains the resistant alleles to *Xap* infection. From the pedigree analysis, it suggests that the resistant alleles may originate from the grandparent 'J.H. Hale' which is a mildly susceptible cultivar (see Figure 3.1 in chapter III). In addition, resistant alleles from susceptible parents were indicated in previous reports for various plant-pathogen interactions (Young et al. 1993; Dirlewanger et al., 1994, 1996; Mestries et al., 1998; Keller et al., 1999; Foulongne et al., 2003). Since 'O'Henry' is highly susceptible to *Xap*, leaf and fruit results suggest existence of recessive alleles in *Xap* resistance in peach. Recessive alleles conferring resistance to pathogens have previously been reported in other plant species, such as pepper and tomato (Stall et al., 2009), and rice (Nino-Liu et al., 2006).

## Conclusions

Introgression of *Xap* resistance or tolerance into peach has been initiated in many breeding programs. However, the polygenic character of *Xap* resistance makes traditional

breeding time-consuming and labor-intensive. Four main QTLs were considered for the marker development and future MAB, including *Xap.Pp.CO-4.1* associated only with leaf resistance; *Xap.Pp.CO-5.1* associated with both leaf and fruit resistance; and two QTLs, *Xap.Pp.CO-1.2* and *Xap.Pp.CO-6.1* associated only with fruit resistance. Our study supports breeding strategies for development of *Xap* resistant peach cultivars based on marker-assisted selection of favorable QTLs in advanced generations. It also suggests that an advisable strategy to ensure a stable level of *Xap* resistance in both leaf and fruit would be to combine favorable alleles at these four QTLs in the same genotype. However, achieving this combination solely through phenotypic selection will be difficult since it is hard to control the environmental condition and pathogen population in the field. Therefore, development of markers associated with *Xap* resistance for application in MAB would be very useful in that regard.

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APPENDICES

Appendix I: Phenotypic data that was obtained from SC and NC used for analysis in Chapter II. Phenotypic data for leaf was collected three times on May, June, and July from A row and B row of SC and C row and D row of NC in two different evaluation years, respectively. Phenotypic data for fruit was collected once on June from from A row and B row of SC and C row and D row of NC in 2011 and 2009, respectively.

| Accession No. | SC | | | | | | | | | | | | | | NC | | | | | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | Leaf | | | | | | | | | | | | Fruit | | Leaf | | | | | | | | | | Fruit | |
| | 2009 | | | | | | 2011 | | | | | | 2011 | | 2008 | | | | | | 2009 | | | | 2009 | |
| | A1 | A2 | A3 | B1 | B2 | B3 | A1 | A2 | A3 | B1 | B2 | B3 | A | B | C1 | C2 | C3 | D1 | D2 | D3 | C1 | C2 | D1 | D2 | C | D |
| 1 | 1 | 1 | 4 | 1 | 0 | 1 | 2 | 5 | 4 | 2 | 3 | 2 | . | 2 | 1 | 2 | 2 | 0 | . | . | 0 | 1 | . | . | . | . |
| 2 | 0 | 0 | 3 | 0 | 0 | 1 | 2 | 4 | 4 | . | . | . | . | . | 1 | 3 | 4 | 0 | . | . | 0 | 2 | . | . | . | . |
| 3 | 1 | 1 | 2 | 1 | 1 | 1 | 3 | 5 | 5 | 3 | 5 | 3 | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 4 | 1 | 1 | 4 | 1 | 1 | 1 | 3 | 4 | 4 | . | . | . | . | . | 0 | 1 | 2 | 0 | 0 | 1 | 0 | 1 | 0 | 2 | . | . |
| 5 | 2 | 1 | 3 | 1 | 0 | 1 | 3 | 5 | 4 | 2 | 3 | 3 | 1 | 4 | 0 | 1 | 2 | . | . | . | 1 | 1 | . | . | 1 | . |
| 6 | 1 | 1 | 3 | 1 | 1 | 1 | 3 | 4 | 3 | 2 | 3 | 3 | . | 1 | 2 | 3 | 4 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | . | . |
| 7 | 0 | 1 | 3 | 0 | 0 | 4 | 3 | 3 | 3 | 4 | 4 | 5 | . | . | 2 | 1 | 3 | 2 | 3 | 4 | 2 | 3 | 0 | 1 | . | . |
| 8 | 0 | 1 | 0 | 1 | 0 | 3 | 3 | 4 | 4 | 3 | 3 | 2 | . | . | 0 | 0 | 1 | 1 | 3 | 3 | 1 | 1 | 1 | 3 | . | 3 |
| 10 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 11 | 1 | 1 | 3 | 1 | 0 | 2 | 3 | 3 | 4 | 3 | 3 | 3 | . | . | 1 | 1 | 1 | 4 | 3 | 3 | 1 | 1 | 1 | 2 | 3 | . |
| 12 | . | . | . | 1 | 0 | 2 | . | . | . | 4 | 4 | 3 | . | 3 | 1 | . | 2 | . | . | . | 0 | 1 | . | . | . | . |
| 13 | 0 | 1 | 3 | 1 | 1 | 3 | 4 | 3 | 3 | 2 | 3 | 4 | 5 | 5 | 2 | 2 | 3 | . | . | . | 3 | 3 | . | . | 1 | . |
| 14 | 1 | 1 | 1 | 1 | 0 | 1 | 4 | 5 | 4 | 2 | 4 | 2 | 5 | 5 | 4 | 4 | 5 | 0 | 1 | 2 | 2 | 2 | . | . | 0 | . |

| 15 | 0 | 2 | 1 | 1 | 1 | 3 | 3 | 3 | 4 | 4 | 3 | 3 | . | 3 | 1 | 2 | 2 | 1 | 3 | 4 | 1 | 1 | 2 | 3 | 3 | . |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 16 | 1 | 1 | 1 | 1 | 1 | 2 | 4 | 3 | 4 | 2 | 4 | 2 | 5 | 4 | . | . | . | 1 | 2 | 4 | . | . | 0 | 2 | . | . |
| 17 | 1 | 1 | 1 | 1 | 0 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 3 | . | . | . | 2 | 3 | 2 | . | . | 0 | 1 | . | 1 |
| 18 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 19 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 20 | 0 | 1 | 1 | 1 | 0 | 4 | 2 | 3 | 3 | 3 | 3 | 2 | 3 | 3 | 2 | 3 | 4 | 2 | 2 | 3 | . | . | 1 | 2 | . | . |
| 21 | 1 | 1 | 3 | 1 | 1 | 3 | 4 | 3 | 3 | 3 | 4 | 3 | 3 | 3 | 0 | 1 | 1 | 1 | 1 | 2 | 1 | 3 | 1 | 1 | . | 0 |
| 22 | 1 | 1 | 2 | 1 | 1 | 3 | 4 | 4 | 4 | 3 | 5 | 3 | 2 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 3 | 1 | 1 | . | . |
| 23 | 1 | 1 | 3 | 1 | 1 | 3 | 4 | 3 | 4 | 2 | 3 | 4 | 3 | 3 | . | . | . | 1 | 1 | 1 | . | . | 1 | 3 | . | 2 |
| 24 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 25 | . | . | . | 1 | 1 | 3 | . | . | . | 2 | 3 | 3 | . | 2 | 1 | 1 | 1 | 0 | . | . | 0 | 1 | . | . | 0 | . |
| 26 | 2 | 1 | 3 | 1 | 1 | 1 | 3 | 4 | 3 | 2 | 3 | 3 | 2 | 2 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 2 | 0 | 1 |
| 27 | 1 | 1 | 2 | 1 | 1 | 1 | 2 | 4 | 2 | 3 | 3 | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | . | 0 |
| 28 | 1 | 1 | 1 | . | . | . | . | 4 | . | . | . | . | . | . | 1 | 2 | 2 | 0 | . | . | 0 | 1 | . | . | 3 | . |
| 29 | 1 | 1 | 4 | 2 | 1 | 1 | 4 | 4 | 3 | 4 | 5 | 4 | 3 | 2 | 1 | 1 | 2 | . | . | . | 0 | 0 | . | . | 1 | . |
| 30 | 2 | 2 | 3 | 1 | 1 | 1 | 4 | 4 | 2 | 4 | 5 | 4 | 4 | 1 | . | . | . | . | . | . | . | . | . | . | . | . |
| **31** | **.** | **.** | **.** | **1** | **1** | **2** | **.** | **.** | **.** | **5** | **4** | **3** | **.** | **0** | **1** | **2** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** |
| 32 | 2 | 1 | 2 | 1 | 1 | 1 | 4 | 4 | 3 | 4 | 5 | 4 | 3 | 1 | 1 | 3 | 3 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 |
| 33 | 0 | 1 | 1 | 0 | 0 | 0 | 4 | 3 | 3 | 4 | 3 | 3 | 5 | . | 2 | 3 | 3 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 |
| 34 | 3 | 2 | 3 | 1 | 1 | 1 | 4 | 3 | 3 | 3 | 5 | 4 | 5 | 1 | . | . | . | 0 | 0 | 0 | . | . | 0 | 1 | . | 2 |
| 35 | 2 | 2 | 3 | 1 | 1 | 1 | 4 | 3 | 4 | 3 | 5 | 5 | 5 | 3 | 3 | 2 | 2 | 0 | 0 | 0 | 2 | 3 | 3 | 3 | 2 | 2 |
| 36 | 1 | 1 | 3 | 1 | 1 | 1 | 4 | 4 | 4 | 2 | 5 | 4 | 4 | 1 | 1 | 0 | 2 | 1 | 0 | 1 | 0 | 1 | 1 | 2 | 2 | 3 |
| 37 | 2 | 1 | 2 | 1 | 1 | 4 | 3 | 5 | 4 | 2 | 3 | 3 | . | . | 1 | 2 | 1 | 0 | 0 | 1 | . | . | 1 | 2 | . | . |
| 38 | . | . | . | 1 | 0 | 4 | . | . | . | 1 | 3 | 3 | . | . | 1 | . | 1 | 1 | 1 | 1 | . | . | . | . | . | . |

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| 39 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 40 | 1 | 2 | 4 | 1 | 1 | 3 | 4 | 5 | 4 | 4 | 4 | 3 | 1 | 2 | 2 | 1 | 3 | 1 | 1 | 2 | 0 | 0 | 1 | 3 | 0 | 1 |
| 41 | . | . | . | 1 | 0 | 3 | . | . | . | 3 | 4 | 3 | . | 1 | 1 | 1 | 3 | 2 | 2 | 2 | 1 | 1 | 1 | 2 | . | 0 |
| 42 | 1 | 1 | 1 | 1 | 1 | 1 | 4 | 4 | 4 | 3 | 3 | 3 | 5 | 4 | 1 | 1 | 3 | 1 | 2 | 2 | 1 | 1 | 2 | 3 | 0 | 1 |
| 43 | 0 | 1 | 0 | 1 | 0 | 1 | 5 | 5 | 4 | 4 | 3 | 3 | 3 | 4 | 1 | . | 3 | 2 | 3 | 3 | 1 | 3 | 3 | 4 | . | 1 |
| 44 | 0 | 1 | 2 | 1 | 3 | 3 | 4 | 3 | 4 | 3 | 3 | 3 | 2 | 4 | 4 | 3 | 4 | . | . | . | 0 | 1 | . | . | 0 | . |
| 45 | 1 | 1 | 1 | 1 | 1 | 1 | 5 | 3 | 3 | . | . | . | . | . | 0 | 0 | 2 | 2 | 2 | 2 | 2 | 3 | 0 | 2 | . | 2 |
| 46 | 1 | 3 | 3 | 1 | 0 | 1 | 3 | 4 | 4 | 2 | 4 | 4 | 5 | 5 | 1 | 1 | 3 | 0 | 1 | 1 | 3 | 3 | 1 | 3 | 3 | 2 |
| 47 | 2 | 1 | 2 | 1 | 1 | 1 | 4 | 4 | 4 | 3 | 4 | 4 | 4 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 3 | 1 | 1 | 1 | 2 |
| 48 | 1 | 3 | 3 | 1 | 1 | 3 | 3 | 4 | 4 | 3 | 4 | 3 | 4 | 4 | 1 | 0 | 1 | 1 | 2 | 1 | 0 | 2 | 1 | 3 | 0 | 2 |
| 49 | 1 | 1 | 2 | 3 | 1 | 2 | 4 | 4 | 3 | 5 | 4 | 3 | 5 | 5 | 1 | 1 | 1 | . | . | . | 0 | 2 | . | . | 1 | . |
| 50 | 1 | 1 | 1 | 1 | 1 | 1 | 3 | 4 | 3 | 4 | 3 | 3 | 4 | . | 1 | 2 | 2 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 3 | 0 |
| 51 | 1 | 1 | 3 | 1 | 1 | 3 | 5 | 3 | 4 | 4 | 4 | 4 | 1 | 2 | 0 | 1 | 1 | 0 | . | . | 1 | 1 | . | . | 1 | . |
| 52 | 3 | 4 | 3 | 3 | 1 | 1 | 4 | 5 | 4 | 5 | 5 | 4 | 1 | 1 | 1 | 1 | 3 | . | . | . | . | . | . | . | . | . |
| 53 | 1 | 1 | 2 | 1 | 0 | 1 | 4 | 4 | 3 | 3 | 3 | 2 | 4 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | . | . | 0 | 1 | . | 0 |
| 54 | 1 | 2 | 2 | 1 | 0 | 1 | 4 | 3 | 4 | 3 | 4 | 5 | 2 | 1 | . | . | . | . | . | . | . | . | . | . | . | . |
| 55 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 56 | 2 | 2 | 3 | 1 | 1 | 1 | 3 | 4 | 4 | 4 | 5 | 4 | 1 | 1 | . | . | . | 2 | 1 | 2 | . | . | 0 | 1 | . | 0 |
| 57 | 1 | 1 | 1 | 1 | 1 | 1 | 3 | 4 | 4 | 4 | 4 | 2 | . | . | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| 58 | 1 | 1 | 1 | 1 | 0 | 1 | 3 | 4 | 4 | 2 | 4 | 3 | 1 | 1 | 2 | 2 | 1 | . | . | . | 0 | 2 | . | . | 3 | . |
| 59 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 60 | 2 | 1 | 1 | 1 | 1 | 4 | 3 | 4 | 4 | 2 | 4 | 3 | 3 | 3 | . | . | . | . | . | . | . | . | . | . | . | . |
| 61 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 62 | . | . | . | 1 | 0 | 1 | . | . | . | 4 | 5 | 4 | . | 2 | 1 | 2 | 3 | . | . | . | 0 | 2 | . | . | 0 | . |

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| 63 | 1 | 2 | 2 | 1 | 0 | 1 | 3 | 3 | 2 | 3 | 4 | 4 | . | . | 0 | 0 | 1 | . | . | . | 0 | 1 | . | . | 1 | . |
| 64 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 65 | 2 | 2 | 3 | 1 | 1 | 4 | 4 | 4 | 4 | 4 | 5 | 4 | 2 | 4 | . | . | . | . | . | . | . | . | . | . | . | . |
| 66 | 2 | 2 | 3 | 1 | 1 | 3 | 4 | 4 | 4 | 3 | 3 | 4 | 3 | 1 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 3 | 0 | 1 | 2 | 1 |
| 67 | 1 | 2 | 3 | 1 | 1 | 3 | 4 | 4 | 4 | 3 | 4 | 4 | 3 | 3 | 1 | 4 | 4 | 1 | 1 | 2 | 0 | 2 | 0 | 0 | . | . |
| 68 | 1 | 1 | 3 | 1 | 1 | 2 | 4 | 4 | 4 | 3 | 3 | 4 | 4 | 2 | 0 | 1 | 2 | . | . | . | 1 | 4 | . | . | . | . |
| 69 | 1 | 1 | 1 | 1 | 0 | 3 | 4 | 3 | 4 | 3 | 3 | 4 | 3 | 1 | 2 | 1 | 1 | . | . | . | 1 | 3 | . | . | . | . |
| 71 | 1 | 1 | 3 | 1 | 1 | 1 | 4 | 4 | 3 | 4 | 5 | 3 | 3 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 2 | 1 | 2 | . | . |
| 72 | 1 | 2 | 2 | 1 | 1 | 3 | 4 | 4 | 4 | 2 | 3 | 2 | 1 | 2 | 1 | 0 | 1 | . | . | . | 0 | 1 | . | . | 0 | . |
| 73 | 1 | 1 | 1 | 1 | 0 | 2 | 3 | 4 | 3 | 3 | 3 | 4 | 3 | 3 | 1 | 1 | 3 | . | . | . | 1 | 3 | . | . | . | . |
| 74 | 3 | 3 | 3 | 1 | 1 | 1 | 4 | 4 | 3 | 4 | 4 | 4 | 4 | 2 | 1 | 1 | 2 | . | . | . | 1 | 2 | . | . | 0 | . |
| 75 | 1 | 1 | 1 | 1 | 0 | 1 | 2 | 4 | 4 | 4 | 4 | 3 | 4 | 2 | . | . | . | . | . | . | . | . | . | . | . | . |
| **76** | **.** | **.** | **.** | **1** | **1** | **1** | **.** | **.** | **.** | **2** | **2** | **2** | **.** | **3** | **0** | **0** | **1** | **.** | **.** | **.** | **1** | **3** | **.** | **.** | **3** | **.** |
| 77 | . | . | . | 1 | 1 | 1 | . | . | . | 4 | 5 | 5 | . | 3 | . | . | . | . | . | . | . | . | . | . | . | . |
| 78 | 1 | . | 2 | 1 | 1 | 1 | 4 | 4 | 3 | 2 | 4 | 3 | 4 | 5 | 4 | 4 | 5 | . | . | . | 1 | 1 | . | . | 1 | . |
| 79 | 2 | 1 | 1 | 1 | 0 | 1 | 3 | 4 | 3 | 3 | 4 | 2 | 3 | . | . | . | . | 1 | 1 | 2 | . | . | 1 | 2 | . | 4 |
| 80 | 2 | 2 | 3 | 1 | 1 | 1 | 3 | 4 | 4 | 4 | 3 | 3 | 3 | 0 | 3 | 3 | 5 | . | . | . | . | . | . | . | . | . |
| 81 | 2 | 1 | 1 | . | . | . | 1 | 5 | 5 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 82 | 1 | 1 | 1 | 1 | 1 | 1 | 4 | 4 | 4 | 4 | 3 | 5 | 1 | 2 | 1 | 2 | 2 | 1 | 2 | 3 | 1 | 2 | 3 | 3 | . | 3 |
| 83 | 1 | 2 | 2 | 1 | 1 | 1 | 3 | 4 | 4 | 4 | 4 | 3 | . | 3 | 2 | 2 | 5 | 2 | 2 | 4 | 1 | 2 | 0 | 1 | 0 | 0 |
| 84 | 3 | 2 | 1 | 1 | 2 | 1 | 4 | 5 | 4 | 4 | 3 | 5 | 0 | 3 | 0 | 2 | 3 | . | . | . | 2 | 3 | . | . | 0 | . |
| 85 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 86 | 2 | 2 | 1 | 1 | 1 | 1 | 3 | 4 | 4 | 4 | 4 | 4 | 0 | 2 | . | . | . | 3 | 3 | 3 | . | . | 0 | 3 | . | . |
| 87 | 0 | 2 | 1 | 1 | 1 | 1 | 3 | 3 | 5 | 3 | 4 | 3 | 1 | . | . | . | . | 2 | 4 | 4 | . | . | 0 | 2 | . | 1 |

| 88 | 1 | 1 | 1 | 1 | 2 | 1 | 4 | 3 | 3 | 4 | 5 | 5 | 1 | 3 | 0 | 2 | 4 | . | . | . | . | . | . | . | . | . |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 89 | . | . | . | 1 | 1 | 1 | . | . | . | 5 | 3 | 4 | . | . | 0 | 0 | 1 | . | . | . | 1 | 3 | . | . | . | . |
| 90 | 1 | 1 | 0 | 1 | 2 | 1 | 4 | 4 | 4 | 4 | 3 | 3 | 1 | 5 | 3 | 4 | 4 | 2 | 3 | 4 | 2 | 5 | 1 | 2 | 4 | 1 |
| 91 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 92 | . | . | . | 1 | 0 | 1 | . | . | . | 3 | 3 | 4 | . | 1 | 0 | 0 | 1 | 2 | 3 | 4 | 1 | 3 | 0 | 1 | . | 1 |
| 93 | 1 | 1 | 1 | 1 | 2 | 1 | 3 | 4 | 4 | 4 | 4 | 4 | 2 | 2 | 1 | 1 | 2 | 2 | 3 | 4 | 0 | 2 | 1 | 1 | 0 | . |
| 94 | . | . | . | 1 | 1 | 0 | . | . | . | 5 | 4 | 5 | . | 1 | 1 | 2 | 3 | 2 | 2 | 3 | 1 | 1 | 1 | 1 | 1 | 0 |
| 95 | 0 | 1 | 1 | 1 | 4 | 1 | 4 | 5 | 4 | 4 | 3 | 4 | 1 | 2 | . | . | . | 0 | 0 | 1 | . | . | 0 | 0 | . | 0 |
| 96 | 0 | 1 | 0 | 1 | 3 | 3 | 5 | 4 | 4 | 3 | 1 | 3 | 2 | 3 | 1 | 1 | 3 | . | . | . | 0 | 1 | . | . | 0 | . |
| 97 | . | . | . | 1 | 1 | 1 | . | . | . | 4 | 4 | 3 | . | . | 0 | 1 | 3 | . | . | . | 1 | 3 | . | . | 4 | . |
| 98 | 1 | 1 | 1 | 1 | 1 | 1 | 3 | 4 | 4 | 2 | 2 | 3 | 1 | 1 | 0 | 0 | 1 | . | . | . | 0 | 1 | . | . | 1 | . |
| 99 | 1 | 1 | 1 | 1 | 1 | 1 | 4 | 3 | 3 | 4 | 4 | 5 | 1 | 2 | 1 | 0 | 2 | 4 | 4 | 4 | 0 | 1 | 0 | 1 | 1 | 0 |
| 100 | 0 | 1 | 1 | 1 | 1 | 1 | 4 | 4 | 3 | 3 | 4 | 4 | 1 | 1 | 0 | 1 | 1 | 4 | 2 | 3 | 0 | 1 | 0 | 0 | . | 0 |
| 101 | 1 | 1 | 1 | . | . | . | 4 | 3 | 4 | . | . | . | 5 | . | 1 | 4 | 4 | 0 | 0 | 3 | 1 | 3 | 2 | 2 | 1 | . |
| 102 | 1 | 1 | 1 | 0 | 1 | 1 | 3 | 3 | 4 | 3 | 3 | 4 | 3 | 0 | 2 | 3 | 4 | 3 | 4 | 4 | 0 | 2 | 0 | 2 | 3 | 3 |
| 104 | 1 | 1 | 1 | 1 | 3 | 3 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 105 | . | . | . | 1 | 1 | 2 | 5 | 5 | 4 | 3 | 4 | 4 | 2 | 3 | 2 | 2 | 3 | 4 | 5 | 3 | 1 | 3 | 2 | 3 | 1 | . |
| 107 | 0 | 1 | 1 | 1 | 1 | 1 | . | . | . | 4 | 3 | 4 | . | 3 | . | . | . | . | . | . | . | . | . | . | . | . |
| 108 | 0 | 1 | 1 | 1 | 3 | 1 | 1 | 4 | 4 | 3 | 5 | 5 | . | . | . | . | . | 4 | 1 | 2 | . | . | 0 | 0 | . | . |
| 109 | . | . | . | . | . | . | 4 | 4 | 5 | 1 | 3 | 3 | 2 | 1 | . | . | . | . | . | . | . | . | . | . | . | . |
| 110 | 1 | 1 | 1 | 1 | 2 | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 111 | 1 | 1 | 1 | 1 | 3 | 2 | 4 | 4 | 4 | 3 | 3 | 2 | 0 | . | . | . | . | 1 | 1 | 1 | . | . | 1 | 1 | . | 0 |
| 112 | 1 | 1 | 1 | 1 | 3 | 1 | 4 | 3 | 3 | 3 | 4 | 4 | 1 | 1 | 2 | 1 | 3 | . | . | . | 0 | 1 | . | . | 1 | . |
| 113 | 1 | 1 | 1 | 1 | 1 | 1 | 4 | 3 | 3 | 4 | 5 | 5 | 3 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 |

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| 114 | 3 | 1 | 1 | 1 | 3 | 1 | 3 | 3 | 3 | 3 | 4 | 4 | 1 | 2 | 2 | 3 | 3 | . | . | . | 1 | 2 | . | . | 1 | . |
| 115 | 1 | 1 | 1 | 1 | 1 | 1 | 3 | 3 | 4 | 3 | 4 | 4 | 0 | 2 | . | . | . | . | . | . | . | . | . | . | . | . |
| 116 | 1 | 2 | 1 | 1 | 1 | 1 | 4 | 3 | 4 | 3 | 4 | 4 | 1 | 1 | 3 | 2 | 3 | . | . | . | 0 | 1 | . | . | 0 | . |
| 117 | 1 | 2 | 2 | 1 | 1 | 1 | 3 | 4 | 4 | 3 | 3 | 4 | 0 | 1 | 1 | 1 | 3 | . | . | . | 0 | 1 | . | . | 0 | . |
| 118 | 1 | 1 | 1 | 1 | 1 | 1 | 3 | 3 | 4 | 2 | 3 | 4 | 3 | 4 | 1 | 3 | 4 | . | . | . | 0 | 1 | . | . | . | . |
| 119 | 0 | 1 | 1 | 1 | 1 | 1 | 3 | 4 | 4 | 3 | 5 | 5 | 5 | 2 | . | . | . | 3 | 3 | 3 | . | . | 2 | 3 | . | 3 |
| 120 | 1 | 1 | 1 | 1 | 3 | 4 | 4 | 4 | 4 | 2 | 3 | 3 | 3 | 3 | . | . | . | 3 | 3 | 3 | . | . | 2 | 2 | . | 1 |
| 121 | 1 | 1 | 1 | 1 | 1 | 1 | 4 | 3 | 3 | 3 | 4 | 5 | 3 | . | 1 | 3 | 3 | 1 | 1 | 1 | . | . | 0 | 1 | . | . |
| 122 | 1 | 1 | 1 | 1 | 3 | 1 | 3 | 4 | 4 | 3 | 5 | 5 | 1 | . | 1 | 3 | 3 | 1 | 1 | 2 | 1 | 2 | 1 | 1 | 1 | 0 |
| 123 | 0 | 1 | 1 | 0 | 0 | 1 | 4 | 4 | 4 | 2 | 5 | 5 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 2 | 1 | 2 | . | . |
| 124 | 1 | 1 | 1 | 1 | 3 | 1 | . | . | . | 3 | 4 | 3 | . | . | 1 | 0 | 1 | . | . | . | 1 | 3 | . | . | . | . |
| 125 | 0 | 1 | 1 | 1 | 3 | 1 | 3 | 5 | 4 | 4 | 4 | 5 | . | . | 2 | 3 | 3 | 0 | 2 | 2 | . | . | 1 | 1 | . | 1 |
| 126 | 1 | 1 | 1 | 1 | 1 | 1 | 5 | 4 | 5 | 3 | 4 | 5 | 2 | . | 1 | 2 | 2 | 0 | 0 | 1 | 1 | 2 | 0 | 0 | . | 0 |
| 127 | 1 | 1 | 1 | 1 | 1 | 1 | 4 | 5 | 4 | 3 | 4 | 4 | 0 | . | 1 | 2 | 2 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | . | 1 |
| 128 | 1 | 1 | 1 | 1 | 1 | 1 | 3 | 5 | 4 | 3 | 3 | 4 | 1 | 4 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | . | 0 |
| 129 | 1 | 1 | 1 | 1 | 2 | 3 | 4 | 5 | 4 | 3 | 5 | 5 | 1 | 1 | . | . | . | . | . | . | . | . | . | . | . | . |
| 130 | 0 | 1 | 1 | . | . | . | 4 | 4 | 3 | 3 | 3 | 3 | 1 | 1 | . | . | . | 1 | 1 | 2 | . | . | 1 | 3 | . | 2 |
| 131 | 2 | 1 | 1 | 1 | 1 | 4 | 4 | 4 | 5 | . | . | . | 1 | . | 0 | 1 | 2 | 1 | 1 | 1 | 2 | 3 | 1 | 3 | . | 3 |
| 132 | 1 | 1 | 1 | 1 | 2 | 2 | 4 | 5 | 5 | 4 | 5 | 4 | . | 1 | 1 | 1 | 2 | . | . | . | 0 | 1 | . | . | . | . |
| 133 | . | . | . | 1 | 1 | 1 | 3 | 4 | 3 | 3 | 4 | 4 | 2 | 2 | 0 | 1 | 2 | 0 | 1 | 2 | 0 | 1 | . | . | . | . |
| 134 | 1 | 1 | 1 | 1 | 1 | 1 | . | . | . | 4 | 3 | 3 | . | 1 | 1 | 1 | 1 | . | . | . | 0 | 2 | . | . | . | . |
| 135 | 1 | 1 | 1 | 1 | 1 | 1 | 4 | 3 | 4 | 4 | 4 | 4 | 2 | 1 | 2 | 2 | 1 | 1 | 0 | 0 | 1 | 3 | 0 | 1 | . | 0 |
| 136 | 0 | 1 | 1 | 1 | 3 | 2 | 3 | 4 | 4 | 4 | 4 | 5 | 3 | 2 | . | . | . | 0 | 0 | 0 | . | . | 0 | 0 | . | 0 |
| 137 | 1 | 1 | 1 | 1 | 1 | 3 | 4 | 3 | 4 | 3 | 5 | 4 | 0 | 2 | . | . | . | . | . | . | . | . | . | . | . | . |

| 138 | 1 | 1 | 1 | 1 | 0 | 1 | 4 | 5 | 5 | 3 | 4 | 3 | 3 | 3 | 1 | 2 | 1 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 139 | . | . | . | . | . | . | 4 | 4 | 4 | 4 | 4 | 5 | 1 | 2 | . | . | . | . | . | . | . | . | . | . | . | . |
| 140 | 0 | 1 | 1 | 1 | 3 | 1 | . | . | . | . | . | . | . | . | 1 | 2 | 3 | . | . | . | 0 | 1 | . | . | 0 | . |
| 141 | 0 | 1 | 1 | 0 | 1 | 1 | 4 | 5 | 4 | 4 | 5 | 4 | 3 | 3 | 0 | 1 | 1 | 1 | 3 | 4 | 2 | 4 | . | . | 2 | . |
| 142 | 0 | 1 | 1 | 1 | 1 | 3 | 4 | 4 | 3 | 3 | 5 | 5 | 2 | 4 | . | . | . | 0 | 3 | 4 | . | . | 0 | 1 | . | . |
| 143 | 1 | 1 | 2 | 1 | 1 | 3 | 3 | 4 | 4 | 4 | 5 | 5 | 4 | 4 | 2 | 1 | 1 | 1 | 2 | 2 | 1 | 2 | 0 | 2 | . | . |
| 144 | 2 | 1 | 1 | 1 | 2 | 1 | 4 | 5 | 5 | 4 | 4 | 5 | 3 | 4 | 1 | 2 | 3 | 1 | 3 | 2 | 0 | 0 | 1 | 1 | . | 0 |
| 145 | 1 | 1 | 1 | 1 | 1 | 1 | 4 | 5 | 4 | 4 | 3 | 2 | 5 | 3 | . | . | . | 1 | 2 | 2 | . | . | 1 | 1 | . | 1 |
| 146 | 1 | 1 | 1 | 0 | 1 | 2 | 4 | 5 | 4 | 3 | 3 | 5 | 3 | 3 | 0 | 1 | 2 | 1 | 1 | 1 | 0 | 0 | 0 | 2 | . | 3 |
| 147 | 1 | 1 | 1 | . | . | . | 3 | 4 | 4 | 3 | 3 | 3 | 4 | 5 | . | . | . | . | . | . | . | . | . | . | . | . |
| 148 | 0 | 0 | 0 | 0 | 1 | 1 | 4 | 4 | 4 | . | . | . | 5 | . | 0 | 1 | 2 | 0 | 1 | 1 | 0 | 3 | 0 | 1 | 4 | 0 |
| 149 | . | . | . | . | . | . | 4 | 4 | 3 | 4 | 5 | 3 | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 150 | 1 | 2 | 2 | 0 | 1 | 1 | 4 | 4 | 5 | 3 | 4 | 4 | 4 | 1 | 2 | 3 | 3 | 0 | 1 | 1 | . | . | 2 | 3 | . | 2 |
| 151 | 2 | 3 | 3 | 1 | 1 | 3 | 4 | 4 | 4 | 3 | 3 | 5 | 4 | 4 | . | . | . | 0 | 0 | 1 | . | . | 1 | 1 | . | 1 |
| 152 | . | . | . | 1 | 0 | 1 | . | . | . | 3 | 5 | 3 | . | . | . | . | . | 1 | 0 | 2 | . | . | 1 | 3 | . | 2 |
| 153 | 0 | 1 | 1 | 0 | 4 | 1 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 3 | 0 | 1 | 1 | . | . | . | 0 | 2 | . | . | . | . |
| 154 | 1 | 1 | 1 | 1 | 1 | 2 | 4 | 5 | 5 | 3 | 4 | 3 | 1 | 3 | 1 | 2 | 3 | 1 | 1 | 1 | 0 | 2 | 1 | 1 | 1 | . |
| 155 | 1 | 2 | 3 | 1 | 1 | 2 | 4 | 4 | 4 | 3 | 4 | 4 | 4 | 4 | 2 | 2 | 2 | 1 | 2 | 4 | 1 | 5 | 1 | 1 | 3 | 0 |
| 156 | 1 | 1 | 1 | 1 | 1 | 1 | 4 | 5 | 4 | 4 | 5 | 4 | 3 | 2 | . | . | . | 0 | 1 | 2 | . | . | 1 | 1 | . | 0 |
| 157 | 0 | 1 | 1 | 1 | 1 | 1 | 4 | 4 | 4 | 4 | 3 | 4 | 2 | 2 | . | . | . | 3 | 4 | 4 | . | . | 1 | 1 | . | 0 |
| 158 | 1 | 1 | 1 | 1 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | . | . | . | 3 | 4 | 3 | . | . | 0 | 0 | . | 0 |
| 159 | 3 | 4 | 4 | 0 | 1 | 1 | 3 | 3 | 4 | 3 | 5 | 4 | 2 | 3 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 2 | 2 | 1 | 2 | 1 |
| 160 | 1 | 1 | 3 | 1 | 1 | 1 | 3 | 4 | 4 | 3 | 3 | 4 | 5 | 4 | . | . | . | 3 | 3 | 3 | . | . | 1 | 1 | . | 1 |
| 161 | 1 | 2 | 2 | 1 | 0 | 1 | 3 | 4 | 3 | 4 | 4 | 4 | 4 | 3 | 1 | 2 | 3 | 1 | 3 | 4 | 1 | 3 | 3 | 5 | . | 2 |

| | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 162 | 5 | 2 | 3 | 1 | 1 | 3 | 4 | 4 | 4 | 3 | 4 | 4 | 3 | 4 | 2 | 3 | 5 | . | . | . | 1 | 5 | . | . | 1 | . |
| 163 | 0 | 1 | 1 | 1 | 1 | 1 | . | . | . | 2 | 4 | 4 | . | . | 0 | 1 | 2 | . | . | . | . | . | . | . | . | . |
| 164 | 0 | 2 | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 165 | 2 | 3 | 5 | 1 | 1 | 3 | 1 | 4 | 4 | 2 | 4 | 4 | . | 3 | . | . | . | 1 | 2 | 2 | . | . | 1 | 1 | . | 1 |
| 166 | 0 | 1 | 1 | 0 | 1 | 1 | 3 | 5 | 5 | 4 | 5 | 3 | 1 | 3 | . | . | . | 1 | 3 | 3 | . | . | 2 | 3 | . | 1 |
| 167 | 0 | 3 | . | . | . | . | 4 | 5 | 4 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 168 | 1 | 1 | 1 | 0 | 1 | 1 | 4 | 4 | 4 | 3 | 3 | 4 | 1 | 3 | 3 | 5 | 5 | . | . | . | 0 | 2 | . | . | 2 | . |
| 169 | 1 | 1 | 1 | 1 | 0 | 1 | 4 | 4 | 4 | 1 | 3 | 3 | 0 | . | 2 | 3 | 4 | 0 | 0 | 1 | 0 | 2 | 2 | 2 | . | . |
| 170 | 1 | 1 | 1 | 1 | 2 | 2 | 4 | 4 | 4 | 3 | 3 | 4 | 2 | 4 | 2 | 3 | 4 | 0 | 1 | 2 | 2 | 5 | 1 | 4 | 4 | 2 |
| 171 | 1 | 1 | 1 | 1 | 2 | 1 | 4 | 4 | 4 | 4 | 4 | 4 | 1 | 4 | 1 | 3 | 2 | 0 | 1 | 1 | 1 | 3 | 1 | 2 | 1 | 0 |
| 172 | 1 | 1 | 3 | 0 | 1 | 1 | 4 | 5 | 5 | 3 | 2 | 5 | . | . | 1 | 1 | 2 | 1 | 3 | 4 | 0 | 2 | 2 | 4 | . | . |
| 173 | 1 | 1 | 1 | 0 | 2 | 1 | 3 | 5 | 4 | 3 | 3 | 4 | 0 | 2 | 1 | 0 | 1 | . | . | . | 0 | 1 | . | . | 1 | . |
| 174 | 0 | 2 | 2 | 1 | 1 | 1 | 4 | 5 | 4 | 3 | 4 | 3 | 1 | 5 | . | . | . | . | . | . | . | . | . | . | . | . |
| 175 | 1 | 1 | 1 | 0 | 3 | 1 | 4 | 4 | 4 | 2 | 3 | 3 | 4 | 3 | 3 | 4 | 4 | 1 | 1 | 2 | 0 | 2 | 2 | 2 | . | 1 |
| 176 | 1 | 1 | 1 | 1 | 3 | 1 | 4 | 4 | 4 | 2 | 3 | 3 | 2 | 2 | 2 | 3 | 3 | 3 | 4 | 4 | 1 | 4 | 1 | 2 | 1 | 0 |
| 177 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| 178 | 1 | 1 | 1 | 0 | 0 | 1 | 4 | 3 | 4 | 1 | 2 | 3 | . | 2 | 0 | 2 | 2 | 1 | 2 | 2 | 0 | 2 | 0 | 0 | . | 0 |
| 179 | . | . | . | 0 | 1 | 1 | . | . | . | . | . | . | . | 1 | 2 | 1 | 2 | 4 | 4 | 1 | 1 | 1 | 0 | 0 | . | . |
| 180 | 0 | 1 | 1 | 1 | 2 | 1 | 4 | 4 | 5 | 4 | 1 | 4 | 1 | 1 | . | . | . | 1 | 2 | 2 | . | . | 2 | 2 | . | 2 |
| 181 | 0 | 1 | 1 | 1 | 4 | 3 | 4 | 3 | 4 | 2 | 1 | 4 | 4 | 4 | . | . | . | 1 | 1 | 2 | . | . | 2 | 3 | . | . |
| 182 | 1 | 1 | 2 | 1 | 4 | 1 | 4 | 2 | 4 | 2 | 2 | 3 | 2 | 2 | 0 | 2 | 1 | . | . | . | 1 | 2 | . | . | 0 | . |
| 183 | . | . | . | 0 | 1 | 1 | . | 4 | . | 3 | . | 5 | . | . | 0 | 4 | 3 | 0 | 1 | 2 | 0 | 1 | 1 | 0 | . | . |
| 184 | 1 | 1 | 1 | 1 | 2 | 1 | 4 | 3 | 4 | 3 | 3 | 3 | 2 | 3 | 1 | 3 | 3 | 4 | 4 | 4 | 0 | 1 | 1 | 1 | 0 | 1 |
| 185 | 0 | 1 | 1 | 1 | 3 | 1 | 4 | 2 | 4 | 3 | 1 | 3 | 1 | 1 | . | . | . | . | . | . | . | . | . | . | . | . |

| | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 186 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 4 | 0 |
| 187 | 1 | 1 | 1 | 0 | 1 | 0 | 2 | 3 | 4 | 3 | . | 3 | . | . | . | . | . | . | . | 2 | 1 | 0 | 0 | . | . | |
| 188 | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 | 0 | 1 | 0 | 1 | 2 | . | . | . | . | . | . | . |
| 189 | 1 | 1 | 2 | 1 | 1 | 1 | 4 | 5 | 4 | 4 | 1 | 5 | 1 | 1 | . | . | . | 1 | 2 | 2 | . | . | 1 | 2 | . | . |
| 190 | 1 | 1 | 1 | 0 | 1 | 1 | 4 | 3 | 4 | 4 | 2 | 4 | 0 | 2 | . | . | . | 1 | 2 | 1 | . | . | 2 | 3 | . | 1 |
| 191 | 1 | 2 | 2 | 1 | 1 | 1 | 4 | 5 | 4 | 4 | 0 | 5 | 3 | 3 | 3 | 5 | 4 | 1 | 2 | 2 | 1 | 2 | 1 | 4 | 1 | 2 |
| **192** | **1** | **1** | **1** | **1** | **1** | **0** | **4** | **5** | **4** | **4** | **1** | **5** | **1** | **1** | **1** | **.** | **.** | **0** | **1** | **1** | **.** | **.** | **1** | **1** | **.** | **0** |
| **Sum** | 154 | 153 | 153 | 164 | 164 | 164 | 150 | 152 | 150 | 160 | 158 | 160 | 126 | 131 | 123 | 119 | 122 | 113 | 108 | 108 | 111 | 111 | 104 | 104 | 68 | 77 |

Appendix II: ANOVA analysis of mean scores on different replications, disease evaluation cycles, years, and locations in Chapter II.

A.  Variance analysis of leaf mean scores on replicate effect

SC 2009-Cycle 1

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 73.792 | 170 | .434 | 1.316 | **.044** |
| Within Groups | 48.500 | 147 | .330 |  |  |
| Total | 122.292 | 317 |  |  |  |

SC 2009-Cycle 2

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 94.981 | 170 | .559 | .777 | .944 |
| Within Groups | 105.000 | 146 | .719 |  |  |
| Total | 199.981 | 316 |  |  |  |

SC 2009-Cycle 3

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 181.364 | 169 | 1.073 | 1.343 | **.034** |
| Within Groups | 117.500 | 147 | .799 |  |  |
| Total | 298.864 | 316 |  |  |  |

SC 2011-Cycle 1

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 119.819 | 167 | .717 | 1.220 | .111 |
| Within Groups | 83.500 | 142 | .588 | | |
| Total | 203.319 | 309 | | | |

SC 2011-Cycle 2

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 131.194 | 168 | .781 | .974 | .566 |
| Within Groups | 113.000 | 141 | .801 | | |
| Total | 244.194 | 309 | | | |

SC 2011-Cycle 3

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 97.855 | 167 | .586 | 1.081 | .318 |
| Within Groups | 77.000 | 142 | .542 | | |
| Total | 174.855 | 309 | | | |

B. Variance analysis of C and D replicates

NC 2008-Cycle 1

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 175.631 | 151 | 1.163 | 1.329 | .076 |
| Within Groups | 73.500 | 84 | .875 | | |
| Total | 249.131 | 235 | | | |

NC 2008-Cycle 2

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 230.817 | 150 | 1.539 | .947 | .616 |
| Within Groups | 123.500 | 76 | 1.625 | | |
| Total | 354.317 | 226 | | | |

NC 2008-Cycle 3

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 247.387 | 151 | 1.638 | 1.469 | **.030** |
| Within Groups | 87.000 | 78 | 1.115 | | |
| Total | 334.387 | 229 | | | |

NC 2009-Cycle 1

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 95.388 | 146 | .653 | 1.367 | .074 |
| Within Groups | 32.500 | 68 | .478 | | |
| Total | 127.888 | 214 | | | |

NC 2009-Cycle 2

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 200.923 | 146 | 1.376 | 1.366 | .074 |
| Within Groups | 68.500 | 68 | 1.007 | | |
| Total | 269.423 | 214 | | | |

C. Variance analysis of fruit mean scores on replicate effect

SC 2011

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 350.418 | 143 | 2.450 | 1.943 | .000 |
| Within Groups | 142.500 | 113 | 1.261 | | |
| Total | 492.918 | 256 | | | |

NC 2009

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 154.007 | 113 | 1.363 | .983 | .546 |
| Within Groups | 43.000 | 31 | 1.387 | | |
| Total | 197.007 | 144 | | | |

D. Variance analysis of leaf mean scores on disease evaluation cycle effect

SC 2009-Row A

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 180.664 | 153 | 1.181 | 2.436 | **.000** |
| Within Groups | 148.333 | 306 | .485 | | |
| Total | 328.998 | 459 | | | |

SC 2009-Row B

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 112.933 | 163 | .693 | .942 | .665 |
| Within Groups | 241.333 | 328 | .736 | | |
| Total | 354.266 | 491 | | | |

SC 2011-Row A

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 86.850 | 151 | .575 | 1.294 | **.031** |
| Within Groups | 133.333 | 300 | .444 | | |
| Total | 220.184 | 451 | | | |

SC 2011-Row B

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 189.500 | 159 | 1.192 | 1.692 | **.000** |
| Within Groups | 224.000 | 318 | .704 | | |
| Total | 413.500 | 477 | | | |

NC 2008-Row C

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 345.797 | 122 | 2.834 | 3.512 | **.000** |
| Within Groups | 194.500 | 241 | .807 | | |
| Total | 540.297 | 363 | | | |

NC 2008-Row D

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 398.442 | 112 | 3.558 | 5.851 | **.000** |
| Within Groups | 131.333 | 216 | .608 | | |
| Total | 529.775 | 328 | | | |

NC 2009-Row C

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 152.874 | 110 | 1.390 | 1.160 | .218 |
| Within Groups | 133.000 | 111 | 1.198 |  |  |
| Total | 285.874 | 221 |  |  |  |

NC 2009-Row D

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 153.981 | 103 | 1.495 | 2.221 | **.000** |
| Within Groups | 70.000 | 104 | .673 |  |  |
| Total | 223.981 | 207 |  |  |  |

E.  Variance analysis of leaf mean scores on location effect

SC and NC 2009

|  | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 209.981 | 170 | 1.235 | 1.518 | **.000** |
| Within Groups | 985.394 | 1211 | .814 |  |  |
| Total | 1195.375 | 1381 |  |  |  |

F.  Variance analysis of year effect on leaf mean scores

SC 2009 and 2011

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 2991.358 | 339 | 8.824 | 13.258 | .000 |
| Intercept | 10320.009 | 1 | 10320.009 | 15505.655 | .000 |
| Year | 2459.277 | 1 | 2459.277 | 3695.027 | **.000** |
| Tree# | 160.816 | 173 | .930 | 1.397 | .001 |
| Year * Tree# | 147.233 | 165 | .892 | 1.341 | **.004** |
| Error | 1026.300 | 1542 | .666 | | |
| Total | 15144.000 | 1882 | | | |
| Corrected Total | 4017.658 | 1881 | | | |

NC 2008 and 2009

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 787.493 | 298 | 2.643 | 2.616 | .000 |
| Intercept | 1935.314 | 1 | 1935.314 | 1915.478 | .000 |
| Year | 36.354 | 1 | 36.354 | 35.982 | **.000** |
| Tree# | 427.403 | 152 | 2.812 | 2.783 | .000 |
| Year * Tree# | 268.543 | 145 | 1.852 | 1.833 | **.000** |
| Error | 832.533 | 824 | 1.010 | | |
| Total | 4062.000 | 1123 | | | |
| Corrected Total | 1620.027 | 1122 | | | |

Appendix III:  Primers derived from 48 Resistance gene analogs (RGAs) used for analysis in Chapter III.

| Name | Forward primer sequence | Reverse primer sequence |
|---|---|---|
| NBS1 | GAAGATTGAGGAGCGCTTTG | GGGAGGCCCTTACACTTCTC |
| NBS3 | TTTTGACAGCATCCGTGCTA | TCCACTCCAAATGTGCTCAG |
| NBS4 | GGTGTGGGAAAGACGACAAT | TAGCGTATTGCACGTTCTGC |
| NBS5 | GGGGTTTCTCAACGTGGTAA | GGAGGCCTCTGGCATAATTT |
| NBS9 | GGGGGAGTGGGAAAGACTAC | TTTCTTAAAGGCATGTCGCC |
| NBS10 | CTGAGAATGCTTTGCTGCTG | ATTCCACGAAGCAAACAAGG |
| NBS11 | AGGAGGGAAAGACTTGGAGC | GTCTTTCTCCTGGAAAGGCA |
| NBS15 | GGAGCTCTTTAGTTGGCACGCT | GAGCCAGGGGAAGCCCTCCA |
| NBS16 | AGGGATTTGGCGATGACGAGGC | CCTTGAGGGCGAGTGGAAGGC |
| NBS17 | AGCTGAGAATGCTTTGCCGC | TGGGTGGGCTTGGGAAAACGA |
| NBS18 | CTTGAGCGCCAGGGGCAGTC | TGGAGCTCTTCAGTTGGCACGC |
| NBS19 | AGGGCGAGTGGGAGACCCTT | CGGGAAGACCACCCTTGCGG |
| NBS20 | AGCTCCCACGCATCACCCCT | CGTGTGGGATGTCCACCTTTGGG |
| NBS21 | GGGATGGGTGGGCTGGGAAA | GCAAGTGGGAGGCCTTGAGCA |
| NBS22 | TGGTTTGGTTCAGGCAGCAGA | CTAGGGGGAGCCCGTCAGCA |
| NBS23 | GGTGCGATTGGTTTGGTTTGGGC | GCAAGGGGCAGACCTCCAGC |
| NBS25 | GGGGGAGTGGGAAAGACTACACT | AGAGCGAGGGGGAGGCCTTT |
| NBS26 | ACGGGTTCTCCTTGTTCTCGATG | CGAGGGGGAGTCCTCCGCAA |
| NBS28 | GGGATGGGCGGATTGGGCAAA | CGAGGGGTAGGCCTCTGGCA |
| NBS29 | GCGCTAGGGGAAGGCCATCAG | GGTGTGGACCAATTGGGGCAGTT |
| NBS30 | GGGAAAGACTTGGAGCTGGCCC | AGGGCTAGAGGTAGGCCTCG |
| NBS32 | GGGAGGGGTGGGTAAGACGACC | AGGTTCCCCGCTGTTCATCCCA |
| NBS33 | TGTTCTCGAGCATTCACGTC | TGCTTGTTTTCTCGCAAATG |
| NBS34 | AGCAGCTTTCTTGCAAATGT | CTTCCCAAACCAAAGCAGTC |
| NBS35 | CGATCATGGTCACCAACAAG | ATCAGAACAAACGGGTGGAC |
| NBS36 | CCACAAGTTCCCGAGCTAAA | GATGTCCACCTTTGGGAGAA |
| NBS37 | CCCAACCAATCATTTCCAAC | TAGCCACTTTCTTCCCGATG |
| NBS38 | CGGCTTCCTTCATAAAACCA | AAATTTCAGCAGGGCATGAG |
| NBS39 | GCTAACGGTAAGCCTCGACA | TGGCCCTGGAAGTAGAATTG |
| NBS40 | TCCTGGATGAAGCCATTCTC | GATCTCCAAAAGGGGAAAGC |
| NBS41 | CCAAACCAATCACCATTTCC | TCGAAGCTCATGGTTTCCTT |
| NBS42 | CATCCATTTCTTCAGCGACA | CAAGACGAAGGACGTTGGTT |
| NBS43 | CAGGGAAAGCTGATCCTGAG | TGGCAGCCATGTTTAGATCA |
| NBS44 | CTGCTTATCTAGCCCGGATG | TGACGGTGCAGGTTTGAGTA |
| NBS45 | TCATCGTCTGCTACGTCGTC | GGCTATGTTGGTGCCTGACT |
| NBS46 | CCTGCCAAGAAAGGTGTCAT | TTGACTCTGATCCCATGCTG |
| NBS47 | TGGACTATCCCCAGATCGAG | TCAACATCAATGGCCTGAAA |
| NBS48 | TGGAACATGTCAGCTTCTGC | GAATTGCTGAAGTGGTGCAA |
| NBS49 | CCCTTGAGGGTATCAAGCAA | CACGGCTTCTCATCTTGTCA |
| NBS50 | GTGCTAATGGAGGGAAGTGG | ACGTTGACAACTGCTCCACA |
| NBS51 | CTGTGGGTGTTGGAGCACTA | TGCCGTAACCATGTTTCTGA |

| NBS52 | TTTGCTGCAGGAGGAACTTT | AATGCACCATATTGCGATCA |
|-------|----------------------|----------------------|
| NBS53 | CCTCAATTTGCCCTTGGATA | GTCCAAAAAGGTGGACGAAA |
| NBS54 | TCCCACAATTTGAAACCACA | TCAGGGAGGTCAAGATACCG |
| NBS55 | CACTTGCCAAGGCTCTTTTC | TTGCTTCTCATACGCAATCG |
| NBS56 | GAAGTAATGCGAGCGTGTCA | ACCTTGGCTGAATTGACTGC |
| NBS57 | GGGTTGGTAAGACCACCCTTA | CGTGAGCCTTTTCGAGTTGT |
| NBS58 | GGAGCAACTCGAAGCGATAG | GCCGACAAAAGAAGCTCAAG |

Appendix IV: Genotyping plot of the mapped markers in the C x O linkage map for Chapter III. The a allele is from O'Henry, and b allele is from Clayton.
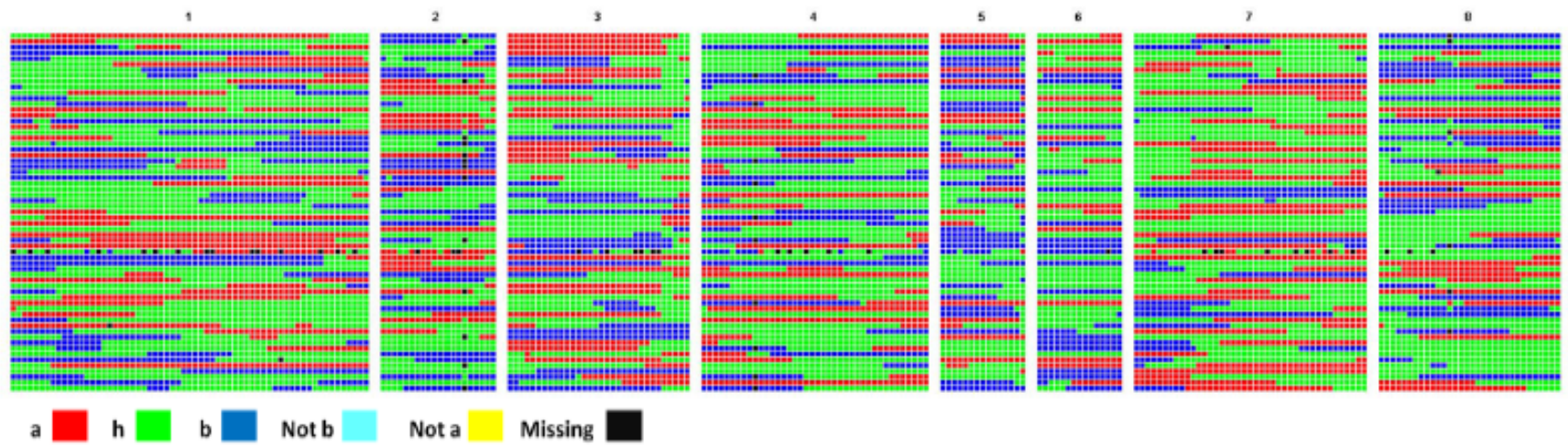
Appendix V: Annotation of Putative Nucleotide-binding sequences in Peach for Chapter III, the coding sequences were acquired from GDR.

| Seq. Name | Annotation |
| --- | --- |
| ppa023316m | (CC-)NBS |
| ppa000645m | (CC-)NBS-LRR |
| ppa024389m | (CC)NBS-LRR |
| ppa024764m | (CC)-NBS-NBS |
| ppa019132m | (NBS-LRR) |
| ppa001003m | CC-NBS |
| ppa014755m | CC-NBS |
| ppa015104m | CC-NBS |
| ppa015789m | CC-NBS |
| ppa016009m | CC-NBS |
| ppa016604m | CC-NBS |
| ppa017700m | CC-NBS |
| ppa017971m | CC-NBS |
| ppa019094m | CC-NBS |
| ppa019235m | CC-NBS |
| ppa020719m | CC-NBS |
| ppa021797m | CC-NBS |
| ppa021874m | CC-NBS |
| ppa023411m | CC-NBS |
| ppa023686m | CC-NBS |
| ppa023899m | CC-NBS |
| ppb014066m | CC-NBS |
| ppb014730m | CC-NBS |
| ppb016299m | CC-NBS |
| ppb025386m | CC-NBS |
| ppa001007m | CC-NBS-CC |
| ppa000247m | CC-NBS-LRR |
| ppa000274m | CC-NBS-LRR |
| ppa000335m | CC-NBS-LRR |
| ppa000343m | CC-NBS-LRR |
| ppa000391m | CC-NBS-LRR |
| ppa000407m | CC-NBS-LRR |
| ppa000457m | CC-NBS-LRR |
| ppa000953m | CC-NBS-LRR |
| ppa000961m | CC-NBS-LRR |

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|---|---|
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| ppa001008m | CC-NBS-LRR |
| ppa001076m | CC-NBS-LRR |
| ppa001090m | CC-NBS-LRR |
| ppa001182m | CC-NBS-LRR |
| ppa001212m | CC-NBS-LRR |
| ppa001282m | CC-NBS-LRR |
| ppa001346m | CC-NBS-LRR |
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| ppa014680m | CC-NBS-LRR |
| ppa014872m | CC-NBS-LRR |
| ppa014877m | CC-NBS-LRR |
| ppa014998m | CC-NBS-LRR |
| ppa015043m | CC-NBS-LRR |
| ppa015125m | CC-NBS-LRR |
| ppa015185m | CC-NBS-LRR |
| ppa015274m | CC-NBS-LRR |
| ppa015461m | CC-NBS-LRR |
| ppa015499m | CC-NBS-LRR |
| ppa015658m | CC-NBS-LRR |
| ppa015762m | CC-NBS-LRR |
| ppa015899m | CC-NBS-LRR |
| ppa016036m | CC-NBS-LRR |
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| ppa016635m | CC-NBS-LRR |
| ppa016692m | CC-NBS-LRR |

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| ppa016937m | CC-NBS-LRR |
| ppa016959m | CC-NBS-LRR |
| ppa017078m | CC-NBS-LRR |
| ppa017126m | CC-NBS-LRR |
| ppa017163m | CC-NBS-LRR |
| ppa017399m | CC-NBS-LRR |
| ppa017506m | CC-NBS-LRR |
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| ppa017615m | CC-NBS-LRR |
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| ppa018247m | CC-NBS-LRR |
| ppa018378m | CC-NBS-LRR |
| ppa018388m | CC-NBS-LRR |
| ppa018463m | CC-NBS-LRR |
| ppa018717m | CC-NBS-LRR |
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| ppa018842m | CC-NBS-LRR |
| ppa018885m | CC-NBS-LRR |
| ppa018920m | CC-NBS-LRR |
| ppa019012m | CC-NBS-LRR |
| ppa019037m | CC-NBS-LRR |
| ppa019071m | CC-NBS-LRR |
| ppa019097m | CC-NBS-LRR |
| ppa019412m | CC-NBS-LRR |
| ppa019824m | CC-NBS-LRR |
| ppa019872m | CC-NBS-LRR |
| ppa019887m | CC-NBS-LRR |
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| ppa019915m | CC-NBS-LRR |
| ppa019936m | CC-NBS-LRR |
| ppa020292m | CC-NBS-LRR |
| ppa020323m | CC-NBS-LRR |
| ppa020375m | CC-NBS-LRR |
| ppa020437m | CC-NBS-LRR |

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| ppa020740m | CC-NBS-LRR |
| ppa020745m | CC-NBS-LRR |
| ppa020993m | CC-NBS-LRR |
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| ppa021194m | CC-NBS-LRR |
| ppa021541m | CC-NBS-LRR |
| ppa021551m | CC-NBS-LRR |
| ppa021741m | CC-NBS-LRR |
| ppa021839m | CC-NBS-LRR |
| ppa022119m | CC-NBS-LRR |
| ppa022198m | CC-NBS-LRR |
| ppa022439m | CC-NBS-LRR |
| ppa022498m | CC-NBS-LRR |
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| ppa022876m | CC-NBS-LRR |
| ppa022946m | CC-NBS-LRR |
| ppa023090m | CC-NBS-LRR |
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| ppa023894m | CC-NBS-LRR |
| ppa024157m | CC-NBS-LRR |
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| ppa024377m | CC-NBS-LRR |
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| ppa024705m | CC-NBS-LRR |
| ppa024822m | CC-NBS-LRR |
| ppa024868m | CC-NBS-LRR |
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| ppa025169m | CC-NBS-LRR |
| ppa025202m | CC-NBS-LRR |
| ppa025265m | CC-NBS-LRR |

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| ppa026747m | CC-NBS-LRR |
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| ppa027039m | CC-NBS-LRR |
| ppa1027168m | CC-NBS-LRR |
| ppa1027175m | CC-NBS-LRR |
| ppa1027210m | CC-NBS-LRR |
| ppb015994m | CC-NBS-LRR |
| ppb017543m | CC-NBS-LRR |
| ppb019479m | CC-NBS-LRR |
| ppb021897m | CC-NBS-LRR |
| ppb024266m | CC-NBS-LRR |
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| ppa019783m | CC-NBS-LRR-CC |
| ppa017578m | CC-NBS-NBS |
| ppa000373m | CC-NBS-NBS-LRR |
| ppa015771m | CC-NBS-NBS-LRR |
| ppa016027m | CC-NBS-NBS-LRR |
| ppa024346m | CC-NBS-NBS-LRR |
| ppa025816m | CC-NBS-NBS-LRR |
| ppa014815m | LRR-TIR-NBS-LRR |
| ppa000799m | NBS |
| ppa014763m | NBS |
| ppa015025m | NBS |
| ppa015199m | NBS |
| ppa015368m | NBS |
| ppa015433m | NBS |
| ppa016137m | NBS |
| ppa017563m | NBS |
| ppa017627m | NBS |
| ppa017946m | NBS |

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| ppa018793m | NBS |
| ppa020607m | NBS |
| ppa022724m | NBS |
| ppa023927m | NBS |
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| ppa024986m | NBS |
| ppa026249m | NBS |
| ppa026842m | NBS |
| ppa027225m | NBS |
| ppa1027204m | NBS |
| ppb017370m | NBS |
| ppb023374m | NBS |
| ppa020893m | NBS-CC |
| ppa020065m | NBS-CC-NBS-LRR |
| ppb022444m | NBS-CC-NBS-LRR |
| ppa000600m | NBS-LRR |
| ppa000828m | NBS-LRR |
| ppa000891m | NBS-LRR |
| ppa000935m | NBS-LRR |
| ppa001015m | NBS-LRR |
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| ppa001461m | NBS-LRR |
| ppa001712m | NBS-LRR |
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| ppa016037m | NBS-LRR |
| ppa016232m | NBS-LRR |
| ppa016391m | NBS-LRR |
| ppa016517m | NBS-LRR |
| ppa017004m | NBS-LRR |
| ppa017857m | NBS-LRR |
| ppa017999m | NBS-LRR |
| ppa018003m | NBS-LRR |
| ppa018088m | NBS-LRR |
| ppa018298m | NBS-LRR |

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| ppa019283m | NBS-LRR |
| ppa019580m | NBS-LRR |
| ppa019683m | NBS-LRR |
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| ppa019834m | NBS-LRR |
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| ppa020855m | NBS-LRR |
| ppa021061m | NBS-LRR |
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| ppa024365m | NBS-LRR |
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| ppa025033m | NBS-LRR |
| ppa025035m | NBS-LRR |
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| ppa025442m | NBS-LRR |
| ppa025617m | NBS-LRR |
| ppa026159m | NBS-LRR |
| ppa026937m | NBS-LRR |
| ppb015774m | NBS-LRR |
| ppa023610m | NBS-LRR (cc?) |
| ppa026786m | NBS-LRR-CC |

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| ppa024390m | NBS-NBS |
| ppb017898m | NBS-NBS |
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| ppa023165m | NBS-NBS-LRR |
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| ppa026289m | NBS-NBS-LRR |
| ppa022016m | TIR-LRR |
| ppa015945m | TIR-NBS |
| ppa017752m | TIR-NBS |
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| ppa019613m | TIR-NBS |
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| ppa025905m | TIR-NBS |
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| ppa026276m | TIR-NBS |
| ppa027032m | TIR-NBS |
| ppa021230m | TIR-NBS-BED |
| ppa021538m | TIR-NBS-BED |
| ppa022772m | TIR-NBS-BED |
| ppa026962m | TIR-NBS-BED |
| ppa000268m | TIR-NBS-LRR |
| ppa000477m | TIR-NBS-LRR |
| ppa000489m | TIR-NBS-LRR |
| ppa000501m | TIR-NBS-LRR |
| ppa000524m | TIR-NBS-LRR |
| ppa000525m | TIR-NBS-LRR |
| ppa000551m | TIR-NBS-LRR |
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| ppa001315m | TIR-NBS-LRR |
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| ppa014797m | TIR-NBS-LRR |
| ppa014887m | TIR-NBS-LRR |

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|---|---|
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| ppa015956m | TIR-NBS-LRR |
| ppa016158m | TIR-NBS-LRR |
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| ppa016634m | TIR-NBS-LRR |
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| ppa017550m | TIR-NBS-LRR |
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| ppa017937m | TIR-NBS-LRR |
| ppa017983m | TIR-NBS-LRR |
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| ppa019742m | TIR-NBS-LRR |
| ppa020033m | TIR-NBS-LRR |

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| ppa023596m | TIR-NBS-LRR |
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| ppa023936m | TIR-NBS-LRR |
| ppa023967m | TIR-NBS-LRR |
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| ppa024249m | TIR-NBS-LRR |
| ppa024258m | TIR-NBS-LRR |

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| ppa024688m | TIR-NBS-LRR |
| ppa024831m | TIR-NBS-LRR |
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| ppb015618m | TIR-NBS-LRR |
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| ppa017814m | TIR-NBS-NBS-LRR |
| ppa023180m | TIR-NBS-NBS-LRR |
| ppa023486m | TIR-NBS-NBS-LRR |
| ppa024381m | TIR-NBS-NBS-LRR |
| ppa026531m | TIR-NBS-NBS-LRR |
| ppa021062m | TIR-TIR-NBS-LRR |