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ENABLING MARKER-ASSISTED BREEDING (MAB) FOR BLUSH IN PEACH
[*Prunus persica* (L.) Batsch]

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Horticulture

---

by
Terrence J. Frett
May 2012

Committee Members:
Dr. Ksenija Gasic, Committee Chair
Dr. Gregory Reighard
Dr. Desmond Layne
Dr. Douglas Bielenberg

ABSTRACT

Phenotyping is a crucial component for using DNA-based tools in gene discovery and marker development. Phenotypic and genotypic data are essential for linking genetic variation with biological function, thus documenting gene function. However, phenotypic data gathering is not keeping pace with the immensely increasing amount of available genomic information, brought forth by current High Throughput technologies. Standardized phenotyping protocols for peach [*Prunus persica* (L.) Batsch] have been developed for 6 productivity traits (on the tree) and 16 fruit quality traits. Documentation of fruit quality phenotypes has been performed applying developed standardized phenotyping protocol in two seasons, at nine locations, on 513 peach and almond accessions, cultivars, advanced selections, lines, and or populations.

In this study blush (i.e. red skin pigmentation) inheritance and associated genes were investigated extensively. Blush is an important trait for marketing peaches. The red skin pigmentation develops through the flavonoid and anthocyanin pathways. Both genetic and environmental stimuli and their interaction (genotype x environment) control the regulation of this pathway. Sunlight induces the progression of red skin development.

To study the genetic control of blush in peach a controlled cross between two cultivars with contrasting phenotypes, Zin Dai (~30% red) and Crimson Lady (~100% red), was made. One F_1 hybrid, BY02p4019, with intermediate levels of blush (~65% red) was selfed to generate a segregating F_2 blush population (ZC^2). The segregating population was phenotyped for blush for four years (2007, 2008, 2010, 2011) using a

visual rating scale (0-5) and in 2011 using a colorimeter (L*, a*, b*). Twenty-five individuals, exhibiting a blush range from 0 (0% red) to 5 (100% red) and a normal distribution for this trait, were genotyped with an IPSC 9K peach SNP array v1.

A ZC^2 genetic linkage map was constructed with 1,335 SNP markers, comprising 14 linkage groups. This map covers a genetic distance of ~452.51 cM with an average marker spacing of 2.38 cM/marker and an average number of 95 markers per LG. A major QTL for blush has been located on LG3, denoted Blush.Pp.ZC-3.1. This QTL spanned 21-41cM on LG3 and explained on average 72% of the phenotypic variation for the trait. QTL analysis for four different seasons confirms the identification of this major QTL for blush in peach, and supports its stability. Three minor QTL were located on LG's 4, and 7 indicating the presence of minor genes involved with blush development.

Candidate genes involved in skin and flesh coloration of cherry (*PavMYB10*) and apple (*MdMYB10*) are located within the interval of the major QTL on LG3 suggesting the same genetic control for color development in the Rosaceae family. A standardized protocol for collecting phenotypic data in peach will facilitate discovery of genes associated with fruit quality and other agronomically important traits.

RESUMEN

La caracterización del fenotipo es un componente crucial para usar herramientas de ADN en el descubrimiento de genes y el desarrollo de marcadores. Los datos de fenotipo y genotipo son esenciales para conectar la variación genetica con la función biológica. Sin embargo, la colección de datos de fenotipo no avanza al mismo ritmo de la información genética que se genera a travé de tecnologías de secuenciación de siguiente generación (Next Generation Sequencing). Se han desarrollado protocolos estandarizados para la caracterización del fenotipo de duraznos [*Prunus persica* (L.) Batsch] para 6 caracteres de productividad (en el árbol) y 16 caracteres de calidad del fruto. Se documentó el fenotipo de calidad de frutos durante dos años en nueve localidades, en 513 accesiones, cultivares, selecciones avanzadas, líneas, y o poblaciones de duraznos y almedras.

Investigamos extensivamente la herencia y genes asociados con el "rubor"(i.e. pigmentación roja de la piel). El rubor es una característica importante en el mercado de los duraznos. La pigmentación roja de la piel se desarrolla a través de las rutas de flavonoides y antocianinas. Estímulos tanto genéticos como ambientales y su interacción (genotipo x ambiente) controlan la regulación de estas rutas. La luz del sol induce la progresión del desarrollado de la piel roja.

Para estudiar el control genético del rubor en duraznos, se hizo un cruce controlado entre dos cultivares con fenotipos contrastantes, Zin Dai (~30% rojo) y Crimson Lady (~100% rojo). Se auto-fertilizó un híbirido F_1, BY02p4019, con niveles

promedios (~65% rojo) para generar una población F_2 que segrega para rubor (ZC^2). Se caracterizó el fenotipo para rubor de la población segregante durante cuatro anos (2007, 2008, 2010, 2011) utilizando una escala visual (0-5) y en 2011 utilizando un colorímetro (L*, a*, b*). Se caracterizó el genotipo de veinticinco individuos con rubor entre 0 (0% rojo) y 5 (100% rojo) y distribución normal para este carácter, utilizando un IPSC 9K SNP Array v1 para durazno.

Se construyó un mapa genético ZC^2 con 1,335 marcadores de SNP que contienen 14 grupos del ligamiento. Este mapa cubre una distancia genética de ~452.51 cM con un promedio de espaciamiento de marcadores de 2.38 cM/marcador y un promedio de 95 marcadores por LG. Un loci de caracteres cuantitativos (QTL) mayores se localizó un en LG3, denotado Blush.Pp.ZC-3.1. Este QTL atravesado 21-41cM en LG3 y explicado en el promedio 72% de variación fenotípica para el rasgo. El análisis de QTL para cuatro anos diferentes, confirma la identificación de este QTL mayor para rubor en durazno, y confirma su estabilidad. Tres QTL menores se localizaron en LG's 4, y 7 indican la presencia de genes menores implicados con el desarrollo del rubor.

Se localizaron genes candidatos involucrados en la coloración de la piel y la carne de la (*PavMYB10*) y la manzana (*MdMYB10*) en el intervalo del QTL mayor en LG3 sugiriendo el mismo control genético para el desarrollo del color en la familia Rosaceae. Un protocolo estandarizado para la colección de datos fenotipicos de falicitar el descubrimiento de genes asociados con la calidad de fruto y otros caracteres agronomicos importantes.

DEDICATION

I dedicate this thesis to my family: my father Terry Frett, mother Lauree Frett, and sister's Christina and Hannah Frett. Their support and positive encouragement, especially during the stressful times, enabled me to complete this work. My family will forever be the most important part of my life. To show my graditute, I look forward to supplying them and my future family with endless amounts of fruit I work with in my future career.

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First and foremost I would like to thank my advisor Dr. Ksenjia Gasic for her guidance, patience, and opportunity to perform this study. Secondly I want to express my gradititude for the willingness of Dr. Bielenberg, Dr. Layne and Dr. Reighard to serve on my committee. Their guidance, and insightful feedback, positively shaped my education here at Clemson in a great way. I am extremely greatful for all they and Dr. Gasic have done for me, without their help this thesis would not have been possible. They all went above and beyond by giving up their time to not only help me on my thesis but also to give me valuable advice on career paths and helped me set up internships that will significantly impact my future. Through my work with them they have all become lifelong colleages and more importantly friends.

I would also like to express my gratitude for Dr. William Okie, USDA peach breeder, for creating the blush population and allowing us to propogate it at the Musser farm. Thanks to all the Musser Farm research crew (Jeff Hopkins, Billy Newall, Kathy Brock, Sam Hudson, David Ouellette and Trey Burrell) and all greenhouse staff members for all their assistance in peach tree maintenance.

Special thanks to all the colleages I have gained through RosBREED (http://www.rosbreed.org/), the National Association of Plant Breeders (NAPB), the Plant Breeding Coordinating Committee (PBCC), and the newely formed NAPB Graduate Student Working Group. I have learned a lot working with all of you and I look forward to future collaborations! I would like to commend all the faculty, staff, and fellow gradute

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CHAPTER I

LITERATURE REVIEW

Introduction to Peach: Decline in Peach Industry

Peach [*Prunus persica* (L.) Batsch] is a commercially important fruit tree species. This species is native to China and a member of the Rosaceae family, subfamily Prunoideae. The Rosaceae family encompasses several economically significant temperate fruits: *Malus domestica* (apples), *Fragaria* × *ananassa* (garden strawberries), *Pyrus communis* L. (European pears), *Rubus sp.* (blackberries and raspberries) and ornamental plants such as roses (*Rosa sp.* L.). The subfamily Prunoideae comprises the largest genus of the Rosaceae family, Prunus. Members of the Prunus genus are known as stone fruits (drupes), because they contain a fleshy mesocarp, enclosing a hard or stony endocarp. The most economically important fruit and nut Prunus species include: peach (and nectarine), *P. avium* and *P. cerasus* (sweet and sour cherry), *P. domestica* and *P. salicina* (European and Japanese plum), *P. armeniaca* (apricot), and *P. dulcis* (almond) respectively.

In the Rosaceae family, peach is second in temperate fruit production only to apple, with 10 million tons produced globally (Fideghelli et al., 1998). China became the main producer of peaches around 1993, and their production continues to grow to this day (Huang et al., 2008). In fact, in 2006, China was responsible for the production of 44% of the total global supply, while the other top producers of peaches: Italy, Spain, the

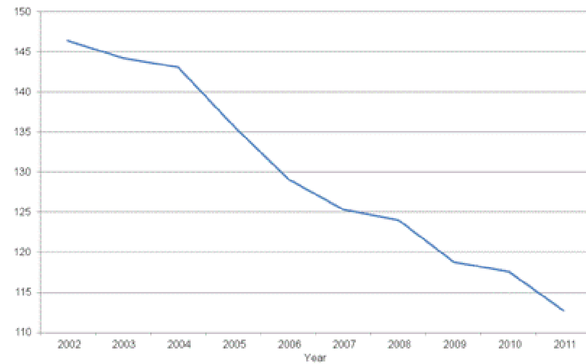
USA and Greece, only produced 10%, 7%, 5%, and 5%, respectively (Huang et al., 2008).

In the U.S.A. peach is a very important economic fruit. The peach industry in the U.S.A. has been dominated by California, followed by South Carolina, Georgia and New Jersey. Across 2008, 2009, and 2010, California accounted for 74% of the peaches and nectarines produced in the U.S.A., while South Carolina, New Jersey and Georgia accounted for 7.23%, 3.10% and 2.95% respectively (Perez et al., 2011). In 2011, California, South Carolina, Georgia, and New Jersey were projected to account for 72 %, 8%, 4% and 3%, respectively, of the total production of peaches and nectarines in the U.S.A (Perez et al., 2011). Throughout the past decade the U.S. peach industry has seen a decline (Figure 1), attributed to two main factors: [1] the need to harvest peaches at immature stages for storage and shipment purposes, negatively impacting fruit quality, and [2] low fruit quality (Crisosto et al., 1995; Crisosto 2002; Crisosto and Costa, 2008).

The primary reason for the decline of the U.S. peach industry is attributed to the necessity to harvest peaches at immature stages for storage and shipment purposes (Fideghelli et al., 1998; Crisosto 2002; Sansavini et al., 2006; Crisosto and Costa, 2008). Several vital fruit quality traits including size, flavor (high sugar and moderate to low acidity), color, and blush (red skin pigmentation) develop as a peach ripens on the tree. Harvesting a peach at an immature stage limits the full development of essential fruit quality traits. Low peach consumption in the USA therefore can be overcome by finding a more precise balance with respect to fruit quality and the maturity stage at harvest (Bielenberg et al., 2009).

[A]

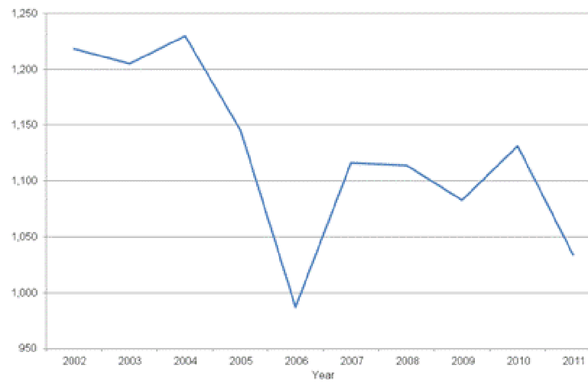
Peach Bearing Acreage
United States, 2002-2011



USDA-NASS
3-22-12

[B]

Peach Utilized Production
United States, 2002-2011



USDA-NASS
3-22-12

Figure 1. [A] USA Peach Bearing Acreage 2002-2011 (http://www.nass.usda.gov/Charts_and_Maps/Noncitrus_Fruits/histbape.asp); [B] USA Peach Utilized Production 2002-2011 (http://www.nass.usda.gov/Charts_and_Maps/Noncitrus_Fruits/histuppe.asp). (*Note: Y-axis does not start at zero).

Fruit quality can be improved through the development of new cultivars. During the last century peach breeding programs have worked diligently to develop and release hundreds of new cultivars throughout the world (Sansavini et al., 2006). However, the cultivars trace back through their pedigree to similar founders with a very narrow genetic base. This is of concern because a high amount of diversity or, variability, is desired in order to breed for specific traits. New cultivar development depends on using diverse peach germplasm to satisfy evolving consumer demands (Byrne, 2005). Currently, peach breeding programs aim to produce cultivars with improved fruit quality traits such as size, flavor with high sugar and moderate to low acidity, low pubescence, appealing color, increased blush (red skin pigmentation), improved texture, slow softening, and increased firmness to resist damage associated with shipping (Howad et al., 2005; Okie et al., 2008; Bielenberg et al., 2009).

Peach Blush: Introduction and Importance

Improving several fruit quality traits offer the potential to promote the consumption of peaches in the U.S.A. A particular trait that is essential for improving the marketability of peaches is the improvement of blush, which will enhance the aesthetic appeal to consumers. Blush is associated with specific “anthocyanin” compounds. These compounds serve to provide flavor and nutrients important for the human diet (Parr and Bolwell, 2000; Sun et al., 2002; Balasundram et al., 2006). Thus, blush is important because it can improve the appearance, flavor, and nutrition of peaches, which are all

necessary factors to stimulate repeat customers and invigorate the declining peach industry (Figure 1; Crisosto 2002; Crisosto and Costa, 2008).

Anthocyanins are a specific group of flavonoids produced through the flavanoid and anthocyanin pathways which are regulated by sun light. These polyphenol, secondary metabolites are important in several biological processes of plants (Schijlen et al., 2004). The anthocyanin pigments in flowers and seeds serve to attract pollinators and seed dispersers (Koes et al., 1994: Mol et al., 1998). Plants need sunlight in order to perform photosynthesis, however, exposure to excess UV radiation can cause significant damage. Anthocyanin pigments aid in this delicate balance by acting as a shield to protect the plant and absorb the toxic photoproducts caused by excess UV light in the epidermal cells of the plant (Schmelzer et al., 1988). Anthocyanins are crucial to this process because they are powerful antioxidants that break down harmful, highly chemically active secondary messenger reactive oxygen species (ROS) produced from excess light exposure. Degradation of these ROS by anthocyanins and other antioxidants is very important in preventing the ROS from causing oxidative stress that would damage cellular components. Flavonoids, and specifically anthocyanins, also help defend plants against pathogenic microorganisms and are important in plant sexual reproduction; they have been found in anthers and pistils of plants (Koes et al., 1994: Mol et al., 1998). Interestingly, some flavonoids have been shown to mediate the symbiotic interaction between plants and bacteria or parasites (Koes et al., 1994). Flavonoids and specific anthocyanins are known to be present in great quantities in seed coats, bark, leaves and roots of plants and thus help with plant structure. Lastly, anthocyanin compounds are

known to combat the development of cancer, cardiovascular, and other health problems related to aging in humans (Parr and Bolwell, 2000; Sun et al., 2002; Schijlen et al., 2004; Howad et al., 2005; Balasundram et al., 2006).

Peaches with a high level of red skin coloration (caused by increased levels of anthocyanins) visually appeal to the consumer and provide them with heightened nutrients, essential components of the human diet (Parr and Bolwell, 2000). For these reasons, private and public breeders have emphasized the creation of fresh market peaches with high red blush (Scorza and Sherman, 1996; Okie et al., 2008).

Blush Development

The progression of blush development is linked to the stage of peach development. While peaches ripen on the tree, their background color changes from green to yellow or other hues. Then, during the final swell of peach development (stage III), different levels of red skin pigmentation emerge over this background color (Delwiche and Baumgardner, 1983; Delwiche and Baumgardner, 1985; Byrne et al., 1991; Marini et al., 1991; Layne et al., 2001). The red over color develops in different intensities and patterns, depending on the genotype (mottled, striped, variegated, etc.).

In addition, the phenotypic variation of blush is controlled by genetic (genotype dependent) and environmental factors (light throughout the canopy; Layne et al., 2001), along with a genotype*environment interaction. Together, these three factors regulate the highly conserved, flavonoid and further anthocyanin biochemical pathways (Schijlen et al., 2004). Specifically, sunlight induces particular MYB transcriptional factor genes

which regulate the activation (transcription and translation) of specific structural genes. The resulting enzymes encoded by the structural genes transripts chemically modify the flavonoid compounds, thus changing their structural conformation. These structual modifications generates new compounds that perform diverse functions. The flavanoid pathway contains three precursors which through structural modifications lead into the anthocyanin pathway: delphinidin, pelargonidin and cyanidin (Kui et al., 2010). In peach the structural conformation of the cyanidin precursor is converted by enzymes encoded by specific cyanidin structural genes, resulting in the production of two specific anthocyanins associated with blush: cyanidin 3-glucoside and cyanidin 3-rutinoside (Hsia et al., 1965; Van Blaricom and Senn, 1967; Chaparro et al., 1995; Tomás-Barberán et al., 2001; Byrne et al., 2004; Wu and Prior, 2005; Cevallos-Casals et al., 2006; Vizzotto et al., 2006; Vizzotto et al., 2007; Cantín et al., 2009).

The flavonoid pathway is highly conserved in plants (Schijlen et al., 2004). All flavonoids share the same basic chemical structure of two aromatic rings (A and B), each with six carbon atoms, and a third ring (C), containing three carbon atoms (Schijlen et al., 2004). Different classes of flavonoids are created by enzymatic structural modifications of the third ring. Flavonoids are synthesized through the phenylpropanoid metabolic pathway. Through this pathway, phenylalanine is converted to p-coumaroyl-CoA. The enzyme chalcone synthase (CHS) next catalyzes a reaction which condensates three acetate units from malonyl-CoA with p-coumaroyl-CoA, to yield tetrahydroxychalcon (naringenin chalcone; a yellow colored chalcone), the backbone of flavonoids (Holton and Cornish, 1995). Tetrahydroxychalcon is then structurally modified further by

different enzymes encoded by different structural genes to form different classes of flavonoids: flavanones, dihydroflavonols, and anthocyanins.

Chalcone isomerase (CHI) isomerizes tetrahydroxychalcon (Schijlen et al., 2004), forming the colorless flavanone naringenin. Next, flavanone-3-hydroxylase (F3H) hydrolyzes the third position on the carbon of flavanone naringenin, generating dihydrokaempferol (DHK), a dihydroflavonol. DHK can also be converted by flavonoid 3'-hydroxylase (F3'H) to yield dihydroquercetin (DHQ), or by flavonoid 3',5'-hydroxylase (F3'5'H) to construct dihydromyricetin (DHM) (Holton and Cornish, 1995). Next, dihydroflavonol 4-reductase (DFR) and the cofactor NADPH, reduce DHK, DHQ and DHM into leucoanthocyanidins, which are precursors for anthocyanins. The leucoanthocyanidins are oxydized, dehydrated, and glycosylated by anthocyanidin synthase (ANS), UDP-glucose, and 3-O-glucosyltransferase (3GT) (Holton and Cornish, 1995). This in turn stabilizes the anthocyanidin, allowing them to serve as water-soluble pigments in the plant's vacuoles (Holton and Cornish, 1995).

Hydroxylation of the B aromatic ring of anthocyanidin converts the molecule into three main groups of anthocyanins, responsible for pigmentation in plants: delphinidin, pelargonidin and cyanidin. These pigments are found in the vacuole of plants. Anthocyanins are odorless, and nearly flavorless, they only slightly contribute to a moderate astringency. Each of the three anthocyanins shows a different color, depending on pH and the amount of hydroxyl groups in their B-carbon ring. The anthocyanins visible absorption maximum increases as the amount of hydroxyl groups on the molecules B ring increases and this results in different colors (Zuker et al., 2002).

Delphinidin-derived pigments produce blue or purple, while pelargonidin-derived pigments generate orange pink or red, and cyaniding-derived pigments create red (Zuker et al., 2002). Each can be further modified in the anthocyanin pathway into specific types of anthocyanins.

Anthocyanin Synthesis in Plant Species

Anthocyanins have been a focus of study in plants for many years. Wu and Prior (2005), characterized the specific anthocyanin which develop in 25 different fruits through high-performance liquid chromatography analysis (HPLC). Concord grapes showed the highest diversity of anthocyanin with 31 different types, followed by blueberry with 27, cranberry with 13, red grapes with 11, blackberry with 9, plum with 8, raspberry with 7, strawberry with 6, sweet cherry with 6, apple with 6, and peaches and nectarines with only 2 types (Wu and Prior, 2005).

Anthocyanin Specific for Blush in Peach

Several studies have identified and quantified these two main anthocyanin in peach: cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside (Hsia et al., 1965; Van Blaricom and Senn, 1967; Chaparro et al., 1995; Tomás-Barberán et al., 2001; Byrne et al., 2004; Wu and Prior, 2005; Cantín et al., 2009; Cevallos-Casals et al., 2006; Vizzotto et al., 2006; Vizzotto et al., 2007). Cyanidin 3-O-acetyl glucoside and cyaniding 3-O-galactoside were also located in peach, however in very minute amounts (Tomás-Barberán et al., 2001; Wu and Prior, 2005).

The concentrations of these two anthocyanin vary depending on the type of peach and specific tissue (exocarp vs. mesocarp). Tomás-Barberán et al., (2001) and Vizzotto et al., (2006; 2007) showed that red fleshed cultivars contained significantly higher levels of anthocyanin than white or yellow fleshed peaches (no differences were found between white and yellow fleshed peaches). Interestingly in general, the peach skin (exocarp) was found to contain three times or greater levels of phenolic compounds (anthocyanins and flavonols) than the flesh (mesocarp; Chang et al., 2000; Tomás-Barberán et al., 2001; Gil et al., 2002; Gorinstein et al., 2002; Cevallos-Casals et al., 2006; Vizzotto et al., 2006; Vizzotto et al., 2007). The skin is therefore a highly concentrated source of these compounds, however, it only represents ~8% of the total fruit weight. Therefore, the complete distribution of phenolic compounds in the skin and flesh for each fruit is ~30% and 70% (Cevallos-Casals et al., 2006).

Traditional Breeding Limitations & Blush

Throughout the last century peach breeding programs worldwide have released many new cultivars (Sansavini et al., 2006). Traditional breeding has eventually led to the development of peach cultivars with increased levels of blush, such as: ‘Blazeprince’ (USDA-ARS, Byron, GA), ‘Crimson Lady’ (Bradford and Bradford, 1991), ‘Goldcrest’ (USDA-ARS, Fresno, CA, 1983), ‘Red Globe’ (USDA, Beltsville, MD, 1954), ‘Redskin’ (Maryland Agricultural Experiment Station, College Park, MD, 1994) and ‘Springprince’ (USDA-ARS, Byron, GA, 1998). However, several fruit quality traits, including blush, are quantitative in nature and thus present practical challenges in selection (Bliss, 2010).

These difficult to select traits show low heritability, where the vast proportion of the phenotypic variance is due to environmental factors (Bliss, 2010). Environmental factors ultimately mask the genes effect on the phenotypic trait of interest.

Furthermore, traditional breeding is a time consuming process, taking 15 years or more, until a new cultivar can be released. The breeder must wait at least three years for peach trees to mature to fruit bearing capacity before taking fruit quality data on the progeny (Dirlewanger et al., 1998, 2004b, 2007). Once the trees bear fruit, it can then take 10-15 years of phenotypic analysis, selection, and regional testing to develop a new cultivar. Moreover, peach farms require a significant amount of space and continuous maintenance such as herbicide, pesticide and fungicide spraying, planting, pruning, thinning, and watering. Overall, traditional fruit tree breeding is a very time consuming, expensive, and laborious process.

Using Molecular Markers to Complement the Traditional Breeding Process

Marker assisted breeding (MAB), a genomic approach, holds vast potential to compliment and accelerate traditional breeding techniques, and would increase the efficiency of breeding new peach cultivars with superior fruit quality traits.

Moreover, consumer preference is known to change throughout time. Therefore, new cultivar development incorporating MAB as a tool, will enable more informed decisions, thus save resources, and enhance the traditional breeding process. Incorporating MAB into the traditional breeding process will enable more efficient cultivar development that will ensure the peach industry appeals to evolving consumer demands (Byrne, 2005).

Steps to Discover Markers Associated with Fruit Quality in Peach

1. Create a population which segregates for trait of interest.
2. Characterize the phenotype variation in the population.
3. Characterize the genetic variation in the population.
4. Use genotypic data to perform linkage analysis for linkage map construction.
5. Quantitative Trait Loci (QTL) analysis - incorporating linkage map and phenotypic data.
6. Candidate gene approach - Characterization of genes co-locating within QTL.
7. Validation (i.e. testing) of marker/s.
8. Use of markers in selection.

1. Population Creation

A population which segregates for the traits of interest must be generated to perform linkage analysis and later QTL analysis. In general, two parents with contrasting phenotypes are selected for crossing. One of the seedlings (termed Filial generation 1, i.e. F_1) intermediate for the trait of interest is selected for selfing. Through selfing, the maximum possible recombination of the parental alleles occurs resulting in the Filial generation 2 (F_2). This F_2 population is used to discover marker/s associated with the trait of interest.

2. Phenotypic Data

Phenotyping is a crucial component for quantitative trait loci (QTL) analysis. It connects genetic variation with biological activity thus documenting gene function

(Bassil and Volk, 2010). A standardized protocol for phenotyping for fruit quality in peach is explained in chapter II.

3. Genotypic Data

A genetic marker is a specific sequence of DNA which characterizes the genotype. The two main types of genetic marker include [a] morphological markers, and [b] molecular markers (Collard et al., 2005). All genetic markers that show differences between genotypes are useful for generating genetic linkage maps. Morphological markers were the first genetic marker used to construct genetic linkage maps, however, their limited numbers and variablity due to environmental effects, hindered creation of extensive linkage maps (Winter & Kahl, 1995). All genetic markers can be shown to be polymorphic or monomorphic for a specific population. Polymorphic markers are informative co-dominant or dominant markers which discriminate between individuals by distinguishing between different genotypes, and therefore can be used for constructing linkage maps (Collard et al., 2005). On the other hand, monomorphic markers are non-discriminatory, and therefore are not useful for constructing linkage maps.

[a] Morphological Markers

Morphological markers are observable, qualitative phenotypic traits, associated with a major gene. These major genes were initially discovered by Gregor Mendel, the founder of modern genetics. Mendel made specific hybridizations of a model organism, *Pisum sativum* (the common pea plant), to generate pea populations segregating (shows an observable difference between members of a family) for different visible phenotypic

traits. From these crosses, he observed that certain pea traits followed particular inheritance patterns, which were later termed the laws of Mendelian inheritance (Law of Segregation & the Law of Independent Assortment). In his work with pea he discovered the genetic control of seven discrete morphological traits: floral color (purple or white), floral position (axial or terminal), pod shape (inflated or constricted), pod color (green or yellow), seed shape (round or wrinkled), seed color (yellow or green), stem length (long or short).

In the genus Prunus, the position of 28 major gene controlling important agronomic traits (physiological fruit quality, productivity, and disease resistant traits) have been located on the Prunus reference 'Texas' almond x 'Earlygold' (TxE) genetic map (Dirlewanger et al. 2004b). A total of 19 of these major gene were discovered in peach, and have been located to their specific positions on the eight linkage groups (LG). of the TxE reference map.

Nine major genes **[1-9]** controlling fruit quality traits have been linked tightly to molecular markers (<5 cM) in the peach genome (Dirlewanger et al., 2004b; Dirlewanger et al., 2006; Mingliang et al. 2007) (**gene**; LG; *molecular marker*; <u>distance from marker (cM)</u>): **[1]** fruit flesh color - white/yellow (**Y**; LG1; *UDP98-407*; <u>2.2</u>; Bliss et al., 2002; Mingliang et al. 2007); **[2]** red around the pit - red/no red (**Cs**; LG3; *OPO2/0.6*; <u>12.4</u>; Yamamoto et al., 2001); **[3]** flesh adhesion to pit - freestone/clingstone (**F**; LG4; *UDAp-431/b*; <u>1.2</u>; Dirlewanger et al., 2006; *BPPCT009/b*; <u>2.2</u>; *AG12 & AG16b*; <u>2.0</u>; Dettori et al., 2001); **[4]** acidity - non-acid/acid fruit (**D**; LG5; *pTC-CTG/a* & *pGT-TTG/a*; <u>0</u>; Dirlewanger et al., 1998; Dirlewanger et al., 1999; Etienne et al., 2002; Dirlewanger et al.

2006); **[5]** pubescence - nectarine/peach (**G**; LG5; *eAC-CAA/a*; 0; Dirlewanger et al., 2006; *UDP96-018*; 4.5; Mingliang et al., 2007); **[6]** fruit shape - flat/round (**S**; LG6; *MA040a*; 0; Dirlewanger et al., 1998; Dirlewanger et al., 1999; Dirlewanger et al., 2006); **[7]** fruit skin color (**Sc**; LG6; *UDP96-015*; 3.7; Yamamoto et al., 2001); **[8]** blood flesh (**bf**; LG4; *C41H*; 10.3; Gillen and Bliss 2005); and **[9]** aborting fruit (**Af**; LG6; *MA040a*; 0; Dirlewanger et al., 2006). Fruit flesh color, flesh adhesion to pit, acidity, pubescence, fruit shape, fruit skin color, and aborting fruit, are linked tight enough (<5 cM) to their respective molecular marker, which gives them the potential to be used for MAB (Collard et al., 2005; Dirlewanger et al., 2006).

Interestingly, in two studies by Beckman, major gene have been shown to be probable in association with the inheritance of blush. Beckman and Sherman (2003) showed that 100% red skin color in peach is under the control of a single recessive gene (fr/fr). The 100% red over color was shown to develop even in the absence of light (Beckman and Sherman, 2003). Likewise, Beckman et al. (2005) also found that a single gene recessive trait (h/h) is associated with qualitative suppression of red skin color in a peach. These two major gene can be located and linked by marker/s, to enable MAB of 100% blush in peach, ideal for the fresh market, and MAB for 0% blush, fit for the canning industry.

[b] Molecular Markers

Molecular markers are specific sequences of DNA associated to a particular region in the genome (Winter & Kahl, 1995; Jones et al., 1997). They develop through several types of DNA mutations during meiosis (point, insertion or deletion, and

replication error mutations, etc.; Paterson, 1996). These mutations vary from individual to individual, thus screening molecular markers on genotypes of a population can be used to determine if the marker is polymorphic (different between genotypes) or monomorphic (all the same) for the population. These DNA molecular markers hold distinct advantages over morphological markers, in that they are highly abundant, can be analyzed at any time in the lab, and thus are not influenced by the stage of plant development and or the environment (Winter and Kahl, 1995; Jones et al., 1997; Collard et al., 2005). For these reasons, DNA-based markers have come to be the genetic markers most commonly used for linkage analysis, QTL analysis and ultimately MAB (Bliss, 2010).

DNA molecular markers are divided into three classes; hybridization-based, PCR-based, and DNA sequence-based (Winter and Kahl, 1995; Jones et al., 1997; Collard et al., 2005). Important types of these DNA molecular markers include but are not limited to random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphic DNA (AFLP), simple sequence repeats (SSR), and single nucleotide polymorphisms (SNP).

RAPD offer a quicker, simpler and more cost effective marker for use in genetic studies. This molecular marker, first documented by Williams et al. (1990), are arbitrary fragments of genomic DNA that contain single primers of random nucleotide sequence. A major advantage is that random RAPD can be amplified through PCR. The major disadvantages of RAPD markers include low reproducibility, and non-transferability (Winter and Kahl, 1995; Penner, 1996; Jones et al., 1997; Collard et al., 2005).

RFLP are restriction enzyme sites which vary among individuals. They were the first genetic markers used, along with small numbers of morphological markers, to produce extensive genetic linkage maps of Rosaceae species (Peace and Norelli, 2009). They are co-dominant, highly reproducible and transferable molecular markers. However, use of RFLP are limited because they are time consuming and expensive to run (Beckmann and Soller, 1986; Tanksley et al., 1989; Kochert, 1994; Winter and Kahl, 1995; Jones et al., 1997; Collard et al., 2005).

Another type of DNA markers are AFLP. These are dominant, population-specific molecular markers and use a similar technique to RFLP, only differing in that their PCR amplification technique is selective to specific restriction fragments. The DNA is first digested by restriction enzymes into restriction fragments. The sticky ends of the fragments are ligated to oligonucleotide adapters. Next, selected restriction fragments are amplified, and separated through gel electrophoresis, to determine the AFLP banding pattern (Vos et al., 1995). Sometimes AFLP can result in several bands, which only few are of significance. When this occurs these significant bands can be cut out from the gel sequence, and primers can be designed to only amplify those specific bands, which can be implemented into genetic mapping. This process is known as cutting out Sequence Characterized Amplified Region (SCAR). Downsides of AFLP are that they require amounts of DNA and are relatively complicated to screen (Vos et al., 1995; Winter and Kahl, 1995; Jones et al., 1997; Collard et al., 2005).

SSR are highly polymorphic, PCR based markers, which arise from tandem repeat duplications of a specific string of two to six DNA nucleotides (Edwards et al., 1991).

These are the marker of preference for developing genetic linkage maps, because of their co-dominant nature, frequent polymorphisms, and high density in all plants genomes (Powell et al., 1996; Taramino and Tingey, 1996; McCouch et al., 1997). Specific forward and reverse primers can be generated in order to screen the SSR markers on plant DNA (Winter and Kahl, 1995; Paterson, 1996; Powell et al., 1996; Taramino and Tingey, 1996; Jones et al., 1997; McCouch et al., 1997; Collard et al., 2005). Downsides to SSR is that you need sequence information to design the primers and the majority are species specific.

SNP are co-dominant, bi-allelic markers (present or absent) that develop over time through single nucleotide change mutations. SNP can be caused by insertions or deletions, transitions or transversions (Vignal et al., 2002). These single nucleotide changes will vary for each individual of a species (Vignal et al., 2002). SNP markers are by far the most abundant and are currently used to highly saturate the Prunus linkage map. In Rosaceae, SNP hold an estimated frequency of 1/100 in intronic (non-coding) sequences and 1/225 and in exonic (coding) sequences, respectively (Sargent et al. 2009; Illa et al. 2010). SNP is the marker currently being used by genetic groups studying numerous plant speicies around the world, because of their high density in plant genomes and relative ease in screening vast numbers.

4. Linkage Analysis

Linkage maps approximate the genomic position and genetic distances between genetic markers (Paterson, 1996; Jones et al., 1997; Collard et al., 2005). A segregating population is required for linkage map development. The construction of a genetic

linkage map is based off the events of meiosis. During meiosis, genetic recombination occurs between homologous chromosomes and leads to the development of recombinant genotypes. Segregating populations will contain parental and recombinant genotypes (SNP, RFLP, RAPD, SSR, AFLP, isozyme and EST). The recombination frequency (RF) between molecular markers in a segregating population are calculated based off the frequency of recombinant genotypes. The RF is used to determine the order and specific distances between the markers. Computer software is required to calculate the RF between the markers and consequently determine the position of the markers in the genome. The lower the RF between the molecular markers, the closer they are on the linkage group (Paterson, 1996; Jones et al., 1997; Collard et al., 2005). When markers show a RF >50%, they are termed unlinked and most likely located on different linkage groups. The linkage group approximates all the alleles or markers which are linked on the same chromosome and excluding cross over events, remain together during meiosis.

History of Linkage Maps in Prunus and Peach

The first genetic map for peach was developed by Chaparro et al., (1994). After this, an almond peach genetic map ('T x E') was generated, later used as the Prunus reference map (Joobeur et al., 1998; Pozzi and Vecchietti, 2009). The 'T x E' Prunus reference map was developed through an interspecific F_2 cross between almond ('Texas') and peach ('Early Gold') (Joobeur et al., 1998; Pozzi and Vecchietti, 2009). The 'T x E' map saturated the Prunus genome with 235 RFLP's and 11 isozymes (Joobeur et al., 1998; Aranzana et al., 2003). This 'T x E' linkage map showed all 8 linkage groups and spanned a total distance of 491 cM. The reference map currently holds a total distance of

524 cM with 826 molecular markers leading to an average map density of .63cM / marker (Dirlewanger et al., 2004b; Howad et al., 2005).

The ‘T x E’ Prunus reference map provides anchor markers (i.e. transferable markers throughout Prunus) with known map locations (Dirlewanger et al., 2004b; Howad et al., 2005; Pozzi and Vecchietti, 2009). These anchor markers enabled comparative genomics throughout peach and Prunus which facilitated in the development of eight intraspecific peach linkage maps and several interspecific Prunus linkage maps (Dirlewanger et al., 2004b; Howad et al., 2005; Pozzi and Vecchietti, 2009).

Currently a total of eight linkage maps **[1-8]** generated for peach, can be found on the Genomic Database for Rosaceae (GDR; http://www.rosaceae.org/): **[1]** 'Ferjalou Jalousia' x 'Fantasia', F_2 (‘J x F’; Dirlewanger et al., 1998; Pozzi and Vecchietti, 2009); **[2]** Peach *Prunus persica* x *P. ferganensis*, *BC1* (‘PxF’; Dettori et al., 2001; Verde et al., 2005; Pozzi and Vecchietti, 2009) **[3]** ‘Lovell’ x ‘Nemared’, F_2 (‘L x F’; Lu et al., 1998; Sosinski et al., 1998; Pozzi and Vecchietti, 2009); **[4]** ‘Guardian’ x ‘Nemaguard’, F_2 (‘G x N’; Blenda et al., 2007; Pozzi and Vecchietti, 2009); **[5]** ‘Akame’ x ‘Juseito’, F_2 (‘A x J’; Yamamoto et al., 2001, 2005; Pozzi and Vecchietti, 2009); **[6]** ‘Suncrest’ x ‘Bailey’, F_2 (‘Sc x B’; Sosinski et al., 1998; Pozzi and Vecchietti, 2009); **[7]** ‘Harrow Blood’ x ‘Okinawa’, F_2 - PMP2 (‘HB x Oki’; Gillen and Bliss., 2005; Pozzi and Vecchietti, 2009); **[8]** ‘New Jersey Pillar’ x ‘KV77119’, F_2 (‘NJ x KV’; Sosinski et al., 1998; Pozzi and Vecchietti, 2009) (F_2 = second generation population; *BC1*= backcross 1 population). There are several additional peach linkage maps are not currently housed on GDR. These

include, but are not limited to ‘Contender’ x ‘Fla.92-2C’, F_2 (‘A-popultion’; Fan et al., 2010) and ‘Dr. Davis’ x ‘Georgia Belle’ F_2 (Pop-DG; Ogundiwin et al 2009).

The additional interspecific Prunus reference maps include: *Prunus persica* x *Prunus dulcis* (Joobeur et al. 1998); *Prunus persica* x *Prunus dulcis* (Jáuregui et al. 2001); *Prunus persica* x *Prunus dulcis* (Foolad et al. 1995); *Prunus persica* x *P. ferganensis* (Quarta et al. 1998; Dettori et al., 2001); *Prunus persica* x *P. davidiana* (Dirlewanger et al. 1996); *Prunus cerasifera* x [*Prunus dulcis* x *Prunus persica hybrid*] (Dirlewanger et al. 2004a).

These genetic linkage maps serve as powerful tools for the localization and identification of QTL and or gene associated with the control of important qualitative and quantitative fruit quality traits (Tanksley et al., 1989; Winter & Kahl, 1995; Paterson, 1996; Jones et al., 1997; Collard et al., 2005).

5. QTL Mapping

Functional Genomics – Phenotype to QTL/Gene

Reverse and forward genetics can be used to study functional genomics. Reverse genetics starts with a known gene and tries to associate it with a specific phenotype. This can be done if the sequence of a gene is known. The sequenced gene can be transformed into a plant, and then the phenotype associated with the gene can be observed. Reverse genetics would be used with a procedure such as transgenics (inserting genes into the genome of an organism). Transgenics is commonly performed two ways: indirectly through Agrobacterium-mediated transformation or directly using a gene gun. A gene can be designed and inserted into an Agrobacterium’s genome. The Agrobacterium then

transfers the gene into the plant host cells. The gene gun, shoots small particles along with the gene and physically inserts the gene into the host cells genome.

The genes associated with most quantitative traits have not been localized. Therefore forward genetics will be exploited to locate the QTL/gene associated with blush and other peach quality traits. Contrary to reverse genetics, forward genetics involves molecular mapping; associating a known phenotype to a specific QTL or gene flanked by molecular markers. To perform this, a segregating population must be phenotyped for the trait of interest, genotyped by polymorphic molecular markers, and a linkage map must be generated through linkage analysis of the genotypic data. Computer software programs use the linkage map and phenotypic data to identify a QTL or gene associated with the phenotypic trait (Tanksley et al., 1989; Winter & Kahl, 1995; Paterson, 1996; Jones et al., 1997; Collard et al., 2005).

Three main types of QTL analysis include: single-marker analysis, simple interval mapping (SIM), and composite interval mapping (CIM) (Tanksley 1993; Liu, 1998). Single-marker analysis, the most basic QTL mapping tool, incorporates an analysis of variance (ANOVA) and linear regression to detect QTL associated with single molecular markers (Collard et al., 2005). Unlike single-marker analysis, the SIM QTL method is more powerful because it evaluates intervals in between adjacent linked markers along linkage maps simultaneously (Lander and Botstein, 1989; Liu, 1998). Considering all three QTL methods, the CIM is the most powerful and precise QTL mapping technique because it combines linear regression and interval mapping and also incorporates

additional molecular markers (Jansen, 1993; Jansen and Stam, 1994; Zeng et al., 1993; Zeng et al., 1994).

Identified QTL for Fruit Quality Traits

Contrary to mapped qualitative peach quality traits, most agronomically important fruit quality traits, such as blush, exhibit continuous phenotypic variation indicating more complex, polygenic control.

Several major fruit quality traits have been associated with QTL (Abbott et al., 1998; Dirlewanger et al., 1999; Quarta et al., 2000; Etienne et al., 2002; Peace et al., 2006; Pozzi and Vecchietti, 2009; Cantín et al., 2010). QTL controlling hexose content have been identified (Abbott et al., 1998; Pozzi and Vecchietti, 2009). Additionally, QTL for soluble solid content (SSC) were located on LG's 1 and 2 (Quarta et al., 2000; Pozzi and Vecchietti, 2009). The 'Ferjalou Jalousia' x 'Fantasia', ('J x F') F_2 population was used to uncover QTL for fresh weight, color, pH, titratable acidity (TA), SSC, acidity, and sugar (Dirlewanger et al., 1998; Dirlewanger et al., 1999; Pozzi and Vecchietti, 2009). All of these QTL were improved by Etienne et al. (2002) (Pozzi and Vecchietti, 2009). QTL for peach fresh weight were located near the fruit shape locus (S; Dirlewanger et al., 1998; Dirlewanger et al., 1999; Dirlewanger et al., 2006) on LG6 (Dirlewanger et al., 1999; Etienne et al. 2002; Pozzi and Vecchietti, 2009). Three QTL were uncovered for TA on LG 1, 5 and 6 (Dirlewanger et al., 1999; Etienne et al. 2002; Pozzi and Vecchietti, 2009). QTL for malic acid also mapped to LG 1, 5 and 6, and a citric acid QTL was found on LG9 (Dirlewanger et al., 1999; Etienne et al. 2002; Pozzi and Vecchietti, 2009). Using the 'Dr. Davis' and 'Georgia Belle' (Pop-DG) F_2

population, Peace et al. (2006), located a major QTL for peach fruit browning on LG5 explaining 61% of the phenotypic variance for the trait (Pozzi and Vecchietti, 2009). A QTL for mealiness was mapped on LG4 and also explained 61% of the phenotypic variance for the trait (Peace et al., 2006). Additionally, a QTL for peach bleeding (red pigmentation in the flesh) was found on LG4, and explained 43% of the phenotypic variance (Peace et al., 2006). Recently QTL for SCC, pH, TA, firmness, endocarp staining, suture diameter, cheek diameter, bleeding, fruit weight, mealiness, graininess, leatheriness, and *blush* were all localized on LG4 on the linkage map generated by analysis of an F_1 progeny from a cross between 'Venus' x 'BigTop' (V×BT; Cantín et al., 2010). The phenotypic variation explained by all of these traits ranged from 26% to 92% (Cantín et al., 2010).

QTL Studies for Blush in Prunus

A few studies have focused on the discovery of possible QTL associated with the production of red skin and blush in peach. Quilot et al., (2004) reported a QTL responsible for red skin coloration (SRColor2) close to RFLP marker AC108 on linkage group 5 of the *Prunus* genome. Ogundiwin et al. (2007; 2008; 2009) discovered a QTL (qP-Brn5.1^{m}) on linkage group 5 responsible for PpLDOX leucoanthocyanidin dioxygenase, associated with browning. Leucoanthocyanidin dioxygenase also serves as an important structural gene in the anthocyanin pathway. This QTL, qP-Brn5.1^{m} was located in the same general location as that of the QTL SRColor2 (Quilot et al., 2004). Additionally, the QTL for blush located on LG4 (LOD peak position of 52.8 cM) of the

VxBT F_1 map has been associated with 68.7% of the phenotypic variance (Cantín et al., 2010).

QTL studies have also been performed in other Rosaceae plants to determine specific regions in the genome responsible for the biosynthesis of anthocyanins. The investigation of red skin pigmentation has been extensively performed in apple (*Malus domestica*) (Takos et al., 2006; Ban et al., 2007; Chagné et al., 2007; Espley et al., 2007; Espley et al., 2009), cherry (*Prunus avium* L.) (Sooriyapathirana et al., 2010), octoploid strawberry (*Fragaria x ananassa*; Zorrilla-Fontanesi et al., 2011) raspberry (*Rubus idaeus*; McCallum et al., 2010), and grape (*Vitis vinifera*) (Kobayashi et al., 2004; Walker et al., 2007; Kobayashi, 2009).

6. <u>Candidate Gene Approach for Anthocyanin Pigmentation Gene/s</u>

The candiadate gene (CG) approach is used when assumptions are made in regards to the biological function of genes of interest (Byrne and McMullen 1996; Pflieger et al., 2001). Previously sequenced structural or transcriptional regulating gene which co-locate within mendelian or major QTL are useful in characterization of the major loci function. Primers can be designed for CG which co-locate within identified mendelian or major QTL. Thus the CG can be screened on different germplasm to validate its ability to predict the phenotypic variation of important traits of interest (Pflieger et al., 2001).

The genetic study of anthocyanin synthesis began with Mendel's study on flower color in peas (Holton and Cornish, 1995). This landmark study is still being investigated today. Studies are being conducted to locate genes responsible for the regulation of the

structural genes in the anthocyanin biosynthetic pathway in Rosaceae plants (Allan et al., 2008). These structural genes controlling the biosynthesis of anthocyanin are ultimately under the control of MYB transcription factors encoded by regulatory genes (Stracke et al., 2001). In all plant species anthocyanin biosynthesis is regulated through a class of MYB transcription factors (Baudry et al., 2004). These MYB transcription factors also control several other diverse pathways, including developmental signal transduction and disease resistance pathways (Jin and Martin, 1999). This class of regulatory proteins encompasses DNA-binding proteins which regulate transcription of genes in plants. The MYB proteins contain a specific string of approximately 52 amino acids, which conform into a helix-helix-turn-helix domain that is able to bind to DNA (Jin and Martin, 1999). These MYB proteins are further classified into three subfamilies according to the number of repeats of the MYB DNA-binding domain; those with one repeat are considered 'MYB1R factors', while those with two and three repeats are designated as, 'R2R3-type MYB' and 'MYB3R factors' (Jin and Martin, 1999; Stracke et al., 2001).

In the Rosaceae family, the two-repeat R2R3 MYB transcription factor class has been associated with the activation of the anthocyanin biosynthesis pathway (Allan et al., 2008; Kui et al., 2010).

In apples (*Malus* x *domestica*), the major gene MYB10/MYB1/MYBA was mapped in populations segregating for red skin (Ban et al., 2007) and red flesh (Chagné et al., 2007; Espley et al., 2007; Espley et al., 2009). MYB10 expression was strongly correlated with anthocyanin production in the flesh (Espley et al., 2007; Espley et al., 2009), while MYBA and MYB1 were shown to regulate anthocyanin production in the

skin of apples (Ban et al., 2007; Takos et al., 2006). Espley et al., (2009) showed that a rearrangement resulting in a minisatellite of five tandem multiple repeats in the regulatory region of the MYB10 gene, causes increased levels of anthocyanin throughout the plant. This minisatellite was located in all apple cultivars tested with red flesh and foliage. However, it was absent in apple cultivars with green foliage and white flesh. In studies on red skin pigmentation, red skin apple cultivars contained significantly higher levels of transcripts from MYBA and MYB1 genes in comparison to non-red skin cultivars (Ban et al., 2007; Takos et al., 2006).

In sweet cherry (*Prunus avium* L.) the candidate gene, PavMYB10 (homologous to apple MdMYB10 and Arabidopsis AtPAP1) co-located within the major QTL on LG3 for skin and flesh color (Sooriyapathirana et al., 2010). This provides substantial evidence that PavMYB10 could be the major gene responsible for the production of red skin and flesh in sweet cherry.

Since apple, cherry and peach are all members of the Rosaceae family, it is likely that the major gene MYB10/MYB1/MYBA has also been conserved and can be associated with the production of anthocyanin in peach skin and flesh. The peach MYB polypeptide chain PprMYB10 was aligned through a protein sequence alignment with the Rosaceae MYB10 (Kui et al., 2010). The PprMYB10 sequence was shown to be homologous with only an 18 amino acid deletion in the C terminus, which did not hinder the ability of the transcription factors to regulate the anthocyanin biosynthetic pathway. This demonstrates that the MYB10 has been conserved in peach, and has the potential to regulate the amount of anthocyanin production in peach skin and flesh. Despite this, the

genes which code for the MYB10, MYB1, and MYBA transcription factors remain to be mapped in a peach population segregating for different anthocyanin pigmentation levels in the flesh and skin (Kui et al., 2010).

These previous studies paved the way for indentification of the precise transcription factor genes responsible for the genetic regulation of the anthocyanin biosynthetic pathway in Rosaceae. In this family, the two-repeat R2R3 MYB transcription factor class has been associated with the activation of the anthocyanin biosynthesis pathway (Allan et al., 2008; Kui et al., 2010). In apple the major gene MdMYB10/MdMYB1/MdMYBA was mapped in populations segregating for red skin (Ban et al., 2007; Takos et al., 2006) and red flesh (Chagné et al., 2007; Espley et al., 2009; Espley et al., 2007). Kui et al. (2010) showed that these three MYB activators of apple anthocyanin (MYB10/MYB1/MYBA) are expected alleles of each other. They then blasted these key genes to locate homologs across the Rosaceae family. Over-expression of these genes in apple and strawberry correlated with elevated levels of anthocyanins in the fruit and flowers (Kui et al., 2010).

In sweet cherry a population segregating for skin and flesh color was used to locate a major QTL for red skin pigmentation on LG3 (Sooriyapathirana et al., 2010). The candidate gene, PavMYB10, shown to be homologous to the apple MdMYB10 gene and Arabidopsis AtPAP1 (Kui et al., 2010) was located within the major QTL interval for red skin pigmentation in sweet cherry. This showed that PavMYB10 is likely to be the major gene responsible for the production of red skin and flesh in sweet cherry (Sooriyapathirana et al., 2010).

The production of anthocyanins has also been studied thoroughly in the Vitaceae family. In grapes (*Vitis vinifera*) the production of red anthocyanins is controlled by a single genetic locus containing two MYB genes, VvMYBA1 and VvMYBA2 (Kobayashi et al., 2004; Walker et al., 2007; Kobayashi, 2009). White grapes on the other hand, hold mutations in these two genes; a retro transposon induced mutation in the promoter of VvMYBA1 and two mutations in the coding region of VvMYBA2. These mutations cause of loss of function of the genes which results in no production or formation of anthocyanins and ultimately the formation of white grapes.

7. Validation of Marker/s

Markers which show the potential to be used for MAB of a specific trait need to be tested on diverse germplasm to validate that they accurately depict the trait (Sharp et al., 2001; Spielmeyer et al., 2003; Collard et al., 2005).

8. Use of markers in selection.

Markers which accuratly depict the trait of interest can be used for marker assisted parent selection (MAPS) and marker assisted seedling selection (MASS). MAPS will enable quick genotypic screening of peach germplasm, and lead to more informed decisions on efficient cross combinations. The parents to use in a cross, are identified through discovery of favorable alleles with efficient combining abilities. After the cross is made, MASS can be used to screen the seedlings, and decide on which seedlings to grow in the field and which to discard (Collard et al., 2005).

Current Status of MAB for Quality Traits in Peach and Other Rosaceae Species

Molecular marker/s linked to fruit quality traits are being developed, and several QTL affecting peach quality have been identified (Dirlewanger et al., 1999; De Pascual-Teresa et al., 2010). However, further QTL mapping must be performed to enable the use of MAB throughout the Rosaceae family.

Peach has been selected as the model species for Rosaceae genomics studies because of its relatively simple genomic structure and high amount of developed genetic resources (Abbott et al., 2002). Peach is a self-fertile diploid species (2n = 16), with a base chromosome number of x = 8. It has a small genome (~220Mb) and a short juvenility period (~2-3 years) in comparison to several other tree fruit species. Peach is the best genetically characterized Prunus species with 19 major genes mapped to specific loci on the highly saturated Prunus genetic reference map, 'T x E' (Etienne et al., 2002; Sansavini et al. 2006; Pozzi and Vecchietti, 2009). In addition the 'Lovell' di-haploid peach genome sequence v1.0, has recently been released. Furthermore, a high-throughput Illumina Infinium® IPSC 9K SNP v1 genotyping array has been developed (Verde et al. 2012). A Genomic Database for Rosaceae (GDR) (http://www.rosaceae.org/) houses all of this information enabling genetic studies of this family.

Despite the growing availability of genomic resources in peach, the use of markers for molecular breeding in peach is still in its infancy. The necessary level of collaboration between geneticists and breeders to implement the use of molecular markers in peach breeding has yet to be established (Bliss 2010; Iezzoni et al. 2010). A USDA funed project, RosBREED (http://www.rosbreed.org/), is promoting multi-

disciplinary collaboration between geneticists and breeders to search for QTL related to fruit quality, and ultimately discover markers linked to fruit quality traits. Through collaboration, more markers will be used to uncover additional QTL and or tighter linkages (Dirlewanger et al., 1999; De Pascual-Teresa et al., 2010) to enable broad adaptation of marker assisted parent and seedling selection (MAPS and MASS), providing the potential to produce higher quality peaches in a timely process that meets consumer demands. This will provide an efficient procedure to allow effective parent selection and determine which progeny to further screen. This approach should lead to savings of time, money, and space (Bliss, 2010).

MAB Enabled for Fruit Texture and Adherence

Freestone/clingstone and melting/non-melting traits were effectively mapped to a single locus, containing three genes that control the development of the different flesh and adherence types (Peace et. al, 2005, 2007). This endoPG locus contains at least two copies of endopolygalactose genes which code for proteins that break down the cell wall leading to softening of the peach. This discovery has enabled quick genotypic screening of peach parents (MAPS) and seedlings (MASS), since peaches contain different alleles at this locus. MAPS and MASS can now be used to determine the endoPG genotypes that should correlate with four possible phenotypes (freestone melting flesh = FMF; clingstone melting flesh = CMF; clingstone non-melting flesh = CNMF; clingstone non-softening flesh = CNSF) that each parent or seedling should embody in the future (Peace et al., 2005; Peace et al. 2007). Each of these phenotypic traits are important for distinct markets. Fresh market peach breeders develop FMF peach cultivars while peach breeders

in the canning market breed for CNMF or CNSF varieties. The fresh market peach breeders are now able to screen for parents and progeny with the specific genotype that should result in the FMF phenotype, while, on the other hand, peach breeders in the canning market can screen for CNMF and CNSF peaches. This endoPG test is a recently developed model example of how to implement MAB.

Status of MAB for Blush in Peach

The production of blush in peach has not been investigated sufficiently to enable MAB for the trait. Only a few studies have focused on the discovery of possible QTL associated with the production of blush in peach (Quilot et al., 2004; Ogundiwin et al., 2008; Ogundiwin et al., 2009; Illa et al., 2010).

Molecular markers linked to important QTL associated with the production of blush must be located to enable MAB for blush and other fruit quality traits. Similar to the MAB of flesh and adherence, MAB for blush would provide an economic benefit to peach breeders of both fresh and canning markets. Both could screen their germplasm, and fresh market breeders could keep peaches with the marker depicting high blush, whereas canning market breeders could keep the genotypes lacking the marker. Ultimately, MAB would enable quicker development of peach cultivars that meet consumer demands.

Enabling MAB for Blush in Peach

There is a need to develop MAB for blush in peach. The investigation of definitive QTL, and even further, structural and regulatory candidate genes associated

with the production of blush has been extensively performed in apple (*Malus domestica*) (Takos et al., 2006; Ban et al., 2007; Chagné et al., 2007; Espley et al., 2007; Espley et al., 2009), cherry (*Prunus avium* L.) (Sooriyapathirana et al., 2010) and grape (*Vitis vinifera*) (Kobayashi et al., 2004; Walker et al., 2007; Kobayashi, 2009). However, there has only been a few initial studies focused on the discovery of possible QTL associated with the production of blush in peach (Quilot et al., 2004; Ogundiwin et al., 2008; Ogundiwin et al., 2009; Illa et al., 2010).

Importance of MAB for Blush?

Peach cultivars with higher levels of blush should increase the ability to market peaches catering to consumer demands. In this study and future studies, molecular markers tightly linked to blush and other peach quality traits will be identified. These markers will become tools that will allow breeders to perform MAPS and MASS for blush and other fruit quality traits in peach, thus saving time, money, and space (Bliss, 2010). This should enable the quicker development of future improved peach cultivars with extensive blush and other enhanced fruit quality traits in providing consumers with high quality fruit that may lead to increased demand.

Since the markers developed will be available to the peach and Rosaceae breeding community, other fruit breeders and fruit industries could benefit from the uncovered markers as well. Peach is a model organism for Rosaceae, therefore, comparative genomics can be applied. Blush and other peach quality trait QTL can be compared to other Rosaceae species and thus allow for further studies to locate markers tightly linked to genes associated with these fruit quality traits in other members of the Rosaceae family

(Dirlewanger et al. 2004b). Ultimately MAB for blush and other fruit quality traits in economically important Rosaceae species will be enabled as well as in peach.

Project Objectives

1. To develop and implement a standardized phenotyping protocol for peach.
2. To use an F_2 population segregating for blush to identify QTL associated with red skin pigmentation in peach:
3. This will enable testing of the identified marker/s on several peach populations to determine their accuracy in depicting blush.
    i. An accuracy of ~75% will be sought for the identified marker/s ability to depict blush development. Locating marker/s tightly linked to gene/s associated with the production of blush will ultimately enable routine MAB for blush in peach.

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CHAPTER II

STANDARDIZED PHENOTYPING FOR FRUIT QUALITY IN PEACH [*PRUNUS PERSICA* (L.) BATSCH]

Introduction

Peach [*Prunus persica* (L.) Batsch] is a commercially important fruit tree species. It is native to China and a member of the Rosaceae family. Despite a significant decrease in production in the past decade, peach is second in temperate fruit production only to apple (*Malus domestica*), with 10 million tons produced globally (Fideghelli et al., 1998; Figure 1). Harvesting peaches at immature stages for storage and shipment purposes (Fideghelli et al., 1998; Sansavini et al., 2006), negatively impacts peach quality. Peach consumption in the USA can be increased if fruit are consistently harvested at optimal maturity for the market (Crisosto et al., 1995; Crisosto 2002; Crisosto and Costa, 2008; Bielenberg et al., 2009; Figure 1).

However, present day cultivars span back through their pedigree history to similar founders with a very narrow genetic base. This is of concern because a high amount of diversity and / or variability is needed to breed for specific traits. New cultivar development depends on using diverse peach germplasm to satisfy new consumer demands as the requirements in the peach industry change (Byrne, 2005). Currently, peach breeding programs aim to produce cultivars with improved traits such as large size, high sugar, moderate to low acidity, low pubescence, appealing color, increased blush (red skin pigmentation), improved texture, slow softening, and increased firmness to resist shipping damage (Howad et al., 2005; Okie et al., 2008).

Developing new peach cultivars to meet current demands solely through traditional techniques is very time consuming and can take 15 to 20 years before release of a new cultivar. The breeder must wait at least three years for peach trees to mature to fruit bearing capacity before taking fruit quality data on the progeny (Dirlewanger et al., 1998; Dirlewanger et al., 2004; Dirlewanger et al., 2007). Once the trees bear fruit, it generally takes 10-20 years of analysis, selection, and regional testing to develop a new cultivar. Moreover, most of the quality traits of interest are quantitative in nature, which present practical challenges in selection (Bliss, 2010). Furthermore, peach farms require a significant amount of space and continuous maintenance including herbicide, pesticide and fungicide spraying, planting, pruning, thinning, and watering.

To mitigate these problems marker-assisted breeding (MAB), a genomic approach to enhance crop improvement, holds vast potential to compliment and accelerate traditional breeding techniques. However, the necessary level of collaboration between geneticists and breeders to implement the use of molecular markers in peach breeding has yet to be established (Bliss 2010; Iezzoni et al. 2010).

The majority of agriculturally important traits, such as fruit quality in peach, are controlled by multiple genes and quantitatively inherited. Phenotyping is a crucial component for quantitative trait loci (QTL) analysis because it connects genetic variation with biological function (Bassil and Volk, 2010). This process plays a crucial role in documenting gene function. However, quality and quantity of available phenotypic data is not keeping pace with the immensely increasing amount of available genomic information, brought forth by current Next Generation Sequencing (NGS) technologies.

This lack of phenotypic documentation hinders our ability to associate genotypic with phenotypic data, and thus limits QTL mapping and further gene function discovery (Bassil and Volk, 2010). In fact, barely two-thirds of genes have been associated with biochemical functions, and fewer still have been associated with a phenotype (Bochner, 2003). In order to combat this deficiency in phenotypic data, protocols should be standardized across different institutions, personnel, and environments (Bassil and Volk, 2010). The development of these standardized phenotypic traits should be done through collaborations among interested parties (Volk, 2010).

The idea of standardized phenotyping was first implemented in the mouse community (Abbott, 1999). It has since been practiced in several other studies focused on enhancing plant quality, productivity and biotic and abiotic stress resistance traits including disease resistance genes (Postman et al., 2010), fruit quality characteristics in tomato (*Solanum lycopersicum*) and potato (*S. tuberosum*) (Scott, 2010), and postharvest fruit quality traits for population comparisons (Rudell, 2010). Standardized phenotyping has been applied to several other plant species, including certain tree fruits (Peace and Norelli, 2009), and has lead to consistent data collection and efficient transfer of information across four U.S. peach breeding institutions, as well as research and germplasm programs (Rudell, 2010).

Scientific research has become more nationally and internationally integrated further emphasizing the importance of standardized phenotyping (Bassil and Volk, 2010). Cooperation to generate standardized phenotypic data assimilation must significantly increase to realize the potential of vast genotypic data available. Productive means of

storage, organization and retrieval of this information is necessary for it's efficient utilization. Currently there is a large amount of data stored in public and private scientific databases: biological collections in museums, herbaria, genebanks, botanical gardens, breeder plots, and research institutions (Volk, 2010). The majority of these databases were developed individually, or locally, to promote effective means of data accumulation, organization, and retrieval for independent studies. Different means of data collection and ontologies (nomenclatures, or vocabularies) limits the ability to compile data from separate databases (Volk, 2010). Several databases have been established for storage and retrieval of genomic and genetic data; GenBank, the NIH genetic sequence database at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/), Europe's primary nucleotide sequence resource, The European Molecular Biology Laboratory (EMBL) at the European Bioinformatics Institute (http://www.ebi.ac.uk/embl/), and the DNA Database of Japan (DDBJ) at the Center for Information Biology in Japan (http://www.ddbj.nig.ac.jp/). Despite recognized efforts to organize genomics and genetics data, storage of phenotypic data remains mainly individualistic.

Recent efforts on data for Rosaceae species is an exception and underscores the importance of standardized phenotyping, centralized data storage and regulated ontologies for trait classifications. A Genome Database for Rosaceae (GDR: http://www.rosaceae.org/) has been created for centralized storage, organization and access to Rosaceae genomics and genetics data and recently for phenotypic data. GDR contains standardized phenotypic descriptors created for pertinent economic traits of

peach as well as other Rosaceae species [strawberry (*Fragaria* × *ananassa)*, apple (*Malus domestica*), sweet (*Prunus avium L.*) and sour cherry (*Prunus cerasus*)]. Efficient exploration of gene function for Rosaceae species is being facilitated by standardized data collection regulated ontologies for trait classifications, and centralized data storage (Bassil and Volk, 2010).

The recently funded USDA-SCRI multi-institutional and trans-disciplinary project, RosBREED (http://www.rosbreed.org/), has developed standardized phenotyping protocols for several Rosaceae crops: apple, peach, cherry and strawberry. Phenotypic data have been collected in various locations following standardized phenotyping protocols to facilitate Pedigree-Based Analysis (PBA) and discovery of molecular markers linked to Quantitative Trait Loci (QTL) controlling complex fruit quality traits (Bink, 2004, 2005, 2008). Development and application of standardized phenotyping is vital for using pedigree connected diverse germplasm via PBA in discovering QTL for traits such as fruit quality for Rosaceae species. The demonstration peach breeders within the RosBREED project (located at four universities throughout the United States: Clemson, UC Davis, Arkansas and Texas A&M) have worked together in collaboration with Rosaceae community breeders to develop and implement a standardized phenotyping protocol with emphasis on fruit quality traits. We are reporting on development and application of the standardized phenotyping protocol for fruit quality traits in peach.

Materials and Methods

A peach reference germplasm set of 513 accessions was selected for phenotyping (Table 1). The accessions are present at the following locations: Clemson University Musser Fruit Research Center, Seneca, SC; The Sandhill Research and Education Center, Columbia, SC; USDA Fruit and Nut Research Center, Byron, GA; University of Arkansas Fruit Research Station, Clarksville, AR; National Clonal Germplasm Repository for Fruit and Nut Crops, Davis, CA; UC-Davis Wolfskill Orchard, Winters, CA; Foundation Plant Services Orchard, UC-Davis, Davis, CA; Texas A&M University, College Station, TX; and The Burchell Nursery, Oakdale, CA. Standardized phenotyping for peach fruit quality evaluations has been initiated in 2010 at nine locations.

The Phenotyping protocol included productivity and fruit quality traits. Productivity traits were evaluated for each accession: 50% bloom date, bloom type (showy or non-showy), leaf gland type (reniform, globose, eglandular), fruit set, and ripe date. Quality traits were further divided into non-destructive/organoleptic (pubescence, blush %, ground color, ground color L*, ground color R, ground color theta, flesh color, flesh color L*, flesh color R, flesh color theta, red in flesh, red around the pit) and destructive measures (diameter, weight, flesh firmness, soluble solids concentration (SSC; brix %), pH, malic acid/titratable acidity, fruit texture, adherence to pit, pit weight, percentage split pit). Ten to twenty fruits from each of the 513 accessions have been collected at tree ripe stage and analyzed for 16 fruit quality traits (Table 2; http://www.rosbreed.org/resources/fruit-evaluation).

Using a RosBREED procedure, the data quality was checked. Quality checking consisted of five steps: 1 – cross checking of data for line shifts; 2 – cross checking data for outlier identification by calculating maximum and minimum values and developing histogram/box-plots; 3 – correcting outliers; 4 – multi-year data checking for additional outliers by calculating differences in traits between years; and 5 – multi-year data checking for additional outliers by generating scatterplots between years to determine correlations and identified data points far outside of correlations.

Table 1. Accessions included in peach crop reference set.

Name[§]	Seed-Parent[¶]	Pollen-Parent	Location[#]
2000_16_133	F8_5_159	F8_5_159	CA[2]
2000_2_8	Loadel	P.argentea	CA[2]
2000_2_9	Loadel	P.argentea	CA[2]
2000_3_205	Andross	MissionxScoparia	CA[2]
2001_7_180	Andross	P.argentea	CA[2]
2003_1_329	Dr.Davis	P.mira19	CA[3]
2005_16_191	H_6_55	98_13_17	CA[3]
2005_20_139	2000_3_205	2000_3_205	CA[3]
2005_20_141	2000_3_205	2000_3_205	CA[3]
2005-19_139	19_2_72	2000_3_205	CA[3]
54P455	GoldenGlory	Bonanza	CA[3]
91_17_195	18_6_33	87_13_13	CA[2]
99_12_155	Woltemade	91_17_195	CA[2]
AdmiralDewey	*	*	CA[1]
Andross	Fortuna	Dix_5A_1	CA[3]
Arrington	A_178	A_232	AR[1], SC[1]
Blazeprince	BY81P2840	OP	SC[1]
Bolinha	*	*	CA[3]
Bradley	A_190	A_178	AR[1], SC[1]
BY01P6245	Contender	Fla92-2C	SC[1]
CA_Pop_5_10_XXX	Dr.Davis	D62_193	CA[3]
CA_Pop_5_11_XXX	Loadel	99_12_155	CA[3]
CA_Pop_5_16_XXX	O'Henry	F8_1_42	CA[3]
CA_Pop_5_17_XXX	Goodwin	Vilmos	CA[3]
CA_Pop_5_17_XXX	Carson	persXdavidiana	CA[3]

Table 1 continued

Name[§]	Seed-Parent[¶]	Pollen-Parent	Location[#]
CA_Pop_5_18_XXX	2001_7_180	2001_7_180	CA[3]
CA_Pop_7_12_XXX	2000_16_133	2000_16_133	CA[3]
CA_Pop_7_13_247	2000_3_205	2000_3_205	CA[3]
CA_Pop_7_13_248	2000_3_205	2000_3_205	CA[3]
CA_Pop_7_13_249	2000_3_205	2000_3_205	CA[3]
CA_Pop_7_13_250	2000_3_205	2000_3_205	CA[3]
CA_Pop_8_13_XXX	Loadel	2003_1_329	CA[3]
CA_Pop_8_3_XXX	Loadel	Yumyeong	CA[3]
CAF2	P97_14	Y150_13	TX[1]
CAF3	P91_23	Y142_75	TX[1]
CAF4	Y140_77	Y142_194	TX[1]
Candor	Redhaven	ErlyRedFre	SC[1]
Carmel	Nonpareil	Mission	CA[3]
Carmen	Elberta	FamilyFavorite	CA[1]
CarolynG	Libbee	Lovell	CA[3]
Carson	Leader	Maxine	CA[3]
ChinaPearl	Contender	PI134401	SC[1]
ChineseCling	*	*	CA[1], SC[1], SC[3]
Clayton	Pekin	Candor	SC[1], SC[2], SC[3]
Conserva458	*	*	CA[1]
Contender	Winblo	NC64	CA[1], SC[1]
CrimsonLady	RedDiamond	Springcrest	CA[1], SC[1]
Cumberland	GeorgiaBelle	Greensboro	CA[1]
D62_193	NJC83	Conserva485	CA[2]
Diamante	*	*	CA[1]
Dixon	AustralianMuir	OrangeCling	CA[2]
Dr.Davis	D25_9E	G40_5E	CA[3]
E22_59	18_8_11	OP	CA[3]
EarlyCrawford	*	*	CA[1]
Elberta	ChineseCling	EarlyCrawford	CA[3], SC[1]
Everts	Dix_22A_5	Dix_5A_1	CA[3]
F10C_12_28	F8_72_33	OP	CA[2]
F10C_20_51	F8_76_45	OP	CA[2]
F8_1_42	90_1_4	90_1_4	CA[2]
F8_5_166	90_10_91	90_10_91	CA[2]
Flordaprince	Fla2-7	Maravilha	TX[1]
Galaxy	P34_106	D33_1	CA[1], SC[1]
GeorgiaBelle	ChineseCling	OP	SC[1]
Goldprince	Loring	FV3_257	SC[1]
Goodwin	Dr.Davis	11_11_37	CA[3]
Greensboro	*	*	SC[1]

Table 1 continued

Name[§]	Seed-Parent[¶]	Pollen-Parent	Location[#]
Hakuho	Hakuto	TachibanaWasa	SC[1]
Halford	*	*	CA[3]
Hesse	Riegels	Riegels	CA[3]
Hiley	*	*	CA[1]
Jefferson	*	*	SC[3]
JHHale	Elberta	OP	SC[3]
Jordanolo	Nonpareil	Harriott	CA[2]
Kakamas	StHelena	F_Kakamas	CA[2]
Klampt	Dixon	Wiser	CA[3]
LateCrawford	*	*	CA[1]
LateRoss	Ross	*MUT	CA[3]
Lilliland	Ross	R1-1	CA[3]
Loadel	Lovell	F_Loadel	CA[3]
Lola	*	*	CA[1]
Loring	Frank	Halehaven	SC[1]
Lovell	*	*	CA[3], SC[1]
Mayfire	Armking	F_Mayfire	TX[1]
Mission	*	*	CA[2]
Mission_BF	Mission	*MUT	CA[2]
Nickels	CP_5_33	Nemaguard	CA[3]
Nonpareil	*	*	CA[3]
O'Henry	MerrillBon	F_O'Henry	CA[1], SC[1], TX[1]
Ogawa	90_10_91	90_10_91	CA[2]
OldmixonFree	OldmixonCling	OP	SC[3]
OrangeCling	*	*	CA[3]
P.mira19	*	*	CA[1]
Panamint	BabcockxBoston	GoldminexROG	CA[1]
Peento	*	*	SC[1], CA[1]
persXdavidiana	Peach	P.davidiana	CA[1]
Redhaven	Halehaven	Kalhaven	SC[1]
Redskin	JHHale	Elberta	SC[1]
Riegels	Jungerman	Everts	CA[3]
RioOsoGem	LateCrawford	F_RioOsoGem	CA[1]
Rizzi	Everts	F_Rizzi	CA[2]
Ross	D_30_3E	GH_8_14	CA[3]
Saturn	Pallas	602903	CA[1], SC[1]
SC_Pop0804_XXX	Contender	BY92P2710	SC[1]
SC_Pop0809_XXX	ChinaPearl	Bolinha	SC[1]
SC_Pop0814_XXX	Intrepid	Blazeprince	SC[1]
SC_Pop0815_XXX	Intrepid	Bolinha	SC[1]
SC_Pop0817_XXX	O'Henry	Cascata1006	SC[1]

Table 1 continued

Name[§]	Seed-Parent[¶]	Pollen-Parent	Location[#]
SC_Pop0821_XXX	BY92P2710	Bolinha	SC[1]
SC_Pop0824_XXX	BY86P2609	Westbrook	SC[1]
SC_Pop0825_XXX	BY86P2609	Bradley	SC[1]
SC_Pop0826_XXX	BY86P2609	WhiteRiver	SC[1]
SC_Pop0836_001	Contender	Bolinha	SC[1]
SC_PopB_XXX	BY02p4019	BY02p4019	SC[1], SC[3]
Slappey	*	*	CA[1]
Sonora	Nonpareil	F_Sonora	CA[3]
Springold	FV89_14	Springtime	SC[1]
Springprince	Springcrest	F_Springprince	SC[1]
Springtime	LHoneyxJElberta	Robin	CA[1]
StJohn	ChineseCling	F_StJohn	CA[1]
Stukey_6_27	Nonpareil	OP	CA[2]
Stukey_6_27H	Nonpareil	OP	CA[2]
Stukey_6_8	Nonpareil	OP	CA[2]
Stukey_6_9BF	Nonpareil	OP	CA[2]
Sunfre	P42_81	P42_91	TX[1]
Sunhigh	JHHale	NJ40CS	TX[1]
TardyNonpareil	Nonpareil	*MUT	CA[2]
TropicBeauty	Fla3-2	Flordaprince	TX[1]
TX_Pop2	TX2B136	CAF2	TX[1]
TX2293_3	TropicBeauty	Goldprince	TX[1]
TX2B136	Hermosillo	TXW1293_1	TX[1]
TXW1293_1	TropicBeauty	TropicBeauty	TX[1]
UFGold	Fla84-18C	Fla9-20C	SC[1]
Vilmos	F10C_12_28	*VP	CA[2]
Westbrook	A_172	A_176	AR[1], SC[1]
WhiteCounty	A_392	A_433	AR[1]
WhiteRiver	Loring	NJ257	AR[1]
Winblo	Redskin	Redskin	AR[1], SC[1]
Woltemade	Kakamas	OP	CA[2]
Yumyeong	*	*	CA[1]
ZinDai	*	*	SC[1], SC[3]

List of symbols and acronyms

[§]XXX – represents a seedling population of multiple trees
Seed parent = Female parent, Pollen parent = Male parent, asterisk (*) means parent(s) unknown; OP – open pollination; *MUT – mutation; *VP – vegetatively propagated.
[#]Location in which the accessions are present (state and orchard):
AR[1] = University of Arkansas Fruit Research Station
CA[1] = USDA-National Clonal Germplasm Repository for Fruit and Nut Crops

CA[2] = UC-Davis Wolfskill Orchard
CA[3] = Foundation Plant Services Orchard
SC[1] – Clemson University Musser Fruit Research Farm
SC[2] = Sandhill Research and Education Center
SC[3] = USDA Fruit and Nut Research Center
TX[1] = The Burchell Nursery
TX[2] = Texas A&M University

Results and Discussion

A standardized protocol for collecting phenotypic data for fruit quality and productivity traits was developed in collaboration with RosBREED demonstration peach and Rosaceae community breeders (Table 2).

A standardized protocol has been used to phenotype peach crop reference set (CRS) consisting of 513 accessions linked through pedigree (Table 1). Selected material included peach and almond cultivars, breeding lines, and seedlings and comprised cultivars (45%), advanced selections (4%) and seedlings (51%). Samples with pure peach and almond ancestry accounted for 82% and 2%, respectively, while 16% of genotyped material had interspecific backgrounds with almond (9%), and peach and almond wild relatives, 5% and 4%, respectively, in their pedigrees. Some accessions were related *Prunus* species or were known interspecific hybrids: 5% had peach-related (*P. davidiana* and *P. mira*) ancestry, 10% had almond (*P. dulcis*), and 3% had almond-related (*P. argentea* and *P. scoparia*) ancestry.

Fruit quality data have been obtained for two seasons on 22 traits (Table 2). Once each accession reached 50% bloom a Julian date (0-365) was recorded along with the bloom type: showy (1), non-showy (0) and the leaf gland type: Reniform (1), Globose (2), Eglandular (3). Julian dates were incorporated to quantify data based on dates. Fruit set was next calculated following scale 0 to 9 where 0 = none; 5 = full crop, 6-8” (15 - 20cm) spacing between fruit; 7 = 2x fruit needed, 3” (7.5cm) spacing; and 9 = 4x fruit, 1” (2.5cm) spacing (Table 2, http://www.rosbreed.org/resources/fruit-evaluation).

Peach fruit was harvested at the tree ripe stage to ensure uniformity of maturity. Tree ripe stage was determined at the time when a few fruit on the tree were soft/edible. Harvesting the peaches at the same stage is critical for the success of the study, since nearly all peach fruit quality traits are known to change with the ripening stage of the fruit (firmness, internal and external color, acidity, and sugar). When fruit were deemed 'tree ripe', ten to twenty fruits, slightly firmer than tree ripe, were harvested into cardboard or plastic box container (Figure 2).

Keeping the material in an open container allowed the fruit to "breathe" and dry out if there was excess moisture from morning dew or rain. Label depicting accession name/ID, and harvest date in Julian days (0-365) was created and attached to the container. Pubescence level was assessed in the field by estimating visual amount of pubescence for each fruit on a scale from 0 to 7, with a higher numeral indicating a greater degree of pubescence (Table 2; Figure 3). Fruit type was also recorded in the field with 0-1, where peach was designated as 1 and nectarine as 0.

Fruit harvested in the field were brought to the lab for phenotyping of quality traits. Five fruit from each peach accession were selected to be analyzed, using the traits listed in Table 2. A standard Konica Minolta Chroma Meter (CR-400, Konica Minolta Chroma Meter, Tokyo, Japan; or other models) was used to quantify the background and flesh color for each fruit (Figure 4). Measurements were taken on the darkest portions of both cheecks of the skin and flesh of the peach using the 'Light Protection Tube' (glass protection plate CR-A33a, 22mm in diameter, Tokyo, Japan; or other models). Care was taken not to measure the blush or red in the flesh, since red pigmentation complicates

Table 2. Standardized phenotyping protocol for peach

Trait	***Unit of measure***
Productivity traits	
50% Bloom Date(Julian)	0-365 days
Bloom Type	Showy = 1; Non-showy = 0
Leaf gland type	Reniform = 1; Globose = 2; Eglandular = 3
Fruit Set	0=none, 5=full crop, 6-8" (15-20cm) spacing between fruit, 7=2x fruit needed, 3" (7.5cm) spacing, 9=4x fruit, 1" (2.5cm) spacing
Ripe Date (Julian)	0-365 days
Fruit quality traits – Organoleptic phenotyping	
Pubescence	0 = glaborous or nectarine; 3 = slight ; 5 = medium ; 7 = heavy
Blush %	0 = no blush; 1 = 1-20%; 2 = 21-50%; 3 = 51-80%; 4 = 81-99%; 5 = 100%
Ground Color	1= green; 2= cream green; 3= cream; 4= cream yellow; 5= yellow green; 6= yellow; 7= yellow orange; 8= orange; 9= red
Ground Color L* (C)	L*
Ground Color R	R = length of vector
Ground Color Theta (Θ)	Theta (Θ) = angle of vector
Flesh Color	1= green; 2= cream green; 3= cream; 4= cream yellow; 5= yellow green; 6= yellow; 7= yellow orange; 8= orange; 9= red
Flesh Color L*(C)	L*
Flesh Color R	R = length of vector
Flesh Color Theta (Θ)	Theta (Θ) = angle of vector
Red in Flesh	0= no red overlay; 1 = 1-20%; 2 = 21-50%; 3 = 51-80%; 4 = 81-99%; 5 = 100%
Red around Pit	1= red; 0 = no red
Fruit quality traits – Destructive phenotyping	
Diameter	Widest part of the fruit (mm)
Weight	Grams
Flesh Firmness average	Kg/cm2 of force
Brix %	%
pH	#
Malic Acid / Titratable Acidity	#
Fruit Texture	Melting= 1; Non-melting= 2
Adherence to pit	Freestone= 1; Semi-freestone= 2; Semi-clingstone= 3; Clingstone= 4
Pit weight	Grams
Pit Split %	Proportion of split / normal pits

Figure 2. Fruit harvested into cardboard box, labeled and brought into lab for phenotypic analysis.

Figure 3. Fruit with two different levels of pubescence. Left = peach, [7]. Right = nectarine, [0].

Chroma Meter measurements. The Chroma Meter was used to quantify the color content (L*, intensity (-L*, dark; +L*, light), a* (−a*, green; +a*, red) and b* (-b* blue; +b*, yellow). The saturation and hue angle can be more readily determined for data analysis, when the information content for blush is stored using polar instead of cartesian coordinates. Cartesian coordinates show a relative distance between two colors while polar coordinates determine the exact position. For this reason the a* and b* values were

converted from cartesian (x, y) to polar coordinates [r = saturation, theta (θ) = hue angle] using a simple transformation of coordinate systems.

The weight (g) and diameter (mm) for five fruit were measured using any scale, and micrometer caliper. An automatic fruit texture analyzer (FTA, GUSS Manufacturing Pty. Ltd., Strand, South Africa; or other model), with electronic scale and electronic fruit size measure (EFM, GUSS Manufacturing Pty. Ltd., Strand, South Africa; or other model) was also used in some instances. Fruit firmness was measured on both cheeks, after removing approximately 1-cm of the outer flesh, using either an automatic FTA, mounted or hand held penetrometer. The fruit firmness was quantified in kg/cm^2 of force.

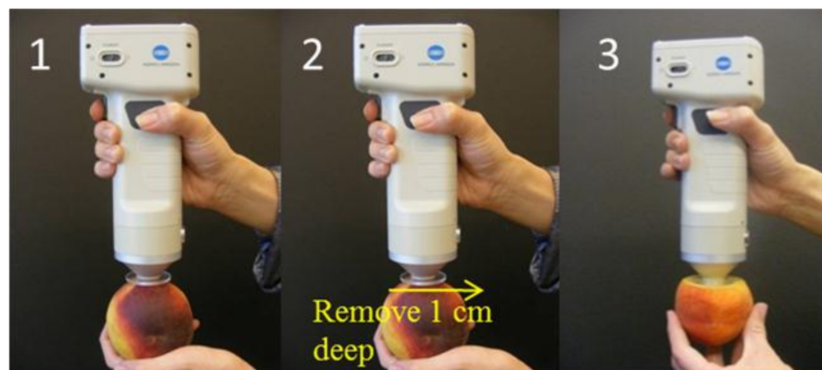

Figure 4. Using Chroma Meter to quantify [1] background color and [3] flesh color.

Care was taken to make sure the fruit was at room temperature (~24°C) before proceeding, since varying temperatures hinder the standardization of the subsequent phenotypic traits (i.e. sugar, titratable acidity and pH all change with temperature fluctuations). A longitudinal slice of each of the five fruit was taken to extract the juice for measurement of SSC, pH and TA. Sampling fruit in this manner is important to

account for variation of sugar levels throughout the fruit, since accumulation of sugars is elevated at the stem end and decrease at the tip end. The five slices were juiced through cheese cloth using a hand presser, or a blender and the composite sample was used to determine soluble solid content (SSC; sugars are the most prominent SSC in fruit juice) of each accession using a digital hand held brix refractometer (Atago USA, INC 3810 PAL-1 Digital Hand-Held Pocket Refractometer, WA, USA; or other model). This instrument quantifies the refractive index, indicating the degree to which the light is bent as it moves through the juice. Six grams of the juice was measured with a pipette and diluted with 50 ml of water. The initial pH and titratable acidity (TA) of each sample was quantified with either a pH meter, phenolphthalein indicator, or automated volumetric titrator (862 Compact Titrosampler, Metrohm AG, Herisau, Switzerland; or other model). Each sample was titrated with 0.1 N NaOH to an end point of 8.2pH, at which the milliliters (mls) of NaOH used were recorded. The TA was calculated using the following formula: % acid = [mls NaOH used] x [0.1 N NaOH] x [milliequivalent factor] x [100] grams of sample. Lastly, the flesh and pits were separated and pits evaluated for tendency to split. Finally weight of five pits was obtained and an average pit weight calculated (Table 2).

A second set of five fruits for each accession was phenotyped for series of organoleptic traits. The percentage of blush covering the fruit skin was approximated using a scale from 0-5; 0 indicating no blush and 5 indicating full red over color. The skin and flesh color were marked following a numerical scale for different colors (1-9; Table 2). Adherence to the pit was noted: The fruit was deemed freestone (flesh easily separates

from the pit), clingstone (strong adherence to the pit), semi-freestone (most of the flesh separates from the pit) or semi-clingstone (medium adherence to the pit). Fruit texture was scored as non-melting if the flesh was firm and intact, or melting if the flesh was smooth, soft and easily fell apart. The amount of red in the flesh was scored on a scale of 0-5; 0 indicating no red and 5 indicating flesh entirely red. Pigmentation at the pit was marked as 1 if red, or 0 if not red (Table 2).

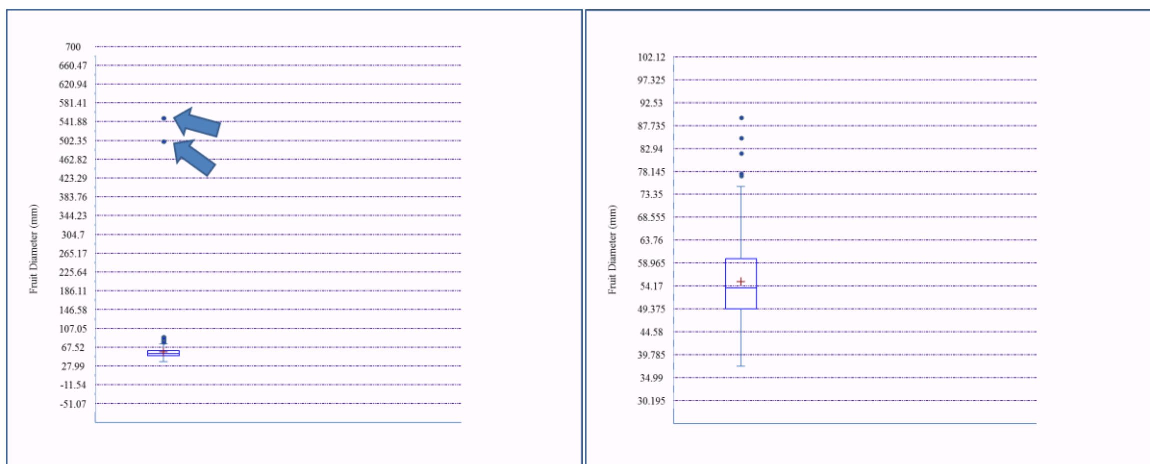

Figure 5. Box Plots of 2010 peach diameter. Left = original data, two outliers identified with blue arrows. Right = two outliers removed.

A quality checking protocol of the phenotypic data was developed and implemented to make sure there are no typing mistakes or outliers. Collected data were quality checked, using Microsoft excel spreadsheet following five steps.

First – checking every 30th data point to check for line shifts that potentially could have occurred during data entry. Second – outlier identification by calculating maximum and minimum values and / or developing histograms/box-plots (Figure 5). If the outliers were spotted they were corrected as shown in Figure (5, 6) outliers identified and deleted. In case multiyear data available additional outliers checked by calculating differences in

traits between years (i.e. a peach scored as white one year, cannot be yellow next year). Finally multi-year data checked for additional outliers by generating scatterplots between years to determine correlations and identified data points far outside of correlations (Figure 6).

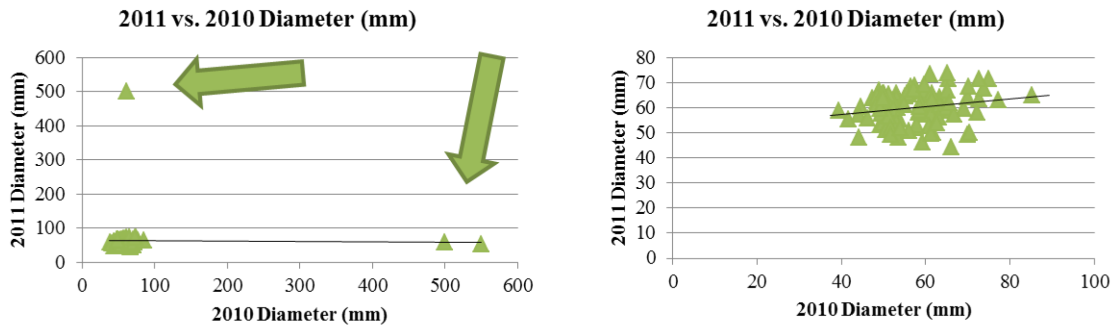

Figure 6. Multi-year data check for additional outliers by generating scatterplots between years to determine correlations and identify data points far outside of correlations. Left = original data, 5 outliers identified with green arrows. Right = 5 outliers removed.

A detailed PowerPoint presentation portraying the standardized peach phenotyping protocol, including pictures (http://www.rosbreed.org/resources/fruit-evaluation) and videos showing each step have been generated (http://www.rosbreed.org/resources/fruit-evaluation/phenotyping-videos/peach).

Conclusions

Developing a standardized phenotyping protocol for peach is a starting point in enabling collection of phenotypic data related to fruit quality across different institutions, environments and countries. Public and private peach breeders shared their expertise and experiences in suggesting the most feasible protocol for collecting fruit quality data. With increasing interest in the peach breeding community for understanding the genetic makeup of many agronomically important traits, this protocol will change and grow. Available genomic data are housed in a freely accessible database, GDR. Phenotyping is a crucial component for QTL mapping because along with genotypic data it allows genetic variation to be associated with biological function (Bassil and Volk, 2010). This connection reveals genotypic expressions and exposes the function of critical structural and or regulatory genes.

One of the potential uses for standardized phenotyping data is to be used in analysis such as PBA where pedigree linked germplasm is phenotyped and genotyped to reveal alleles in pedigree associated with the traits of interest. Molecular markers tightly linked to the significant QTL and or candidate genes controlling the peach traits are identified and tested on several segregating populations to determine the markers success in association with the trait. After the markers efficiency is confirmed it can then be used to enable routine MAB for that trait in peach. This approach should provide an efficient procedure to allow for and effective parent selection and determine which progeny to invest in and grow, thus saving time, labor, money, and space (Bliss, 2010).

This work will help to directly facilitate the development of future peach cultivars with improved fruit quality traits in the hopes of revitalizing a dwindling peach industry. Moreover, this study also has enormous implications in the development of future cultivars for other members of the Rosaceae family. Phenotyping protocols for several other Rosaceae species (strawberry, apple, sweet and sour cherry) have also been developed and are being implemented (Peace et al., 2011). Peach is a model organism for Rosaceae species because it has a relatively short juvenile period, is easy to cross, and has a small genome size ~220-230 Mbp (Abbott et al., 2002; Bielenberg et. al., 2009). Comparative genomics can be applied with the other Rosaceae species, allowing for breeders and other fruit industries to benefit from the uncovered markers.

Uniform efforts, standardized phenotyping, regulated trait ontologies or nomenclatures for trait classifications, and centralized storage organization and access to data will enable results to be compared across years, locations and researchers. These uniform efforts will facilitate efficient exploration of gene function for Rosaceae species (Bassil and Volk, 2010; Volk, 2010). The peach, sour cherry, sweet cherry, apple and strawberry standardized phenotyping protocols, can be used by the national and international community to cover different accessions and environments. Global standardized phenotypic data collection for specific species will drastically increase the availability of phenotypic data for each species. Increasing phenotypic and genotypic data will culminate in an enhanced PBA QTL analysis, due to the increased mapping resolution, more allele segregation, reduced research time, and increased allele numbers

(Yu and Buckler, 2006). Standardized phenotyping efforts in combination with genomic data will lead to discovery of markers that will ultimately enable MAB.

The success of standardized phenotyping, noted herein, and previous standardized phenotyping models (Abbott, 1999; Peace and Norelli, 2009; Bassil and Volk, 2010; Postman et al., 2010; Rudell, 2010; Scott, 2010) should spur its application to improve other important fruit traits as well as fruit quality, productivity and biotic & abiotic stress resistance. Further, other agronomically important plant species can benefit from standardized phenotyping, allowing for efficient discovery of genes that control important agricultural traits. Ultimately, standardized phenotyping in conjunction with genotyping and QTL analysis will enable MAB for several vital agronomic plant traits. The developed markers will become tools, to increase the efficiency of traditional breeding, leading to the release of agricultural cultivars with enhanced traits.

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CHAPTER III

MAPPING QTL FOR BLUSH IN PEACH [PRUNUS PERSICA (L.) BATSCH]

Introduction

Blush is an important fruit quality trait in marketing peaches. The red pigmentation is attractive to the consumer's eye and the anthocyanin compounds associated with blush provide flavor and nutrients, are essential components of the human diet (Parr and Bolwell, 2000; Sun et al., 2002; Balasundram et al., 2006). Moreover, anthocyanin compounds are known to combat the development of cancer, cardiovascular and other health problems related to aging (Parr and Bolwell, 2000; Sun et al., 2002; Schijlen et al., 2004; Howad et al., 2005; Balasundram et al., 2006). For these reasons, breeding efforts in the private and public sectors have been driven towards fresh market peaches with an extensive level of blush (Scorza and Sherman, 1996; Okie et al., 2008).

As a peach ripens, background color changes from green to yellow, or other hues. Throughout the final swell (stage III of peach fruit development) different levels of red skin pigmentation develop over the background color (Delwiche and Baumgardner, 1983; Delwiche and Baumgardner, 1985; Byrne et al., 1991; Marini et al., 1991; Layne et al., 2001). The red over color develops in diverse intensities and patterns depending on the genotype (molted, striped, variegated, spotted, etc.).

The phenotypic variation of blush development is controlled by (i) genetic factors; (ii) sunlight exposure (Layne et al., 2001); and (iii) co-dependent factors of the genotype*environment interaction. These three factors regulate the flavonoid and further anthocyanin biochemical pathways, and are highly conserved in plants (Schijlen et al.,

2004). Numerous structural genes code for proteins that chemically modify the flavonoid compounds which are substrates for the anthocyanin pathway.

Sunlight regulates specific MYB transcriptional regulatory genes which encode transcription factor protiens that activate the expression of the structural genes in the flavonoid and anthocyanin pathways. The enzymes encoded by the structural genes chemically modify the flavonoid compounds, which changes their structural conformation. These structual modifications generates new compounds that perform diverse functions. The flavanoid pathway contains three precursors which through structural modifications lead into the anthocyanin pathway: delphinidin, pelargonidin and cyanidin (Kui et al., 2010). In peach the structural conformation of the cyanidin precursor is converted by enzymes encoded by cyanidin structural genes, resulting in the production of two main anthocyanin associated with blush in peach: cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside (Hsia et al., 1965; Van Blaricom and Senn, 1967; Chaparro et al., 1994; Tomás-Barberán et al., 2001; Byrne et al., 2004; Wu and Prior, 2005; Cevallos-Casals et al., 2006; Vizzotto et al., 2006; Vizzotto et al., 2007; Cantín et al., 2009) (Figure 7).

The concentrations of these two anthocyanin vary depending on the type of peach and specific tissue (exocarp vs. mesocarp). Tomás-Barberán et al., (2001) and Vizzotto et al., (2006; 2007) showed that red fleshed cultivars contained significantly higher levels of anthocyanin than white or yellow fleshed peaches (no differences were found between white and yellow fleshed peaches).

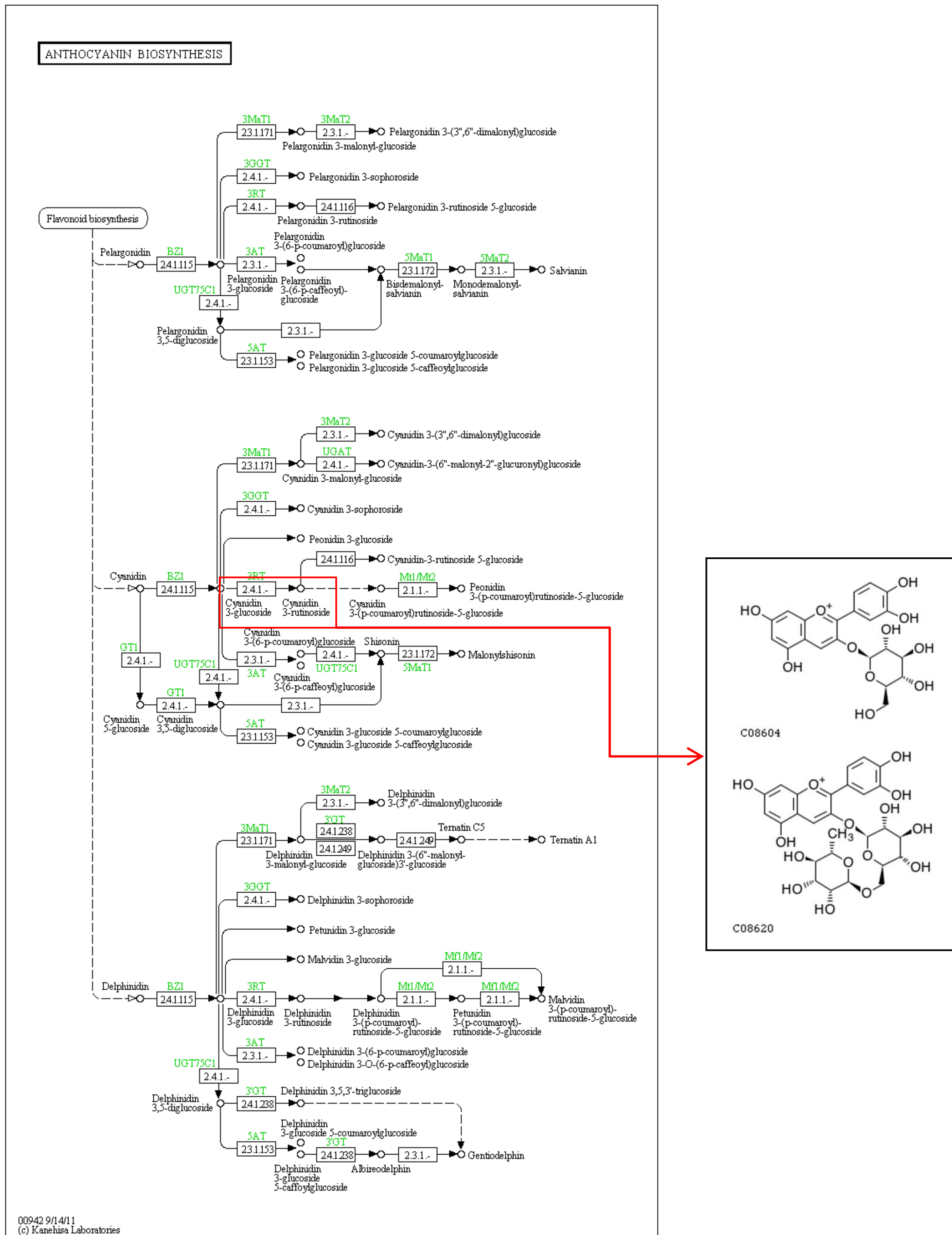

Figure 7. Anthocyanin biosynthesis. Flavonoid pathway leads into the anthocyanin pathway and production of 2 main anthocyanins in peach (CO8604= Cyanidin 3-O-glucoside ; C08620= Cyanidin 3-O-rutinoside) (KEGG, Kanehisa Labs, 1995-2012 http://www.genome.jp/kegg-bin/show_pathway?map00942).

Interestingly in general, the peach skin (exocarp) was found to contain three times or greater levels of phenolic compounds (anthocyanins and flavonols) than the flesh (mesocarp; Chang et al., 2000; Tomás-Barberán et al., 2001; Gil et al., 2002; Gorinstein et al., 2002; Cevallos-Casals et al., 2006; Vizzotto et al., 2006; Vizzotto et al., 2007). The skin is therefore a highly concentrated source of these compounds, however, it only represents ~8% of the total fruit weight. Therefore, the complete distribution of phenolic compounds in the skin and flesh for each fruit is ∼30% and 70% (Cevallos-Casals et al., 2006).

Traditional breeding has been successful in developing peach cultivars with increased levels of blush, such as: 'Blazeprince' (USDA-ARS, Byron, GA), 'Crimson Lady' (Bradford and Bradford, 1991), 'Goldcrest' (USDA-ARS, Fresno, CA, 1983), 'Redglobe' (USDA, Beltsville, MD, 1954), 'Redskin' (Maryland Agricultural Experiment Station, College Park, MD, 1994) and 'Springprince' (USDA-ARS, Byron, GA, 1998) (Figure 8). Like several fruit quality traits blush is quantitative in nature, and thus presents practical challenges in selection (Bliss, 2010). Furthermore, traditional breeding is a time consuming process. To overcome the limitations of traditional breeding and enhance blush in peach cultivars, discovery of molecular marker(s) linked to quantitative trait loci (QTL) associated with the development of blush to facilitate marker assisted breeding (MAB) and enable more efficient selection of this trait.

Figure 8. Images of cultivars depicting various levels of blush (D. Layne; http://www.clemson.edu/hort/peach/index.php?p=73).

Despite the growing availability of genomic resources in peach, the newly released peach genome sequence (http://www.rosaceae.org), high-throughput Illumina Infinium® IPSC 9K SNP v1 genotyping array (Verde et al. 2012), existence of a highly saturated reference map (TxE) (Abbott et al. 2002; Etienne et al., 2002; Dirlewanger et al. 2004), and most of the simple characters being sufficiently marked for selection, the use of molecular markers for commercial breeding in peach is still in its infancy.

Development and inheritance of blush in peach at the molecular level has not been sufficiently investigated to enable MAB.

Recently, some studies focusing on fruit quality in peach have reported discovery of possible QTL associated with the production of red skin and blush in peach (Quilot et al., 2004; Ogundiwin et al., 2007, 2008, 2009). Quilot et al., (2004) discovered a QTL responsible for red skin coloration (SRColor2) close to RFLP marker AC108 on linkage group 5 of the *Prunus* genome. In addition, Ogundiwin et al. (2007, 2008, 2009) also reported a QTL (qP-Brn5.1^{m}) on linkage group 5 associated with Leucoanthocyanidin dioxygenase (PpLDOX), associated with browning. Leucoanthocyanidin dioxygenase also serves as an important structural gene in the anthocyanin pathway. This QTL, qP-Brn5.1^{m} is in the same general location as that reported by Quilot et al. (2004). Additionally, a QTL for blush was localized on LG4 (LOD peak position of 52.8cM) of a genetic linkage map created using a F_1 progeny from a cross between ‘Venus’ x ‘BigTop’ (V×BT; Cantín et al., 2010). This QTL explained 68.7% of the phenotypic variance of blush (Cantín et al., 2010).

QTL studies have also been performed in other Rosaceuos plants to determine specific regions in the genome responsible for the biosynthesis of anthocyanins. The investigation of blush and QTL discovery has been extensively performed in apple (*Malus domestica*) (Takos et al., 2006; Ban et al., 2007; Chagné et al., 2007; Espley et al., 2007, 2009), cherry (*Prunus avium L.*) (Sooriyapathirana et al., 2010) and grape (*Vitis vinifera*) (Kobayashi et al., 2004; Walker et al., 2007; Kobayashi, 2009). These studies were essential for indentification of the transcription factors (TF) responsible for the

genetic regulation of the anthocyanin biosynthetic pathway in Rosaceae. In this family, the two-repeat R2R3 MYB TF class has been associated with the activation of the anthocyanin biosynthesis pathway (Allan et al., 2008; Kui et al., 2010). A major gene MdMYB10/MdMYB1/MdMYBA associated with red skin (Takos et al., 2006; Ban et al., 2007) and red flesh coloration in apple (Chagné et al., 2007; Espley et al., 2007, 2009) was mapped. Kui et al. (2010) demonstrated that the three MYB activators of apple anthocyanin (MYB10/MYB1/MYBA) were expected alleles of each other. Through comparative genomic techniques, they determined that this locus is highly likely to be homologous across the Rosaceae family. Over-expression of these genes in apple and strawberry correlated with elevated levels of anthocyanins in the fruit and flowers (Kui et al., 2010).

In sweet cherry (*Prunus avium* L.) a population segregating for skin and flesh color was used to locate a major QTL for red skin pigmentation on LG3 (Sooriyapathirana et al., 2010). The candidate gene, PavMYB10, located within the major QTL interval for red skin pigmentation in sweet cherry was homologous to the anthocyanin associated genes in apple, MdMYB10, and *Arabidopsis*, AtPAP1, (Kui et al., 2010; Sooriyapathirana et al., 2010). This suggested that PavMYB10 is likely a major TF gene responsible for the production of red skin and flesh in sweet cherry (Sooriyapathirana et al., 2010).

Qualitative inheritance and existence of two single genes controlling development (Beckman and Sherman, 2003) or suppression (Beckman et al., 2005) of red skin in peach has also been reported. The 'full red' genotype in peach is a single gene recessive

trait (fr/fr) where 100% red over color develops even in the absence of light (Beckman and Sherman, 2003). Additionally, Beckman et al., (2005) further proposed existence of so called 'highlighter phenotype' (h/h) in peach, where anthocyanin development appeared to be suppressed only in the fruit tissues. Their research suggested that qualitative control for the suppression of red skin color in a peach fruit is associated with a single gene recessive trait (h/h).

The overall objective of this research is to facilitate MAB for blush in peach via development of a linkage map for a peach F_2 population segregating for blush. This includes both detection and mapping of QTL associated with blush in peach. Potential application of this research to marker-assisted breeding for blush in peach is discussed.

Materials and Methods

Plant Material Used

An F_2 population segregating for red skin pigmentation was used for QTL analysis of blush. An intraspecific cross between two parents with contrasting phenotypes for blush, 'Zin Dai' (~30% blush) x 'Crimson Lady' (~100% blush), was made and the individual F_1 tree (BY92p4019: ~65% blush) was selfed to obtain a segregating F_2 population of 93 individuals (denoted as ZC^2; Figure 9; Figure 10). The seed parent, 'Zin Dai', has white, non-melting and low-acid flesh. 'Zin Dai' originates from China and its parental background is unknown. The pollen donor, 'Crimson Lady' is a yellow, non-melting flesh peach that originated from a cross made at the experimental orchard at Bradford Farms (San Joaquin Valley, CA; U.S. Plant Pat No. 7,953) in 1984 between seed parent, 'Red Diamond' and pollen parent, 'Springcrest'. The single F_1 individual selected for selfing, BY92p4019g, has intermediate blush, yellow, melting and semi-low acid flesh.

The ZC^2 individuals are all clingstone, yellow fleshed, peaches (G/G), with a showy bloom (sh/sh). ZC^2 population segregates for blush (0-100%), flesh texture (melting vs. non-melting) and ripening time (~June 20th to August 20th). This population is located at the USDA Fruit and Nut Research Center in Byron, Georgia. The seedling progeny were planted in a single row at 3ft in-row spacing. Minimum horticultural maintenance was done.

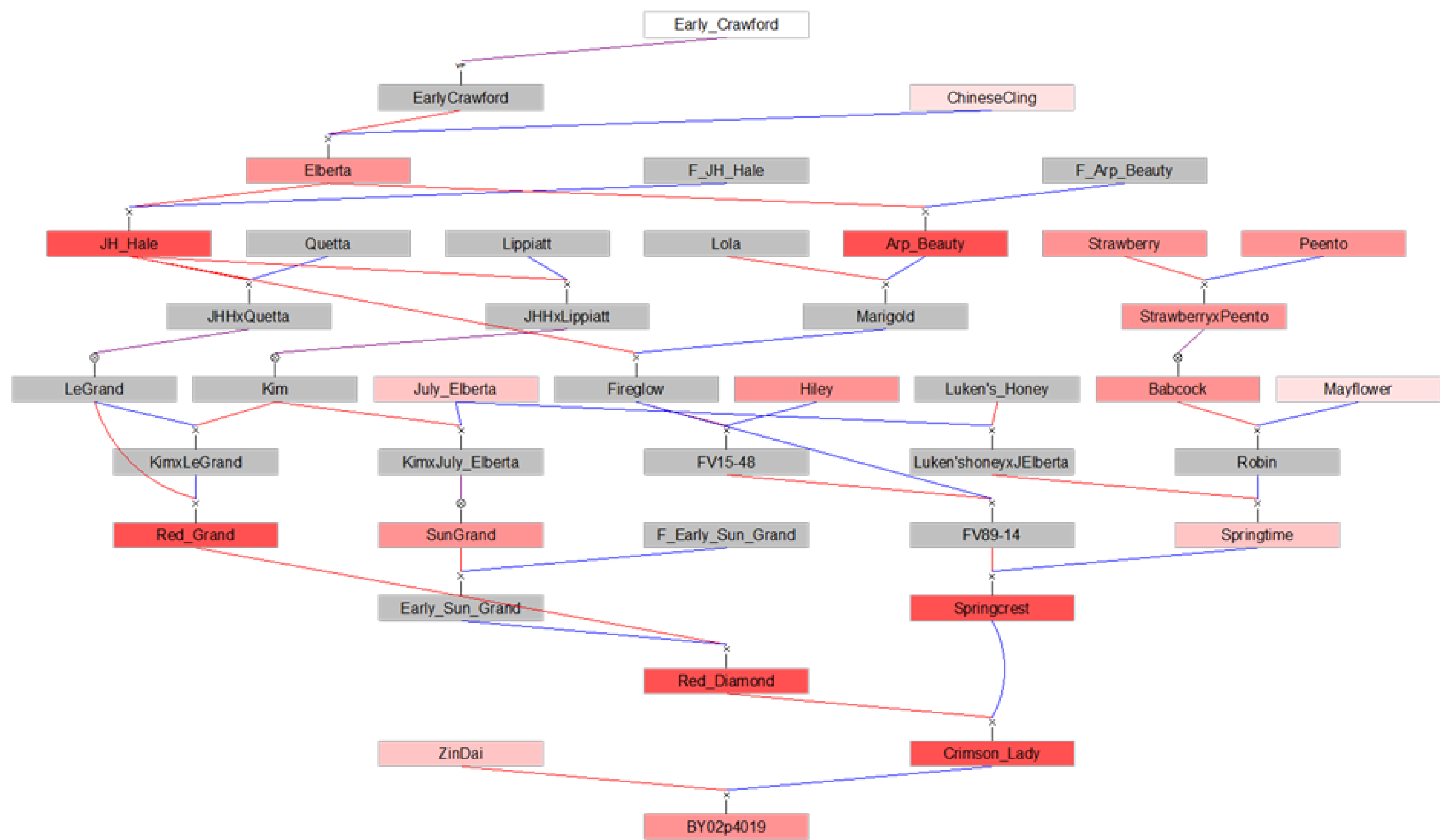

Figure 9. Pedigree of ‘Zin Dai’ x ‘Crimson Lady’ F_2 population. The darker the red, the higher the amount of blush. Grey, no data available. Pedigree analysis was performed using PediMap software (Voorrips 2007). [1](“F_” = OP).

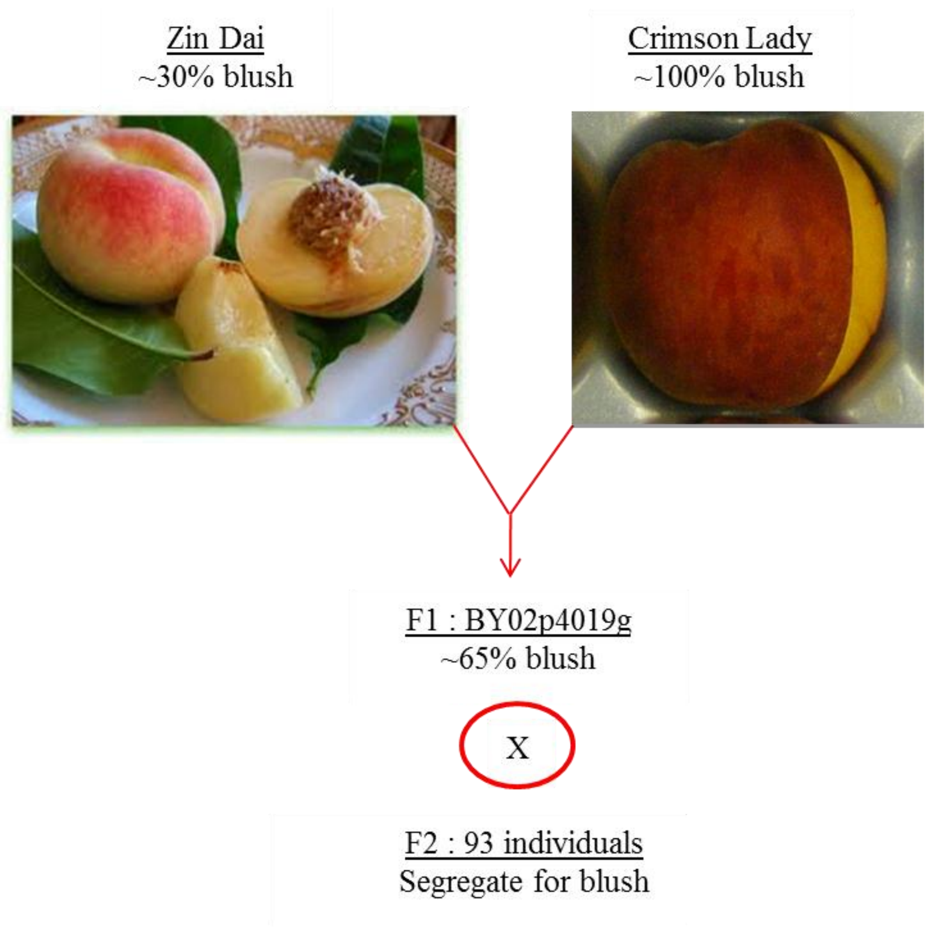

Figure 10. Blush population (BY02p4019g self = Zin Dai Jiu Bao * Crimson Lady).

Trees were hedged during their third year of growth, which resulted in near barren inner part of the tree canopies (almost no foliation), and relatively low foliation on outer parts of canopy. Because trees were not pruned each year, the fruiting wood was in the periphery of the canopy.

Phenotyping

Phenotypic data were recorded over four years (2007, 2008, 2010, 2011) using standardized phenotyping protocol developed for peach and discussed in chapter II. In detail, two methods for blush data collection were used: visual qualitative coverage (0-5 scale) and quantitative intensity using a Chroma meter (CR-400, Konica Minolta, Tokyo, Japan).

Fruit were harvested after a few fruit on the tree became tree ripe (soft to the touch) and the harvest date was marked in Julian days (0-365). An average percentage of the fruit with highest blush was also recorded to account for sunlight variance throughout the canopy and obtain an accurate representation of blush (Figure 11).

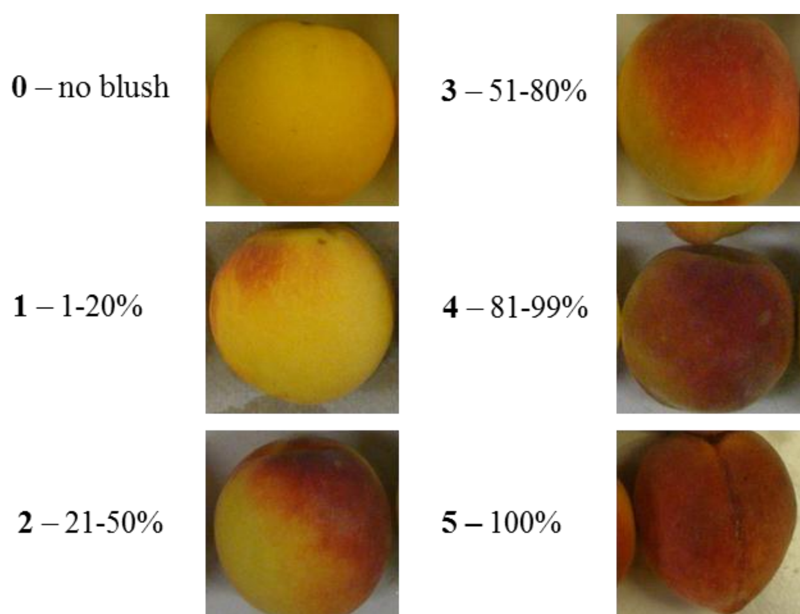

Figure 11. Visual scale for blush phenotyping.

Subsequently, ten to twenty fruits, slightly firmer than tree ripe, were harvested and placed into a cardboard box labeled with the genotype and harvest date and brought to the lab. Five fruit from each accession were selected for analysis of fruit quality traits.

In addition to visual qualitative coverage, blush was also documented in 2011 using Standard Konica Minolta Chroma Meter to record the over color of the skin by placing the 'Light Protection Tube' (glass protection plate CR-A33a; 22mm in diameter) on the most intense area of blush on each peach sample to quantify blush: L* (intensity; -L*, dark; +L*, light), a* (−a*, green; +a*, red) and b* (-b* blue; +b*, yellow). For data analysis, the saturation and hue angle can be more readily determined when the information content for blush is stored using polar coordinates instead of cartesian coordinates. Cartesian coordinates show a relative distance between two colors while polar coordinates determine the exact position. For this reason the a* and b* values were converted from cartesian (x, y) to polar coordinates (r = saturation, theta (θ) = hue angle) using a simple transformation of coordinate systems.

Statistical Analysis

Descriptive statistics, of all blush phenotypic data, both visual qualitative coverage (0-5 scale) and quantitative spectrophotometer readings, L*, a*, b*, r, theta, were calculated using IBM® SPSS® Statistics (19.0.0, 2010). The 0-5 blush scale ratings were averaged across years, and minimum and maximum values were identified. A combined approach was also used where the most abundant visual scoring throughout the four years of data was selected (i.e. four year scores 2, 2, 2, 3 then 2 was selected for

combined data). A paired t test ($P<0.05$) was used to compare the blush means of the two parents.

The descriptive statistics generated included mean, standard deviation, skewness, and kurtosis. Histograms and a normal quantile-quantile (Q-Q) plot were generated, for each data set, in order to determine normality of data graphically. If all data points fell along the 45º line, then the data was not skewed, and it approximated a normal distribution.

Pearson's correlation coefficients were calculated in SPSS for visual blush using 0-5 scale for 2007, 2008, 2010, 2011, and the 2011 L*, a*, b*, r, theta data. Broad-sense heritability (H^2) was approximated using the following formula: $H^2 = \sigma_g^2/(\sigma_g^2 + \sigma_e^2/n)$ (σ_g^2 = genotypic variance; σ_e^2 = environmental variance; n = sample size).

DNA Isolation and Genotyping

Twenty-five of the 93 ZC^2 genotypes (i.e. a subset of ZC^2 population) that exhibited a blush range from 0 (0% red) to 5 (100% red) and a normal distribution for this trait, were selected to be genotyped using IPSC 9K peach SNP array v1. (Verde et al. 2012; Figure 12).

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 25 ZC^2 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 SNP that did not cluster (homozygous) or had ambiguous clustering.

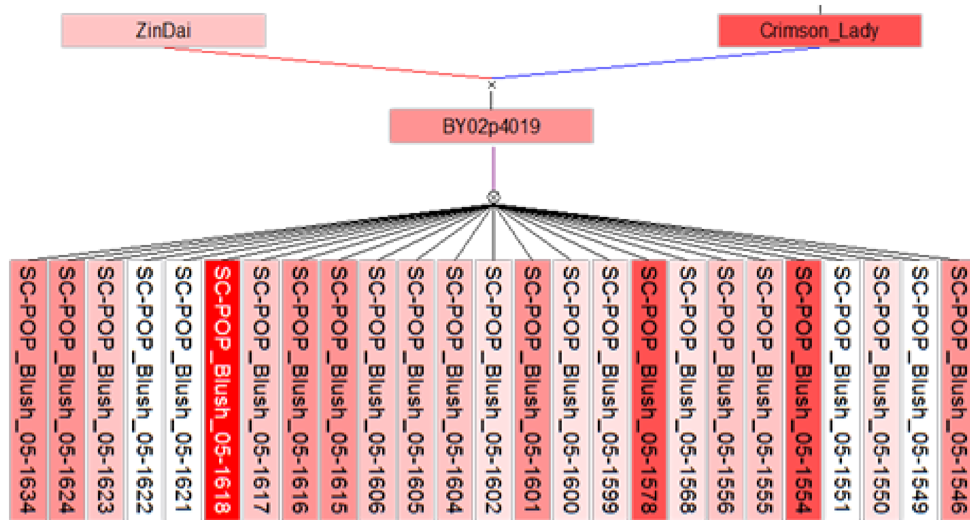

Figure 12. Subset from Pedigree analysis of mapping population depicting blush. The darker the red, the higher the amount of blush. Grey, no data available. Pedigree analysis was performed using PediMap software (Voorrips 2007).

Genetic Linkage Map Construction

SNP homozygous for alternate alleles in two parents as well as SNP homozygous in one and heterozygous in the other parent were considered for mapping. F_2 population type codes were applied (Van Ooijen et al., 2006). Genetic linkage analyses and map construction were performed with JoinMap 4.1 (Van Ooijen et al., 2006). The deviations from Mendelian ratio were tested using Chi-square-goodness-of-fit test ($P<0.05$). Linkage groups (LG's) were established using a minimum 3.0 logarithm of odds (LOD) and maximum recombination frequency of 0.40. Marker distances were calculated using Kosambi (1944) mapping function. Map figures were generated using MapChart 2.2 software (Voorrips 2002).

Linkage Map Comparisons to Peach Physical Map

Based on shared markers, all 14 LG's were compared to the peach physical map to determine their name and orientation. The set of SNP 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.

QTL Analysis and Mapping Blush

Blush data were organized in datasets. Each dataset was created from visual and Chromameter data points. In detail, data collected for each accession in four seasons (2007, 2008, 2010, 2011) included, maximum, minimum average and most consistent value over the four years; and 2011 L*, a*, b*, r, theta data, were organized in 13 datasets for QTL analysis.

The ZC^2 linkage map and phenotypic data sets were used to characterize and map QTL associated with blush in peach. Initially all phenotypic data sets were tested for the normality of distribution using the S-test (i.e., the standard error of mean (SEM)) calculated in Windows-QTL-Cartographer V2.5 (Wang et al. 2007). Those data sets with S values lower than 5.99 ($p<.05$) and 9.21 ($p<.01$) approximated a normal distribution and were used for QTL analysis. Detection of putative QTL was performed separately for each dataset, using composite interval mapping (CIM; Jansen and Stam, 1994; Zeng, 1994). Genome wide QTL threshold values for each data set were determined by a 1,000-permutation test ($p<0.05$). Through this analysis every 1cM of the genome was scanned to approximate LOD curves. Multiple regression (MR) analysis was used to estimate the percentage of phenotypic variation (R^2) explained for each individual QTL and for all QTL (R^2t). The percentage of phenotypic variance (R^2) explained by the QTL was taken as the QTL peak position as determined by WinQTL cartographer 2.5. QTL with an $R^2 >$ 25% were declared major QTL. The QTL with $R^2 <$ 25% were termed minor QTL. The LOD of the peaks were used to indicate the most likely position of QTL effects. QTL intervals were reported in 1LOD ($p<.05$) and 2LOD ($p<.01$) confidence intervals. Figures of the resulting subset ZC^2 linkage map and associated QTL positions were developed using MapChart 2.2 (Voorrips 2002). QTL were named as TTL1-YYYY where TT = trait acronym; L = linkage group number; _1= numbers to identify different QTL for the same trait; YYYY = the year in which the trait was phenotyped, following example from Fan et al. (2010).

Results

Phenotypic Data

Phenotypic data for blush was collected in the ZC^2 population using visual qualitative coverage (0-5 scale; in 2007, 2008, 2010, 2011) and quantitative intensity (CR-400, standard Konica Minolta Chroma Meter, Tokyo, Japan; in 2011) were organized in datasets (Table 3). In addition, field and lab images of fruit from parents and ZC^2 progeny used in linkage map development were obtained (Figure 13, appendix Figure 22, appendix Figure 23, appendix Figure 24). Visual blush (0-5) and L*, a*, b*, r and Θ data showed significant correlations ($P<0.01$) through all years and all data comparisons (Table 4).

These results suggested that blush accumulation did not vary much between years (i.e. the environment does not impact blush as much as genotype). The minimum horticultural maintenance applied to this population, could be a major determinate for such low environmental variation observed. Trees were not pruned to a standard training system, and the first pruning was applied on 3-year-old trees by hedging. This resulted in scarce fruiting wood that escaped to the outer part of the canopy, therefore allowing uninterrupted sun exposure to the fruit. Fruit sampling should also be taken into account since special attention was applied to ensure uniform and homogeneous sample collection from each evaluated tree. Trees were hedged when they reached the third year. Pruning was not performed each year, which resulted in fruiting wood escaping to the outer part of the canopy. The inner part of the tree canopies were relatively barren (near no foliation, and no fruit), and moderately low foliation on outer parts of canopy. This

Table 3. Descriptive statistics for all phenotypic data.

Data set	N	Min	Max	Mean	SD	S-test value	Skewness		Kurtosis	
							Stat.	SE	Stat.	SE
Blush2007	25	.00	5.00	1.88	1.45	1.56	.58	.49	-.03	.98
Blush2008	25	.00	4.00	1.56	1.26	0.75	.12	.49	-1.23	.98
Blush2010	24	.00	5.00	1.67	1.37	1.38	.55	.50	-.05	.92
Blush2011	25	.00	4.00	1.88	1.13	0.47	-.31	.49	-.67	.98
Average	25	.00	4.00	1.88	1.30	0.11	.12	.49	-.65	.98
Combined	25	.00	4.00	1.88	1.24	0.27	.10	.49	-.66	.98
Max	25	.00	5.00	2.24	1.50	0.63	.39	.49	-.37	.98
Min	25	.00	3.00	1.36	1.11	0.93	.19	.49	-1.28*	.98
L* 2011	23	33.39	70.91	51.50	14.24	1.81	.36	.51	-1.63*	1.02
r 2011	23	27.63	63.12	47.36	9.85	0.08	-.05	.51	-.73	1.02
Θ 2011	23	25.70	88.45	54.61	24.15	2.30	.48	.51	-1.67*	1.02
a* 2011	23	1.53	38.80	22.44	14.05	2.32	-.50	.51	-1.65*	1.02
b 2011	23	13.67	62.89	37.00	16.95	1.42	.37	.51	-1.43*	1.02

[1][N, number of analyzed samples; SD, standard deviation; Stat., statistics; SE, standard error. Critical values for the rejection of normality of data sets are 5.99 and 9.21 at the 5% and 1% levels, respectively, for the S test statistics].

enabled a high amount of sun penetration throughout the trees canopy. The fruit was located towards the outer part of the canopies with best sunlight exposure. This decrease in environmental variation was very beneficial for this investigation, since it concentrates on determining the genetic control of blush.

Phenotypic Data Distributions & Descriptive Statistics

Statistically significant differences for visual blush between ‘Zin Dai’ and ‘Crimson Lady’ was observed, p-value of .005 ($p<.01$) (Table 5).

Figure 13. Images of Crimson Lady and Zin Dai.

Table 4. Pearson's correlation coefficients for visual blush % (0-5) 2007, 2008, 2010, 2011 and L*, a*, b*, r and Θ in 2011 using 25 SNP chip individuals.

	2008% (25)	2010% (24)	2011% (25)	2011 L* (23)	2011 r (23)	2011 Θ (23)	2011 a* (23)	2011 b* (23)
2007% (25)	.77**	.87**	.73**	-.80**	-.83**	-.83**	.69**	-.85**
2008% (25)		.88**	.90**	-.82**	-.73**	-.83**	.75**	-.81**
2010% (24)			.79**	-.84**	-.85**	-.86**	.72**	-.87**
2011% (25)				-.79**	-.70**	-.79**	.72**	-.76**
L* (23)					.90**	.98**	-.91**	.98**
r (23)						.89**	-.72**	.96**
Θ (23)							-.94**	.98**
a* (23)								-.88**

[1][** Correlation is significant at the 0.01 level (2-tailed)].

Table 5. Paired sample T-Test results, comparing visual blush coverage of both parents.

	Mean	N	Std. Deviation	Std.Error Mean
Zin Dai	1.50	4	.58	.29
Crimson Lady	4.50	4	.58	.29

Paired Differences

Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference: Lower	95% Confidence Interval of the Difference: Upper	t	df	p value
-3.00	.82	.41	-4.30	-1.70	-7.35	3	.005

Phenotypic data collected for visually scored blush (0-5 scale) and quantified blush data sets obtained with a chroma meter in all years were normally distributed (Table 3; Figure 14 and Figure 15). Graphical representation of data normality shown in Q-Q plots for all phenotypic data sets indicate that 4 out of 5 chroma datasets were not normally distributed containing high deviations from the 45° line, except for BlushR2011 dataset (Figure 16). However, all datasets passed the required normal distribution test (S-test; Table 3) for the Win QTL software and subsequently were used for QTL analysis.

Broad-sense heritability (H2) was highly significant (p<.001) in all data sets of visually scored blush (0-5 scale) (Table 6), suggesting no influence of the year on blush development. Therefore, one might conclude that blush development in ZC^2 population is controlled by the genotype, and that the environment does not play a significant role. Studies on blush in cherry also estimated high broad-sense heritability, ~0.96 and 0.95 for red skin color in cherry (Sooriyapathirana et al., 2010). The minimum horticultural maintenance applied to this population could be a major cause for observing such low

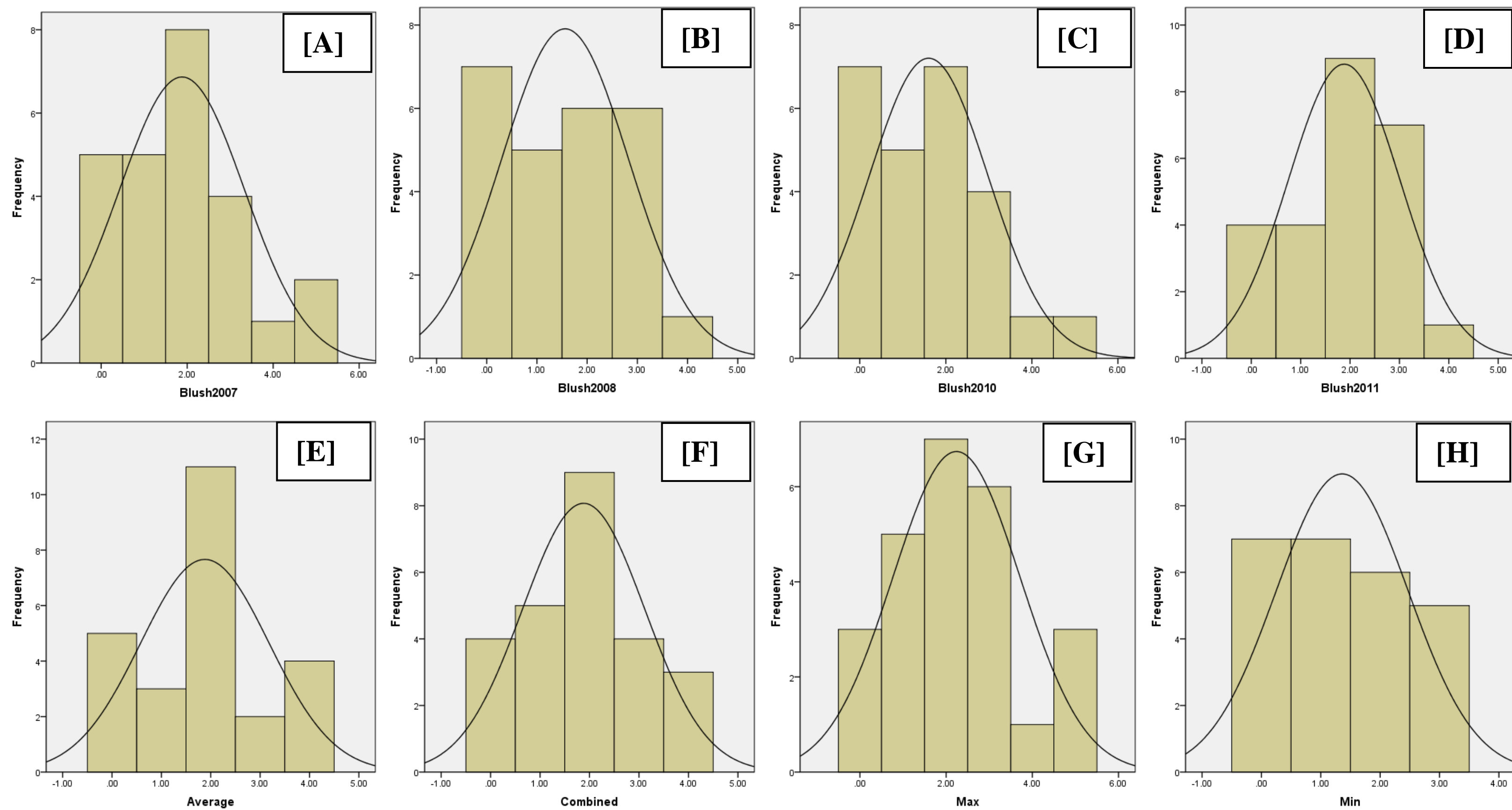

Figure 14. Distribution of phenotypic data organized in 8 datasets for subset of ZC^2 population. [A] Visual blush 2007; [B] Visual blush 2008; [C] Visual blush 2010; [D] Visual blush 2011; [E] Average visual blush; [F] Combined visual blush; [G]; Max blush; [H] Min blush; ZD, Zin Dai; CL, Crimson Lady.

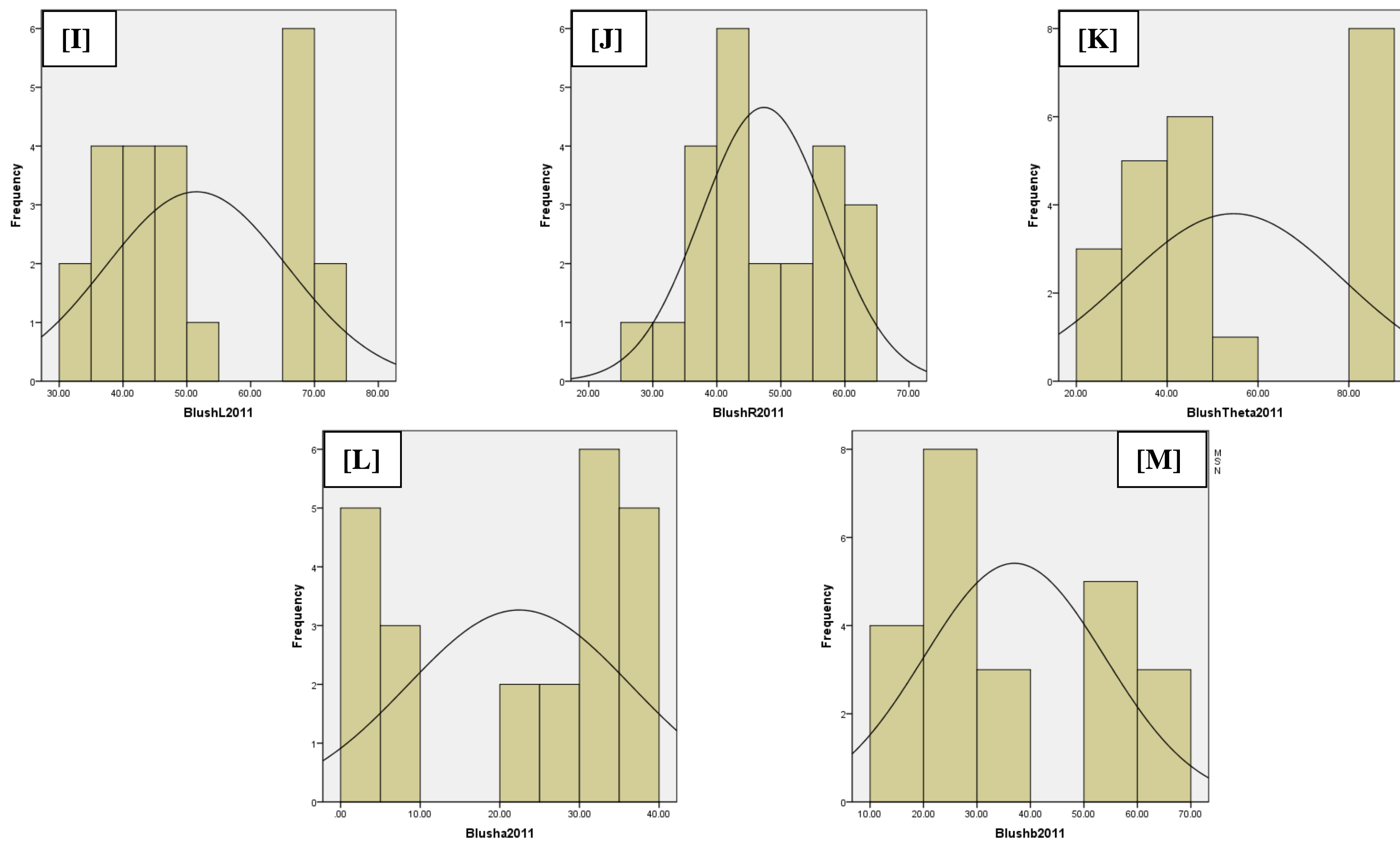

Figure 15. Distribution of phenotypic data organized in 5 datasets for subset of ZC^2 population. [I] L*; [J] r; [K] theta; [L] a*; [M] b*. ZD, Zin Dai; CL, Crimson Lady.

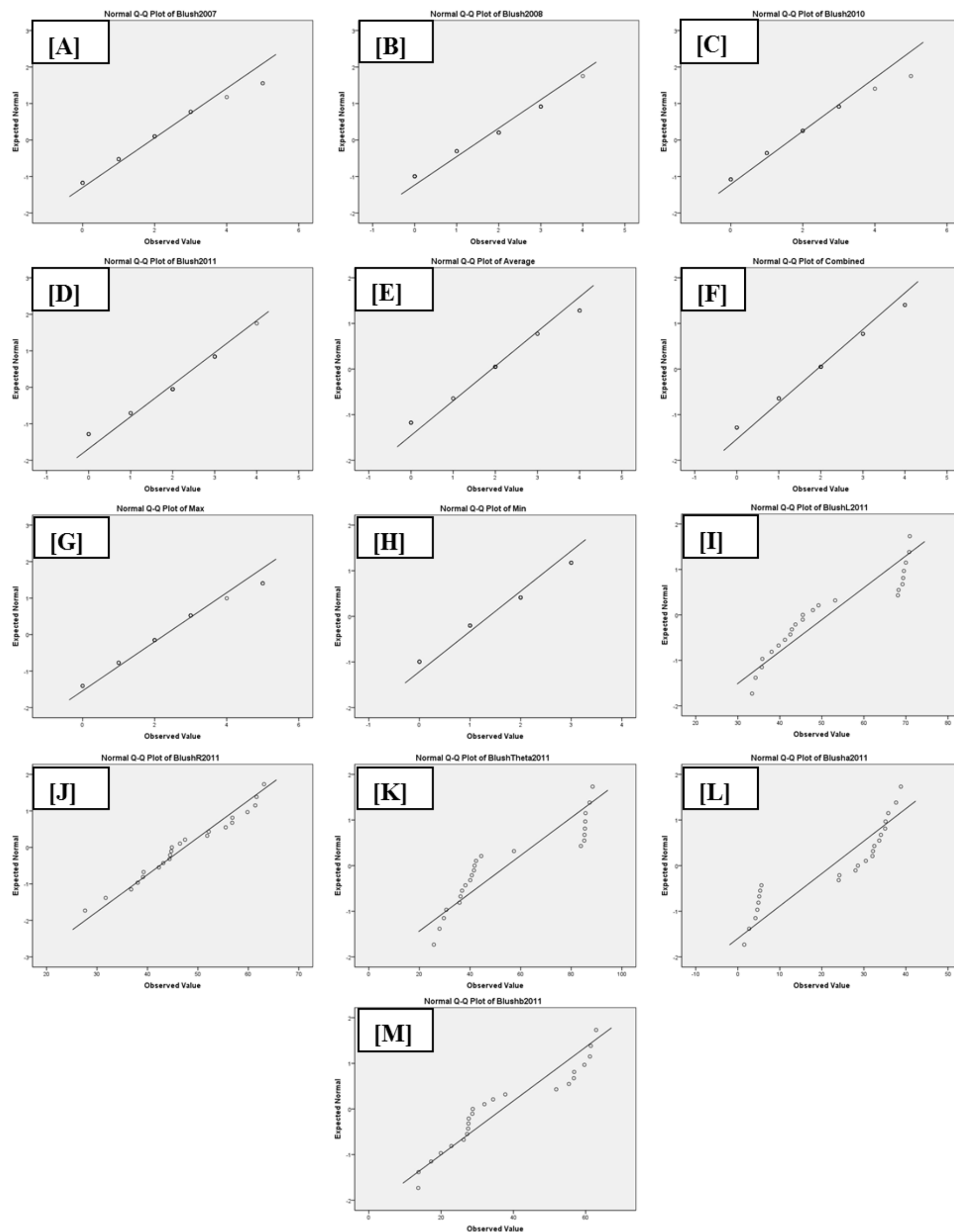

Figure 16. Normal Q-Q plots for phenotypic data ([A] Visual blush 2007; [B] Visual blush 2008; [C] Visual blush 2010; [D] Visual blush 2011; [E] Average visual blush; [F] Combined visual blush; [G]; Max blush; [H] Min blush; [I] L*; [J] r; [K] theta; [L] a*; [M] b*). Data sets with data points following 45 trend line are known to be normally distributed. Data sets with data points deviating from 45 trend line are known to be skewed, i.e. non-normally distributed.

environmental variation. The hedged trees, resulted in a relatively barren inner part of the tree canopies (near no foliation, and no fruit), and moderately low foliation on outer parts of canopy, which enabled a high degree of sun penetration. The fruit was located towards the outer part of the canopy, where the highest concentration of light was. This decrease in environmental variation was very beneficial for this investigation, since it concentrates on determining the genetic control of blush.

Table 6. Broad sense heritability (H2) for all years of visually scored blush.

Data set	Population size (n)	Variance (VF_2)	Broad sense heritability (H2)
Blush2007	25	3471.32	0.99***
Blush2008	25	2472.47	0.99***
Blush2010	24	2372.33	0.99***
Blush2011	25	2971.32	0.99***
average	25	3221.32	0.99***
combined	25	3121.32	0.99***
max	25	3019.77	0.99***
min	25	1898.07	0.99***

[1][***= p<.01; i.e. highly significant) [VF_2 = additive variance + dominance variance + environmental variance].

Genetic Linkage Map

Out of 8,144 SNP on the IPSC peach 9K SNP array v1, 5059 (62.12%) were polymorphic between ‘Zin Dai’ and ‘Crimson Lady’ (GenTrain score of ≥0.4). Of these, 1,370 (27.08%) were informative in the blush progeny, and thus used to construct the

ZC^2 SNP genetic linkage map. A total of 1,335 SNP markers (97%) were successfully grouped in 14 groups creating ZC^2 linkage map (Table 7). Four groups corresponded to LG3, 6, 7 and 8 and 10 of them corresponded to LG1, 2, 4 and 5, representing all 8 peach linkage groups (Table 7; Figure 17). LG1 consisted of three groups, 1_1, 1_2, 1_3; LG2 of two, 2_1, 2_2; LG4 of three 4_1, 4_2, 4_3; and LG5 of two groups 5_1, 5_2. The linkage groups that have more than one group of linked markers were designated as G1, 2, 4 and 5 in further text. Mapped SNP markers did not significantly deviate from Chi-square expectations. Approximately 86% of the mapped SNP shared map position, due to the absence of recombination caused by the small number (25) of accessions genotyped (Table 7). Therefore, 190 unique mapped positions were represented with single SNP marker for each unique position and map figures produced (Figure 17).

Development of SNP 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; Verde et al., 2012).

The ZC^2 linkage map spanned 452 cM with 2.38 cM / marker the average marker density, considering 190 markers (Table 7, Figure 17), which is in agreement with latest published size for the *Prunus* reference map, 519 cM (Dirlewanger et al., 2004). The number of unique map positions, mapped on each linkage group, ranged from 9 on G5 to 36 on LG3, with an average of 24 markers per LG/G (Table 7). The length of LGs was variable, with LG3 being the largest, 108 cM, and G5 covering the shortest distance, 16.7 cM (Table 7). LG3 and G1 had the highest number of unique positions, 36 and 30, respectively, while the lowest number of unique positions was observed on G5 and G7

Table 7. Description of ZC^2 linkage map.

Group	Length (cM)	Mapped markers	Uniquely mapped positions	SNP mapped to the same position	Largest gap (cM)	Smallest gap (cM)
LG1_1	6.1	24	4	20	2.1	2
LG1_2	45.7	78	22	56	6.4	0.3
LG1_3	6.1	10	4	6	2.1	2
G1	**57.9**	**112**	**30**	**82**	**6.4**	**0.3**
LG2_1	47.4	259	22	237	6.4	0.9
LG2_2	4.1	14	3	11	2.1	2
G2	**51.5**	**273**	**25**	**248**	**6.4**	**0.9**
LG3	**108.0**	**162**	**36**	**126**	**16.5**	**0.3**
LG4_1	39.0	133	18	115	4.2	2
LG4_2	19.8	90	5	85	13.7	2
LG4_3	4.1	10	5	5	1.2	0.9
G4	**62.9**	**233**	**28**	**205**	**13.7**	**0.9**
LG5_1	8.2	27	5	22	2.1	2
LG5_2	8.5	6	4	2	4.3	2
G5	**16.7**	**33**	**9**	**24**	**4.3**	**2**
LG6	**49.8**	**167**	**19**	**148**	**8.7**	**2**
LG7	**46.1**	**178**	**14**	**164**	**6.4**	**2**
LG8	**59.6**	**177**	**29**	**148**	**6**	**0.4**
ZC^2 map	452.5	1335	190	1145	16.5	0.3
Average [1]	56.6	167	24	143	-	-

[1]Averages were calculated considering 8 linkage groups

(9 and 14). The largest gaps, 16.5 cM and 13.7 cM were observed on LG3, between SNP_IGA_350488 and SNP_IGA_364100, and on G4 (LG4_2), between SNP_IGA_511285 & SNP_IGA_540776, respectively (Table 7; Figure 17).

The IPSC peach 9K SNP v1 array contains 8,144 high quality SNP covering all eight peach chromosomes with an average spacing of 26.7 kb between SNP and 31.5 kb between only polymorphic SNP (Verde et al., 2012). 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 SNP per cM for the array (Verde et

al., 2012). In the ZC^2 genetic map, the SNP marker density was estimated from 166 kb/cM to 458 kb/cM and average distance between markers ranged between 1.86 and 3.29 cM with an average of 2.4 cM per marker. This is comparable to other peach genetic maps, 3.3 cM in J × F (Dirlewanger et al., 2006), 4.7 cM in 'Guardian®' x 'Nemaguard' (Blenda et al., 2007), 4.2 cM 'Contender' × 'Fla.92-2C', (Fan et al., 2010), and 4.0 cM in 'Dr. Davis' × 'Georgia Belle' map (Ogundiwin et al., 2009) and 0.92 cM marker density reported in the *Prunus* reference map (www.rosaceae.org/).

Comparison of ZC^2 Linkage Map and Peach Physical Map

Linkage positions of the 82% of SNP markers in the ZC^2 linkage map were in agreement with their positions on the pseudomolecules/scaffolds of peach genome v 1.0 (Table 8; Figure 18 and Figure 19). Eighteen regions in ZC^2 map, involving five markers on LG1 (4/LG1_2 and 1/LG1_3), six on LG2 (LG2_1), eight on LG3, seven on LG4 (1/LG4_1, 4/LG4_2, and 2/LG4_3), two on LG6, and six markers on LG8, appeared inverted relative to the physical map (Table 8; Figure 18 and Figure 19).

Linkage groups 5 and 7 exhibit high homology with the 'dhLovell' physical map. The physical length of ZC^2 linkage map was estimated to cover 61.63% of the pseudomolecules of peach genome v 1.0. The largest coverage of 99.07% was achieved between LG3 and pseudomolecule one and the lowest between LG5 and pseudomolecule six (17.06%). In addition, the estimated average coverage per marker on the pseudomolecules ranged from ~1/400 kb on LG1 to 1.2/200 kb on LG4 (Table 8; Figure 18 and Figure 19).

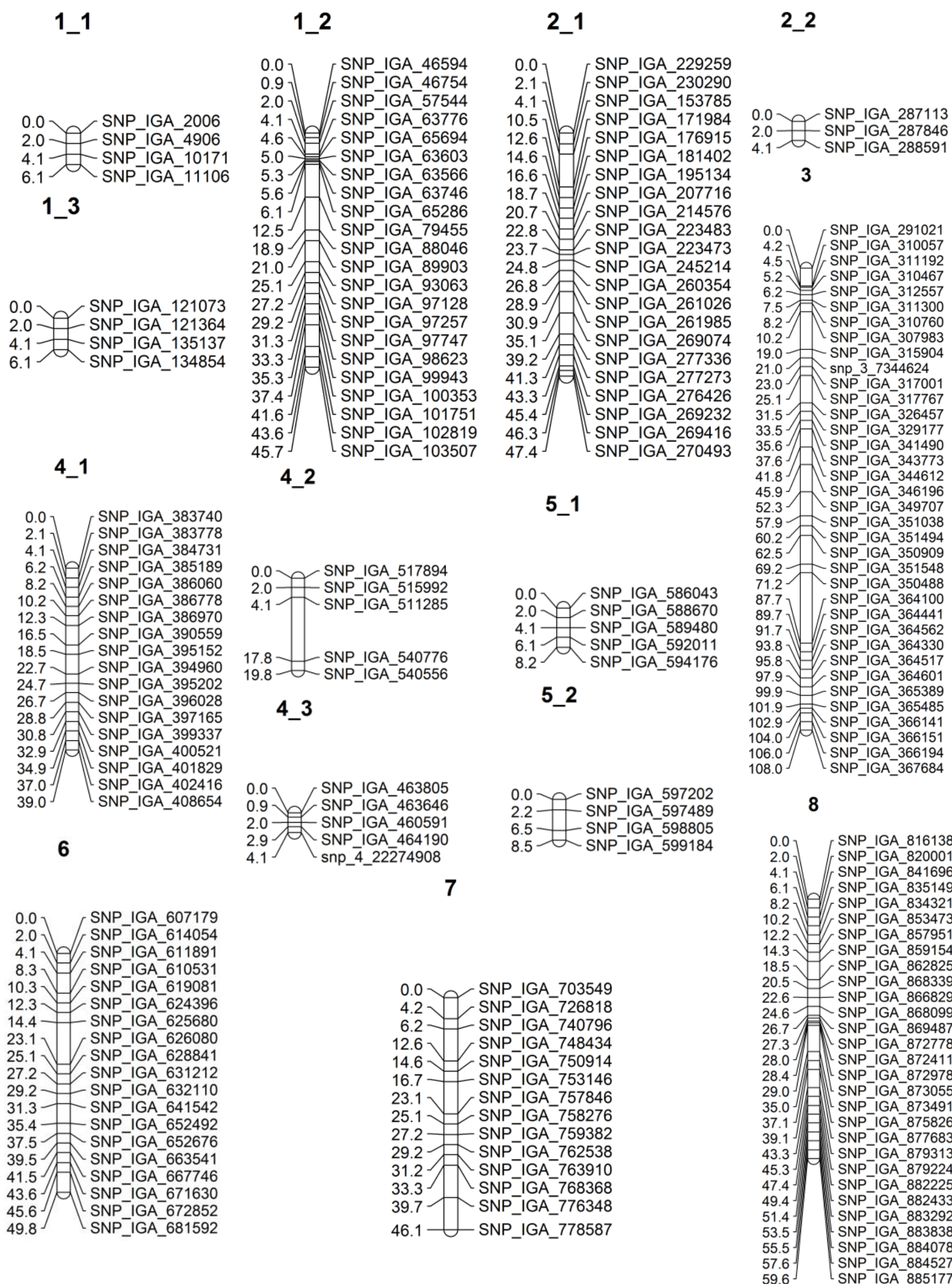

Figure 17. ZC^2 linkage map (1 SNP per loci), generated through Joinmap 4.1 (distances in cM).

Table 8. Comparison of ZC^2 linkage to peach physical map.

LG #	ZC^2 linkage map			Physical Coverage (%)	Marker Density		Average coverage (kb/cM)
	#SNP	Genetic distance (cM)	Physical length (Mb)		cM	kb	
G1	30	57.90	26.49	56.66	1.93	883.0	457.51
G2	25	51.47	18.21	68.17	2.06	728.4	353.59
LG3	36	108.04	21.70	99.07	3.00	602.8	200.93
G4	28	62.90	10.48	34.89	2.25	374.3	166.35
G5	9	16.72	3.11	17.06	1.86	345.6	185.78
LG6	19	49.80	21.61	75.51	2.62	1137.0	434.11
LG7	14	46.07	15.42	68.02	3.29	1101.0	334.78
LG8	29	59.61	16.58	76.60	2.06	571.7	277.54

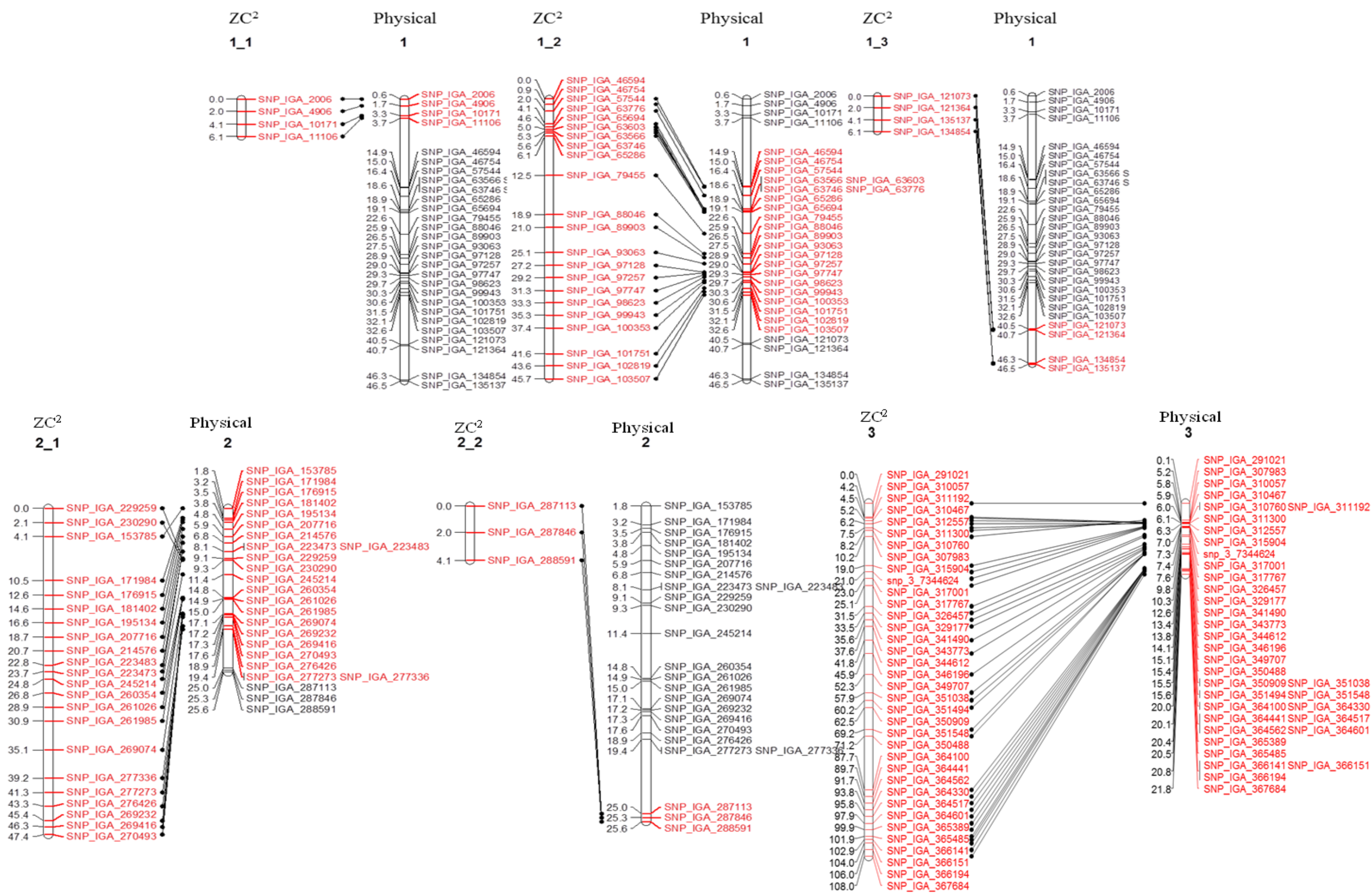

Figure 18. Comparison of preliminary ZC^2 map and peach genome v1.

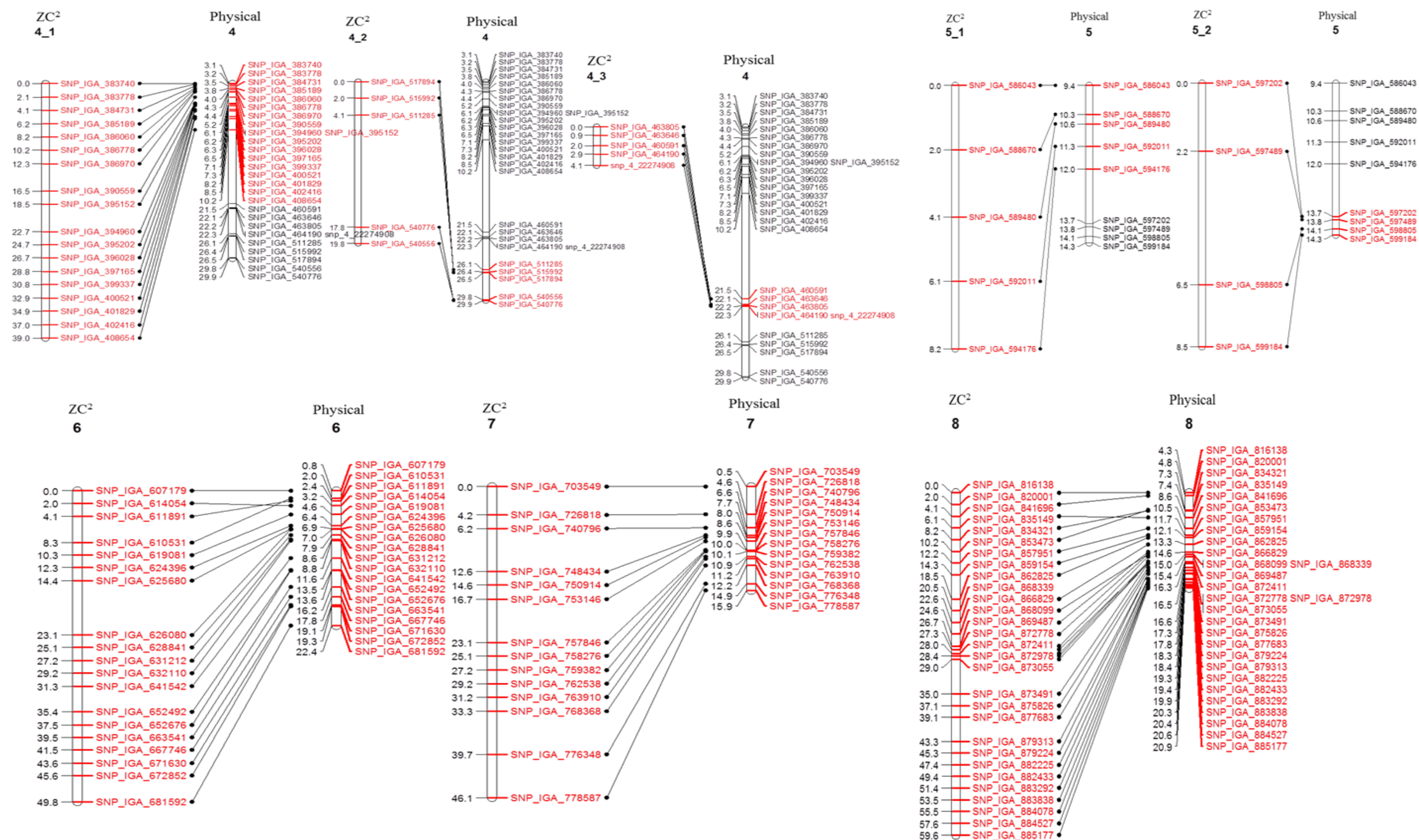

Figure 19. Comparison of preliminary ZC^2 map and peach genome v1, continued.

QTL Analysis

All blush data sets exhibited normal distribution, based on S-test values calculated in Windows-QTL-Cartographer V2.5 (Wang et al. 2007), and thus were used for QTL mapping (Table 3). Results from all years and analysis types were compared to verify accuracy of QTL detection. Twelve out of thirteen datasets depicted QTL (Table 9 and 10; Figure 20 and Figure 21).

Two significant major QTL were identified on LG3 using all visual blush ZC^2 data sets (Table 9; Figure 20 and Figure 21). All 8 visual blush data sets (Blush_2007, Blush_2008, Blush_2010, Blush_2011, combined, average, max, min) uncovered the same major QTL for blush on LG3 peaking at 35.6cM, spanning ~8.94cM (LOD2), and explaining on average ~74% of the phenotypic variance of blush (R^2). Data set 'average' located a second major QTL very close to the first on LG3, spanning 6.4cM (LOD2), with a peak at 27cM, which explained ~75% of phenotypic variance for blush. The four years of visual blush data and four statistical data sets (Blush_2007, Blush_2008, Blush_2010, Blush_2011, combined, average, max, min) approximated this same major QTL, explaining ~74% of the phenotypic variance for blush. The total area covered by single QTL ranged from 6 to 15 cM (LOD2). The area associated with blush considering all QTL ranged from 25 to 41 cM of LG3. The overlap of areas covered by individual QTL, confirms the identification of this major QTL for blush in peach, and supports its stability (Table 9; Figure 20 and Figure 21).

Four minor QTL were located on LG's 3, 4, and 7 indicating the presence of minor genes involved with blush development (Table 9). The 2008 visual blush data set

BL_2008_1 uncovered a minor QTL on LG3 explaining 9.2% of phenotypic variance of blush, peaking at 21cM, and spanning 9.7cM (LOD2). Visual 2011 blush data set, approximated two minor QTL on LG4 (Table 9; Figure 20 and Figure 21). The *VB4_1_2011-2'* QTL explains ~12.85% of R^2 for blush, peaks at 4.1cM and spans ~5.9cM (LOD2). The second QTL, *VB4_1_2011-3'*, peaks at 12.3cM, spanning ~5.8cM (LOD2), and explains ~13.52% of R^2 for blush. The minimum phenotypic data set approximated another minor QTL on LG7 explaining ~1.23% of phenotypic variation for blush, peaking at 41.7cM and spanning ~9.1cM (LOD2). Phenotypic variation explained by major QTL depicted with visual datasets varied from 61 to 97% (Table 9).

Six major and two minor QTL also on LG3 were revealed with all chroma data sets, excluding R dataset (Table 10; Figure 20 and Figure 21). The major QTL detected from each data set were: QBL*3-2011 with a peak at 27.1cM, spanned 5 cM, and explained 60.8% R^2 of blush; QBΘ3-2011-2, explained 63.6% R^2 of blush, peaked at 22cM, spanned 1.7cM; QBΘ3-2011-3, with a peak at 31.5cM, explained 83.9% phenotypic variance of blush, spanned 11.2cM; QBΘ3-2011-4 explained with 81.8% phenotypic variance of blush, with a peak at 38.6cM, spanned 3.2cM; QBa*3-2011-2, with a LOD peak of 27.1cM, spanned 5.5cM, and explained 61.2% of blush phenotypic variance.

Table 9. Summary of the QTL detected for blush using visual blush (VB) data sets by Composite Interval Mapping.

QTL	QTL peak position in cM & (closest marker)	LOD at QTL peak	R2 (%)	LOD2-left[1]	LOD1-left	LOD1-right	LOD2-right	Add.
VB3-2007	**35.6 (SNP_IGA_341490)**	**6.06****	**69.61**	**25.1**	**30.4**	**37.2**	**39.5**	**-1.60**
VB3-2008-1	*21 (snp_3_7344624)*	*4.94**	*9.10*	*14.4*	*19.2*	*22.8*	*24.1*	*0.32*
VB3-2008-2	**35.6 (SNP_IGA_341490)**	**7.53*****	**65.32**	**31.5**	**33.9**	**37**	**37.6**	**-1.97**
VB3-2010	**35.6 (SNP_IGA_341490)**	**4.13***	**60.64**	**32**	**27**	**37.3**	**40.8**	**-1.35**
VB3-2011-1	**35.6 (SNP_IGA_341490)**	**10.97*****	**87.98**	**30.8**	**33.9**	**37**	**37.6**	**-1.43**
VB4_1_2011-2	*4.1 (SNP_IGA_384731)*	*7.61****	*12.85*	*0.2*	*0.7*	*5.6*	*6.1*	*0.40*
VB4_1_2011-3	*12.3 (SNP_IGA_386970)*	*4.92**	*13.52*	*10.5*	*11.6*	*15.3*	*16.3*	*0.17*
VB3combined	**35.6 (SNP_IGA_341490)**	**6.13*****	**68.01**	**30.1**	**30.1**	**37**	**37.6**	**-1.52**
VB3average-1	**27.1 (SNP_IGA_317767)**	**7.22*****	**75.43**	**25.1**	**25.6**	**30.8**	**31.5**	**-0.81**
VB3average-2	**35.6 (SNP_IGA_341490)**	**6.28*****	**72.89**	**31.5**	**31.5**	**37**	**37.6**	**-1.40**
VB3max	**35.6 (SNP_IGA_341490)**	**6*****	**71.64**	**25.1**	**31.1**	**37.5**	**40.5**	**-1.67**
VB3min-1	**35.6 (SNP_IGA_341490)**	**8.09*****	**97.26**	**31.2**	**33.8**	**37**	**37.6**	**-1.42**
VB7min-2	*41.7 (SNP_IGA_776348)*	*5.3***	*1.24*	*35.6*	*39.9*	*44.7*	*44.7*	*0.20*

[1](*1 LOD, ** 2 LOD, *** 3 LOD values significant at P<0.05, P<.01, P<.001 - based on 1,000 permutation tests). [2](**bolded** = Major QTL significant at P<0.05 or lower. *italicized* = Minor QTL significant at P<0.05 or lower; Add. = additive effects). [3](QTL were named following this format TTTL-YYYY-1 ['TTT'= trait acronym; 'L' = linkage group number; 'YYYY'=the year in which the trait was phenotyped; '1'= numbers to identify different QTL for the same trait]).

Table 10. Summary of the QTL detected blush using chroma quantified blush (QB) data sets (QBL*,QBa*,QBb*, QBr, QBΘ) by Composite Interval Mapping (CIM).

QTL	QTL peak position in cM (closest marker)	LOD at QTL peak	R2 (%)	LOD2-left	LOD1-left	LOD1-right	LOD2-right	Add.
QBL*3-2011	**27.1 (SNP_IGA_317767)**	**7.81*** **	**60.84**	**26.1**	**26.9**	**31.1**	**31.1**	**13.23**
QBΘ3-2011-1	*15.3 (SNP_IGA_315904)*	*5.87**	*18.45*	*11.9*	*12.2*	*17.3*	*18.3*	*8.69*
QBΘ3-2011-2	**22 (snp_3_7344624)**	**9.19*** **	**63.65**	**21**	**21**	**22.4**	**22.7**	**22.28**
QBΘ3-2011-3	**31.5 (SNP_IGA_326457)**	**14.2*** **	**83.89**	**25.9**	**27.1**	**36**	**37.1**	**28.91**
QBΘ3-2011-4	**38.6 (SNP_IGA_343773)**	**9.91*** **	**81.81**	**37.8**	**38.2**	**40.7**	**41**	**26.27**
*QBa*3-2011-1*	*16.3 (SNP_IGA_315904)*	*7.2*** *	*16.78*	*11*	*11.3*	*18.3*	*18.3*	*-11.18*
QBa*3-2011-2	**27.1 (SNP_IGA_317767)**	**12.93*** **	**61.20**	**25.6**	**25.9**	**31.1**	**31.1**	**-14.56**
QBa*3-2011-3	**34.5 (SNP_IGA_329177)**	**12.24*** **	**39.35**	**33.5**	**33.5**	**34.9**	**35.2**	**-14.12**
QBb*3-2011-1	**21 (snp_3_7344624)**	**6.49** **	**87.04**	**21**	**21**	**23.1**	**23.1**	**20.78**
QBb*3-2011-2	**31.5 (SNP_IGA_326457)**	**9.88*** **	**83.25**	**25.7**	**26.5**	**36.7**	**37.3**	**22.186**

[1](*1 LOD, ** 2 LOD, *** 3 LOD values significant at P<0.05, P<.01, P<.001 - based on 1,000 permutation tests. [2](**bolded** = Major QTL significant at P<0.05 or lower. *italicized* = Minor significant at P<0.05 or lower; Add. = additive effects). [3](QTL were named following this format TTTL-YYYY-1 ['TTT'= trait acronym; 'L' = linkage group number; 'YYYY'=the year in which the trait was phenotyped; '1'= numbers to identify different QTL for the same trait]).

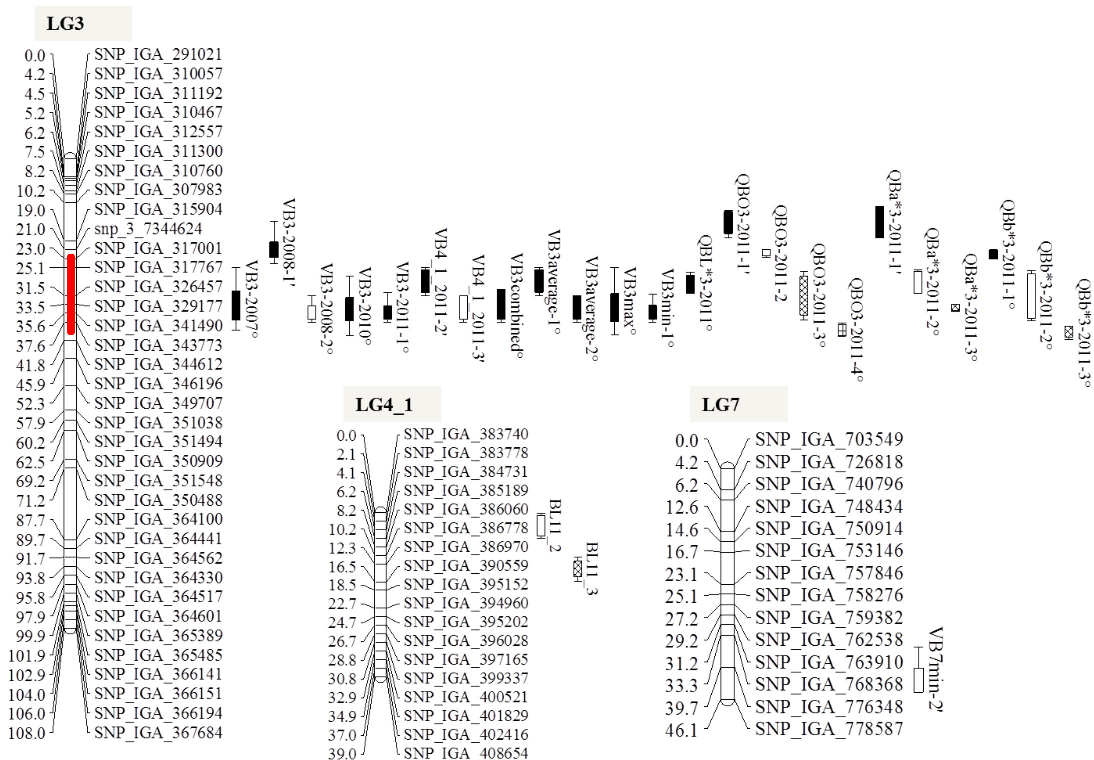

Figure 20. Location of QTL for visual blush (VB) and quantified blush (QBL*,QBa*,QBb*, QBr, QBΘ) on peach ZC^2 SNP linkage map using Composite Interval Mapping (CIM). Thick (1-LOD) and thin (2-LOD) bars mark significance areas of QTL (p<.05; p<.01). QTL were named following this format TTL-YYYY-1. ('TT'= trait acronym; 'L' = linkage group number; '_1'= numbers to identify different QTL for the same trait; 'YYYY'=the year in which the trait was phenotyped: LOD scores and phenotypic variability explained by QTL (R^2) depicted in Table 9 and 8).

QBa*3-2011-3, showed a LOD peak of 34.5cM, spanned 1.7cM and explained 39.3% of blush R^2; QBb*3-2011-1, LOD peak of 21cM, explained 87.0% blush variance, and spanned 2.1cM; QBb*3-2011-2 located a peak at 31.5cM, spanned LOD2 interval of 11.6cM, and explained 83.3% blush variance; and QBa*3-2011-3, with a LOD peak of 39.5cM, explained 76.6% blush variance, and spanned a LOD2 interval of 3cM (Table 10; Figure 20 and Figure 21). The total area covered by single QTL ranged from 5 to 12

cM (LOD2). The area associated with blush considering all QTL ranged from 21 to 41 cM of LG3 similar to QTL located by visual datasets.

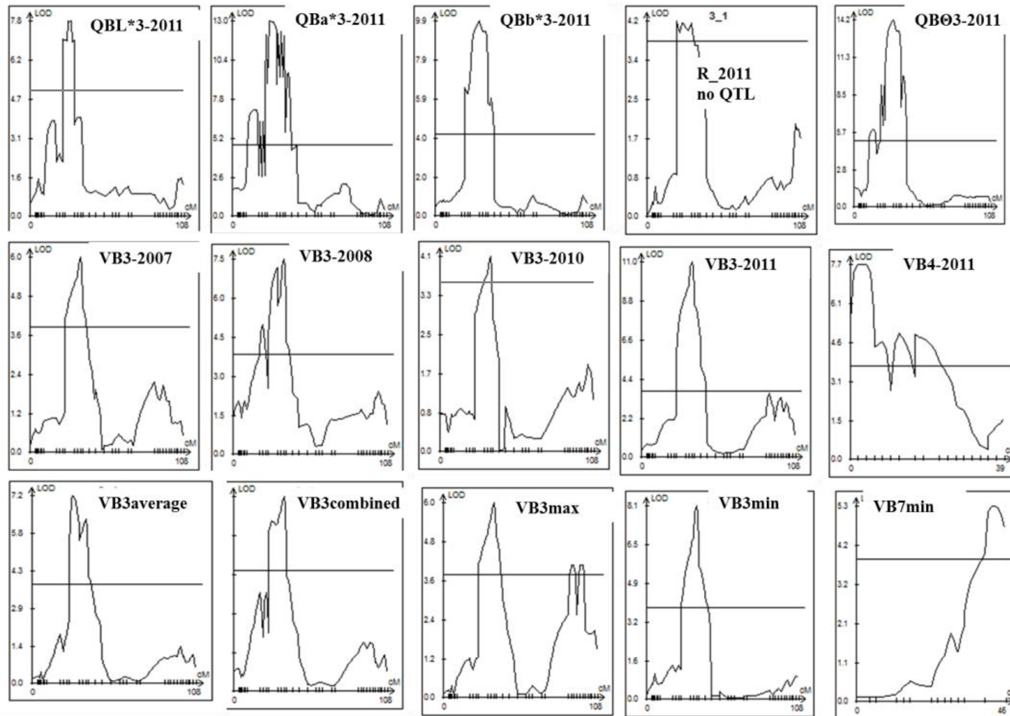

Figure 21. Blush QTL detected from 12 of 13 blush phenotypic data sets. QTL were named following this format TTL1-YYYY. [1](‘TT’ = trait acronym; ‘L’ = linkage group; ‘YYYY’=the year in which the trait was phenotyped number; ‘_1’= numbers to identify different QTL for the same trait). [2](X-axis = specified linkage group; Y-axis = LOD score; horizontal line = LOD threshold for QTL).

Phenotypic variation explained by the major QTL depicted with chroma datasets varied from 39 to 87% (Table 10).

Major QTL were approximated on LG3 using all 8 visual blush data sets and four out of five chroma data sets (L*, a*, b*, theta) (Table 9 and Table 10; Figure 20 and Figure 21). The relative positions of all detected major QTL were in a centralized position between QTL QBΘ3-2011-2 and QBΘ3-2011-4, spanning from 21 (LOD 1-left)

to 41cM (LOD2-right) on LG3 (Table 9 and Table 10; Figure 20 and Figure 21). Based on the above we propose one major blush QTL designated as *Blush.Pp.ZC-3.1*, spanning 21 - 41cM on LG3 explaining on average 72% of phenotypic variation for the trait. Phenotypic variation explained by this major QTL region varied from 39 to 97%.

Minor QTL were also located on LG3 using chroma data sets, Blush 2008, theta, and a* data sets. (Table 9 and Table 10; Figure 20 and Figure 21). The relative positions of all detected minor QTL were between *QBa*3-2011-1* and *VB3-2008-1*, spanning an LOD2 interval of 11 to 24cM. Minor QTL region overlaps with major blush QTL, *Blush.Pp.ZC-3.1*, for 3cM extending the region of LG3 associated with blush 10cM (Table 9 and Table 10; Figure 20 and Figure 21).

A candidate gene approach has been incorporated to uncover the gene/genes within the QTL associated with blush. The major transcription factor R2R3 MYB10/MYB1/MYBA has been associated with the activation of the anthocyanin biosynthesis pathway in the Rosaceae leading to the development of red skin pigmentation (Kui et al., 2010). Through comparative genomics Kui et al., (2010) demonstrated that this orthologous major TF gene R2R3 MYB10/MYB1/MYBA has been conserved throughout the Rosaceae family. This TF gene was first located in apple (Takos et al., 2006; Ban et al., 2007; Chagné et al., 2007; Espley et al., 2007; Espley et al., 2009; Kui et al. 2010).

Later a QTL for red skin pigmentation in sweet cherry was located on LG3 (Sooriyapathirana et al., 2010). They showed that PavMYB10 co-locates within their

QTL and thus designed primers and screened PavMYB10 on their F_2 cherry population segregating for blush (Sooriyapathirana et al., 2010).

Following this model the complete coding sequence of *Prunus persica* R2R3 MYB transcription factor (PprMYB10) was obtained from GenBank (EU155160.1) and located in the peach genome v1.0 assembly (GDR) (Kui et al. 2010). This major transcription factor gene collocates within the major QTL for blush on LG3 of ZC^2 SNP linkage map.

Also a QTL for blush was localized on LG4 of a genetic linkage map created using F_1 progeny from a cross between 'Venus' x 'BigTop' (V x BT; Cantín et al., 2010). The *VB4_1_2011-2'* and *VB4_1_2011-3'* QTL in this study also located to LG4.

In addition, Quilot et al., (2004) located a QTL for blush (termed SRColor2) on LG5 of their genetic linkage map. This QTL only depicts ~21% of red skin coloration/pigmentation, and therefore is most likely associated with a structural gene in the anthocyanin pathway. Ogundiwin et al. (2007, 2008, 2009) reported a QTL (qP-Brn5.1^m) on LG5 associated with a structural gene in the anthocyanin pathway, Leucoanthocyanidin dioxygenase (PpLDOX). This qP-Brn5.1^m co-locates with the SRColor2 QTL for blush and therefore shows that PpLDOX could be the major gene associated with this QTL. If the ZC^2 population size is increased to at least 50 individuals, it is highly likely that this minor QTL will be identified on LG5.

Conclusions

A genetic linkage map using a subset of ZC^2 population has been generated in this study and used for mapping of genes responsible for blush in peach. The linkage map consisted of 14 groups corresponding to 8 peach chromosomes and spanned a total length of ~452 cM with an average density of 2.4cM / marker.

Six minor QTL for blush were detected in this study, three located on LG3, two on LG4 and one on LG7, indicating the presence of minor genes involved with blush development. The *VB4_1_2011-2'* and *VB4_1_2011-3'* minor QTL both located to LG4 (*VB4_1_2011-2'* = LOD peaks at 4.1cM and explains ~12.85% variance blush; *VB4_1_2011-3'* = LOD peaks at 12.3cM and explains ~13.52% variance blush). Cantín et al., (2010) also located a QTL for blush on LG4. They used a different peach linkage map (V x BT), and their QTL explained 68.7% of the phenotypic variance of blush (Cantín et al., 2010). These QTL could potentially be the same and be associated with a candidate structural gene involved in the anthocyanin pathway.

Lastly a major QTL for blush in peach designated as *Blush.Pp.ZC-3.1,* has been located on LG3, using both visual and chroma blush data. *Blush.Pp.ZC-3.1* encompasses 21 - 41cM region on LG3 explaining on average 72% of phenotypic variation for the trait and is supported with four years and two different types of phenotypic data. Recently a major QTL associated with red skin pigmentation in sweet cherry (*Prunus avium* L.) also on LG3 was reported (Sooriyapathirana et al., 2010). The candidate gene, *PavMYB10*, homologous to the anthocyanin associated genes in apple (MdMYB10) and Arabidopsis (AtPAP1) co-locates within the QTL region (Kui et al., 2010; Sooriyapathirana et al.,

2010). This suggested that red coloration of skin and flesh in *Prunus* and Rosaceae is under the control of the same transcriptional factor MYB10.

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CHAPTER IV

OVERALL CONCLUSIONS AND FUTURE WORK

Overall Conclusions

The overarching goal of this study was to demonstrate the potential of MAB to augment our understanding of fruit quality traits in peach. Markers that are developed will become tools to efficiently complement traditional breeding methods and expedite the development of future peach cultivars with improved fruit quality traits. MAB combined with traditional breeding techniques will help to ensure the development of high quality peach cultivars in a timely manner to increase consumer demand for this important crop.

Standardized Phenotyping

A standardized phenotyping protocol for peach was generated and implemented. This protocol merged peach phenotypic data from a vast array of genotypes from different institutions, environments and countries. Studying phenotypic and genotypic data concurrently is essential to link genetic variation with biological function and document gene function. However, proper documentation of phenotypic data is not keeping pace with the immensely increasing amount of available genomic information. The success of standardized phenotyping, demonstrated in this thesis, and in previous studies should spur the collaboration and collection of more phenotypic data from peach breeders around the world. A natural extension of this thesis will be to perform pedigree based QTL analysis (PBA) to discover precise markers associated with vital peach

quality traits. If brought to fruition, the developed markers will serve as useful tools to increase the efficiency of traditional breeding and development of improved peach cultivars. It is emphasized that the development of standardized phenotyping is not restricted to peaches. The successful development and implementation of MAB can be incorporated to improve economically important traits of all plant species.

MAB for Blush

The genetic control of blush was extensively investigated to enable MAB. Blush is a quantitative trait, which develops through the flavonoid and anthocyanin pathways. These pathways are regulated by sunlight an genetic factors. Sunlight regulates specific MYB transcriptional regulatory genes which encode transcription factor protiens that activate the expression of the structural genes in the flavonoid and anthocyanin pathways.

A major QTL for blush, named Blush.Pp.ZC-3.1, was detected on LG3 through QTL analysis of a subset ZC^2 population and LG3 using all 8 visual blush data sets and four out of five chroma data sets (L*, a*, b*, theta). Blush.Pp.ZC-3.1 spanned 21-41cM on LG3 and explained on average 72% of phenotypic variation for the trait. Results from previous studies have suggested that a specific candidate transcriptional factor gene is involved in skin and flesh coloration of cherry (*PavMYB10*) and apple (*MdMYB10*). The complete coding sequence of the peach homolog of, R2R3 MYB transcription factor (PpMYB10) was obtained from GenBank (EU155160.1) and located in the peach genome v1.0 assembly (GDR). PpMYB10 co-locates on LG3 of the subset ZC^2 linkage map within the interval of the major QTL for blush in peach. These preliminary results

suggest that this major transcription factor gene is conserved through the Rosaceae family and provides the same genetic control for color development throughout the family.

Future Work

Increase ZC^2 Population Size

To further investigate the location of the QTL for blush noted herein and other fruit quality traits, the whole ZC^2 population will be genotyped to develop a complete linkage map. Increasing the number of individuals will effectively decrease the size and increase confidence in the QTL location. The population size will be increased by:

(1) Adding SNP data for remaining 68 individuals using genotype by sequencing method (GBS; Elshire et al., 2011) or IPSC 9K peach SNP array v1. (Verde et al., 2012).

2) Genotype ZC^2 population with SSR markers to allow comparison with other *Prunus* maps via anchored SSR markers.

Furthermore, recombination events will be increased and lead to the development of a more precise linkage map. In theory, this should decrease the size of blush QTL, increase its significance, and potentially increase the amount of phenotypic variance it explains. Additionally, since blush is quantitative in nature, an increased population size will most likely lead to identification of additional minor QTL for blush that might be associated with structural genes of the flavonoid and anthocyanin pathway.

Pedigree-Based QTL Analysis (PBA) Approach

A Pedigree-Based QTL Analysis (PBA) is a powerful statistical approach used to simultaneously identify marker-trait associations, validate their robustness and

applicability in individual breeding programs, and discover alleles for functional diversity (van de Weg et al., 2004). PBA identifies networks of major genes and QTL that determine genetic variation in horticulturally important traits. This approach also elucidates their interaction and mines their functional allelic diversity (van de Weg et al., 2004). The PBA strategy integrates marker and phenotypic data over past, current, and future generations within and across breeding programs. Therefore, it creates a flexible and continuously expanding platform for marker identification, validation, and use (van de Weg et al., 2004).

The PBA approach is based on two complementary statistical approaches. The first identifies QTL regions based on Markov chain Monte Carlo simulations and Bayesian statistics. The second is based on "Identity By Descent" values of each allele of a genotype, taking the different alleles of founding cultivars as factors in statistical analysis (Bink et al 2008). The use of multiple populations holds several key advantages over linkage analysis performed on a single population. The chance of locating segregation of QTL alleles is enhanced because more than one population may segregate for the trait. Thus, a larger genetic background enhances the ability to detect QTL action. This culminates in an enhanced QTL analysis due to the increased mapping resolution, allele segregation, and reduced research time, which can be compared to the QTL uncovered in this study (Yu and Buckler, 2006). The PBA will be used to functionally characterize alleles and detect QTL, thus providing further confirmation of major and minor QTL associated with blush in peach.

Testing of Blush Markers

The key candidate TF gene (PprMYB10) located inside the major QTL for blush will be tested in breeding populations to test the accuracy of the genes in explaining blush in peach. PCR primers will be designed to screen predicted PprMYB10 on the entire blush population of 93 individuals as well as an extensive set of peach germplasm to determine if the candidate gene accurately depicts blush in peach. The accuracy of PprMYB10 in depicting parents that pass on genes associated with blush (MAPS) and whether the seedlings contain the associated blush genes (MASS) will be tested. An accuracy of ~72% will be sought for the markers ability to depict blush development. Other populations and germplasm that segregate for blush will be used to further test the markers. More significantly a second blush population created by Dr. Okie will be used for marker validation. This population, BY02p3997 self = Zin Dai Jiu Bao * BY96p2591 (=Sunprince*BY92P2459 (=L75-A50-20*BY87P2208), contains 385 individuals and shares the same mother (Zin Dai) as the ZC^2 blush population.

Ultimately, if PpMYB10 is confirmed to accurately explain blush, MAPS and MASS will be initiated for blush in peach. These molecular tools have the potential to play an indispensable role in efficiently breeding the next generation of peach cultivars with an extensive level of blush. This will provide a well supported procedure to allow for informed parent selection and determine progeny to invest in. This will realize a reduction in the expenditure of valuable resources such as time, money, and space (Bliss, 2010). Faster turn-around times will lead to quicker development of future peach cultivars, with enhanced fruit quality traits that can keep up with the pace of evolving

consumer demands. Peach cultivars with fruit quality traits that cater to consumer demands offer the potential to enhance the peach industry.

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APPENDICES

Appendix I:

Figure 22. Images of 10 blush F_2 individuals used for subset ZC^2 linkage map.

Appendix II-CTD:

Figure 23. Images of 9 blush F_2 individuals used for subset ZC^2 linkage map.

Appendix III-CTD:

Figure 24. Images of 6 blush F_2 individuals used for subset ZC^2 linkage map.