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MAPPING QUANTITATIVE TRAIT LOCI ASSOCIATED WITH CHILLING REQUIREMENT AND BLOOM DATE IN PEACH

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Genetics

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by
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May 2010

Accepted by:
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ABSTRACT

Chilling requirement (CR), together with heat requirement (HR), determines bloom date (BD), which impacts climatic distribution of genotypes of temperate tree species. The molecular basis of floral bud CR is poorly understood despite its importance to fruit tree adaptation and production. A peach F_2 populations developed from two genotypes with contrasting CR values was used for QTL mapping for CR, HR and BD. Using the Contender × Fla.92-2c population, 20 QTLs with additive effects were identified for three traits including one major QTL for CR and two major QTLs for BD. Particularly, one genomic region of 2cM pleiotropic for the three traits overlaps with the sequenced peach *evg* region. Association approaches and candidate-gene approaches were used to explore and refine the detected QTL regions. Seven Polycomb group and their associated protein encoding genes in/close to QTL regions and three genes (including *DAM4* and *DAM6*) in *evg* locus were identified as potential candidate genes regulating CR and BD. In addition, we established the use of plum as transgenic system to test peach candidate genes for CR and BD. Transgenic plums overexpressing *DAM6* showed dwarfing and more branching phenotype.

DEDICATION

I dedicate this dissertation to my family. To my parents, thank you for the never ending supports in all aspects of my life even when I think I have already grown up. To my sister, thank you for being with our parents and taking care of them. To my wife, thank you for giving up your job in China and accompanying me in US.

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CHAPTER ONE

LITERATURE REVIEW

Dormancy and Chilling Requirement in Temperate Tree Species

Lang (1987) defined plant dormancy as "the temporary suspension of visible growth of any plant structure containing a meristem" and further distinguished three types/stages of dormancy as endo-dormancy, para-dormancy and eco-dormancy. The prefix *endo* is used to describe dormancy when the initial reaction leading to growth control is a specific perception of an environmental or endogenous signal in the affected structure alone. The prefix *para* is used to describe dormancy when the initial reaction leading to growth control involves a specific signal originating in or initially perceived in a different structure from the one in which dormancy is manifested. Para-dormancy is also referred as apical dominance or correlation inhibition. The prefix *eco* is used to describe dormancy when one or more factors (temperature, water, etc.) in the basic growth environment are unsuitable for overall growth metabolism (Lang, 1987). Temperate tree species have the ability to cease meristem activity in the fall and establish an endo-dormancy state in which the meristem is rendered insensitive to growth promoting signals before it is released (Rohde et al., 2007). Endo-dormancy overlaps with para-dormancy and eco-dormancy in its beginning and ending period (Faust et al., 1995; Faust et al., 1997). Despite the clearly different definitions of three types of dormancy, Rohde & Bhalerao (2007) proposed that endo-dormancy might be derived

from the evolutionarily older para-dormancy and still share molecular mechanisms with it.

The release of temperate trees from endo-dormancy requires exposure to low temperatures (chilling requirement, CR). CR prevents trees from initiating growth in response to transient warm temperatures thus avoiding damage by subsequent frost(s) in the late winter or early spring. CR is the result of long term climatic adaption of genotypes of tree species developed in different regions. Conversely, it limits the climatic distributions of genotypes of temperate fruit trees (Sherman & Beckman, 2003).

Many models have been developed to evaluate the CR of genotypes of temperate tree species. Most of these models fall into two categories: chilling hour models and chilling unit models (Cesaraccio et al., 2004). The chilling hour models count the number of hours when the air temperature is in a certain range, and assume that all air temperatures in this range are equally effective. The $<7.2^{o}C$ (Weinberger, 1950) and 0-$7.2^{o}C$ models (Eggert, 1951) are two most often used models in this category. In the chilling unit models, different weighting factors are assigned to temperatures in different ranges. High temperatures above a limit are considered to reverse the chilling effects of lower temperatures and negative chill units are assessed for them (Cesaraccio et al., 2004). The Utah model (Richardson, et al., 1974) and Low Chill model (Gilreath & Buchanan, 1981) are two popular chilling unit models in temperate regions (Cesaraccio et al., 2004).

The Dynamic model (Fishman et al., 1987; Erez et al., 1988) is a two-step chilling unit model developed for evaluating CR of tree species in warm winter regions such as Israel or California in the US. It assumes a biochemical basis for endo-dormancy release. The first step produces a reversible intermediate of the substance for endo-dormancy release and the second one fixes the intermediate by an irreversible transition. This model can account for not only the apparent negative effect of the high temperature, but also the varying effect of the same temperature in different daily temperature cycles (Erez et al., 1988).

We should keep in mind that, because of lack of knowledge of biochemical or physiological mechanisms controlling CR, almost all CR models were developed empirically or statistically to fit the responses (mainly bloom dates) of tree species to local weather conditions. Special caution is needed in selecting an appropriate model to evaluate the CR of different genotypes of particular tree species/region.

Interrelationships among Chilling Requirement, Heat Requirement and Bloom Date

CR is the major factor determining bloom date (BD) in *Prunus* (Egea et al., 2003; Ruiz et al., 2007; Alburquerque et al., 2008). Genotypes with low CR bloom early in cold regions/years and are susceptible to late frost damage (Scorza & Okie, 1990). Genotypes with high CR could suffer inadequate chilling in warm regions/years resulting in irregular floral and leaf bud break and thus poor fruit set, which is potentially problematic with the current global warming trend (Topp et al., 2008). On the other hand, in temperate or subtropical regions, early ripening cultivars are often preferred because of better early

market prices for their fruits (Ruiz et al., 2007; Topp et al., 2008). Breeding for earlier BD (often associated with low CR) is one approach to getting earlier ripening fruit with adequate size.

Heat requirement (HR) is another factor determining the BD of cultivars of *Prunus* (Richardson et al., 1974; Citadin et al., 2001). It is unclear whether heat accumulation for floral or vegetative bud break starts before or after the release of endo-dormancy. It has also been reported that extended chill (more than CR) resulted in the reduction of HR of tree buds (Scalabrelli & Couvillon, 1986; Citadin et al., 2001; Harrington et al., 2009). These two issues complicate the quantification of the variation of HR among different genotypes. The growing degree hour (GDH) model developed by Richardson et al. (1975) is most widely used (Citadin et al., 2001; Egea et al., 2003; Ruiz et al., 2007; Alburquerque et al., 2008), but it only counts the heat accumulation from endo-dormancy release to full bloom.

Among the three inter-related traits, BD is considered to be quantitatively inherited in most fruit species (Anderson & Seeley, 1993), CR is considered to be semi-qualitatively inherited in apple (*Malus* × *domestica* Borkh.) (Hauagge & Cummins, 1991), and no study has yet been reported on the genetic nature of HR.

Couvillon & Erez (1985) pointed out that extended chilling in several fruit tree species results in 90% of HR variations among different cultivars with different CRs and there is no actual (genetic) difference in HR for bloom among different cultivars. Okie & Blackburn (2008) confirmed that artificially supplied, incremental chilling dramatically

reduced HR for bud break in peach when shoots were under-chilled, but they found the effects diminished when buds received more chilling. Recently, Harrington et al. (2009) proposed a model, whereby for all plants with an obligate chilling requirement, there is a minimum number of chilling units required (critical CR) for possible budburst even with very high heat units, and an optimum number of chilling units required (optimum CR) after that additional chilling will not accelerate budburst. Between the critical CR and optimum CR, many combinations of chilling units and forcing (heat) units could make bud-break possible, implying a possible overlapping period of CR and HR fulfilling after a tree's critical CR was met.

If Couvillon & Erez (1985) were right about proposing no genetic differences for HR among fruit tree cultivars/genotypes, then among genotypes of tree species of different CR, low CR genotypes would be over-chilled and require less heat accumulation for the bloom than would high CR genotypes. However, Ruiz et al. (2007) reported a negative correlation between CR and HR in different apricot genotypes. Scorza & Okie (1990) also found that some peach selections from Aguascalientes, Mexico have low CR, but late BD. These results suggested the existence of the different HRs among genotypes/cultivars and a potential genetic contribution to this character.

Peach Floral Bud Development in dormancy Period

Baggliolini (1952) defined a series of phonological stages in peach floral buds after bud establishment and gave these a nomenclature: Stage A, winter resting bud, a long period without apparent change; Stage B, swelling buds, the indication of dormancy

release; Stage C, visible calyx, the protective bracts begin to separate gradually and sepals become visible; Later stages, occurring very rapidly, in a few days the flower will be open.

Reinoso et al. (2002) studied anatomical changes in the peach floral buds in different phonological stages according to Baggliolini (1952)'s nomenclature. They found that the peach floral buds showed a continuous anatomical development during the late autumn and winter dormancy. Sterile whorls (sepals and petals) differentiated rapidly in late summer through early autumn. Fertile whorls (stamen and gynoeciums) developed slowly during winter and rapidly in later winter to early spring. The androecium developed throughout the winter, while the gynoecium developed in late winter. By late winter, the anthers began microsporogenesis and microgametogenesis and ovaries had formed ovules. Vascular connections between flower primordial and branch wood were complete by late winter. Based on observation, Reinoso et al. (2002) concluded that there was a combination of ongoing cell division, enlargement and differentiation that results in organogenesis during the entire "dormancy" period and they defined this process as a "slow maturation phase (corresponding to "Stage A"), in contrast to the "rapid maturation phase" denoting the rapid development from the end of winter (corresponding to "Stage B-F").

In comparison, peach vegetative buds are fully differentiated in late summer and progressively enter a dormant state (Reinoso et al., 2002).

Control of *Arabdopsis thaliana* Flowering and Its Value for Research on Woody Perennial Chilling Requirement and Bloom Date

Genetic analysis has identified many pathways that control the timing of floral transition in *A. thaliana*. Downstream of many floral pathways are a set of floral pathway integrators. The activation of these floral pathway integrator genes triggers the floral transition. In turn the integrators activate floral meristem identity genes, which encode proteins that promote floral development (Henderson et al, 2004).

The multiple pathways that regulate the floral integrators in *A. thaliana* are classified as promotion, enabling, and resetting pathways (Boss et al, 2004). The photoperiod pathway is one of the promotion pathways. Long day photoperiods promote flowering by activating the B-box transcription factor CONSTANTS (CO), which is required for the up-regulation of the floral integrator genes. *CO* mRNA exhibits rhythmic, diurnal expression controlled by the circadian clock. This rhythm is reinforced through different photoreceptors acting on CO protein stability: PHYB promotes the degradation of CO protein, whereas PHYA, CRY1 and CRY2 stabilize it. In contrast to the promotion pathways, the enabling pathways determine the activity of repressors of the floral pathway integrators (FLC). Vernalization is one of the independent pathways which down-regulates FLC. The vernalization process is initiated by VIN3 and maintained by VRN1 and VRN2. Once acquired, the vernalized state is 'remembered' by the plant during subsequent growth, suggestive of an epigenetic basis (Henderson, et al, 2004).

Some evidences suggest that at least some components of flowering control mechanisms in *A. thaliana* are shared by woody perennials. The CO/FT module of photoperiod pathway found in *A. thaliana* also controls the flowering in aspen trees. Moreover, it controls short-day-induced growth cessation and bud set occurring in the fall (Bohlenius et al, 2006). Homologous cDNA fragments of *CO*, *FT*, and *FAR1* (encodes a nuclear protein specific to PHYA signaling in *A. thaliana*) were also identified in almond and *FAR1* was genetically mapped to a QTL controlling almond flowering time (Silva, et al. 2005). This suggests that CO/FT module may also control growth cessation and/or dormancy induction and flowering time in *Prunus* species.

The obvious similarities between vernalization requirement in annual plants and chilling requirement in woody perennials imply that some genes might be involved in controlling both pathways. Both vernalization and chilling requirement are the characteristics required for plants to tolerate winter low temperatures and align flowering in spring favorable conditions. Both vernalization and dormancy breaking require exposure to chilling for enabling but not promoting flowering (Chouard, 1960). One contradiction to this idea is that vernalization occurs effectively only in actively dividing cells, whereas chilling is required to release endodormancy of woody perennials after termination of cell division (Rohde & Bhalerao, 2007). However, this is not necessarily correct. Reinoso et al (2002) found that although there were not macroscopic changes, the peach floral bud shows a continuous anatomical development during the winter dormancy period.

In *A. thaliana*, FLC is the central player of vernalization pathway. Allelic variation of *FLC* gene contributes to natural variation in the vernalization requirement (Gazzani et al. 2003). Prior to vernalization, a high steady state level of FLC is acquired via the interplay of endogenous ABA with the protein FCA or ABH1 (Rohde, et al, 2007). The down regulation of *FLC* RNA during vernalization is a quantitative process, with longer period of cold exposure leading to progressively lower *FLC* RNA expression (Sheldon, et al, 2000). Recently, Chen & Coleman (2006) reported a differential expression of *FLC*-like genes during the completion of the chilling requirement in vegetative buds of poplar. This might suggest that these genes play a similar role in dormancy breaking in woody perennials.

Summary of Previous Genetic Studies on Chilling Requirement, Heat Requirement and Bloom Date in Woody Plants

There have been no reported results on successfully mapping QTLs associated with CR for floral bud break in temperate tree species. However, two genetic studies suggested that CR was in control of at least one major gene with dominant low CR allele(s) in apple and apricot (Hauagge &Cummins, 1991; Tzonev & Erez, 2003). As for the HR, almost no genetic studies have been reported. It is even unclear if HR is an intrinsic characteristic of several fruit tree species (Couvillon & Erez, 1985; Ruiz et al., 2007).

QTL mapping results for BD in various genomic regions in *Prunus* has been reported. Using the terminology of the almond cv. Texas × peach cv. Earlygold (T × E)

Prunus reference map on linkage groups (G), four QTLs on G1, G4, G6 and G7 were detected by Joobeur (1998) in an almond × peach F_2 population, two QTLs on G2 and G7 by Dirlewanger et al. (1999) in a peach F_2 population, one major gene (*Late blooming or Lb*) on G4 by Ballester, et al. (2001) in an almond F_1 population, and one QTL on G4 by Verde et al. (2002) in a peach backcross (BC_1) population. A candidate gene approach associated only two out of ten candidate genes homologous to *LEAFY* and MADS-box genes in *A. thaliana* with two QTLs in almond (Silva et al., 2005), suggesting that direct application of the knowledge of the genetic control of flowering time of annual plants to the perennial tree species may be more complicated than expected.

Growing *Prunus* Genomic Resource

The rapidly growing *Prunus* genomic resource consists of three fundamental units: the physical map, integrated genetic marker maps, and mapped ESTs. A physical map would serve as the foundation on which the genetic markers (SSR or RFLP, etc.) and ESTs could be layered (Georgi et al, 2002).

To date, 20 genetic maps have been constructed for peach and other *Prunus* species (GDR web, http://www.rosaceae.org). Map comparisons using transferable genetic markers showed that *Prunus* species share nearly identical genome organization (Abbott et al, 2006). A stepwise saturated linkage map developed with the almond 'Texas' × peach 'Earlygod' F_2 population, was recognized as a *Prunus* reference map providing a set of transferable markers and a common linkage group terminology and

marker order in each linkage group (Joobeur et al, 1998; Aranzana et al, 2003; Dirlewanger et al., 2004). The T×E *Prunus* reference map has 562 codominant markers including 11 isozymes, 185 SSRs, 361RFLPs and 5 STSs. It consists of eight linkage groups in agreement with the haploid chromosome number of the *Prunus* genus, and covers a genetic distance of 519 cM with an average marker density of 0.92 cM/per marker. Subsequently, Howad et al (2005) placed 264 additional SSRs on the T×E map using a "bin mapping" approach. The transferable SSR markers mapped on reference *Prunus* map enriched by "bin" map strategy were used to "saturate" (increase marker density) in specific genomic regions on a peach linkage map, which we developed for chilling requirement and bloom date QTL mapping at the center of the research in this thesis. Additionally, they served as "anchor markers" to integrate the peach CR QTL linkage map with *Prunus* reference map and thus, allow access to the candidate gene infrastructure of the peach physical map/EST database.

A genome-wide framework physical map was constructed for peach, a *Rosaceae* model species (due to its small genome size, diploidy, colinearity of genome with other *Prunus* species). It contains 2138 contigs composed of 15,655 clones from two complementary BAC libraries. The total physical length of all contigs is estimated at 303Mbp or 104.5% of the peach genome. The total physical length of anchored contigs is estimated at 45.0Mbp. 2636 markers including genetic markers, peach unigene ESTs, gene specific and overgo probes, were incorporated into framework physical map. Among these 2636 markers, the common RFLP and SSR markers integrated the peach framework physical map with the *Prunus* reference map (Zhebentyayea et al, 2008). The

integrated physical/genetic map was of critical importance for high throughput EST mapping, efficient map-based cloning of important genes and peach whole genome sequence assembly.

Prior to 2006, 35 (6 species, 10 tissues, 17 development stages) *Prunus* cDNA libraries had been constructed. Prior to 2008, 92,421 EST sequences in *Prunus* species were available and resolved into 24,307 putative unigenes (GDR web, http://www.rosaceae.org). Currently, 2239 peach unigenes have already been positioned onto the integrated physical/genetic map.

In our current study, the growing genomic resources not only provided us anchor SSR information for linkage map construction, but also help us in scanning and cataloging genes and SSRs in specific genomic regions for further association mapping and candidate-mapping.

Advantages of Using Peach F_2 Population to Map Agriculturally Important Traits

In temperate and subtropical regions, peach is widely grown and economically important. As a proposed tree model species (Abbott et al., 2002), its self-compatibility and short generation cycle (2-3 years) enable relatively easy development of true F_2 populations and early characterization of floral and seed-related traits. Its diploidy and the availability of a large number of mapped simple sequence repeat (SSR) markers transferable within *Prunus* greatly facilitate linkage map construction. The small genome size (~220Mbp, Sosinski B, North Carolina State University, Personal communication) and extensive genomics/genetics resources available at the Genome Database for

Rosaceae website (GDR web, http://www.rosaceae.org) enable map-based cloning and annotation of genes controlling important agronomic traits for tree arboriculture, and development of markers inside or tightly linked with these genes for marker-assisted breeding applications. However, to achieve these goals, it is critical to have detailed resolution of the location of genomic regions (QTL) harboring these genes.

Project Overview

This research is a part of USDA BARD program "Structural and functional genomics approaches for marking and identifying genes that control chilling requirement in apricot and peach trees". The major objective of this research is to identify QTLs associated with CR and CR-related traits using two approaches, linkage mapping and association mapping.

Specific Objectives of the Project

1. A peach F_2 population derived from two genotypes with contrasting CR values was used for linkage map construction and QTL mapping for CR and BD.

2. A collection of 65 peach germplasm accessions with different CR values was chosen for association mapping for validating QTL positions and refining QTL regions with large genetic effects.

3. Having identified robust reproducible QTL loci, we focused on integrating, genome sequences, physically mapped markers, ESTs and previous work in other

systems to identify potential candidate genes in the major QTL intervals that could be tested to determine their role in CR and BD.

With completion of these objectives, the following results are presented in this thesis:

1) We identified genomic regions (QTLs) associated with CR, HR and BD and provided the first picture of the genetic inter-relationships among these traits in *Prunus* species.

2) We developed transferable genetic markers tightly linked with QTL regions.

3) We refined the QTL regions enabling the identification of putative candidate genes controlling these traits.

4) We established plum (in cooperation with Dr. R. Scorza's group, ARS) as a potential transgenic system to quickly test CR candidate genes so that verification of the role of important genes controlling these traits would be possible.

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CHAPTER TWO

MAPPING QUANTITATIVE TRAIT LOCI ASSOCIATED WITH CHILLING REQUIREMENT, HEAT REQUIREMENT AND BLOOM DATE IN PEACH

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Shenghua Fan conducted linkage map construction and QTL analysis, participated CR phenotyping and composed the manuscript. Douglas G. Bielenberg maintained the mapping population and organized CR and BD phenotyping. Tetyana N. Zhebentyayeva helped with linkage map construction. Gregory L. Reighard helped with maintaining the mapping population. William R. Okie developed the mapping population. Doron Holland collaborated in the BARD program which funded this research. Albert G. Abbott supervised the whole project.

Summary

Chilling requirement (CR), together with heat requirement (HR), determines bloom date (BD), which impacts climatic distribution of genotypes of tree species. The molecular basis of floral bud CR is poorly understood despite its importance to fruit tree adaptation and production. Also, the genetic nature of HR and genetic inter-relationships among CR, HR and BD remain unclear.

A peach F_2 population of 378 genotypes developed from two genotypes with contrasting CR values was used for linkage map construction and QTL mapping. Floral bud CR and HR of each genotype were evaluated in two years and BD scored in four years.

20 QTLs with additive effects were identified for three traits including one major QTL for CR and two major QTLs for BD. The majority of QTLs co-localize with QTLs for other trait(s). Particularly, one genomic region of 2cM pleiotropic for the three traits overlaps with the sequenced peach *evg* region.

This first report on floral bud CR QTL mapping will facilitate marker assisted breeding for low CR cultivars and map based cloning of genes controlling CR. The extensive co-localization of the QTLs suggests one unified temperature sensing and action system regulating CR, HR and BD together.

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Introduction

Temperate tree species have the ability to cease meristem activity in the fall and establish a dormant state (endo-dormancy or true dormancy) in which the meristem is rendered insensitive to growth promoting signals before it is released (Rohde & Bhalerao, 2007). Chilling requirement (CR) refers to the duration of low temperatures required for the release of temperate trees from endo-dormancy. CR prevents trees from initiating growth in response to transient warm temperatures thus avoiding damage by subsequent frost(s) in the late winter or early spring. CR is the result of long term climatic adaption of genotypes of tree species developed in different regions. Conversely, it limits the climatic distributions of genotypes of temperate fruit trees (Sherman & Beckman, 2003). CR is the major factor determining bloom date (BD, also referred to as flowering time) (Egea et al., 2003; Ruiz et al., 2007; Alburquerque et al., 2008), which is an important agronomic trait affecting seed and fruit development of temperate fruit tree species. Genotypes with low CR bloom early in cold regions/years and are susceptible to late frost damage (Scorza & Okie, 1990). Genotypes with high CR could suffer inadequate chilling in warm regions/years resulting in irregular floral and leaf bud break and thus poor fruit set, which is potentially problematic with the current global warming trend (Topp et al., 2008). On the other hand, in temperate fruit tree species, early ripening cultivars are often preferred because of better early market prices for their fruits (Ruiz et al., 2007; Topp et al., 2008). Breeding for earlier BD (often associated with low CR) is one approach to getting earlier ripening fruit with adequate size.

Heat requirement (HR) is another factor determining the BD of cultivars in temperate tree species (Richardson et al., 1974; Citadin et al., 2001). It is unclear whether heat accumulation for floral or vegetative bud break starts before or after the release of endo-dormancy. It has also been reported that extended chill (more than CR) resulted in the reduction of HR of tree buds (Scalabrelli & Couvillon, 1986; Citadin et al., 2001; Harrington et al., 2009). These two issues complicate the quantification of the variation of HR among different genotypes. The growing degree hour (GDH) model developed by Richardson et al. (1975) is most widely used (Citadin et al., 2001; Egea et al., 2003; Ruiz et al., 2007; Alburquerque et al., 2008), but it only counts the heat accumulation from endo-dormancy release to full bloom.

Among the three inter-related traits, BD is considered to be quantitatively inherited in most fruit tree species (Anderson & Seeley, 1993), CR is considered to be semi-qualitatively inherited in apple (*Malus* × *domestica* Borkh.) (Hauagge & Cummins, 1991), and no study has yet been reported on the genetic nature of HR.

QTL mapping results for BD in various genomic regions in *Prunus* has been reported. Using the terminology of the almond (*Prunus amygdalus* L.) (cv.Texas) × peach [*Prunus persica* (L.) Batsch] (cv. Earlygold) map (T×E *Prunus* reference map) on linkage groups (G-), four QTLs on G1, G4, G6 and G7 were detected by Joobeur (1998) in an almond × peach F_2 population, two QTLs on G2 and G7 by Dirlewanger et al. (1999) in a peach F_2 population, one major gene (*Late blooming or Lb*) on G4 by Ballester, et al. (2001) in an almond F_1 population, and one QTL on G4 by Verde et al.

(2002) in a peach backcross (BC_1) population. A candidate gene approach associated only two out of ten candidate genes homologous to *LEAFY* and MADS-box genes in *Arabdopsis* with two QTLs in almond (Silva et al., 2005), suggesting that direct application of the knowledge of the genetic control of flowering time of annual plants to the perennial tree species may be more complicated than expected.

There have been no reported results on successfully mapping QTLs associated with CR for floral bud break in temperate tree species. However, two genetic studies indicated that CR was in control of at least one major gene with dominant low CR allele(s) (Hauagge & Cummins, 1991; Tzonev & Erez, 2003). As regard to the HR, almost no genetic studies have been reported. It is even unclear if HR is an intrinsic character in several fruit tree species (Couvillon & Erez, 1985; Ruiz et al., 2007).

In temperate and subtropical regions, peach is widely grown and economically important. As a proposed tree model species (Abbott et al., 2002), its self-compatibility and short generation cycle (2-3 years) enable relatively easy development of true F_2 populations and early characterization of floral and seed-related traits. Its diploidy and the availability of a large number of mapped simple sequence repeat (SSR) markers transferable within *Prunus* greatly facilitate linkage map construction. The small genome size (~220Mbp, Sosinski, North Carolina State University, Raleigh, Pers. Comm..) and extensive genomics/genetics resources available at the Genome Database for Rosaceae website (GDR, www.bioinfo.wsu.edu/gdr/) enable map-based cloning and annotation of genes controlling important agronomic traits for tree arboriculture, and development of markers inside or tightly linked with these genes for marker-assisted breeding

applications. However, to achieve these goals, it is critical to have detailed resolution of the location of genomic regions (QTL) harboring these genes.

The major objective of this research was to identify QTLs associated with CR and CR-related traits using a peach F_2 population derived from two genotypes with contrasting CR values: the high CR cv. 'Contender' and the low CR selection 'Fla.92-2C'. The F_2 progenies segregate in a continuous fashion for a variety of traits including CR, HR and BD. Utilizing this mapping population, we obtained the first data on the genomic regions (QTL) determining floral bud CR and HR and provided the first picture of potential genetic inter-relationships among CR, HR and BD in temperate tree species.

Materials and Methods

Plant Materials

A peach F_2 population with 378 different genotypes was developed at ARS-USDA, Southern Fruit and Tree Nut Research Lab (Byron, GA, USA) by crossing two peach genotypes with high and low CR values and selfing the resultant F_1 hybrid 'BY01p6245'. The female grandparent 'Contender' is a commercial peach cultivar in the southeastern US developed by North Carolina Agricultural Service (Raleigh, NC, USA) and requiring approximately 1050 chilling hours (CH) of CR. The male grandparent 'Fla.92-2C' is a selection from the University of Florida's (Gainesville, FL, USA) low chilling peach breeding program requiring approximately 300 CHs of CR. Both grandparents have cv. 'Candor' and 'Pekin' as distant ancestors in their pedigrees. F_2 seeds were stratified, germinated and pot-planted in a greenhouse in 2003 and

transplanted to Clemson University's Musser Fruit Research Center (Seneca, SC, USA) in 2004. Three to four clones of each genotype were made by rooting the shoot cuttings from seedling trees and planted in a second plot at the same site in 2006. This population segregates for multiple quantitative traits including CR, HR and BD. It also segregates for ripening date and the qualitative trait Non-Showy/showy flower (Sh/sh) in a 3:1 ratio.

Phenotyping

Chilling requirement (CR) For deciduous fruit trees, two methods are routinely employed to determine when their CR is fulfilled for blooming. One is to expose the cuttings harvested from different times to a controlled warm condition for a period of time with subsequent scoring of the status of floral bud break (Gibson & Reighard, 2002). Another is to measure and compare the weight of floral buds before and after these cuttings are exposed to a warm condition for a period of time (Tabuenca, 1964). Because of the necessity of large scale rapid screening, the first method was used in this study.

Floral bud CR data for the F_2 population obtained in winter 2007/spring 2008 and winter 2008/spring 2009 were designated as CR2008 and CR2009, respectively. Average temperatures in 10 min intervals were continuously recorded by the temperature data loggers placed in the canopy of the experimental trees starting in the middle of October when air temperature drops to below 7.2^{o}C, and ending in late March of the next year. The <7.2^{o}C (Weinberger, 1950) model was chosen to determine the times to sample branches and evaluate chilling fulfillment. The number of hours below 7.2^{o}C (CH) was counted. Starting with the time of 300 CH, the branches of each genotype were harvested

approximately every 100 CH till the time of 1000 CH (2007/2008) or 1100 CH (2008/2009). For each genotype, three clones grown in natural field conditions were sampled and three branches (generally longer than 40cm and populated with floral buds) were taken from each clone. Branch cuttings were placed into 1% "Floralife (Fresh Flower Food)" solution (Floralife, Inc., Walterboro, SC, USA) in a greenhouse at Clemson University campus at 25°C to force floral bud break under a 16-hour photoperiod. After 14 days, the progression of floral bud break of the branches was evaluated. A genotype's chilling requirement was considered satisfied at a specific sampling time if 50% of floral buds on the branch cuttings opened (pink stage).

After CR evaluation based on the $<7.2^{o}$ C model was finished, CR of each genotype was recalculated based on the 0-7.2^{o} C model (Eggert, 1951), the Utah model (Richardson et al., 1974), the Low Chill model (Gilreath & Buchanan, 1981) and the Dynamic model (Fishman et al., 1987; Erez et al., 1988).

Chilling accumulations calculated by different models on each sampling date in years (winter/spring) 2007/2008 and 2008/2009 are listed in Supporting Information Table S2. 1.

Heat requirement (HR) Floral bud HR data for the F_2 population obtained in winter 2007/spring 2008 and winter 2008/spring 2009 were designated as HR2008 and HR2009, respectively. HR of each F_2 genotype was evaluated with the Growing Degree Hour (GDH) model developed by Richardson et al. (1975). GDHs for a specific genotype was determined by subtracting 4.5°C (below which no growth or development of peach buds occurs) from the hourly temperature, and accumulating the balance from

the time of CR completion to full bloom. Temperatures above 25°C were treated as 25°C because of no extra heat benefit for the tree (Anderson et al., 1986).

Bloom date (BD) BD of each F_2 genotype was evaluated as the date when 50% of floral buds have reached the full bloom stage in the springs of 2006, 2007, 2008 and 2009. For each genotype, the whole tree of one clone was observed every one or two days in the spring to determine BD. BD was recorded and analyzed as the number of days from January 1st to the date of bloom.

Non-Showy/Showy flower (Sh/sh) Sh/sh was evaluated in the spring of 2006 as two classes: Non-Showy (flower with small petals, dominant) and Showy (flower with large petals, recessive).

Statistical Analysis of Phenotypic Data

Statistical analyses of the phenotypic data were performed with the Statistical Analysis System (SAS) 9.2 package (SAS Institute INC., Cary, N.C., USA). The "UNIVARIATE" procedure of SAS was used to test for normality of phenotypic data distributions. The "CORR" procedure of SAS was used to test correlations between different traits. The Spearman correlation coefficient (r) from SAS output was chosen due to the non-normal distribution of all traits. The range of "r" was interpreted empirically: the correlation between two variables was considered "weak" if "r" ranged 0-0.3; "moderate" if "r" ranged 0.31-0.7; and "high" if "r" ranged 0.71-1.0.

Genotyping

SSR markers A set of 370 SSRs isolated from different *Prunus* species was tested for polymorphism in the F_2 mapping population using the female grandparent 'Contender' and the F_1 tree 'BY01p6245'. The origins and references of these SSRs were listed in Supporting Information Table S2. 2. Segregation analysis was carried out in the entire F_2 population for polymorphic SSR markers with clear segregation patterns as outlined in Zhebentyayeva et al. (2003), with preference for those mapped onto the T×E *Prunus* reference map (Dirlewanger et al., 2004) and peach "bin map" (Howad et al., 2005).

Amplified fragment length polymorphism (AFLP) markers AFLP marker analysis was essentially performed as outlined in Vos et al. (1995). In total, 206 *EcoR*I/*Mse*I primer combinations were tested for polymorphism in the F_2 population with the female grandparent 'Contender' and F_1 tree 'BY01p6245'. Selective amplification was performed using an *EcoR*I-end primer with two selective nucleotides and a *Mse*I-end primer with three selective nucleotides. Segregation analysis was then carried out in the entire F_2 population for the primer combinations showing polymorphisms and clear segregation patterns. Following the manufacture's manual, the size of AFLP fragments was determined by the DNA ladders generated from fmol DNA Cycle Sequencing System (Promega Corp., Madison, WI, USA). A dominant AFLP marker was named EXXMYYY(a) and a codominant AFLP marker EXXMYYY(a/b), with "XX" being the selective nucleotides for *EcoR*I-end primers, "YYY" the selective nucleotides for *Mse*I-end primers, and "a" or "b" the number of base pairs of AFLP fragment(s).

Map Construction

Genetic mapping of the F_2 population was performed using the JoinMap 3.0 software (Van Oojjen & Voorrips, 2001). Kosambi's mapping function was applied for map distance calculation (Kosambi, 1944). Segregation distortion of individual markers was revealed by the Chi-square test of JoinMap. Markers showing skewed segregation ($P<0.05$) were still utilized for mapping after the verification of the genotypic data. Linkage groups (G-) were constructed and marker order determined using default parameters of JoinMap. Only marker order and distances generated by the first or second run of mapping were adopted. Finally, the name and orientation of all linkage groups, except G4, were dictated by the *Prunus* reference map (Dirlewanger et al., 2004) based on the SSR markers shared by two maps (Supporting Information Fig. S2.1). G4 shared only one SSR marker with the T × E *Prunus* reference map and its orientation was dictated by the peach "bin" map (Howad et al., 2005).

The Sh/sh trait was mapped as a dominant phenotypic marker since it segregates in a 3:1 (Non-Showy: Showy) ratio. Generally, SSRs were scored and mapped as codominant markers, and AFLPs as dominant markers. In the case of possible multi-locus SSR markers or codominant AFLP markers, all separated PCR bands were first scored as dominant markers and processed by JoinMap 3.0 together with other markers. In dominant scoring, if a pair of PCR bands from the same primer combination was mapped to the same locus, the pair was considered allelic and then rescored and mapped as a codominant marker. The SSR marker names standardized in GDR website

(www.bioinfo.wsu.edu/gdr/) were adopted. In the case of multi-locus SSR markers amplified with the same pair of primers, a capital letter was added to the end of the marker name for each locus. The selection of letters was consistent with that for the T×E *Prunus* reference map (Dirlewanger et al., 2004), if these markers had also been mapped on it.

QTL Analysis

Composite interval mapping (CIM) (Jansen & Stam, 1994; Zeng, 1994) was performed using PLABQTL version 1.2bic (Utz & Melchinger, 2006): a QTL mapping software based on a multiple regression approach with flanking markers described by Haley & Knott (1992).

Different years of phenotypic data for the same trait were analyzed separately. Cofactors (markers best accounting for QTL effects) for QTL mapping in each trait were selected by a stepwise regression procedure. A pure additive model for each trait was chosen by fitting phenotypic and marker data with different gene action models (different combinations of additive, dominance and epistatic effects) and selecting the model with the minimal Bayesian Information Criterion (BIC) value after the "final simultaneous fit" procedure (simultaneous multiple regression using all detected QTLs and their estimated positions). Threshold of logarithm of the odds (LOD, 2.85) for QTL detection at a genome-wise error rate of 5% was obtained by 1000 iterations of permutation test for all traits. LOD curves were created by scanning every 1cM of the genome.

Once all parameters for CIM were set, the "final simultaneous fit" procedure was carried out again to obtain final estimates of the additive effects for each QTL, the proportion of the phenotypic variance explained by each QTL (Partial R^2) and the proportion of the phenotypic variance explained by all QTLs with adjustment for the number of QTL terms in the full regression model (Adjusted R^2) (Hospital et al., 1997). The additive effect is half of the difference between two homozygotes. The allele from the low CR male grandparent of the F_2 population was assumed superior. If it was actually weaker, then a negative additive effect was assessed. The additive effects divided by the phenotypic standard deviation (standardized additive effects) were reported. Partial R^2 for each QTL term was calculated as the change in R^2 of the regression model with that term removed from the model: Partial R^2= [R^2 (full model) -R^2 (reduced model)]/ [1-R^2 (reduced model)]. Note that the denominator of the formula above is different for each Partial R^2 calculated. Therefore, the Partial R^2 value will not sum up to the Adjusted R^2 for the full model (Utz, 2000; Wassom, et al., 2008).

The two way analysis of variance (ANOVA) for genotype × environment interaction was performed with multiple years of phenotypic data of each trait by the "QTL-ANOVA" procedure of PLABQTL. Broad sense heritability (H^2) and mean squares from different sources (genotypes, genotype × environment, etc.) were reported based on PLABQTL output. Mean squares from source of environments were calculated manually according to the method described by Lynch & Walsh (1998).

One- or two-LOD intervals (approximately 95% or 99% confidence interval) (Lynch & Walsh, 1998) for QTL detection were reported. The QTL graphs were prepared using MapChart 2.2 (Voorrips, 2002). The QTLs with Partial R^2 greater than 30% were arbitrarily declared as major QTLs.

A detected QTL is named as qXXYa-ZZZZ, with "XX" being the trait acronym, Y the number of the linkage group on which the QTL was detected, "a" the letter to specify different QTLs if more than one QTLs were detected for the same trait on one linkage group, "ZZZZ" the year in which the trait was phenotyped.

Results

Distribution and Correlation Analysis of Phenotypic Data and Heritability

Both years (2007/2008 and 2008/2009) of CR data of the F_2 population showed bimodal distributions, while the bimodality of CR2008 was more obvious (Fig. 2. 1a, b). Both CR2008 and CR2009 were right skewed, i.e., low CR genotypes dominate the F_2 population. CRs evaluated by the different models highly (or perfectly) correlated with each other (r=1; P <0.001) in both years. The two years of CRs were highly correlated (r=0.723; P <0.001) (Table 2. 1).

Both years (2007/2008, 2008/2009) of HRs showed single peak but skewed distributions (Fig. 2. 1c, d). Two years of HRs were moderately correlated (r=0.379; P <0.001) (Table 2. 1).

All four years of BDs showed multimodal distributions (Fig. 2. 2). The ranges of BDs varied from 16 days (year 2006) to 53 days (year 2007). The distribution of BD was right-skewed in year 2006 and left skewed the other years. The four years of BDs were highly correlated with each other (r=0.704-0.831; P <0.001) (Table 2. 1).

In addition, the Kolmogorov-Smirnov test by the "CORR" procedure of SAS 9.2 also confirmed that the distributions of all three phenotypic traits departed significantly (P <0.01) from normality.

Both years of CRs (<7.2°C model) were moderately correlated with BDs (r=0.698, 0.672; P <0.001) and moderately or highly correlated with HRs (r=-0.653, -0.820; P <0.001); the correlation with BD was positive and that with HR negative. HRs had non-significant (year 2008, r=-0.014, P >0.793) or weak (year 2009, r=-0.188, P <0.001) correlations with BDs (Table 2. 1).

The broad sense heritability (H^2) was 79.5% for CR (<7°C model), 54.0% for HR and 85.2% for BD (Supporting Information Table S2. 3).

Linkage Map

A linkage map composed of 96 SSR markers (of which six are dominant), 30 AFLPs (of which four are co-dominant) and one phenotypic marker (Sh) was constructed. Markers were organized into eight linkage groups that are consistent with the number of chromosomes in the peach genome. G1 covers the longest genetic distance of 96.4cM, while G3 covers the shortest genetic distance of 51.7cM. The total map length

of 535cM was established, corresponding to an average interval of 4.2cM between adjacent markers. Due to a lack of segregating markers in certain genomic regions, there are three gaps of 24-29cM in G2, G4 and G5 (Fig.2. 3). Marker orders in each linkage group were in good agreement with those in the T×E *Prunus* reference map with a few minor differences detected. Out of 36 SSRs shared by two maps, 32 were mapped in the same linkage groups and orders with those on the reference map. Two more SSRs (pchgms3 and CPPCT026) were mapped in the same region in G1, but with a different orientation (Supporting Information Fig. S2. 1). The agreement with the reference map implies high quality for the newly constructed linkage map and forms a solid basis for further QTL analysis.

Most loci (77.8%) exhibited genotype ratios as expected for a segregating F_2 population (1:2:1 for codominant markers or 3:1 for dominant markers). Among 28 markers with significantly skewed genotypic ratios ($P<0.05$), a cluster of 17 were mapped in G1 from 68 cM to the end of the group with an overrepresentation of the alleles inherited from the low CR male grandparent, the other 11 were randomly distributed onto G1, G2, G3, G6, G7, G8 (Fig. 2. 3).

Mapping QTL

QTLs for CR Using <7°C CR evaluation model, in both years, four QTLs (qCR1a, qCR4b, qCR5, qCR7) were detected in the same or largely overlapping genomic regions and considered as the same QTLs. Among these, qCR1a and qCR7 showed very prominent effects. qCR1a explained 40.5-44.8% of phenotypic variance and was declared

as major QTL. qCR7 explained 17.8-24.9% of the phenotypic variance (Table 2. 2, Fig. 2. 3). Additionally, four year-specific QTLs were detected for CR, explaining 4.2-9.7% of the phenotypic variance (Table 2. 2, Fig. 2. 3).

The full regression model for CR QTLs explained 55.7% and 54.3% of the phenotypic variance in each year, respectively (Table 2. 2).

CR2008 calculated by five different CR models were subjected to QTL analysis and yielded very similar results, except that when the <7°C and 0-7°C model were used, one more QTL (qCR6-2008) was detected. qCR6-2008 displayed a minor effect, only explaining 4.2% (the <7°C model) or 3.8% (the 0-7°C model) of phenotypic variance (Table 2. 2). When other three models were used, LOD peaks in the position of qCR6-2008 also showed up. Only because the peak values (1.97, 2.42 or 2.67) were lower than the significant LOD threshold of 2.85, it was mis-detected. Besides qCR6-2008, the other six QTL showed very similar two-LOD CI, LOD peak scores and the proportions of explained phenotypic variances with all five CR models.

CR2009 calculated by the different CR models also yielded very similar QTL mapping results, except that two minor QTLs (qCR4b-2009 and qCR8-2009) were not consistently detected when different CR models were used (Table 2. 2).

QTLs for HR In both years, qHR1 were detected in overlapping genomic regions and considered as the same QTL. qHR1 explained 7.1% and 11.2% of phenotypic variance in years 2007/2008 and 2008/2009, respectively. Another QTL was detected only in year 2007/2008, explaining 3.1% of phenotypic variance (Table 2. 3, Fig. 2. 3).

The full regression models for HR QTLs explained 8.6% and 10.7% of phenotypic variance in years 2007/2008 and 2008/2009, respectively (Table 2. 3).

QTLs for BD In all four years, four QTLs for BD (qBD1a, qBD2, qBD4 and qBD7a) were detected in the same or largely overlapping genomic regions and considered as the same QTLs. Among these, qBD1a and qBD7a were two QTLs having very prominent effects. Except for qBD1a in year 2006, both QTLs explained more than 30% of phenotypic variance in different years and were declared as major QTLs. qBD4 also explained a relatively large portion of the phenotypic variance ranging from 8.5-19.9% (Table 2.4, Fig. 2.3). In two years, one QTL (qBD5) was detected in the same genomic region on G5 and also considered as the same QTL. Additionally, five year-specific QTLs were detected for BD, explaining 3.5-12.8% of the phenotypic variance in different years (Table 2.4, Fig. 2.3).

The approximate locations of BD QTLs in the T×E *Prunus* reference map, detected in this and previous studies in *Prunus*, were shown in Supporting Information Fig. S2.2. Among 10 BD QTLs detected in this study, four (qBD2, qBD4, qBD7b-2007, qBD7a) have overlapping intervals with previously reported QTLs, two on G1 (qBD1c-2007, qBD1d-2008) closely flanked a previously reported QTL. The other two QTLs, qBD1a (overlapping with *evg* locus) and qBD5, were in the similar positions with two QTLs poster-reported by Howad & Arús in 2007 Plant & Animal Genome XV Conference (not shown in Supporting Information Fig. S2.2). No QTL found in this study harbors the *Lb* locus (Supporting Information Fig. S2.2).

The full regression models for BD QTLs explained 52 to 74.1% of phenotypic variances in different years (Table 2.4).

Comparison across traits Based on one- and two-LOD confidence intervals (CIs), all QTLs were diagrammed in Fig. 2.3. Comparison of QTL CIs indicated that all CR QTLs essentially shared the same or overlapping genomic regions with BD QTLs, except two with minor effects (qCR4b, qCR8). Among year-recurrent QTLs, one major CR QTL (qCR1a) and one CR QTL with a large effect (qCR7) shared common genomic regions with two major BD QTLs (qBD1a and qBD7a). Four BD QTLs did not have overlapping CIs with any CR QTLs. However, only one (qBD2) of these four is a year-recurrent QTL.

The year-recurrent HR QTL (qHR1, G1/87) shared the same genomic region with one major CR QTL (qCR1a) and one major BD QTL (qBD1a), while the year-specific HR QTL (qHR8-2008) only shared the same genomic region with one CR QTL (qCR8-2008).

All QTLs for CR and BD, except two on G6 (qCR6-2008 and qBD6-2008), had negative additive effects, while both QTLs for HR had positive additive effects (Table 2. 2, 2. 3, 2. 4). Since QTL alleles inherited from the male grandparent were assumed superior when calculating additive effects, this result could be interpreted as QTL genotypes for BD having the same direction with those for CR, but the opposite with those for HR, i.e., QTL alleles from the high CR grandparent favored higher CR and later BD, but lower HR. This was consistent with positive correlations between BD and CR

and negative correlations (albeit not significant or weak) between BD and HR (Table 2.1).

Two QTLs on G6 showing minor effects exhibited exactly the opposite behavior, i.e. QTL alleles from the high CR grandparent favored low CR and earlier BD (Table 2.2, 2.4).

Discussion

Influence of CR Evaluation Models on CR QTL Mapping

In woody plants, many models have been developed to evaluate the CR for the release of endo-dormancy. Most of these models fall into two categories: chilling hour models and chilling unit models (Cesaraccio et al., 2004). The chilling hour models count the number of hours when the air temperature is in a certain range, and assume that all air temperatures in this range are equally effective. The $<7.2^{o}$C (Weinberger, 1950) and 0-7.2^{o}C models (Eggert, 1951) are two most often used models in this category. In the chilling unit models, different weighting factors are assigned to temperatures in different ranges. High temperatures above a limit are considered to reverse the chilling effects of lower temperatures and negative chill units are assessed for them (Cesaraccio et al., 2004). The Utah model (Richardson, et al., 1974) and Low Chill model (Gilreath & Buchanan, 1981) are two popular chilling unit models in temperate regions (Cesaraccio et al., 2004).

The Dynamic model (Fishman et al., 1987; Erez et al., 1988) is a two-step chilling unit model developed for evaluating CR of tree species in warm winter regions such as Israel and California in US. It assumes a biochemical basis for endo-dormancy release. The first step produces a reversible intermediate of the substance for endo-dormancy release and the second one fixes the intermediate by an irreversible transition. This model can account for not only the apparent negative effect of the high temperature, but also the varying effect of the same temperature in different daily temperature cycles (Erez et al., 1988).

We should keep in mind that, because of lack of knowledge of biochemical or physiological mechanisms controlling CR, almost all CR models were developed empirically or statistically to fit the responses (mainly bloom dates) of tree species to local weather conditions. A model appropriate for one species/genotype growing in one area may not necessarily fit another species/genotype growing in another area. In warm winter regions, the reliability of different CR models is different (Erez et al., 1990). The southeastern US, where our peach mapping population is maintained and phenotyped, is a variable warm winter region with potential low or high chilling accumulations in different years. If we choose an inappropriate CR model, the resultant CR phenotypic data may not accurately show the differences among genotypes and significantly affect the accuracy of CR QTL mapping. In order to resolve this issue, we evaluated CR based on two chilling hour models (the $<7^{o}C$ and $0\text{-}7^{o}C$ models) and three chilling unit models (the Utah, Low Chill and Dynamic models). CR phenotypic data based on different models were significantly and highly correlated ($r=1$, $P<0.001$). This correlation could be

due to a lack of long periods of warm and fluctuating temperatures, so that chilling accumulations based on different models all steadily increased in a similar trend through the two winters (Supporting Information Fig. S2.3). The variable weather also tends to cancel out the differences among different models, e.g. the $<7^{o}$C model does not count temperatures above 7°C but counts sub-freezing temperatures, in contrast to the Utah and LC models (Cesaraccio et al., 2004). The high correlations of CR phenotypic data resulted in very similar QTL mapping results. Except for one (year 2007/2008) or two (year 2008/2009) QTLs showing minor effects, QTL positions and magnitudes mapped with these CR data were nearly the same (Table 2.2). Based on these results, we believe that, at least in years 2007/2008 and 2008/2009 in the experimental site, the influence of different CR models for CR QTL mapping was minor and our results reliable.

Genetic Control of CR

Previous genetic studies in apple and apricot indicated the dominance of low CR character resulting from the involvement of at least one (major) dominant gene (Oppenheimer & Slor, 1968; Hauagge & Cummins, 1991; Tzonev & Erez, 2003). At first glance, our research appeared to show the dominance of low CR character as well: low CR genotypes obviously dominate in the F_2 mapping population (Fig. 2.1a, b). However, a pure additive model of gene action best fits the CR phenotypic data, which means none of the detected QTLs for CR showed significant dominance or even partial dominance favoring low CR alleles. Interestingly, distorted marker genotypic ratios provide a valuable hint to resolve the contradiction in this experiment. A cluster of 17 markers

mapped to a large genomic region (68-96.4cM) in the bottom part of G1 was found to have seriously distorted genotypic ratios favoring the allele from the low CR grandparent. This region covers the confidence interval (CI) of qCR1a, a major QTL explaining more than 40% of phenotypic variance of CR (Fig.2.3, Table 2.2). Apparently, it was the distorted genotypic ratio of the CR major QTL alleles, instead of the dominance of the QTL allele favoring low CR trait, that cause the phenomenon of the low CR dominance in peach. More evidence is needed to know if this also occurs in other tree species mentioned above.

It is not clear what causes the distortion of the marker genotypic ratio in this large genomic block. Very likely, this region might harbor the gene(s) controlling important traits such as gamete fertility, seed formation or seed germination (seed dormancy). The tight linkage of the allele(s) of this (these) gene(s) having better fitness with the allele of the major QTL favoring low CR could explain the contradiction above. Another interesting hypothesis is that maybe both the stratification requirement for seed dormancy breaking and CR for winter bud dormancy breaking are controlled by a similar set of genes. Therefore, seeds with low stratification requirement germinate more easily, which result in more trees (genotypes) with low CR. However, these hypotheses need to be tested by future studies.

To our knowledge, this is the first successful and comprehensive report on floral bud CR QTL analysis in a perennial tree species. The detection of the CR QTLs, especially two year-recurrent QTLs with large effects (qCR1a and qCR7), not only will

facilitate the marker assisted breeding for low CR cultivars, but also pave the way for future fine mapping and map-based cloning of genes controlling CR. The two-LOD CI of the major CR QTL (qCR1a) spans only 2 cM, which overlaps with the peach *evg* region (Fig. 2.3). The peach *Evergrowing* (previously known as *Evergreen*) mutant was originally identified in Mexico. In temperate regions, its terminal apices keep growing until they are killed by subfreezing winter temperatures (Rodriguez et al., 1994). The *evg* locus was genetically mapped as a "recessive gene" (Wang et al., 2002; Bielenberg et al., 2008). A 132kb genomic region around *evg* was cloned, sequenced and annotated utilizing the peach 'Nemared' BAC library. The mutant harbors a sizable deletion, which spans all or part of four MADS box genes. Two additional MADS box genes adjacent to the deletion are also not expressed in the mutant (Bielenberg et al., 2004; Bielenberg et al., 2008). Although it is still unclear whether this nondormant (or very low CR) mutation affects the induction of endo-dormancy or has something to do with CR, the co-localization of a CR major QTL and the sequenced *evg* region makes the six identified MADS-box genes promising candidate genes for CR of peach floral buds.

Currently, the CR QTL mapping on another peach F_2 population derived from two different grandparents and association mapping using peach germplasms with different CR are in progress. With these efforts, we aim to verify and refine the CR QTL regions to better suit the needs of maker assisted breeding and map-based cloning of important genes for CR.

Co-localization of QTLs for CR, HR and BD

Bloom date (BD) in *Prunus* is determined by the cultivar's CR needed to break endo-dormancy as well as HR (Andrés & Durán, 1999). While it is known that CR and BD of *Prunus* are genetically controlled (Anderson & Seeley, 1993; Tzonev & Erez, 2003), genetic characterization has not been reported and controversy exists as to whether genetic components are involved in the HR for bloom in *Prunus*. It was found that prolonged exposure to low temperature reduces HR (Couvillon & Erez, 1985; Citadin et al, 2001). Couvillon & Erez (1985) pointed out that excessive chilling in several fruit tree species results in 90% of HR variations among different cultivars with different CRs and there is no actual (genetic) difference in HR for bloom among different cultivars. Okie & Blackburn (2008) confirmed that artificially supplied, incremental chilling dramatically reduced HR for bud break in peach when shoots were under-chilled, but they found the effects diminished when buds received more chilling. Recently, Harrington et al. (2009) proposed a model, whereby between the critical CR and optimum CR, many combinations of chilling units and forcing (heat) units could make the budbreak possible, implying a possible overlapping period of CR and HR fulfilling after a tree's critical CR was met.

If Couvillon & Erez (1985) are correct in proposing that no genetic differences for HR among fruit tree cultivars, then in our study, low CR genotypes would be over-chilled and require less heat accumulation for the bloom than high CR genotypes. In fact, low CR genotypes have high HRs for bloom and we found a significant negative correlation between CR and HR in the mapping population (Table 2.1). The negative correlation between CR and HR was also reported in apricot (Ruiz et al., 2007) and some peach

selections from Aguascalientes, Mexico were found to have low CR, but late BD (Scorza & Okie, 1990). These results suggest the existence of different HRs among genotypes/cultivars and a potential genetic contribution to this character.

In our study, HR segregated in a wide range (Fig. 2. 1c, d). The analysis of variance for HR indicated a significant genotypic effect ($p<0.01$) (Supporting Information Table S2.3). Two QTLs for HR, accounting for 8.6 -10.7% of phenotypic variance, were detected (Table 2.3). Therefore, we believe that the genetic components played some limited roles in determining HR of each genotype in our mapping population.

In our study, the distribution of BD varied dramatically across years (Fig. 2.2). Both environmental (year) effects and genotype (QTL) × environment interaction effects for BD significantly contributed to the variation of this character (Supporting Information Table S2.3). The variable chilling and heat accumulations in different years could be the major sources of environmental effects (Supporting Information Fig. S2.3, S2.4). Exactly how the genotype × environment interaction influences BD is unknown. But very possibly the variable temperatures interact with different genotypes and affect their CR and HR and finally BD, because the genotype × environment interactions for CR and HR are also significant (Supporting Information Table S2.3).

The extensive overlapping of CIs of QTL for different traits was illustrated in Fig. 2.3. Two major BD QTLs (qBD1a and qBD7a) co-localize with one major CR QTL (qCR1a) and one CR QTL with a large effect (qCR7). Moreover, despite the negligible or

weak correlations between HR and BD (Table 2.1), the HR QTL qHR1 co-localizes with the major QTL for CR and BD. Furthermore, among all 20 QTLs for three traits, only three BD QTLs and one CR QTL neither co-localize nor overlap with any QTL for other traits (Fig. 2.3). These non-co-localized QTLs either explained a small portion of phenotypic variance or were detected only in one year (Table 2.2, 2.3, 2.4; Fig. 2.3), implying that the non-co-localization could be due to: a low power of detection for QTLs with minor effects for some traits; unavoidable human errors in phenotyping; or the fact that these QTLs are not real. The co-localization of the majority of the detected QTLs might suggest that in each co-localization case, the genes regulating different traits are tightly linked together. But considering the significant correlation of phenotypic data between CR and HR or CR and BD, more probably, it suggests the pleiotropy of these QTLs and the existence of one unified temperature sensing and action system, of which some components regulate both CR and HR, and others only regulate CR. The regulation of gene expression in this system should generally guarantee late BDs for high CR cultivars and early BDs for low CR cultivars. It should also up-regulate the HR for low CR peach cultivars so that they could be generally protected from flower or fruit damages by late spring frosts.

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Table 2.1 Spearman correlation coefficients (r) of chilling requirement (CR, <7.2ºC model), heat requirement (HR) and bloom date (BD) in the Contender×Fla.92-2C peach population in different years

	CR2009	HR2008	HR2009	BD2007	BD2008	BD2009
CR2008	0.723	-0.653			0.698	
CR2009			-0.820			0.672
HR2008			0.379		-0.014	
HR2009						-0.188
BD2006				0.738	0.735	0.704
BD2007					0.831	0.784
BD2008						0.821

All correlations are significant ($p<0.001$), except for that between HR2008 and BD2008 ($p=0.793$). CR2008 and CR2009, CR data obtained in winter2007/spring2008 and winter2008/spring2009, respectively; H2008 and HR2009, HR data obtained in winter2007/spring2008 and winter2008/spring2009, respectively

Table 2.2 Quantitative trait loci (QTLs) detected for chilling requirement (CR) calculated by the different CR models with the Contender×Fla.92-2C peach population in different years (winter/springs)

Year	QTL	G/Pos	CI	Co-factor	LOD	Part R^2%	Add	R^2%
2007/2008								
<7.2°C	qCR1a-2008	G1/87	86-88	Pchgms29	44.52	44.8	-0.83	55.7
	qCR4a-2008	G4/7	4-19	ssrPaCITA6	9.77	9.7	-0.32	
	qCR4b-2008	G4/54	41-62	AMPA103	2.95	4.1	-0.20	
	qCR5-2008	G5/32	24-38	ssrPaCITA21	3.79	4.5	-0.20	
	qCR6-2008	G6/41	35-43	EPPISF002	3.29	4.2	0.19	
	qCR7-2008	G7/48	43-59	UDAp-409A	16.95	17.8	-0.46	
	qCR8-2008	G8/51	39-54	PacC13	3.60	4.4	-0.20	
0-7.2°C	qCR1a-2008	G1/87	86-88	Pchgms29	44.87	42.6	-0.81	53.9
	qCR4a-2008	G4/5	1-12	MD205a	9.00	8.9	-0.31	
	qCR4b-2008	G4/54	41-62	AMPA103	4.27	4.4	-0.21	
	qCR5-2008	G5/32	19-35	ETGMCAG(80)	3.73	4.3	-0.21	
	qCR6-2008	G6/41	35-43	EPPISF002	3.14	3.8	0.18	
	qCR7-2008	G7/49	44-60	UDAp-409A	15.89	17.1	-0.45	
	qCR8-2008	G8/51	41-54	PacC13	4.48	5.2	-0.23	
LC	qCR1a-2008	G1/87	86-88	Pchgms29	46.08	43.5	-0.81	55.0
	qCR4a-2008	G4/6	0-12	MD205a	8.18	6.4	-0.26	
	qCR4b-2008	G4/50	41-62	AMPA103	3.90	4.0	-0.22	
	qCR5-2008	G5/34	24-38	ssrPaCITA21	4.34	6.1	-0.23	
	qCR7-2008	G7/49	44-57	UDAp-409A	19.54	20.1	-0.50	
	qCR8-2008	G8/51	40-54	PacC13	4.57	5.2	-0.22	
Utah	qCR1a-2008	G1/87	86-88	Pchgms29	47.62	43.6	-0.81	55.6
	qCR4a-2008	G4/6	0-12	MD205a	8.74	7.9	-0.29	
	qCR4b-2008	G4/58	41-62	AMPA103	4.11	4.2	-0.21	
	qCR5-2008	G5/34	24-38	ssrPaCITA21	4.23	6.0	-0.22	
	qCR7-2008	G7/49	44-57	UDAp-409A	20.04	20.4	-0.50	
	qCR8-2008	G8/51	41-54	PacC13	4.81	5.4	-0.23	
Dynamic	qCR1a-2008	G1/87	86-88	Pchgms29	49.54	44.7	-0.82	56.3
	qCR4a-2008	G4/6	1-12	MD205a	9.26	8.2	-0.29	
	qCR4b-2008	G4/58	41-62	AMPA103	4.35	4.5	-0.22	
	qCR5-2008	G5/33	24-38	ssrPaCITA21	4.43	6.2	-0.23	
	qCR7-2008	G7/49	44-57	UDAp-409A	19.89	20.1	-0.49	
	qCR8-2008	G8/51	41-54	PacC13	4.47	5.0	-0.22	
2008/2009								
<7.2°C	qCR1d-2009	G1/6	0-13	UDA-053	6.71	7.6	-0.29	54.3
	qCR1a-2009	G1/87	86-88	pchgms40	18.37	40.5	-0.78	
	qCR4b-2009	G4/46	40-55	M12a	2.92	5.9	-0.26	
	qCR5-2009	G5/33	24-38	ssrPaCITA21	3.96	4.6	-0.20	
	qCR7-2009	G7/47	43-51	CPPCT033	25.68	24.9	-0.58	

Table 2.2 Quantitative trait loci (QTLs) detected for chilling requirement (CR) calculated by the different CR models with the Contender×Fla.92-2C peach population in different years (winter/springs) (Continued)

Year	QTL	G/Pos	CI	Co-factor	LOD	Part R^2%	Add	R^2%
2008/2009								
0-7.2°C	qCR1d-2009	G1/6	0-13	UDA-053	6.75	7.7	-0.29	54.5
	qCR1a-2009	G1/87	86-88	pchgms40	18.65	40.8	-0.78	
	qCR4b-2009	G4/45	40-55	M12a	2.91	5.9	-0.25	
	qCR5-2009	G5/34	24-38	ssrPaCITA21	4.12	4.7	-0.20	
	qCR7-2009	G7/47	43-51	CPPCT033	25.76	25.0	-0.58	
LC	qCR1a-2009	G1/87	86-88	pchgms40	7.95	40.5	-0.81	50.2
	qCR5-2009	G5/34	24-38	ssrPaCITA21	3.40	3.3	-0.18	
	qCR7-2009	G7/48	44-52	CPPCT033	24.28	22.0	-0.56	
	qCR8-2009	G8/52	36-54	PacC13	2.87	2.6	-0.17	
Utah	qCR1d-2009	G1/5	0-13	UDA-053	6.58	7.9	-0.29	55.0
	qCR1a-2009	G1/87	86-88	pchgms40	19.19	41.4	-0.79	
	qCR4b-2009	G4/46	40-55	M12a	3.55	5.5	-0.25	
	qCR5-2009	G5/34	24-38	ssrPaCITA21	4.00	4.8	-0.20	
	qCR7-2009	G7/48	44-52	CPPCT033	25.52	25.2	-0.58	
Dynamic	qCR1d-2009	G1/5	0-13	UDA-053	6.47	8.0	-0.29	55.9
	qCR1a-2009	G1/87	86-88	pchgms40	19.01	42.3	-0.79	
	qCR4b-2009	G4/46	40-55	M12a	3.46	6.2	-0.26	
	qCR5-2009	G5/34	24-38	ssrPaCITA21	3.87	4.6	-0.20	
	qCR7-2009	G7/48	44-52	CPPCT033	25.46	26.3	-0.59	
	qCR8-2009	G8/53	36-54	PacC13	3.11	3.4	-0.18	

G/Pos, linkage group/QTL position (cM); CI, two-LOD or approximately 99% confidence interval (cM); Part R^2%, percentage of phenotypic variance explained by one QTL when other QTL effects are fixed; Add, additive QTL effect divided by the SD of the trait value, the male grandparent is assumed to carry the superior QTL allele; R^2%, percentage of the phenotypic variance explained by all QTLs with the adjustment for the number of QTL terms in the full regression model.

Table 2.3 Quantitative trait loci (QTLs) detected for heat requirement (HR) with the Contender×Fla.92-2C peach population in different years (winter/springs)

Year	QTL	G/Pos	CI	Co-factor	LOD	Part R^2%	Add	R^2%
2007/2008	qHR1-2008	G1/87	86-89	pchgms29	6.06	7.1	0.37	8.6
	qHR8-2008	G8/50	36-54	PacC13	2.94	3.1	0.25	
2008/2009	qHR1-2009	G1/87	86-88	Pchgms40	7.81	11.2	0.47	10.7

G/Pos, linkage group/QTL position (cM); CI, two-LOD or approximately 99% confidence interval (cM); Part R^2%, percentage of phenotypic variance explained by one QTL when other QTL effects are fixed; Add, additive QTL effect divided by the SD of the trait value, the male grandparent is assumed to carry the superior QTL allele; R^2%, percentage of the phenotypic variance explained by all QTLs with the adjustment for the number of QTL terms in the full regression model.

Table 2.4 Quantitative trait loci (QTLs) detected for bloom date (BD) with the Contender×Fla.92-2C peach population in different years (springs)

Year	QTL	G/Pos	CI	Co-factor	LOD	Part R^2%	Add	R^2%
2006	qBD1b-2006	G1/50	43-56	Pchgms3	12.71	12.8	-0.42	52.0
	qBD1a-2006	G1/87	86-88	Pchgms40	15.78	15.2	-0.45	
	qBD2-2006	G2/27	20-31	ECAMCCG(99)	3.88	4.5	-0.26	
	qBD4-2006	G4/11	6-28	ssrPaCITA6	6.75	9.5	-0.31	
	qBD7a-2006	G7/44	43-47	CPPCT033	31.27	30.4	-0.66	
2007	qBD1c-2007	G1/33	27-42	UDP-005	7.84	10.0	-0.26	73.1
	qBD1a-2007	G1/87	86-88	Pchgms40	11.95	49.4	-0.73	
	qBD2-2007	G2/31	23-37	EPPCU4962A	3.35	4.5	-0.16	
	qBD4-2007	G4/9	4-23	ssrPaCITA6	18.16	19.6	-0.37	
	qBD7b-2007	G7/18	13-22	CPPCT022	3.90	3.5	-0.17	
	qBD7a-2007	G7/44	43-47	CPPCT033	24.90	41.5	-0.72	
2008	qBD1d-2008	G1/0	0-1	CPPCT10B	2.94	4.0	-0.14	74.1
	qBD1a-2008	G1/87	86-89	Pchgms29	34.56	54.5	-0.77	
	qBD2-2008	G2/22	20-31	ECAMCCG(99)	6.77	8.6	-0.23	
	qBD4-2008	G4/10	5-24	ssrPaCITA6	16.43	19.9	-0.36	
	qBD5-2008	G5/27	24-38	ssrPaCITA21	3.37	4.2	-0.16	
	qBD6-2008	G6/35	34-42	UDP-412	3.43	4.0	0.14	
	qBD7a-2008	G7/43	41-44	UDAp-460	45.95	55.2	-0.81	
2009	qBD1a-2009	G1/87	86-88	Pchgms40	24.34	41.3	-0.76	58.0
	qBD2-2009	G2/23	20-31	ECAMCCG(99)	6.52	7.0	-0.26	
	qBD4-2009	G4/21	8-33	ssrPaCITA6	8.17	8.5	-0.33	
	qBD5-2009	G5/31	24-38	ssrPaCITA21	4.91	6.4	-0.24	
	qBD7a-2009	G7/43	40-44	UDAp-460	32.54	32.6	-0.65	

G/Pos, linkage group/QTL position (cM); CI, two-LOD or approximately 99% confidence interval (cM); Part R^2%, percentage of phenotypic variance explained by one QTL when other QTL effects are fixed; Add, additive QTL effect divided by the SD of the trait value, the male grandparent is assumed to carry the superior QTL allele; R^2%, percentage of the phenotypic variance explained by all QTLs with the adjustment for the number of QTL terms in the full regression model.

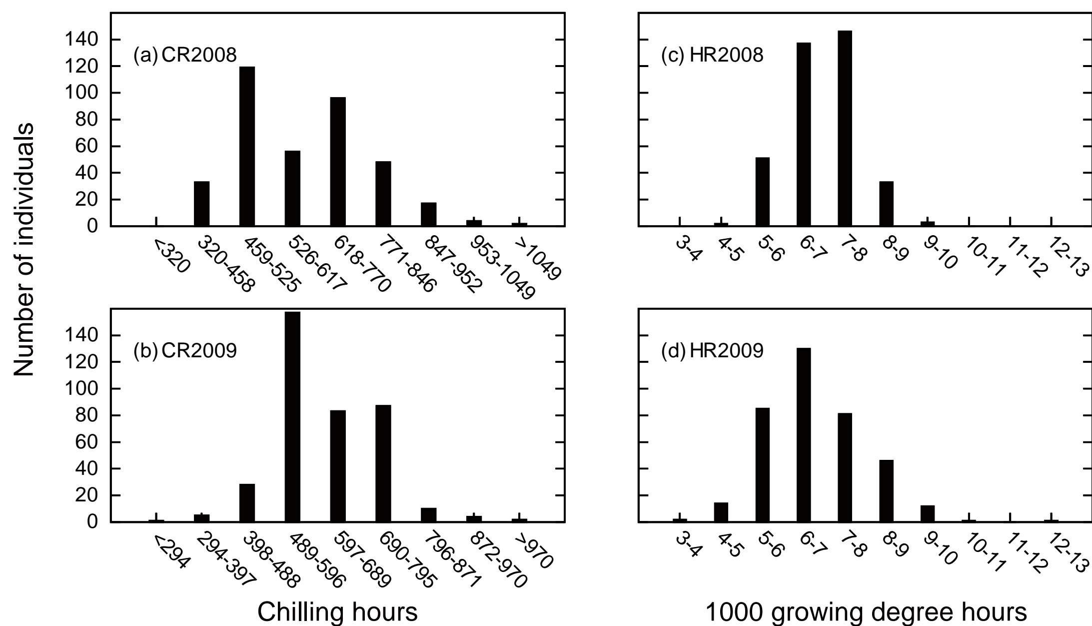


Fig. 2.1 Frequency distributions of chilling requirement (CR) and heat requirement (HR) for floral bud break in the Contender×Fla.92-2C peach population. (a, b) CR evaluated in year (winter/spring) 2007/2008 (CR2008) and 2008/2009 (CR2009) with approximately 100 chilling hours interval and the <7°C model; (c, d) HR evaluated in year 2007/2008 (HR2008) and 2008/2009 (HR2009) with the growing degree hour (GDH) model.

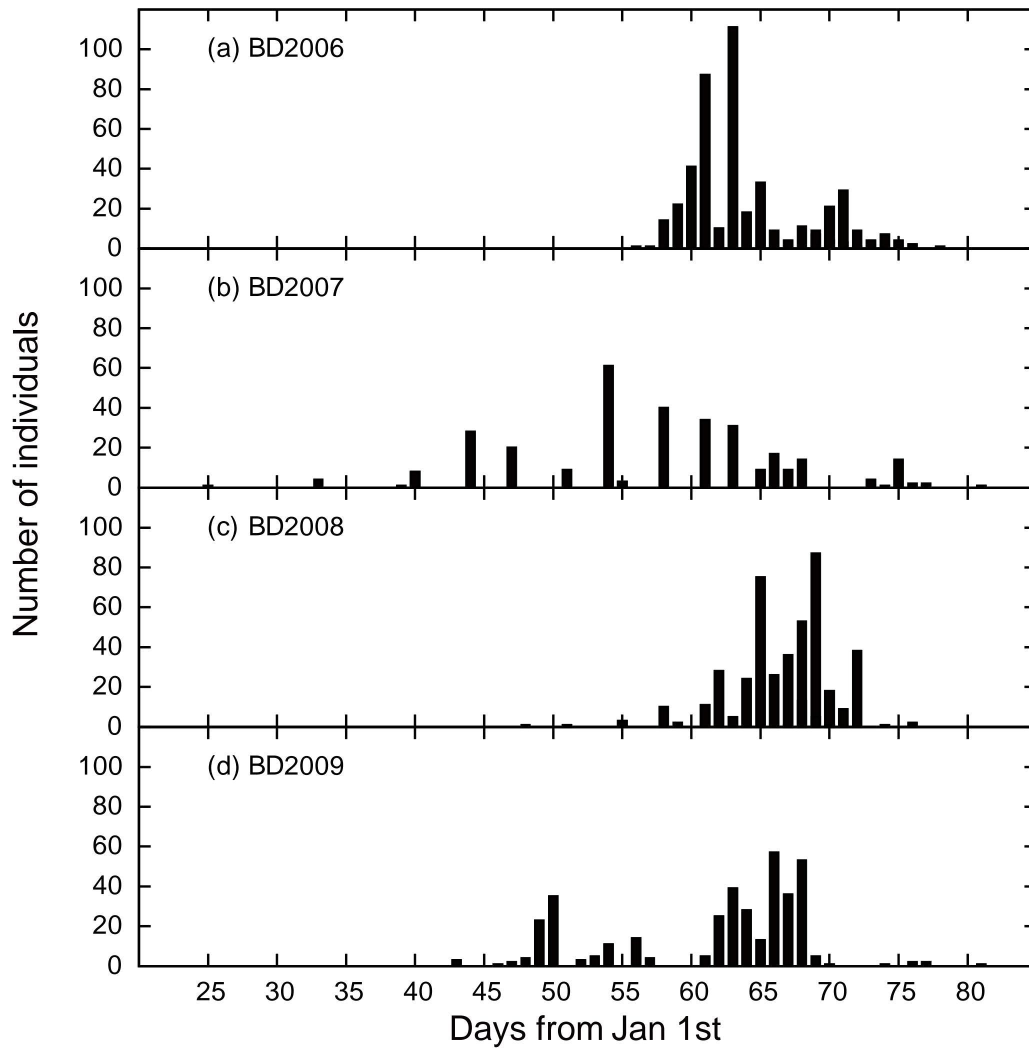
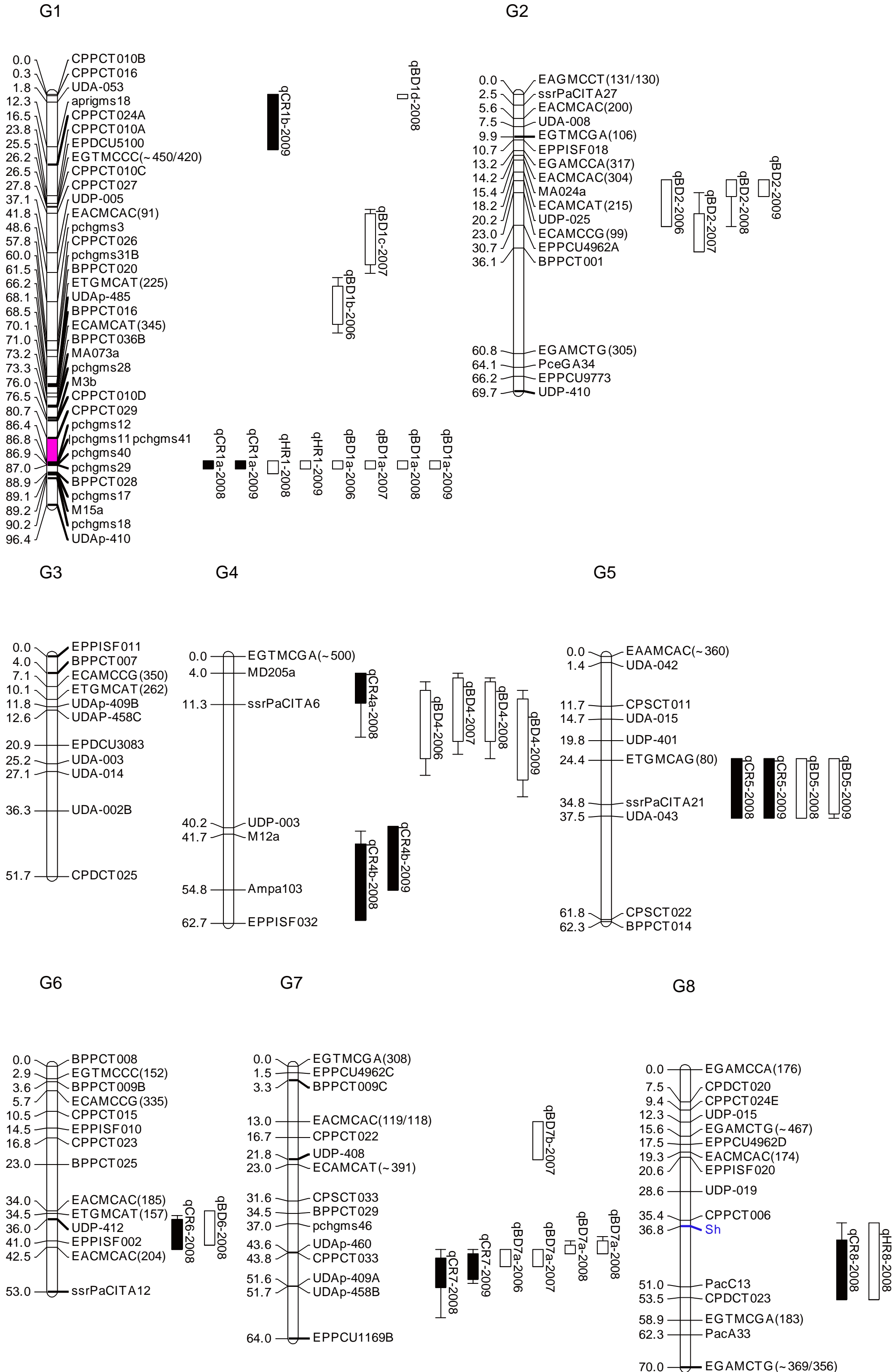


Fig. 2.2 Frequency distributions of bloom dates (BDs) in the Contender×Fla.92-2C peach population scored in year 2006 (a), 2007 (b), 2008 (c) and 2009 (d).



G1
G2
G3
G4
G5
G6
G7
G8

Fig. 2.3 Location of quantitative trait loci (QTLs) for chilling requirement (CR), heat requirement (HR) and bloom date (BD) on the Contender×Fla.92-2C peach map. The solid or whisker parts of vertical bars next to the linkage groups (Gs) indicate one-LOD intervals (approximately 95% confidence intervals) or two-LOD intervals (approximately 99% confidence intervals) of QTLs for different traits, which are differentiated by the styles of solid part of bars: the *filled black* for CR, the *crosshatch* for HR and the *open* for BD. A QTL is named as qXXYa-ZZZZ , with "XX" being the trait acronym, "Y" the number of linkage group, "a" the letter to specify different QTLs for the same trait in one linkage group (G), "ZZZZ" the year in which the trait was phenotyped. Markers with names in *Italic* have significantly distorted genotypic ratios ($P<0.05$). The highlighted fragment of G1 covers *evg* locus (Bielenberg et al., 2008).

Supporting information

Table S2.1 Chilling accumulation calculated by the different chilling requirement (CR) models on each sampling date in different years (winter/springs)

Date	<7.2°C (CH)	0-7.2°C (CH)	Utah (CU)	LC (CU)	Dynamic (CP)
2007/2008					
12/6/2007	320	290	124	222	10
12/21/2007	458	395	212	359	15
12/31/2007	525	455	353	517	22
1/5/2008	617	486	381	550	25
1/18/2008	770	622	483	677	31
1/22/2008	846	674	522	704	33
1/28/2008	952	753	571	751	37
2/7/2008	1049	832	638	857	42
2008/2009					
11/25/08	294	251	203	322	13
12/2/08	397	338	283	389	18
12/7/08	488	394	331	432	21
12/22/08	596	463	410	621	27
1/2/09	689	524	502	766	32
1/13/09	795	596	597	899	39
1/17/09	871	632	633	932	41
1/22/09	970	694	682	970	45

CH, chilling hours; CU, chilling units; CP, chilling portions; LC, Low Chilling model

Table S2.2 Summary of the tested *Prunus* SSR markers

Species	SSR series	Origin	Reference	Tested	Mapped
P. persica	M	cDNA library	Yamamoto et al., 2000	6	3
	EPPB	cDNA library	Dirlewanger, personal comm	4	0
	EPPCU	cDNA library	GDR; Howad, personal comm	24	5
	EPPISF	cDNA library	Vendramin et al, 2007; Verde, personal comm	32	6
	UDP	Genomic library	Cipriani et al., 1999; Testolin et al., 2000	18	9
	pchgms	Genomic library	Sosinski et al., 2000; Wang et al., 2002; Abbott's lab	31	11
	CPPCT	Genomic library	Aranzana et al., 2002; Howad, personal comm	26	15
	BPPCT	Genomic library	Dirlewanger et al., 2002	33	12
	MA	Genomic library	Yamamoto et al., 2002; Yamamoto et al., 2005	17	2
	MD	Gene sequences	Yamamoto et al., 2005	2	1
P. armeniaca	AMPA	cDNA library	Hagen et al., 2004	2	0
	Pac	cDNA library	Decroocq et al., 2003	9	2
	AMPA	Genomic library	Hagen et al., 2004	5	1
	ssrPaCITA	Genomic library	Lopes et al., 2002	21	4
	UDAp	Genomic library	Messna et al., 2004; GenBank	27	7
	aprigms	Genomic library	Lalli et al., 2008	2	1
P. dulcis	EPDCU	cDNA library	GDR	12	2
	EPDC	Genomic library	Howad, personal comm	2	0
	CPDCT	Genomic library	Mnejja et al., 2005	12	3
	UDA	Genomic library	Testolin et al., 2004; GenBank	44	8
P. avium	PS	Genomic library	Joobeur et al., 2000; Sosinski et al., 2000	6	0
	PceGA	Genomic library	Downey & Jezzoni, 2000; Cantini et al., 2001	2	1
	PMS	Genomic library	Cantini et al., 2001	1	0
	UCD-CH	Genomic library	Struss et al., 2003	4	0
	EMPaS	Genomic library	Vaughan & Russell, 2004	10	0
P. salicina	CPSCT	Genomic library	Mnejja et al., 2004	18	3
Total				370	96

GDR, Genome Database for Rosaceae; Tested and mapped, the number of tested and mapped SSR markers.

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Table S2.3. Mean squares (MS) and significance levels of *F*-test from the analysis of variances for chilling requirement (CR), heat requirement (HR) and bloom date (BD) in the Contender×Fla.92-2C peach population in different years

Source	CR(<7°C)	CR(0-7°C)	CR(Utah)	CR(LC)	CR(Dynamic)	HR	BD
E	7.3	248693**	707926**	2899792**	3626**	3.0*	11014**
G	30095**	14205**	16060**	29594**	65.8**	1.5**	118**
QTL	1076163**	499962**	583729**	1062529**	2400.5**	45.5*	6833**
Res	10089**	4914**	5203**	9838**	21.1**	1.2**	27**
G×E	6155**	3231**	2829**	5622**	11.2**	0.7**	17.5**
QTL×E	32251**	21883**	3826	29383**	16.1**	2.7*	460.9**
Res×E	5656	2874	2810	5168	11.1	0.7	10.3
H^2(%)	79.5	77.3	82.4	81	82.9	54	85.2

MS, mean square; E, environments; G, genotypes; Res, residuals; *, Significant at p<0.05 level in F-test; **, significant at p<0.01 level in F-test. H^2, broad sense heritability, due to too few environments (only two years for CR or HR, four years for BD), it could be overestimated. The units for the phenotypes of different traits are as: chilling hours (CH) for CR (<7°C) or CR (0-7°C), chilling units (CU) for CR (Utah) or CR (LC), chilling portions (CP) for CR (Dynamic), 1000 growing degree hours (GDH) for HR, and days after Jan, 1st for BD.

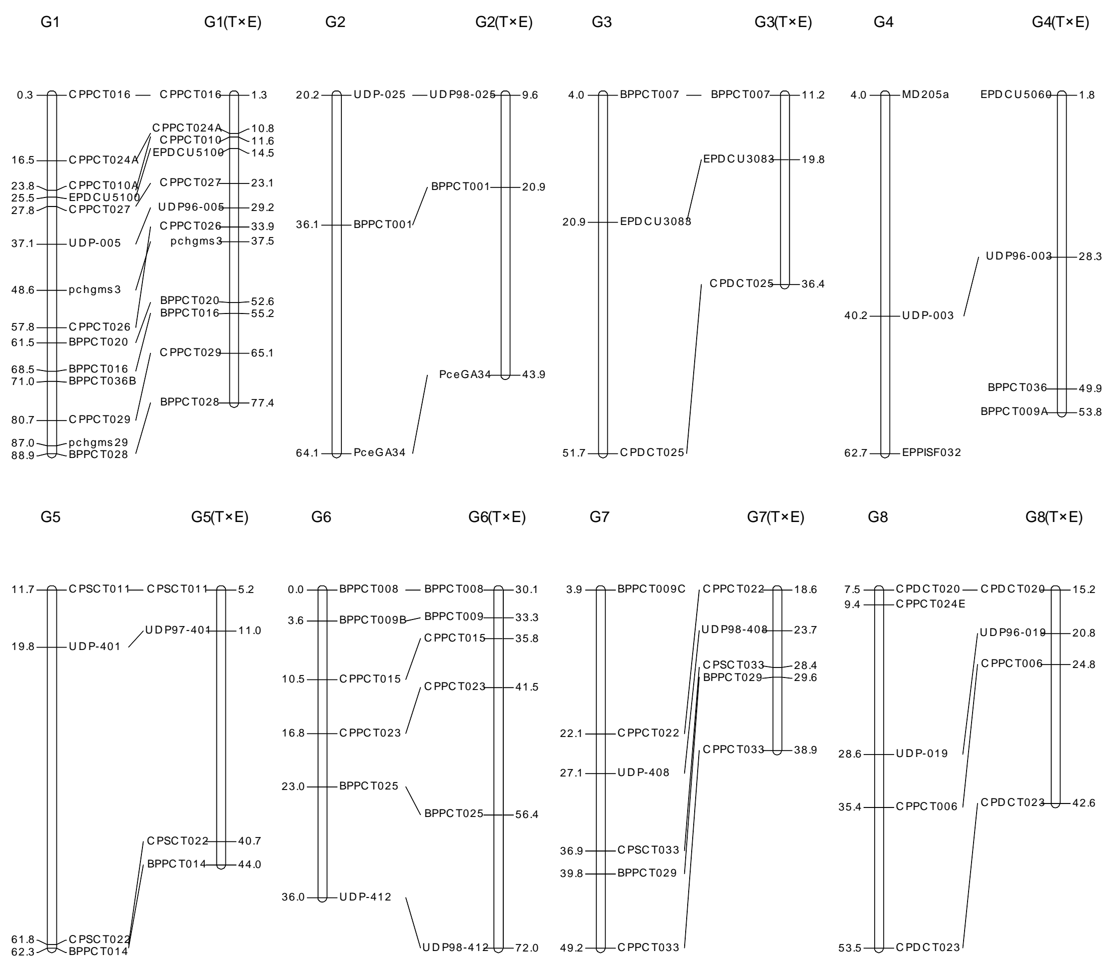

Fig. **S2.1** Alignment of the Contener×Fla.92-2C peach map with the T×E *Prunus* reference map by the shared SSR markers

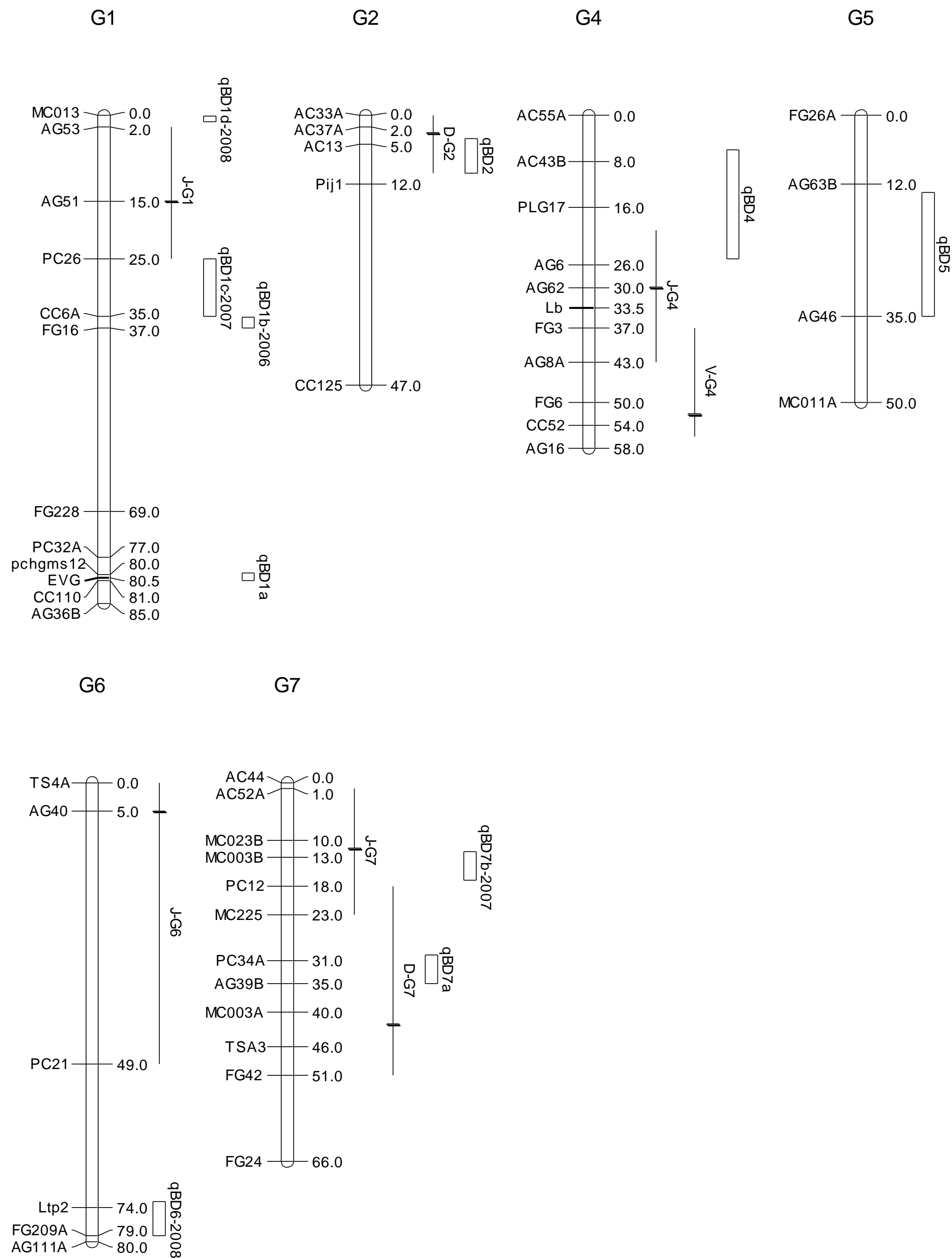

Fig. S2.2 Comparison of BD QTL mapping results in this and previous studies. Linkage groups of the T×E *Prunus* reference map with the approximate locations of quantitative trait loci (QTLs) for bloom date (BD) in *Prunus* detected in this and previous studies were shown.

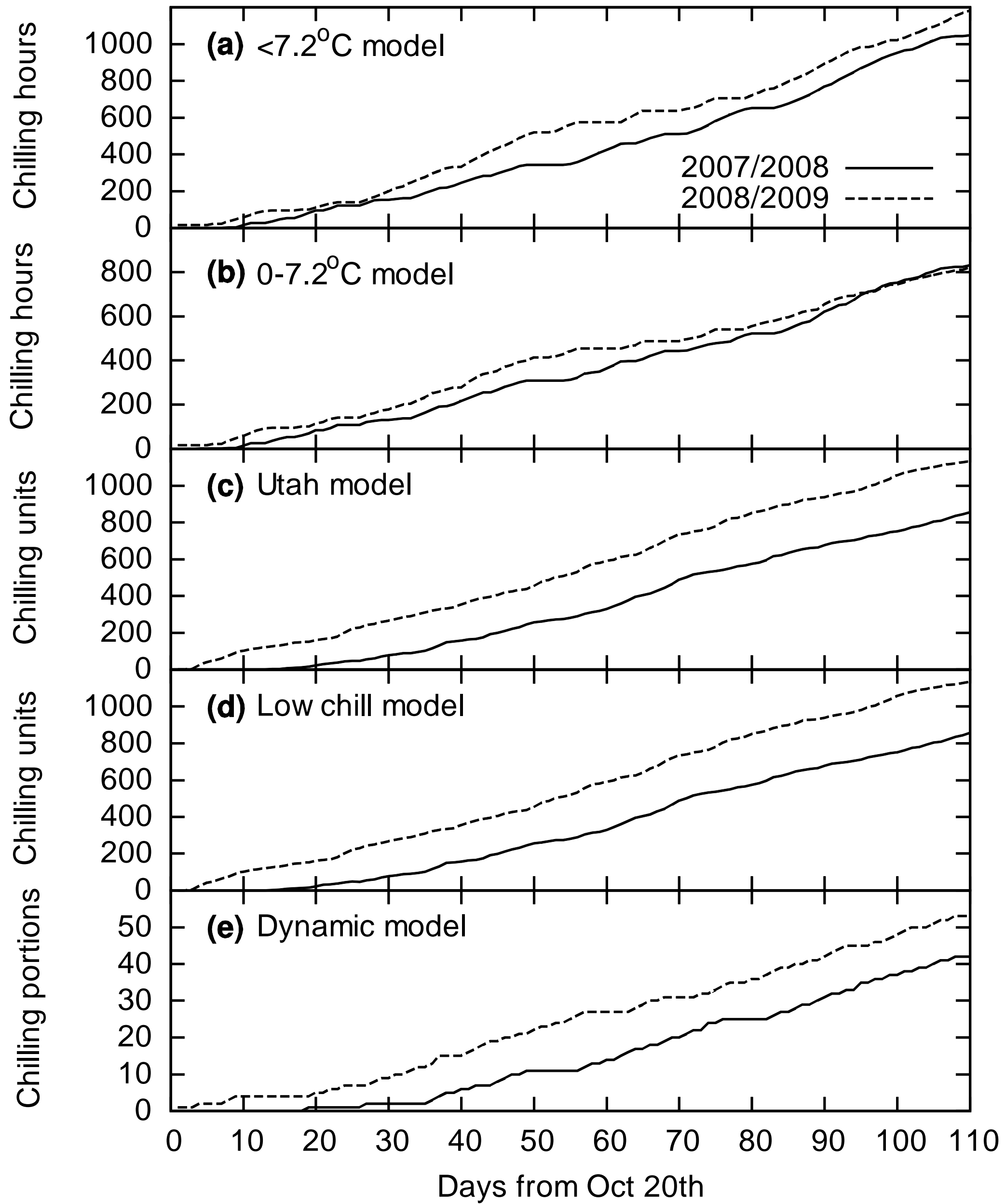

Fig. S2.3 Chilling accumulation calculated with different chilling requirement models in two years (winter/springs) from 20 October 2007/2008 (Seneca, SC, USA)

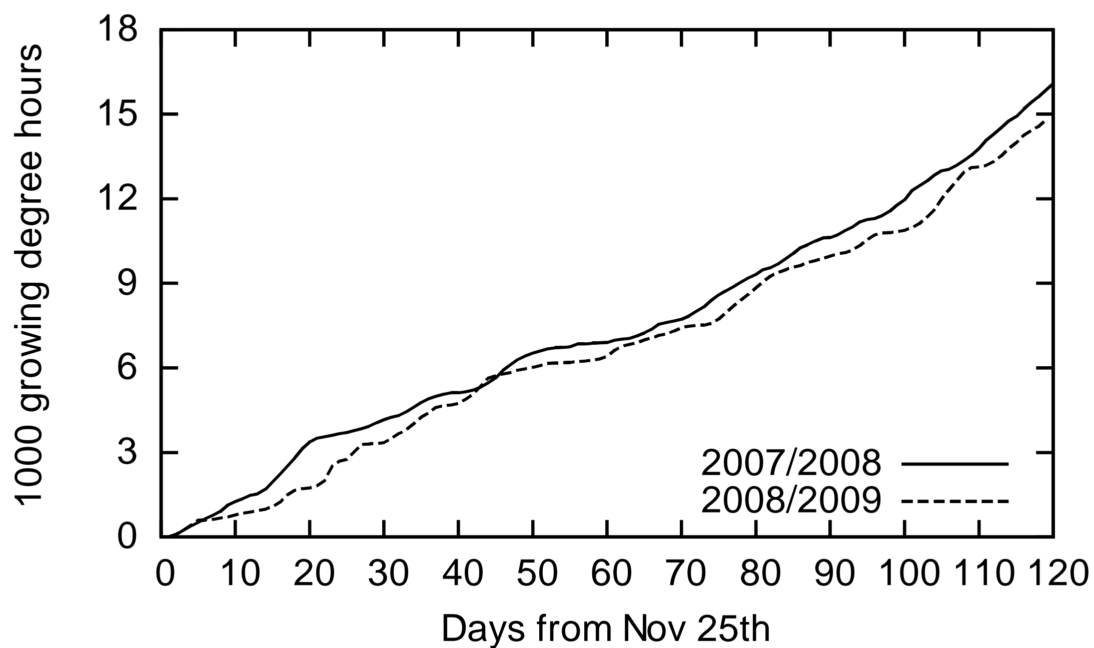

Fig. S2.4 Heat accumulation calculated with growing degree hour model in two years (winter/springs) from 25 November 2007/2008 (Seneca, SC, USA)

CHAPTER THREE

IDENTIFICATION AND FUNCTIONAL TESTING OF CANDIDATE GENES REGULATING CHILLING REQUIREMENT AND BLOOM DATE IN PEACH

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Shenghua Fan conducted association mapping, participated in candidate-gene mapping, engineered the DAM6 overexpressing construct and composed the manuscript. Tetyana N. Zhebentyayeva conducted peach genome sequence mining, SSR marker development and candidate-gene mapping. Douglas G. Bielenberg synthesized DAM6 cDNA. Ralph Scorza conducted peach gene transformation to plum. William R. Okie provided peach germplasm accessions and their CRs for association mapping. Albert G. Abbott supervised the whole project.

Summary

Chilling requirement (CR) is the major factor determining bloom date (BD) in temperate fruit tree species. Both CR and BD are extremely important agronomic traits for adaptation and fruit production in temperate fruit tree species. Although quantitative trait loci (QTLs) for CR and BD in peach have been previously mapped in apricot and peach, the genetic resolution however, was not high enough for the identification of the critical genes controlling the two traits.

Two approaches (candidate-gene mapping and association mapping) were used to investigate candidate genes regulating CR and BD. An Agrobacterium-mediated plum transformation system was used for overexpressing a specific candidate gene, *Dormancy associated MADS-box 6* (*DAM6*).

Seven Polycomb Group (PcG) and associated protein encoding genes were positioned into/close to 2-LOD intervals of previously mapped CR and/or BD QTLs by candidate-gene mapping. Three potential causative genes in/around *evergrowing* (*evg*) region were identified by association mapping. Transgenic plum plants overexpressing *DAM6* showed a dwarfing and more extensive branching phenotype.

In this study, we suggested that PcG and their associated proteins may play roles in controlling CR and BD and *DAM6* is a possible (*FLOWERING LOCUS C*) *FLC* analog in peach. Common components might control both para-dormancy and endo-dormancy. The identification and functional testing of a few important genes regulating CR and BD will lead to the full understanding of CR and BD controlling pathways in the temperate

tree species and also facilitate marker development for the marker assisted breeding in these tree species.

Introduction

Dormancy is “the temporary suspension of visible growth of any plant structure containing a meristem” (Lang, 1987). It can be delineated into three type/stages. “Para-dormancy” is used to describe dormancy when the initial reaction leading to growth control involves a specific signal originating in or initially perceived in a different structure and often referred as apical dominance or correlation inhibition. “Endo-dormancy” is used to describe dormancy when the initial reaction leading to growth control is a specific perception of an environmental or endogenous signal in the affected structure alone. “Eco-dormancy” is used to describe dormancy when one or more factors (temperature, water, etc.) in the basic growth environment are unsuitable for overall growth metabolism (Lang, 1987). Despite the clearly different definitions of three types of dormancy, they often overlap with each other (Faust et al., 1997) and Rohde & Bhalerao (2007) proposed that endo-dormancy might be derived from the evolutionarily older para-dormancy and still share molecular mechanisms with it.

The release of temperate trees from endo-dormancy requires exposure to low temperatures. Chilling requirement (CR) prevents trees from initiating growth in response to transient warm temperatures thus avoiding damage by subsequent frost(s) in the late winter or early spring. CR is the result of long term climatic adaption of genotypes of tree species developed in different regions. Conversely, it limits the climatic distributions of

genotypes of temperate fruit trees (Sherman & Beckman, 2003). In *Prunus*, CR, together with heat requirement (HR) determines bloom date (BD) (Egea et al., 2003; Ruiz et al., 2007; Alburquerque et al., 2008), which is another important agronomic trait affecting seed and fruit development.

Previously, we mapped quantitative trait loci (QTLs) for CR, HR and BD in a peach F_2 population with 378 genotypes. It was found that HR only plays a limited role in affecting BD and almost all QTLs for CR co-localize with those for BD. One major QTL for CR and two major QTLs for BD were detected (Fan et al., 2010). However, most detected QTLs for CR and BD have a 10-20cM of 2-LOD interval, which could harbor hundreds of genes. The major QTLs for CR and BD in G1 cover the shortest 2-LOD interval (2cM) and co-localize with the previously mapped and sequenced peach *evg* region (Wang et al., 2002). The peach *evergrowing* mutant was originally identified in Mexico. It was characterized as insensitivity of shoot tips to day length change and failure of ceasing terminal growth until the apical shoot tip is killed by low temperatures (Rodriguez et al., 1994). The mutant harbors a deletion spanning all or part of four MADS box genes (termed *dormancy-associated* MADS box genes, *DAM*). Two additional *DAM* genes adjacent to the deletion are also not expressed in the mutant (Bielenberg et al., 2004; Bielenberg et al., 2008). Although it is still not clear if endo-dormancy induction or CR were affected by the *evg* mutant, the overlapping of *evg* locus with CR and BD major QTLs makes all genes (especially six MADS-box genes) in *evg* locus promising candidate gene for CR and BD. The *evg* locus harbors 19 annotated genes, which are still many to be functionally tested individually (Wang et al., 2002;

Bielenberg et al., 2004; Bielenberg et al., 2008; Fan et al., 2010). Further effort is needed to significantly shorten the long list and thereby derive a reasonable number of candidate genes for the functional testing with transgenic technologies (overexpression or RNA-inteference).

Association studies using germplasm accessions and cultivated varieties provide an alternative mapping approach that can potentially be used to refine mapped QTL regions and identify candidate genes inside these regions. Association mapping takes advantage of meiotic events that have occurred during past generations in natural populations and can often attain a high genetic resolution for refining coarse QTLs given the potentially low linkage disequilibrium (LD) level among the genotypes in specific genomic intervals. With the integrated peach genetic/physical map (Zhebentyayeva et al., 2008) and assembled whole peach genome sequences (Sosinski B, North Carolina State University, Personal communication), it is possible to exhaustively catalog and genotype all SSRs in QTL intervals and perform association studies to identify potential causative loci (genes) regulating CR and BD.

Candidate-gene study is another approach to identify candidate genes in QTL intervals. In a candidate-gene study, the molecular pathway(s) and candidate genes regulating a complex trait are first hypothesized based on observations made in other plant systems. Markers from these potential candidates are then developed genetically mapped. If these markers are placed into QTL intervals, then these genes become likely candidates for further study (Tabor et al., 2002; Silva et al., 2005). It has long been

known that vernalization of *A. thaliana* is similar to endo-dormancy release of woody perennials in that it requires chilling temperatures to trigger flower bud development (Chouard, 1960). A detailed and systematic knowledge about flowering control in *A. thaliana* is currently available (Bernier & Périlleux, 2005; Henderson & Dean, 2005). Capitalizing on the knowledge gained from this model system researchers working with woody perennial species can only piece together a much more fragmentary picture of this process (Rohde & Bhalerao, 2007). Therefore, genetic pathways controlling flowering in *A. thaliana* unavoidably become genetic models for studying CR and BD in perennial species. In *A. thaliana*, the vernalization pathway is an essential part of the flowering control system (Bernier & Périlleux, 2005) and FLC is the central player in it. A high expression level of *FLC* is induced in the initial stage of vernalization and the steady state level of its transcription is progressively down-regulated with prolonged cold exposure till a stable repression is attained (Sheldon et al., 2000; Rohde & Bhalerao, 2007). It is now becoming clear that the Polycomb group (PcG) proteins control most major regulators of flowering time including *FLC* expression in *A. thaliana*. Presumably, a Polycomb Repressive Complex 2 (PRC2)-like complex (VRN complex, composed of CLF/SWN, FIE, VRN2 and MSI1*)* associates with the plant Homeo Domain (PHD)-finger proteins (VIN3, VRN5, VEL1) to form a PHD-VRN complex and this complex introduces H3K27me3 marks into the *FLC* locus during prolonged cold. Subsequently, a PRC1-like complex (LHP1, AtRING1a, AtRING1b) binds to these methylated marks and establishes stable gene silencing (Hennig & Derkacheva, 2009). Therefore, peach orthologs to the *A. thaliana* genes encoding PcG group and associated proteins could be

the first batch of candidates in this research. With the assembled peach genome sequence, it is possible to exhaustively catalog these peach orthologs and identify SSRs inside/close to them and perform candidate-gene studies to identify potential candidate genes regulating CR and BD. It is also possible to further validate the identified candidate genes through association studies.

Finally, the testing of candidate genes can be performed by overexpressing or down-regulating (RNA interference, etc.) the genes using transgenic plants (Salvi & Tuberosa, 2005). Due to the difficulty and inefficiency of peach transformation (Petri et al., 2009) and availability of highly efficient plum (another *Prunus* species) transformation protocols (Gonzalez-Padilla et al., 2003; Petri et al., 2009), it is necessary to explore the use of plum for functional testing of peach candidate genes. *DAM6* is one of six MADS-box genes cloned in peach *evg* region (Bielenberg et al., 2008). The expression of *DAM6* is missing in peach *evg* mutant (Bielenberg et al., 2008) and its normal expression culminated when dormancy induction is finished and then progressively declines during the fulfillment of the chilling period in peach wild-type plants (Li et al., 2009). Our previous research also indicated that *DAM6* is located in a QTL region pleiotropic for CR, HR and BD in peach (Fan et al., 2010). Therefore, *DAM6* is one of most promising candidate genes controlling the endo-dormancy and bloom date pathways and an ideal candidate for testing in transgenic plum. This work would help to validate the potential role of *DAM6* and at the same time establish transgenic plum as a potential transgenic testing system for *Prunus* species gene candidates.

In this reported research, both candidate-gene approaches and association approaches were used to identify promising candidate genes regulating CR and BD. With the candidate-gene approaches, map positions of peach orthologs of *A. thaliana* genes involved in the vernalization pathway was compared with previously mapped QTL intervals to determine if they co-locate in the QTL intervals thus implicating putative roles in regulating CR and BD. With the association approaches, previously mapped QTLs for CR and BD were validated and a major QTL (peach *evg* region) was dissected allowing specific candidate genes in the interval to be strongly implicated. Lastly, *DAM6* in *evg* region was functionally tested using the *Agrobacterium*-mediated plum transformation system (Petri et al., 2008).

Materials and Methods

Plant Materials

The Contender × Fla.92-2C peach F_2 population described in Fan et al. (2010) was used for mapping candidate genes.

65 peach germplasm accessions with CRs ranged from 150-1250 chilling hours (<7°C CR model, See Supporting Information Fig. S3.1, Table S3.1) were used for this association study. These accessions were maintained at the farm of USDA-ARS, Southern Fruit and Tree Nut Research Lab (Byron, GA, USA).

Candidate Gene Mapping

The cDNA sequences of PcG and associated protein encoding genes in *A. thaliana* reviewed by Hennig & Derkacheva (2009) were retrieved from the GenBank Nucleotide database and a BLAST search was performed against peach whole genome sequences. The BLAST detected peach contig sequences with E-values less than e^{-20} were processed by the gene prediction program Fgenesh (Salamov & Solovyev, 2000). The cDNA and polypeptide sequences of predicted genes were then compared to *A. thaliana* EST and protein databases via BLAST to search for genes with similar sequences and exon-intron structure. Once putative peach orthologous genes were verified, simple sequence repeats (SSRs) in or immediately close to those verified genes were tested and mapped onto the Contender × Fla.92-2C peach map using the method outlined in Fan et al. (2010).

Association Mapping

SSR genotyping 34 SSR markers across whole peach genome and 16 SSR markers in peach *evg* region (Wang et al., 2002; Bielenberg et al., 2008) were tested against 65 peach germplasm accessions. Only polymorphic markers with clear segregation patterns were scored.

Structure analysis of germplasm accessions In order to reduce the false detection of marker-trait association due to the stratification of germplasm accessions, the subgroups of the selected peach germplasm accessions were inferred by *STRUCTURE* software version 2.3 (Pritchard et al., 2000a; Falush et al., 2003). Considering the co-ancestry of germplasm accessions used, the default admixture ancestry model and

correlated allele frequency model were chosen. The λ value, a parameter specifying the allele frequencies in each subpopulation, was estimated by setting the number of subpopulations (K) as one and running the program once and fixed. The length of Burnin period was set 100,000 and the number of MCMC iterations 1,000,000. The optimum K value was determined by running the MCMC scheme for different values of the maximum K and comparing the estimated log probability (Ln Pr(X|K)) to choose the smallest K value when the Ln Pr(X|K) reaches a plateau state.

Association analysis Once the subgroup structure information was obtained, the association of each scored SSR markers with CR was tested using *STRAT* software (Pritchard et al., 2000b) with default settings of all parameters, except setting the number of simulated tests per locus as 10,000 and not pooling rare alleles.

Overexpressing of *DAM6* in Plum

Shoot tissues were sampled from a wild-type genotype (#40) of the peach F_2 population used for *evg* locus mapping (Wang et al., 2002) followed by total RNA extraction and *DAM6*'s cDNA synthesis described in Bielenberg et al. (2008). *DAM6*'s cDNA (protein coding sequence) was isolated by 3' Rapid Amplification of cDNA Ends (RACE) and 5' (RACE) using protocols described in Sambrook & Russell (2002). The cDNA was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced to verify that it has gene specific DNA sequences of *DAM6*. It was then cloned into the EcoRI restriction site in polylinker 1 of the plasmid vector pGA482GGIMCS which places the cDNA under the control of the 35S promoter (Fig. S3. 2). A internal

primer in the cDNA sequence was used for sequencing the DNA insert back to the vector to verify the insert direction. The engineered plasmid was then used to generate transgenic plants from mature seed hypocotyl slices of plum cv. Bluebyrd using an *Agrobacterium*-mediated transformation protocol (Petri et al., 2008).

Results

Candidate-gene Mapping

14 putative genes homologous to *A. thaliana* PcG protein and their associated protein encoding genes were identified from peach genome sequences. To date, seven putative genes have been positioned either onto or close to 2-LOD intervals of CR and BD intervals (Table 3.1, Fig. 3.1). *A. thaliana* homologues to these genes encode five protein components of PRC-2 like complex, one PHD-finger protein and one protein component of PRC-1 like complex (Table 3.1).

Association Mapping

49 SSR markers were tested with all 65 peach germplasm accessions. 34 polymorphic SSR markers were scored. Based on the marker positions in the Contender × Fla.92-2C peach map (Fan et al., 2010), these markers distributed across eight linkage groups. All 34 markers were used for population structure analysis and association analysis.

The Ln Pr(X|K) with different K values were compared in Table S3.1. When K>2, the increase of Ln Pr(X|K) becomes slow. The 65 peach germplasm accessions can

be divided into two subgroups derived from two ancestral sources. Each genotype in one subgroup mainly inherited its genome from one ancestral source (Fig. S3.3). The two ancestral sources do not show clear significance in peach breeding history, except that one has a pedigree related with peach cv. Elberta and cv. Redhaven.

18 SSR markers were detected to be significantly associated with CR ($p<0.05$). All these markers were positioned on the Contender × Fla.92-2C peach map, except for two (pchgms40B and pchgms76) developed from the sequences in/around peach *evg* region. Among these, five markers clustered around the peach *evg* region /major CR and BD QTL interval (qCR1a, qBD1a), six located in six different CR and/or BD intervals and seven did not locate in previously mapped CR and/or BD QTL intervals (Table 3.2; Fig. 3.1).

15 SSR markers distributed in/around peach *evg* region were tested for marker-trait (CR) associations. Five markers were monomorphic and six not significantly associated with CR. The remaining four SSRs were present in the genome sequences of two DAM genes (*DAM4* and *DAM6*) and a predicted gene around *evg* region, indicating that the three genes could be promising candidate genes for further functional testing (Table 3.3, Supporting Information Fig. S3.4).

Overexpressing *DAM6* in Plum

As the data from expression study of the *evg* locus strongly implicated the *DAM6* gene as potentially being involved in CR (Li et al., 2009) and its significant association with CR detected by association approach in this study, it was chosen as a candidate gene

to test in transgenic plum. An overexpression construct of this gene was prepared with the vector pGA482GGIMCS and introduced through agrobacterium mediated transformation using protocols developed in the laboratory of Dr. Ralph Scorza (Petri et al., 2008). This vector drives the expression of introduced genes with the constitutive 35S promoter. Seven transgenic plum plants overexpressing cDNA (protein coding sequence) of *DAM6* were obtained. All transgenic plants showed a clear dwarf phenotype with more branches compared with control wild-type plants Byrd (Fig. 3.2). The influence of overexpressing *DAM6* on CR and BD in plum has not been determined due to time constraint.

Discussion

Association Mapping Dissects a Major CR and BD QTL/*evg* Region

Previously we mapped one major CR QTL (qCR1a) and two major BD QTLs (qBD1a and qBD7a) in a peach F_2 population. The 2-LOD intervals of qCR1a and qBD1a overlap with *evg* region. qCR1a accounted for 40.5-44.8% of phenotypic variance and qBD1a accounted for 41.3-54.5% of phenotypic variance (except in year 2006) (Fan et al., 2010). This tells us that, although there are many loci (genes) regulating CR and BD in peach, one or two major QTLs play critical roles in controlling the two traits. These major QTLs deserve more attention than other loci. Once they are refined, dissected and cloned, a significant portion of phenotypic variance could be captured and manipulated in marker assisted breeding.

In order to significantly cut down the number of candidate genes and search for causative genes for CR and BD in/around *evg* region, we performed a preliminary association study with 65 peach germplasm accessions. Out of 15 tested SSRs, four out of three genes were found to significantly associate with CR phenotype (Table 3.3, Supporting Information Fig. S3.4), suggesting these three genes could be possible causative genes controlling CR and promising candidates for further functional tests. Two of these three genes are *DAM4* and *DAM6*. The third one is a homologue to an *A. thaliana* gene (*AT1G09710*) encoding a DNA binding protein and locates around sequenced *evg* region.

Putative role of Polycomb Group Proteins in Regulating CR and BD in Peach

In this study, we mapped peach orthologs of seven genes encoding PRC2-complex proteins, one gene encoding a PHD-finger protein (VRN5) and one gene encoding a PRC1-like complex protein in or adjacent to the 2-LOD intervals of previously mapped QTLs for CR and BD in G2, G4, G5 and G7 (Fig. 3.1). This suggests that similar genetic pathways may control vernalization in winter annuals and CR in woody perennials. This connection is difficult to verify due to a lack of identified ortholog(s) of *FLC* in peach. There are several reasons that *DAM6* might be the *FLC* substitute in peach. Firstly, *DAM6* is one of the six MADS-box genes cloned in peach *evg* region/CR and BD major QTL interval. Secondly, *DAM6* expression levels progressively decreases during prolonged cold in winter time (Li et al., 2009). Thirdly, allele variation in *DAM6* significantly associates with CR change in the current study (Table 3.2,

Supporting Information Fig. 3.3). However, to conclude that *DAM6* is *FLC* substitute in peach, two more types of evidence are needed: the epigenetic modification of *DAM6* upon dormancy breaking and its removal before dormancy induction, and the CR change observed in transgenic plants of peach or related species overexpressing *DAM6*.

Currently, we are exhaustively cataloguing and positioning more peach orthologs of PcG proteins encoding genes in *A. thaliana*. Also, an association study using peach germplasm accessions aiming to validate the causative relationships between these genes and CR and BD phenotype is underway. Beside, these genes are also being tested with apricot germplasm accession for marker-trait associations. With these efforts, we hope to better understand the roles that PcG proteins play in regulating CR and BD.

Commonality between Endo-domancy and Para-dormancy

Rohde & Bhalerao (2007) noticed an evolutionary trajectory: branching (para-dormancy), bud structure (endo-dormancy), seed (seed dormancy).The acquisition of bud structure about 100-400 million years after the evolution of branching, enables growth cession in only one part of the meristems. The annual life (seed dormancy) was not formed until the rapid warming of the Earth’s climate. Seed dormancy synchronizes the seed germination to the times when seedling establishment is likely to be successful. Based on this trajectory, they proposed that different types of dormancy might share similar molecular mechanisms. Faust et al. (1995) noticed the involvement of apical dominance (para-dormancy) in winter dormancy of apple buds and concluded that winter dormancy starts with para-dormancy, continues with endo-dormancy, and ends with para-

dormancy again, showing that there is no clear boundary between the two conceptually different types of dormancy. Our results seem to support the hypothesis of Rohde & Bhalerao (2007). *DAM6*, one of possible causative gene suggested by association mapping, is similar in sequence with *SHORT VEGETATIVE PHASE* (*SVP*) gene in *A. thaliana*, which plays an important role in the response to ambient temperature change (Lee et al., 2007). Transgenic plum plants over-expressing *DAM6* showed dwarfism and more branches (Fig. 3.2). Evaluation of the CR difference between transgenic plants and wild-type plants is yet to be performed to further confirm this hypothesis.

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Table 3.1 List of candidate genes regulating chilling requirement (CR) and bloom date (BD) in peach proposed by candidate-gene mapping

Peach genes	*Arabdospis* homologues	Category	QTLs
PpCLF	*CLF*	PRC2 complex	qBD7a
PpSWN	*SWN*	PRC2 complex	
PpFIE	*FIE*	PRC2 complex	qCR4b
PpEMF2	*EMF2*	PRC2 complex	qCR4b
PpVRN2	*VRN2*	PRC2 complex	qCR5, qBD5
PpMSI1	*MSI1*	PRC2 complex	qBD2
PpMSI2	*MSI2*		
PpVRN1	*VRN1*		
PpVIN3	*VIN3*	PHD-finger proteins	
PpVEL1	*VEL1*	PHD-finger proteins	
PpVRN5	*VRN5*	PHD-finger proteins	qCR7
PpLHP1	*LHP1*	PRC1 complex	qBD2
PpRING1A	*RING1A*	PRC1 complex	
PpRING1B	*RING1B*	PRC1 complex	

QTLs, QTLs previously positioned onto the Contender × Fla.92-2C peach map and having 2-LOD intervals harboring or close to mapped candidate genes

Table 3.2 *STRAT* test statistic (TS) for markers showing significant associations with chilling requirement (CR) among 65 peach germplasm accessions

Locus	G/Pos	QTLs	DF	TS	SI(P)
aprigms18	G1/12.3	qCR1b-2009	4	4.49	*
UDP-005	G1/37.1	qBD1c-2007	6	65.99	**
BPPCT020	G1/61.5	NA	5	55.02	*
pchgms12	G1/86.4	qCR1a, qBD1a	3	46.94	*
pchgms40	G1/86.9	qCR1a, qBD1a	4	54.88	***
pchgms40B	G1/NA	qCR1a, qBD1a	6	57.27	**
pchgms76	G1/NA	qCR1a, qBD1a	4	56.23	***
BPPCT028	G1/88.9	qCR1a, qBD1a	2	35.09	**
EPPCU9773	G2/66.2	NA	3	37.3	**
ssrPaCITA6	G4/11.3	qCR4a-2008, qBD4	8	77.79	***
UDA-042	G5/1.4	NA	5	48.54	**
ssrPaCITA21	G5/34.8	qCR5, qBD5	4	55.60	***
ssrPaCITA12	G6/53.0	NA	4	46.24	*
UDAp-460	G7/43.6	qCR7, qBD7a	2	31.80	**
EPPCU1169B	G7/64.0	NA	6	67.34	***
UDP-015	G8/12.3	NA	7	80.56	***
CPPCT006	G8/35.8	NA	4	42.21	*
PacC13	G8/51.0	qCR8-2008	5	50.89	**

G/Pos, the linkage group/position in cM of a marker in the Contender × Fla.92-2C peach map; QTLs, the QTL detected with the Contender × Fla.92-2C peach population in the same region; DF, degree of freedom; TS, test statistic ; SI(P), significance level of p value, * 0.05, ** 0.01, *** 0.001

Table 3.3 List of candidate genes regulating chilling requirement (CR) and bloom date (BD) in peach proposed by association mapping

Peach genes	*Arabdospis* homologues	Category	QTLs
DAM4	MIKC MADS-box		qCR1a, qBD1a
DAM6	MIKC MADS-box	PcG target genes?	qCR1a, qBD1a
PpAT1G09710	*AT1G09710*	DNA binding	qCR1a, qBD1a

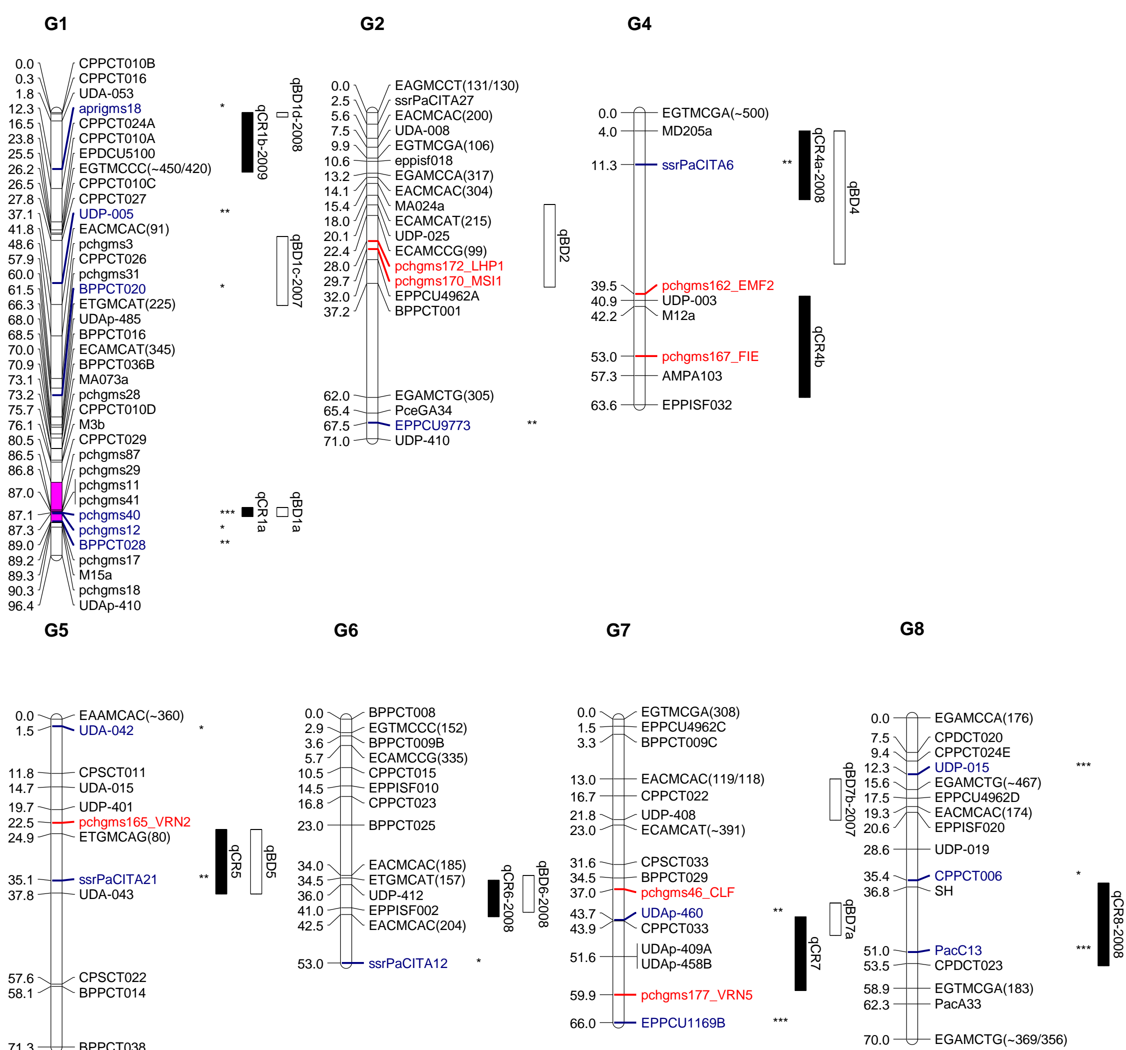

Fig. 3.1 Positions of candidate genes (marker/genes' names in *red*) or SSR markers significant for chilling requirement (CR) in association mapping among 65 peach germplasm accessions (markers with names in *blue* and followed by asterisks are significant: *, p<0.05; **, p<0.01; ***, p<0.001) on the Contender × Fla.92-2C peach map. The vertical bars next to the linkage groups (Gs) indicate 2-LOD interval (approximately 99% confidence interval) of QTLs for CR and bloom date (BD), which are differentiated by the styles of bars: the *filled black* for CR, the *open* for BD. QTLs

with names including year was detected in that particular year. The highlighted fragment of G1 covers *evg* locus (Bielenberg et al., 2008).

Fig. 3.2 Dwarfism phenotype of transgenic plums overexpressing *DAM6* cDNA (protein coding sequence). The plum trees in the middle and two sides are wildtype plum genotype.

Supporting Information

Table S3.1 Chilling requirements (<7°C CR model) of peach germplasm accessions used for association study

Accessions	Chilling units
Flordaguard	150
Flordaprince	150
06-1547	150
UFGold	200
Flordadawn	300
Gulfking	350
Sunsplash	400
Gulfprince	400
Texking	400
Flordaking	400
Peen-to	450
Gulfcrest	500
Texprince	550
Galaxy	600
Juneprince	600
Nemered	650
By01-6245	650
Maycrest	650
Goldcrest	650
Junegold	650
Fackler	700
Havester	750
Gage-Elberta	750
Karla-Rose	750
Galactica	800
Blazeprince	800
Ohenry	800
Q42535C	850
Lovell	850
Durbin	850
J.H.Hale	850
Jerseyqueen	850
RoyGold	850

Table S3.1 Chilling requirements (<7°C CR model) of peach germplasm accessions used for association study (Continued)

Accessions	Chilling units
Rubyprince	850
Julyprince	850
Elberta	850
Redglobe	850
Jefferson	850
Heath-Cling	900
HAKUHO	900
Sureprince	900
Champion	950
Helen-Borche	950
Primerose	950
Clayton	950
Carogem	950
Whiterock	950
Cresthaven	950
Redhaven	950
Ta-Qiao	950
Q36102C	1000
Reliance	1050
Contender	1050
Raritan-Rose	1050
Nector	1050
Surecrop	1050
Carolina-Gold	1050
Hakuto	1050
China-Pearl	1100
86P1079	1100
Chinese-Cling.	1100
93P5030z	1200
Q36019E	1200
Q37434A2	1200
93P4653c	1250

Chilling units were evaluated by ARS-USDA, Southern Fruit and Tree Nut Research Lab.

Table S3.2 Estimated log probabilities (Ln Pr(X|K)) with different K values (maximal number of groups)

K	Ln Pr(X|K)
1	-4649.8
2	-4326.4
3	-4175.5
4	-4043.2
5	-3864.7

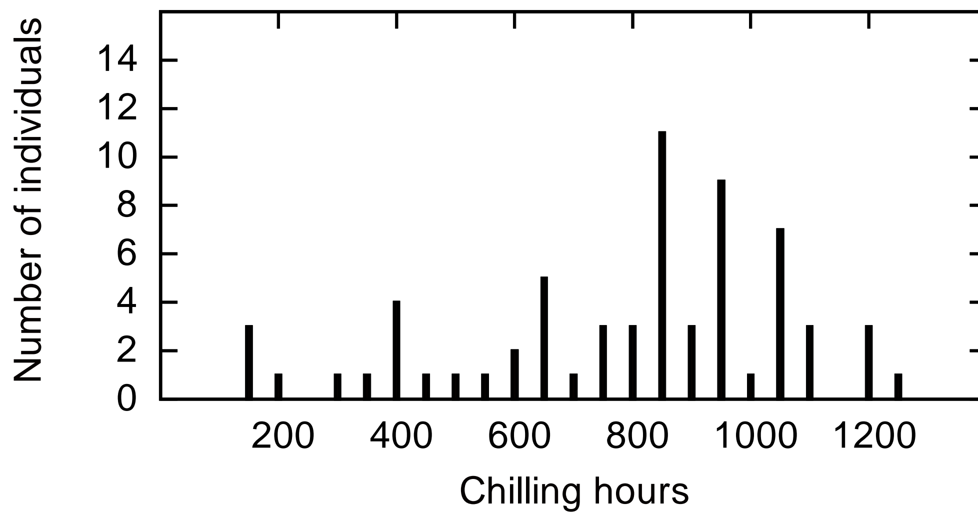

Fig. S3.1 Frequency distributions of chilling requirement (CR) for floral bud break of the peach germplasm accessions

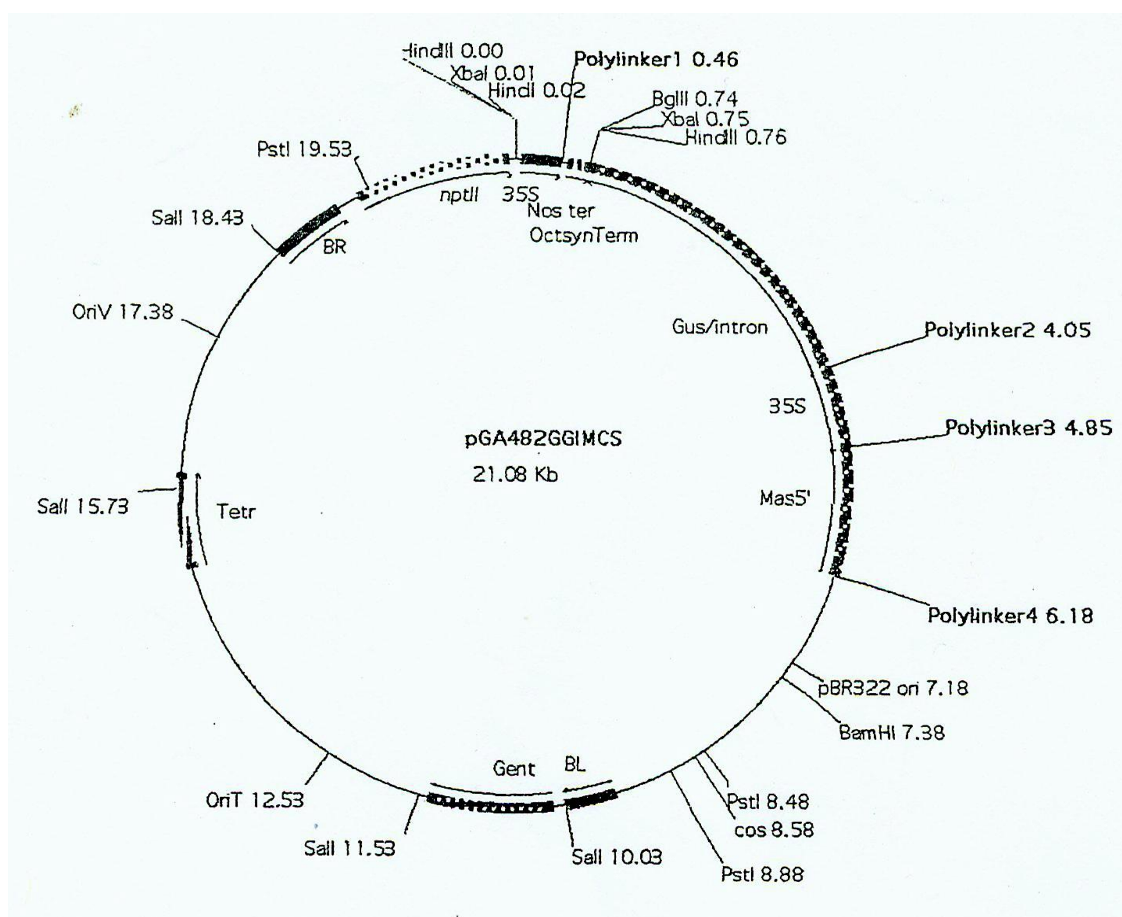

Fig. S3.2 Map of plasmid vector pGA482GGIMCS, a derivative of plasmid pGA482G. The order of restriction sites of polylinker 1 is as: PstI, PvuII, XhoI, EcroI, BamHI, SacII, NotI, PstI.

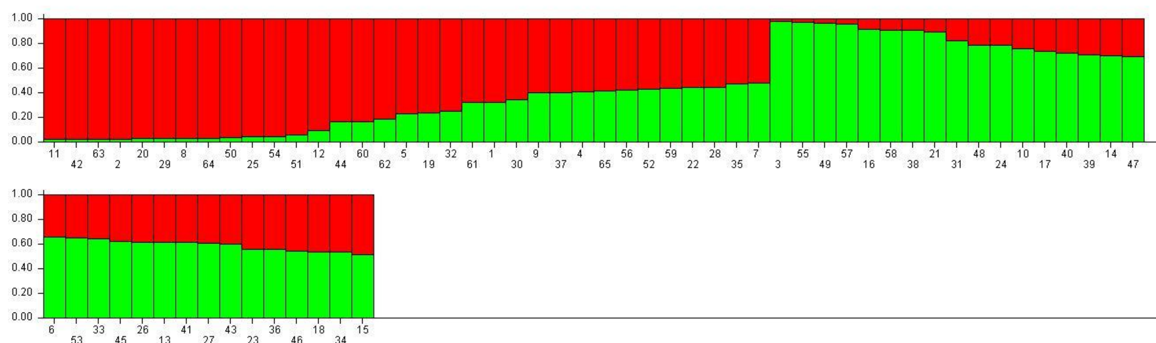

Fig. S3.3 Bar plot of the ancestry of 65 peach germplasm accessions. Each individual is represented by a single vertical line broken into two colored segments, with lengths proportional to the percentage of genome (Y-axis) inherited from one ancestral source.

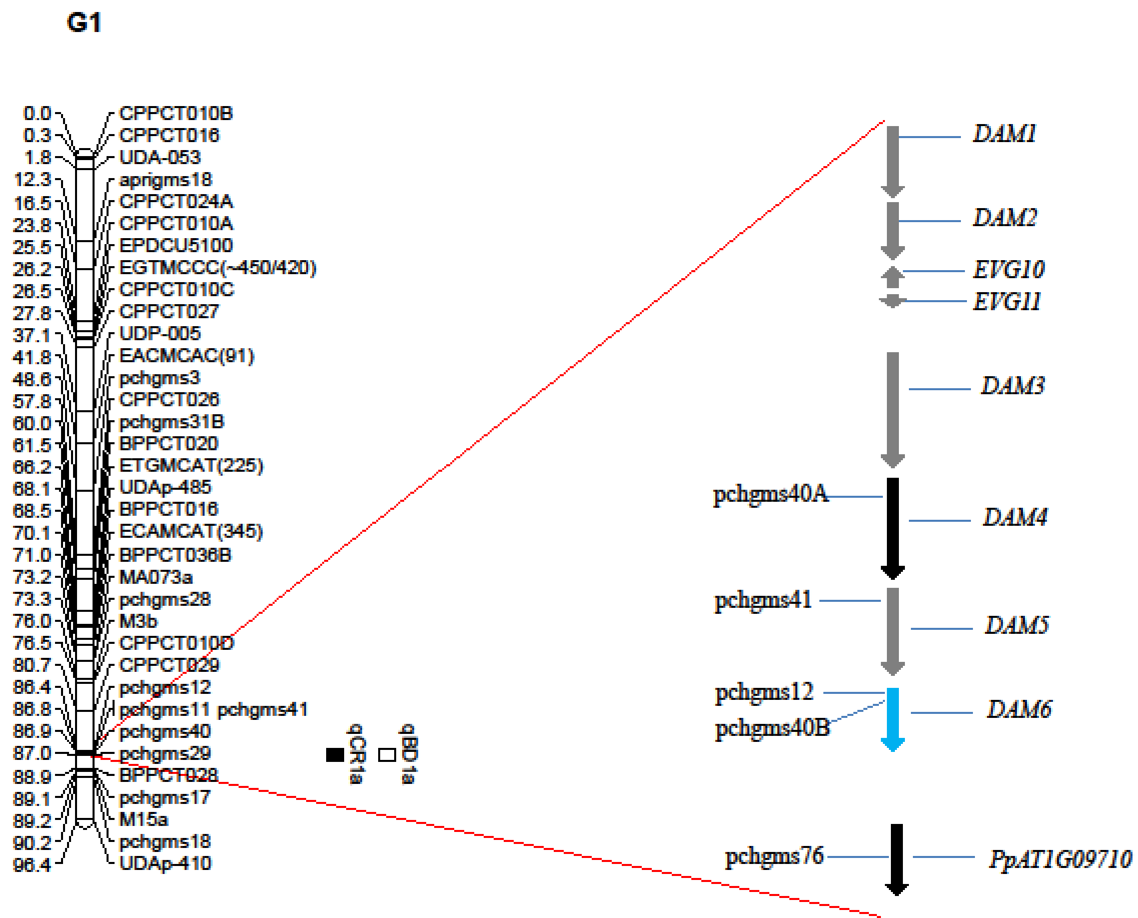

Fig. S3.4 Alignment of candidate genes regulating chilling requirement (CR) identified by association mapping among 65 peach germplasm accessions in/around peach *evg* region. The vertical bars next to the linkage group indicate 2-LOD interval (approximately 99% confidence interval) of QTL for CR (*filled*) and BD (*empty*). Direction and alignment of *evg* genes are according to Fig. 2 in Bielenberg et al. (2008). Colors of line segments representing *evg* genes indicate the significant levels of gene-trait association: the *black* for p<0.001, the *light blue* for p<0.01, the *gray* for p>0.05. The marker order in genes (physical order) is not exactly same with that in genetic map (genetic order).

CHAPTER FOUR

CONCLUSION AND FUTURE PERSPECTIVE

We developed a peach F_2 population with 378 genotypes by crossing high CR cv. Contender (1050 CH) and low CR genotype Fla.92-2C (300 CH) and selfing a resultant F_1 progeny. Using this mapping population, we constructed a genetic linkage map composed of eight linkage groups and 127 markers. The newly constructed map is in good agreement with the almond cv. Texas × peach cv. Earlygold *Prunus* reference map in marker positions and orders. Floral bud CR and HR of each genotype were evaluated in two years and BD scored in four years. In total, we identified 20 QTLs for three traits including one major QTL for CR and two major QTLs for BD. Almost all CR QTLs CR co-localize with BD QTLs. Particularly, one genomic region of 2cM pleiotropic for three traits co-localize with the previously sequenced peach *evg* locus.

The detected CR and BD QTL regions were explored and refined by both candidate-gene approaches and association approaches. With candidate-gene approaches, peach orthologs of seven PcG group and associated protein encoding genes involved in *A. thaliana* vernalization pathway were positioned into/close to 2-LOD intervals of CR and BD QTLs. With association approaches, seven CR and BD QTLs were validated and three potential causative genes in/around *evg* region were identified.

In addition, we successfully explored the highly efficient plum transgenic system to functionally test candidate genes regulating CR and BD in peach. Transgenic plum

plants overexpressing *DAM6* coding cDNA sequence showed a dwarfing and more extensive branching phenotype.

With available peach whole genome sequence and the integrated peach genetic/physical map, genes/SSR markers in/around two genomic regions harboring major QTLs are being exhaustively cataloged. These SSRs could be used for improving the marker density in these regions and hence the resolution of QTL mapping with the Contender × Fla.92-2C population. They also could be used in association mapping to further refine the detected QTLs with peach germplasm accessions to identify the causative genes for CR and BD. Moreover, combining the advantages of both candidate-gene approaches and association approaches, SSRs in/close to candidate genes identified by candidate-gene approaches could be further validated by association approaches.

Finally, more promising genes controlling CR and BD will be identified and functionally tested by over-expression or down-regulation through the *Agrobacterium*-mediated plum transformation system. SSR markers in/close to these genes will be used for marker assisted breeding program for new cultivars fitting with different climate regions.