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APPLICATION OF MICROSATELLITE/SSR MARKERS FOR THE IDENTIFICATION OF PEACH ROOTSTOCKS AND CHROMOSOMAL REGIONS ASSOCIATED WITH THE RESPONSE TO PEACH TREE SHORT LIFE SYNDROME

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APPLICATION OF MICROSATELLITE/SSR MARKERS FOR THE IDENTIFICATION OF PEACH ROOTSTOCKS AND CHROMOSOMAL REGIONS ASSOCIATED WITH THE RESPONSE TO PEACH TREE SHORT LIFE SYNDROME

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ABSTRACT

Peach Tree Short Life (PTSL) is a complicated disease syndrome involving nematodes, temperature, soil conditions, pruning and secondary pathogens. The disease occurs commonly in the southeastern U.S., and possibly in other areas of the U.S., Europe, South America and South Africa as the related Bacterial Canker Complex. PTSL causes premature tree death during the 3rd or 4th year after planting, resulting in large economic losses for growers. Recently, Guardian® 'BY520-9' rootstock was selected for its tolerance to PTSL; however, the genetic basis for this tolerance remains unknown.

Nemaguard, a PTSL susceptible rootstock, and Guardian® selection 3-17-7 were crossed. Each F_1 plant was selfed to create segregating F_2 populations. One hundred and seventy microsatellite/Simple Sequence Repeat (SSR) markers, each uniquely mapped to chromosomal locations on the *Prunus* reference genome, were used to screen the parents and F_1-11. Forty-seven SSR markers showed polymorphism among the parents, and were heterozygous in F_1-11. Segregation data obtained from the F_2-11 population for SSR marker inheritance and PTSL-response were compiled to identify nuclear genomic regions associated with the response to PTSL disease syndrome.

Of the 47 polymorphic SSRs, nine (distributed on 4 linkage groups) were genetically linked with the response to PTSL. Identified SSR markers would be useful in crop improvement and facilitating tolerance rootstock selection. A QTL was associated with the response to PTSL as well. The upper terminus of linkage group 2 appears to be important because both the individual SSR analysis and the QTL analysis linked this region with the response to PTSL. The genes controlling the tolerance or susceptibility of

PTSL may reside in this region. In the future, developing more SSR or other high-resolution markers to saturate this region will further define the specific region, and ultimately lead to identification of the candidate genes.

The second project described in this dissertation is the genotyping peach rootstock seedlings using DNA-fingerprinting with microsatellite/SSR markers. Peach seedling rootstocks are usually derived from open pollination. Seedlings are difficult to distinguish morphologically, and once grafted, typically no above-ground material is available for visual identification. To avoid misidentification and to protect plant varieties and patents, DNA fingerprinting was investigated as a robust rootstock identification tool. The objective of this study was to distinguish among progeny from eight peach seedling rootstocks: Bailey, Halford, Lovell, Nemaguard, Nemared, Guardian® (selection 3-17-7), S-37 and Kakamas.

Each rootstock could be discriminated by at least one SSR marker. No single perfect marker was found to identify all rootstocks. Rootstock seedling identification was conducted by screening open-pollinated seedlings. It is more difficult than parent genotype identification, because heterozygous patterns obtained in a rootstock clone segregate in its seedlings. However, unique segregation patterns were found in the rootstock seedlings. Single SSR markers could identify seedlings of rootstocks Nemared, Bailey, Kakamas and Nemaguard. Marker combinations could identify seedlings of 3-17-7 and S-37. Seedlings of Lovell and Halford can be identified from the other rootstocks. However, there were no SSRs or marker combinations to uniquely differentiate Lovell from Halford seedlings. The SSR markers presented in this study could be used as a

practical fingerprinting system for rootstock seedling identification. This technology is useful to test rootstocks for trueness to type for nursery operators and growers, and also will be helpful in protecting seed propagated proprietary rights (i.e., PVP) for breeders.

DEDICATION

I dedicate this dissertation to my family—my father Guanghui Liu, my mother Xia Jia and my love Yu. It is their love and encouragement that supported me throughout the entire process to complete this dissertation. I will always appreciate it.

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CHAPTER ONE

LITERATURE REVIEW

Peach Industry Introduction

Peach [*Prunus persica* (L.) Batsch] belongs to the subfamily Prunoideae of the Rosaceae. It is a commercially important fruit tree species with 10 million metric tons produced worldwide (Fideghelli et al., 1998). The major peach production countries include China, Italy, Spain, United States, Greece and France (Layne and Bassi, 2008). The United States produces 1,400,000 metric tons of peaches annually, which represents approximately 10% of the total world peach production (USDA 2001).

Both fresh peaches and processed peaches are included when evaluating peach consumption. In 2001, the total peach production in the U.S. included consumption of approximately 700,000 metric tons of fresh peaches, 500,000 metric tons of processed peaches and export of 200,000 metric tons of fresh peaches with a total value estimated at $500,000,000 (USDA 2001).

In the United States, peaches are commercially produced in more than 20 states. California, ranking first in peach production with 90,000 acres, represents 47% of total peach acreage. South Carolina ranks second accounting for 17,000 peach acres (D. Layne, 2008; personal communication). For nearly a century, peach production has been valuable in the southeastern United States for both economic reasons as well as reasons of cultural traditions.

There are more than 60 peach cultivars planted in South Carolina. South Carolina's annual average harvest has an estimated market value of $50,000,000 (D. Layne, 2008. personal communication). Thus, commercial peach production plays an important agricultural and economic role in South Carolina.

Scions and Rootstocks

In commercial peach production, a peach tree is normally composed of two genotypes, a scion and a rootstock. Scions, the above ground portion, are selected for fruit traits such as flesh type, flesh color, sugar content or skin coloration. A dramatic number of scion cultivars have been released (Brooks and Olmo, 1997; Okie 1998).

Rootstocks, the underground portion of the tree, interact with soil and provide nutrients to the whole plant. They play an important role in water and nutrient transportation and in tree survival. Rootstocks are selected for biotic and abiotic stress resistance to specific environmental conditions and to control tree vigor (Brooks and Olmo, 1997; Okie 1998; Reighard and Loreti, 2008).

Peach growers select rootstocks based on the local environmental conditions such as soil pH, humidity, temperature, and the rootstock's compatibility with scion cultivars, nematodes or pathogen resistance. Fewer than 10 rootstocks –Lovell, Halford, Nemared, Nemaguard, Bailey and Guardian® 'BY520-9' have been widely planted in peach orchards in the U.S. (Reighard and Loreti, 2008).

Lovell and Halford are major processing peach cultivars. Lovell originated as a chance seedling in California in the 1880s and 1920s, respectively (Okie 1998). Lovell

produces uniform seedlings that are compatible with all peach cultivars. Scion vigor is strong and productive on Lovell rootstocks. Lovell exhibits better tolerance to ring nematodes (*Mesocriconema xenoplax*) and Peach Tree Short Life (PTSL) syndrome than Nemaguard (Nyczepir et al., 2006; Reighard and Loreti, 2008). However, it is susceptible to root-knot nematodes *Meloidogyne incognita* (Kofoid & White) Chitwood and *M. javanica* (Treub) Chitwood, and root-lesion nematodes *Pratylenchus vulnus* Allen & Jensen and *P. penetrans* (Cobb) Chitwood & Oteifa (Nyczepir et al., 1983). Halford performs similarly to Lovell. It is possibly a sibling or seedling of Lovell (Philip and Davis, 1936; Okie 1998).

Nemared bears red leaves and was released in 1983 by the USDA. It was selected from the F_3 seedlings of a cross between Nemaguard and a red leaf seedling and selected for root-knot nematode resistance (Ramming and Tanner, 1983; Okie 1998). Nemared is tolerant to root-knot nematodes but is susceptible to bacterial canker (*Pseudomonas syringae* pv. *syringae* van Hall) (Reighard and Loreti, 2008). Scions on Nemared rootstock are usually vigorous.

Nemaguard was thought to be a *P. davidiana* x *P. persica* hybrid and released in 1959 (Okie 1998; Reighard and Loreti, 2008). Nemaguard is widely used in California and the southern U.S. for its resistance to root-knot nematodes, its vigor and good compatibility with peach scions. However, Nemaguard imparts adverse effects to scions with respect to cold hardiness and bacterial canker (Nyczepir et al., 1983).

Bailey is a naturalized peach selected from Iowa (Okie 1998). It produces uniform seedlings with good tree vigor. Bailey is used mostly in the northern U.S. and Canada for

its cold-hardiness. Bailey has tolerance to root-lesion nematodes, but is susceptible to root-knot nematodes, fungal root rot, and PTSL (Reighard and Loreti, 2008).

S-37 was used in California approximately 60 years ago for its resistance to root-knot nematodes (Okie et al., 1994a). This rootstock originated from a seedling of an ornamental peach. Seedlings of S-37 segregate for flower color and weeping tree habit (G. Reighard, 2008, personal communication). This rootstock has not been used for many years since it was replaced by Nemaguard. However, it is in the pedigree of Guardian® 'BY520-9' (Okie et al., 1994a).

Guardian® 'BY520-9' was released as a bulked seed lot as a peach rootstock. The pedigree can be traced back to a cross, made between an open-pollinated seedling of S-37 and Nemaguard in 1954 (Okie et al., 1994a). Guardian® 'BY520-9' was selected from F_5 seedlings for its tolerance to ring nematodes, bacterial canker and PTSL (Beckman et al., 1997; Reighard et al., 1997). Thus, this rootstock has been widely used in the southeastern U.S., especially for ring nematode-infested replant orchards. Guardian® 'BY520-9' exhibits resistance to root-knot nematode as well (Nyczepir et al., 2006).

Peach Rootstock Identification

Peach rootstocks with specific characteristics such as pathogen resistance or environmental adaptability are normally developed through years of selection and field evaluation. Once a new rootstock is released, plant variety protection (PVP) or plant patents can be granted to the breeders, which gives them exclusive marketing rights to the

rootstock in the United States (Strachan 1992). However, some growers may buy a few patented trees and propagate them without paying royalties to breeders (Warner 2004). Thus, peach rootstock identification is essential to support a PVP, settle infringement disputes and protect agriculture business from unfair competition (Janick et al., 1983).

Traditional identification evaluations are made based on the observed morphological traits/phenotypes of the mature peach trees such as fruit or flower characters (Arulsekar et al., 1986). Many characters take a long time to be observed and might be affected by environmental conditions and developmental stages of the trees and human judgement (Janick et al., 1983). For peach growers, rootstocks are very difficult to identify morphologically at the seedling stage. In addition, once grafted, any characteristic leaf, floral or fruit traits of rootstock phenotypes will not be visible. Mislabeled, misrepresented rootstocks could lead to huge income losses through orchard replacement and yield loss (Harper and Kime, 2001). Thus, peach rootstock identification would test rootstocks for trueness to type for nursery operators. Growers can then purchase certified rootstocks with confidence. Molecular marker techniques behave as precise, and non-disputable tools in cultivar identification, and would be of considerable benefit to fruit industry.

Molecular Markers and their Application in DNA Fingerprinting

The foundation of cultivar identification via molecular markers is detection of protein or DNA variation/polymorphism among different individuals. Molecular markers give information of allelic variation at a given locus (Schlotterer 2004). Compared with

morphological markers, molecular markers take less space and time and are independent of environmental conditions and developmental stages, more reliable and informative (Aranzana et al., 2003b). There are several types of molecular markers- isozymes, a biochemical based marker system, and DNA based marker systems. Examples of DNA based molecular markers include restriction fragment length polymorphisms (RFLPs), random amplification of polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs)/microsatellites and single nucleotide polymorphisms (SNPs). Both biochemical and DNA based molecular marker systems are different from each other in the number of detected loci, content of information and reproducibility (Weising et al., 2005).

Isozymes:

Isozymes are enzymes that differ in amino acid sequences, but catalyze the same reaction. Isozymes represent isoforms of enzymes that are encoded by homologous genes. The synthesized isozymes share a common substrate but are different from one another in electrophoretic mobility, and the difference can be detected by electrophoresis (Markert and Moller, 1959). The advantages of isozymes are that they are comparatively inexpensive and co-dominant. The disadvantages are that their numbers are limited and developmental-stage dependent (Soltis and Soltis, 1989). Isozymes in early studies showed polymorphism in human and *Drosophila* natural populations (Harris 1966; Johnson et al., 1966). Isozymes were first suggested in the 1960s for use in plant fingerprinting (Scandalios 1969).

Isozyme variability/polymorphism has been used frequently to identify genetic varieties in commercial pea cultivars (Posvec and Griga, 2000), subterranean clover (Collins et al., 1984), sugarcane (Manjunatha et al., 2003), guayule (Estilai et al., 1990) and fruit tree species such as citrus (Rahman et al., 2001). In *Prunus*, isozymes of enzymes including peroxidase, diaphorase, isocitrate dehydrogenase and malate dehydrogenase were reported in peach cultivar identification (Arulsekar et al., 1986; Durham et al., 1987; Messeguer et al., 1987; Agarwal et al., 2001). In sweet and sour cherry, isozymes were reported in cultivar identification (Hancock and Iezzoni, 1988; Kaurisch et al., 1991; Granger et al., 1993; Corts et al., 2008). Additionally, isozyme variability was characterized in apricot (Byrne and Littleton, 1989), plum (Pashkoulov et al., 1995) almond cultivars (Hauagge et al., 1987) and of *Prunus* interspecific hybrid genotypes from plum x peach (Parfitt et al., 1985) and almond x peach hybrids (Chaparro et al., 1987).

Restriction Fragment Length Polymorphisms (RFLPs):

The RFLP technique was developed in 1974 (Grodzicker et al., 1974) and first used in human genetic linkage group construction (Botstein et al., 1980). Later on, Burr et al. (1983) found the prevalence of RFLPs in maize and its potential in genetic map construction. RFLPs derive DNA length polymorphism by variation of the positions of restriction sites along the DNA sequences. Any nucleotide rearrangement, deletion or insertion occurring in restriction sites result in new restriction site creation or original site removal. Once genomic DNA has been digested by restriction enzyme, the fragments separated by electrophoresis, and transferred for Southern blotting, the digested

fragments are hybridized with labeled DNA probes. Polymorphisms of DNA fragments based on the difference of restriction sites can then be detected. RFLPs are co-dominant markers and highly reproducible.

RFLPs have the potential to be used in plant genetic studies such as variety identification, breeders' rights protection, parentage determination and crop improvement by breeding (Tanksley 1983; Soller and Beckmann, 1983). RFLPs were successfully applied in cultivar characterization of avocado (Lavi et al., 1991), rose (Hubbard et al., 1992), tomato (Vosman et al., 1992), grape (Bowers et al., 1993), wheat (Vaccino et al., 1993) persimmon (Maki et al., 2001) and fescue (Busti et al., 2004). In *Prunus*, RFLPs were reported being used to find genetic variability in apricot (de Vicente et al., 1998; Hurtado et al., 2001) and peach (Eldredge et al., 1992). However, RFLP reactions require a relatively large amount of DNA template, and are time-consuming and costly.

Random Amplification of Polymorphic DNAs (RAPDs):

RAPD is a polymerase chain reaction (PCR)-based molecular marker technique and was first reported in 1990 (Williams et al., 1990). It amplified DNA fragments *in vitro* with primers that are designed with arbitrary sequences. Any nucleotide change (i.e., insertion, deletion or substitution) occurring within the regions that the primers amplified is an amplified DNA fragments' length polymorphism. RAPD markers are well suited for genetic map construction, and DNA fingerprinting (Welsh and McClelland, 1990). It provides an efficient way to screen for polymorphisms without knowledge of primer site sequences (Schierwater and Ender, 1993; Schlotterer 2004). RAPD primers are considered universal sets that can be used for a large range of species. However, the

main disadvantage is the low reproducibility (Penner et al., 1993; Benter et al., 1995; Jones et al., 1997), and the dominant nature of each marker that cannot distinguish heterozygous or homozygous loci. RAPDs can be converted to sequence characterized amplified regions (SCARs), which are co-dominant and more reproducible than RAPDs (Paran and Michelmore, 1993).

RAPDs have been widely used to characterize and trace the phylogeny of diverse plant and animal species (Koller et al., 1993; Graham et al., 1994, Gidoni et al., 1994; Schnell et al., 1995; Fabbri et al., 1995; Chessa and Nieddu, 2005). In *Prunus* species, RAPDs were used to study germplasm diversity and cultivar identification in peach (Lu et al., 1996; Warburton and Bliss, 1996; Yang et al., 2001; Cheng 2007), apricot (Mariniello et al., 2002), cherry (Shimada et al., 1999), plum (Heinkel et al., 2000; Boonprakob et al., 2001), almond cultivars (MirAli and Nabulsi, 2003; Shiran et al., 2007), and interspecific hybrids such as commercial and selected clones from *P. persica*, and *P. persica x P. davidiana* hybrids, and *P. cerasifera, P. domestica* and *P. instititia* clones (Casas et al., 1999).

Amplified Fragment Length Polymorphisms (AFLPs):

AFLPs produced DNA fragments polymorphism through PCR mediated selective amplification of DNA fragments (Vos et al., 1995). This technique was developed by Zabeau and Vos (1993) and has three steps. First, genomic DNA is cut by restriction enzymes and double-strand DNA adaptors are ligated to the two ends of the cut fragments. Second, the complementary DNA fragment of the adaptors and restriction site

specific sequences used as DNA primers amplify copies of the target DNA fragments. Third, the amplified DNA fragments are separated by electrophoresis.

AFLP analysis can be performed without knowledge about specific DNA sequences. AFLPs are able to screen thousands of loci in hundreds of individuals for relatively low cost (Bensch and Akesson, 2005). The results of AFLPs are unique and more reproducible than RAPDs (Jones et al., 1997; Hansen et al., 1998; Meudt and Clarke, 2007). However, AFLPs are dominant markers. So, AFLPs produce individually less informative but dramatically the numerous loci in each reaction (Belaj et al., 2003). Based on the advantages discussed above, AFLPs have become widely used as genetic markers with application in population genetics, quantitative trait loci (QTL) mapping and DNA fingerprinting (Mueller and Wolfenbarger, 1999).

AFLPs have been used successfully to identify cultivars of many different plant species, such as bermudagrass (Zhang et al., 1999), mango (Kashkush et al., 2001), apple (Tignon et al., 2000) and sesame (Laurentin and Karlovsky, 2007). In *Prunus*, AFLPs were applied to genetic variety studies and identification of cultivars in apricot (Hagen et al., 2002; Hurtado et al., 2002; Ricciardi et al., 2002; Geuna et al., 2003; Fang et al., 2006; Krichen et al., 2008; Zhebentyayeva et al., 2008) plum (Goulao et al., 2001; Ayanoglu et al., 2007), almond (Sorkheh et al., 2007), cherry (Zhou et al., 2002; Boritzki et al., 2001; Struss et al., 2003; Tavaud et al., 2004) and peach (Manubens et al., 1999; Shimada et al., 1999; Aranzana et al., 2001; Aranzana et al., 2003a; Hu et al., 2005; Xu et al., 2006).

Simple Sequence Repeats (SSRs)/Microsatellites:

SSRs are tandem repeats of two to six nucleotides (Edwards et al., 1991). It has been demonstrated that SSRs are randomly spread in the nuclear genome in human and other eukaryotic species (Litt and Luty, 1989; Luty et al., 1990), and they are highly polymorphic due to variation in length (repeat copy numbers) (Moore et al., 1991). SSR repeats and frequency of occurrence varies among species. For example, AT repeats predominate in plant genomes while AC repeats are common in humans (Powell et al., 1996).

SSRs are PCR-based molecular markers. The unique primer pairs flanking each repeat allele amplify DNA fragments with repeat motif by PCR. The typical repeat region is less than 100 bp, and the amplification can be accomplished by a standard PCR (Schlotterer 2004). The products among individuals vary in length caused by repeat copy number variation. Amplified products can be detected by polyacrylamide gel electrophoresis. The abundance of SSRs existing in the genomes of many species, codominant inheritance, and easy detection by PCR have made them the genetic markers of choice in many genetic diversity studies (Powell et al., 1996; Maghuly et al., 2005). However, the process of primer generation takes time, can be costly (Ellis and Burke, 2007) and primers can only be applied to closely related species.

SSRs have been applied in a genetic resource study in bean (Gonzalez et al., 2005), and DNA fingerprinting in apple rootstocks (Oraguzie et al, 2005), and potato cultivars (Schneider and Douches, 1997; Coombs et al., 2004). In *Prunus*, more than ten series of SSR markers were developed from different fruit species including peach,

almond, apricot and sweet cherry (Table 1.1).

A significant number of studies have been applied to genetic variation, phylogenetic relationships and cultivar/rootstock identification in apricot (Hormaza 2002; Romero et al., 2003; Zhebentyayeya et al., 2003; Chaib et al., 2004; Maghuly et al., 2005; Maghuly et al., 2006; Sanchez-Perez et al., 2005; Krichen et al., 2006), plum (Mnejja et al., 2004; Rohrer et al., 2004), almond (Testolin et al., 2004; Xie et al., 2006; Shiran et al., 2007), sweet and sour cherry (Cantini et al., 2001; Wunsch and Hormaza, 2002; Struss et al., 2003; Ohta et al., 2005; Pedersen 2006; Marchese et al., 2007), peach and nectarine cultivars (Testolin et al., 2000; Aranzana et al., 2001; Aranzana et al., 2002; Dirlewanger et al., 2002; Aranzana et al., 2003b; Yamamoto et al., 2003a; Yamamoto et al., 2003b; Ahmad et al., 2004; Xu et al., 2004; Wunsch et al., 2006; Yoon et al., 2006; Li et al., 2008) and interspecific hybrids (Serrano et al., 2002; Wunsch et al., 2004). The high transferability of SSRs among *Prunus* species makes them a good choice in genetic variety studies and genetic map construction.

Single Nucleotide Polymorphisms (SNPs):

SNPs were developed as the next-generation molecular markers. SNPs are DNA sequence variations caused by single nucleotide changes (i.e., transition, transversion, deletion or insertion) among different members in the same species (Vignal et al., 2002). SNPs are highly abundant in plant and animal genomes, but their density varies dramatically from region to region in each genome (Weising et al., 2005).

Table 1.1. Summary of SSRs developed from several sources (Dirlewanger et al., 2004b).

Series names	Repeat (s)	Species	Origins	References
BPPCT	CT	*P. persica*	Enriched genomic library from 'O' Henry'	Dirlewanger et al. (2002)
CPDCT	CT, GA	*P. dulcis*	Enriched genomic library from 'Texas'	Arus, P. (unpublished)
CPPCT	CT	*P. persica*	Enriched genomic library from 'O' Henry'	Aranzana et al. (2002)
CPSCT	CT, GA	*P. salicina*	Enriched genomic library from 'Suite Rosa'	Mnejja et al. (2004)
EPDCU	CT	*P. dulcis*	cDNA library from 'Nonpareil'	Dandekar et al. (unpublished)
EPDC	AG	*P. dulcis*	Almond pBluescript genomic DNA Library	Graziano, E., Arus,P. (unpublished)
EPPCU	CCA	*P. persica*	cDNA library from 'Loring'	Callahan et al. (unpublished)
M	CT	*P. persica*	cDNA library from 'Akatsuki'	Yamamoto et al. (2002)
MA	GA	*P. persica*	Genomic library from 'Akatsuki'	Yamamoto et al. (2002)
pacita	CT	*P. armeniaca*	Genomic library from 'Ungarische Beste'	Lopes et al. (2002)
PceGA	GA	*P. cerasus*	Genomic library from 'Erdi Botermo'	Cantini et al. (2001), Downey and Iezzoni (2000)
Pchgms Pchcm	CT, CA, AGG	*P. persica*	Genomic library from 'Bicentennial'	Sosinski et al. (2000)
PMS	CT, GA	*P. avium*	Genomic library from 'Valerij Tschakhalov'	Cantini et al. (2001)
PS	GA, GT, GTT	*P. avium*	Enriched genomic library from 'Napoleon'	Joobeur et al. (2000) Cantini et al. (2001)
UDA	AC	*P. dulcis*	Enriched genomic library from 'Ferragne'	Testolin et al. (2004)
UDAp	AG	*P. dulcis* *P. armeniaca*	Enriched genomic library from 'Portici'	Testolin et al. (2004)
UDP	CT, GT	*P. persica*	Two enriched genomic library from 'Redhaven'	Cipriani et al. (1999) and Testolin et al. (2000)

SNPs can be detected by gel-based methods including RFLP and AFLP like assays, single-stranded conformation polymorphism and allele-specific amplification; as well as non-gel based assays including DNA-chips and microarrays, TaqMan assay, molecular beacons and oligonucleotide ligation assay (Gupta et al., 2001). Because development of SNPs is time-consuming and costly, SNPs from only a limited number of plant species with well-studied genomes including *Arabidopsis* (Drenkard et al., 2000; Jander et al., 2002; Schmid et al., 2003), rice (Feltus et al., 2004; Zhang et al., 2005), maize (Batley et al., 2003) and wheat (Somers et al., 2003) have been detected. SNPs have been applied in cultivar identification in barley and for genetic diversity studies in wheat, maize and tree species (Germano and Klein, 1999; Gupta et al., 2001). In *Prunus*, SNPs were detected from AFLP markers and were successfully used to study genetic variation study among 50 *Prunus mume* Sieb et Zucc. accessions (Fang et al., 2006).

Peach Tree Short Life (PTSL) Syndrome

Peach Tree Short Life (PTSL) is a complex and complicated disease syndrome that occurs in the southeastern U.S. It causes peach tree death at a premature or early age (e.g., the third or the fourth year). In early spring after flowering, emerging vegetative shoots throughout the tree collapse due to cambial tissue death, and eventually the entire above ground part of the tree dies. Upon removal of the outer bark, a discolored inner cambium and xylem is observed, along with a strong sour-alcohol odor (Nesmith et al., 1981; Ritchie et al., 1981). Later in the spring, new suckers (adventitious buds) may grow from the rootstock shank below ground.

PTSL is considered to be a rootstock disease syndrome associated with ring nematodes [*Mesocriconema xenoplax* (Raski) Loof and de Grisse] parasitism (Nyczepir et al., 1983; Nyczepir 1988b; Nyczepir 1990; Nyczepir, et al., 1997). The feeding behavior increases susceptibility of peach trees to bacterial canker (*Pseudomonas syringae* pv. *syringae* van Hall) (Lownsbery et al., 1973) or cold injury, or an interaction of both. Many abiotic factors or environmental conditions such as fluctuations in winter temperature, soil pH and cultural practices are closely associated with PTSL (Nyczepir 1988b; Nyczepir 1990). In South Carolina, PTSL affects more than 70% of the peach orchards. It was estimated that a yearly loss of 6 million dollars occurred as the result of this syndrome (Miller 1994).

Ring Nematode Parasitism

Ring nematodes (*M. xenoplax*) develop high populations in soils of high porosity such as sand, well-structured clay loam soils and soils with high moisture (Seshadri 1965). They occur in 100% of PTSL-orchards in Georgia and South Carolina (Nyczepir et al., 1988b).

M. xenoplax is migratory ectoparasite. Feeding behavior on peach roots and root tips starts from inserting single root cortical cells with the nematode' stylet (Wyss et al., 1981). The parasitized cortical cells lie in the first or second layer of the root cortex and are modified by ring nematodes into "food cells" to sustain access to and ingestion of food (Hussey et al., 1992).

In infested root tissues, *M. xenoplax* produce ß-glucosidase to metabolize prunasin, the primary cyanogenic glusoside, into benzaldehyde and cyanide (Patrick et al., 1955). The released metabolites are toxic to both of plants and animals (Kaethler et al., 1982). However, ring nematodes are able to produce ß-cyanoalanine synthase to detoxify the metabolites. This might be one explanation of successful invasion of *M. xenoplax* into peach roots (Nyczepir et al., 1986a; Nyczepir et al., 1988b).

Feeding behavior of ring nematodes is not considered a destructive cellular modification in peach roots (Hussey et al., 1992), but produces minimal root modification. It causes root malformation, discoloration and reduces the number of functional peach feeder roots (Nyczepir and Pusey, 1986b). It also alters root physiological parameters, including root fresh and dry weights, root volume, reducing sugar content and free amino acids (Reilly et al., 1986; Nyczepir et al., 1986a; Nyczepir et al., 1987; Nyczepir et al., 1988a). Nematode feeding decreases the tree's tolerance to biotic and abiotic stresses, leading to tree death that is caused by either cold injury or bacterial canker.

Ring nematode parasitism induces growth hormone alteration. It suppresses indole-acetic acid (IAA) concentration in roots, and fluctuates IAA and abscisic acid (ABA) in shoots (Nyczepir and Lewis, 1980). IAA may affect tree dormancy and thus predisposes the trees to cold injury (Prince 1966; Carter 1976; Nyczepir and Lewis, 1980). Cold injury may cause widespread brown discoloration of the cambial layer on the tree trunk near the soil where the coldest winter temperatures often occur.

Under the conditions of ring nematode parasitization, peach tree susceptibility to *P.*

syringae-mediated bacteria canker increases. The infection originates in the buds and possibly in the leaf scars and epidermal cracks that occur in the fall and spring (Nyczepir et al., 1983). Flower and vegetative buds may fail to open, or open but then stop growing and die. This may occur on individual branches or the entire tree. Removal of the outer bark, the damaged reddish-brown streaks in infected branches can be observed and sour sap odor is usually association with infected tissues (Ritchie et al., 2008).

Control Methods/Orchard Management

Different nematode control methods are used in peach orchards to improve the tolerance of peach trees to PTSL. Soil chemical fumigation (Sharpe et al., 1989; Sharpe et al., 1993), bacteria-based biological controls (Kluepfel et al., 2002), biofumigation (Nyczepir and Rodriguez-Kabana, 2007), crop rotation (Nyczepir et al., 1996) or integration of these control methods are used to control ring nematodes. Because the PTSL disease syndrome is not attributed to any specific organism(s), development of rootstocks with tolerance to ring nematodes through breeding program should be an effective and sustainable method to control this syndrome.

Peach Rootstock Selection and Breeding for Tolerance to Ring Nematodes

A large number of *Prunus* accessions were tested and identified to have host suitability for ring nematodes (Westcott and Zehr, 1991; Westcott et al., 1994). Commonly used rootstocks Lovell and Nemaguard are subject to cold injury and bacterial

canker, and ultimately die from PTSL. Nemaguard is a better host to *M. xenoplax* than Lovell, peach scion cultivars grafted onto Lovell trees survive longer than on Nemaguard (Nyczepir 1990; Nyczepir and Esmenjaud, 2008). Guardian® 'BY520-9' has displayed tolerance to PTSL, causing scions to be less susceptible to bacterial canker and cold injury. Scions on Guardian®'BY520-9' had increased longevity compared with Nemaguard and Lovell (Okie et al., 1994b; Reighard et al., 2004).

The pedigree of Guardian® 'BY520-9' can be traced back to a cross made in 1954. An open-pollinated (OP) seedling of S-37 was crossed with Nemaguard. Seedling F51-25 was selected for its resistance to root-knot nematodes. After three generations of open-pollination from this seedling (F51-25), two seeding selections named BY520-8 and BY520-9, respectively, were tested on severe PTSL sites in South Carolina and Georgia. BY520-9 had the lowest incidence of tree death. OP seeds from 30 genotypes originating from BY520-9 were released under the designation Guardian® 'BY520-9' in 1994 (Okie et al., 1994a; Wilkins et al., 2002). Guardian® 'BY520-9' is comprised of bulked seeds that are not genetically uniform, so individual genotypes may have different levels of tolerance to PTSL or ring nematode infection (Blenda 2003). Selection 3-17-7 was identified as having superior horticultural characteristics (Nyczepir et al., 2006). However, the genetic basis of tolerance to ring nematodes is unknown.

In the long term, introduction of Guardian®'s tolerance to ring nematodes into a breeding program could be the ultimate solution to control PTSL and increase tree longevity. Guardian® 'BY520-9' provided a platform to explore the genetics of the tolerance. Through genetic mapping, genetic markers that closely lie to the tolerance could

be identified. It is the first step to identify the resistance genes through map-based cloning. Eventually, the cloned genes could be integrated into peach rootstock germplasm by transgenetic techniques and through breeding strategies for crop improvement (Lalli 2006). Molecular markers associated with ring nematode tolerance have the potential to be used in marker-assisted selection (MAS) breeding programs. Blenda et al. (2007) constructed the first genetic map based on a population segregating for tolerance to ring nematodes. Thirty-eight AFLPs and 18 SSRs were identified to be associated with the response to PTSL.

Genetic mapping and its development in *Prunus*

Genetic mapping is a useful tool to determine the location of genes and characterize agronomically important traits. Molecular markers—isozymes, RFLPs, AFLPs, RAPDs, SSRs and functionally meaningful markers, such as expressed sequencing tags (ESTs) are used widely in genetic map construction.

In *Prunus*, all species have a basic number of 8 chromosomes (x= 8), but may have different levels of ploidy. Peach, almond and sweet cherry are diploid with 16 chromosomes; sour cherry is tetraploid with 32 chromosomes; and European plum is hexaploid with 48 chromosomes (Dirlewanger et al., 2004b). Peach has a small genome size, with a predicted haploid genome size of 290 Mbp (Baird et al., 1994) and is used as a model plant to study the *Prunus* genome (Abbott et al., 1998).

In peach, the first genetic map was constructed with RAPDs, isozyme and morphological markers (Chaparro et al., 1994). Then, another genetic map was

constructed by Rajapakse et al. (1995) based on a F_2 population. Sixty-five markers including 46 RFLPs, 12 RAPDs and 7 morphological traits were mapped on 8 linkage groups covering 332 cM of the genome with an average marker interval of 8 cM. In 1998, several genetic maps were constructed using peach intraspecific crosses to identify molecular markers tightly linked to fruit quality components (Dirlewanger et al., 1998) and pathogen resistance (Lu et al., 1998). Besides peach, genetic maps were also constructed in almond (Ballester et al., 1998; Joobeur et al., 2000), apricot (Hurtado et al., 2002; Vilanova et al., 2003; Lambert et al., 2004), sweet cherry (Stockinger et al., 1996; Dirlewanger et al., 2004a) and plum (Foulongne et al., 2003a).

A genetic map with highly reproducible and transferable markers using *Prunus* interspecific crosses provided the resource to study the genome structure, make comparisons among different *Prunus* species and provide anchor points for maps made from different populations. In 1998, a genetic map was constructed with 235 RFLP markers and 11 isozymes from a F_2 population generated by a cross between almond 'Texas' and peach 'Earlygold' (Joobeur et al., 1998). This map is regarded as the *Prunus* reference map. All markers were placed in 8 linkage groups with a total of 491 cM coverage of the genome, and the average marker interval is 2 cM. Further, Aranzana et al. (2003a) saturated this map with 96 SSRs, and Dirlewanger et al (2004a) added 126 RFLPs, 89 SSRs and 5 sequence tag sites (STSs), resulting in the *Prunus* reference map having 562 markers and a coverage of 519 cM.

To increase the number of molecular markers on the *Prunus* reference genome, an optimized method called selective mapping (Vision et al., 2000) was used to construct a

Prunus bin map. This strategy is based on two steps. First, a mapping population with a typical size (60-250 individuals) is used to construct a saturated framework with markers placed on a genetic map with high precision. The second step is to map additional markers by screening a subset of the mapping population (less than 10 individuals). This method decreases the precision of the marker loci but saves the effort of screening a large number of the mapping population. Based on the *Prunus* reference map, Howad et al. (2005) mapped two hundred and sixty-four *Prunus* SSRs using this selective mapping concept with six selected F_2 plants from the 'T' and 'E' mapping population.

The 'T' and 'E' map provides a significant number of transferable markers that have facilitated genetic map construction among other crosses and also facilitated the location of major genes studied in different populations in the *Prunus* reference genome. (Dirlewanger et al., 2004a; Abbott et al., 2008). Several genetic maps constructed using inter or intraspecific peach crosses (Dirlewanger et al., 1998; Dettori et al 2001; Foulongne et al., 2003a; Verde et al., 2005; Yamamoto et al 2005; Dondini et al., 2007) and genetic maps of almond (Joobeur et al., 2000), and apricot (Lambert et al., 2004) were constructed with a set of markers selected from the 'T' and 'E' map. The order of genetic markers revealed highly conserved synteny among *Prunus* species by comparison between the 'T' and 'E' map and maps constructed in other *Prunus* species (Abbott et al., 2008). This indicates that gene sequence and position obtained in one species would be identical in others. It also provides a platform to integrate genes studied in different populations into a single map. However, an exception of chromosome rearrangement

(i.e., reciprocal translocation) has been reported in peach germplasm (Jauregui et al., 2001; Dirlewanger et al., 2004b; Yamamoto et al., 2001).

With the development of *Prunus* genetic maps, adequate coverage/density of markers in any genomic regions (Wang et al., 2001; Georgi et al., 2002; Abbott et al., 2008) ensure the use of markers for identification of major genes and quantitative trait loci (QTL). For peach, a limited number of major genes and QTLs controlling fruit quality (Etienne et al., 2002; Dirlewanger et al., 1998; Abbott et al., 1998; Dettori et al., 2001; Yamamoto et al., 2001; Verde et al., 2002), blooming time (Etienne et al., 2002; Verde et al., 2002), flower characters (Chaparro et al., 1994; Joobeur et al., 1998; Bliss et al., 2002; Yamamoto et al., 2001), tree architecture (Abbott et al., 1998) and disease resistance (Abbott et al., 2008) have been mapped on the *Prunus* reference map.

Mapping Disease Resistance Genes in *Prunus*

Identification of disease resistance genes in fruit tree germplasm is a major task in breeding programs. Once the resistance genes are identified, they can be introduced into breeding lines by introgression or transgenic techniques breeding strategies (Lalli 2006). Especially for simply inheritance resistance, molecular markers linked to the resistance genes can be used via MAS breeding, saving time and space compared with traditional morphological character evaluation in the field. For resistance controlled by QTLs, the contribution of each QTL to resistance has to be identified before applying molecular markers in MAS (Abbott et al., 2008).

The genetic basis of disease resistance is thought to be controlled by either single

genes or polygenes/QTLs. Previous research identified a single gene that controlled disease resistance in pumpkin (Paris et al., 1988), apple (Wearing et al., 2003) and soybean (Hill et al., 2006). In peach, root-knot nematode resistance (Lu et al., 1998; Yamamoto et al., 2001; Claverie et al., 2004; Dirlewanger et al., 2004b) was reported controlled by single genes and were mapped on linkage group 2 in peach. MAS has already been used in breeding programs to select root-knot nematode resistance rootstocks (Abbott et al., 2008).

Some disease syndromes exhibit a variable phenotype. Resistance is postulated to be controlled by QTLs, and each QTL has a different contribution to the phenotype. QTLs conferring resistance have been well-studied including rice blast fungus resistance (Wang et al., 1994), the late blight fungus resistance in potato (Leonards-Schippers et al., 1994) and bacterial wilt resistance in tomato (Danesh et al., 1994). In peach, QTLs for powdery mildew resistance were reported being mapped in linkage groups 1, 6 and 8 (Verde et al., 2002; Foulongne et al., 2003b) and QTLs for leaf curl resistance were mapped in linkage groups 3 and 6 (Viruel et al., 1998). In other *Prunus* species, QTLs for *plum pox virus* (PPV) resistance in apricot (Hurtado et al., 2002; Vilanova et al., 2003) and QTLs for mildew and leaf curl resistance in plum (Viruel et al., 1998) have been identified as well. Overall, molecular markers are useful tools in mapping agronomic traits and have potential to be used in breeding programs for cultivar or rootstock selection and improvement.

Project Overview

Development of molecular markers and genetic maps in *Prunus* provides useful information about the peach genome structure. These genetic tools have great potential to locate disease resistance genes in peach genome. In this dissertation, *Prunus* SSRs were applied to explore the genetic basis of tolerance to PTSL syndrome in peach rootstocks. Genomic regions associated with the response to PTSL were characterized. The long-term goal of this project is to identify the genes controlling the tolerance to PTSL and apply markers associated to the tolerance in rootstock selection programs.

Molecular markers have the potential to discriminate genotypes in genetic variety studies and DNA fingerprinting. This dissertation reported applying SSRs to identify peach rootstocks. The target is to develop an efficient and reliable SSR-based DNA fingerprinting system that should identify U.S. commonly used rootstocks by a set of SSR primer pairs. This research has significant implications in breeders' rights protection and certifying genetic quality of rootstock seedlings for the peach industry.

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CHAPTER TWO

MAPPING THE CHROMOSOMAL GENOMIC REGIONS ASSOCIATED WITH THE PEACH TREE SHORT LIFE SYNDROME USING MICROSATELLITE/SSR MARKERS

Introduction

Peach Tree Short Life (PTSL) is a complex disease syndrome occurring primarily in the southeastern U.S. Approximately 70% of the peach acreage in this region shows susceptibility to PTSL. This syndrome kills trees at an early age (e.g., the third or fourth year after planting). PTSL is considered a rootstock disease syndrome that is likely influenced by ring nematode [*Mesocriconema xenoplax* (Raski) Loof and de Grisse] (Nyczepir et al., 1983) feeding injury and to a lesser extent by replant soil conditions and a variety of non-specific secondary pathogens (Nesmith et al., 1981; Ritchie et al., 2008). Cultural practices such as fall pruning and fluctuations in temperature during the winter also contribute to PTSL occurrence and severity (Nyczepir 1988; Nyczepir 1990).

In early spring after flowering, emerging vegetative shoots suddenly collapse, and eventually infection of the cambial tissue by bacterial canker (*Pseudomonas syringae* pv. *syringae* van Hall) progresses down the scaffolds, and often all the way to the soil line, at which time the entire scion can be killed. Less frequently, trees are only affected near the soil line and the trunk cambium becomes less cold hardy and is injured or killed by freezing temperatures.

Different nematode control methods have been used in peach orchards to create tolerance to PTSL, such as pre- and post-plant chemical nematicides (Wehunt et al., 1980), bacteria-based biological control (Kluepfel et al., 2002), soil chemical fumigation (Sharpe et al., 1989; Sharpe et al., 1993), biofumigation (Nyczepir and Rodriguez-Kabana, 2007), crop rotation (Nyczepir et al., 1996) or integration of these methods and rootstock genotype improvement (Reighard et al., 2004). Because PTSL disease syndrome is not attributed to any specific organism(s), development of rootstocks with tolerance to ring nematodes through breeding programs should be an effective and sustainable method to control this syndrome.

Through a germplasm screening program conducted by the USDA and Clemson University, Guardian® 'BY520-9' rootstock gave excellent tree longevity on PTSL sites (Okie et al., 1994). It was released in the 1990s, for its tolerance to PTSL as bulked seeds, and it continues to perform well on PTSL sites and in commercial production. However, the genetic and molecular mechanism of this tolerance is unknown. Once the natural resistance is identified, it can be introduced into peach germplasm by transgenetic technique or introgression of the trait by breeding strategies (Lalli 2006). Molecular markers associated with the resistance genes can be applied in marker-assisted selection (MAS) breeding programs.

Molecular-marker facilitated genetic mapping has been widely used for resistance identification in many plant species such as pumpkin (Paris et al., 1988), apple (Wearing et al., 2003) and soybean (Hill et al., 2006). Peach genome structure has been studied extensively by different DNA-based molecular markers including RFLPs, RAPDs,

AFLPs and microsatellites/simple sequence repeats (SSRs). A number of major genes and QTLs that control fruit quality (Etienne et al., 2002; Dirlewanger et al., 1998; Abbott et al., 1998; Dettori et al., 2001; Yamamoto et al., 2001; Verde et al., 2002), tree architecture (Abbott et al., 1998) and disease resistance have been identified (Abbott et al., 2008). A first genetic map with molecular markers associated with the response to PTSL was reported (Blenda et al., 2007).

Compared with other molecular markers, microsatellites are very reproducible, easily detectable and highly informative (i.e., codominant marker). Approximately 500 SSRs have been developed in recent years. Three hundred SSRs were mapped on the *Prunus* reference map and other *Prunus* maps (Abbott et al., 2008). The information of SSR primer pairs can be accessed through Genome Database for Rosaceae (GDR) (http://www.bioinfo.wsu.edu/gdr/) (Jung et al., 2004). One hundred and seventy SSRs, developed from different species and evenly distributed across the *Prunus* reference genome were used in this investigation.

The ring nematode tolerant rootstock, which was one of the Guardian® 'BY520-9' selections (e.g., 3-17-7), and a ring nematode susceptible rootstock (e.g., Nemaguard) were crossed in the late 1990s. Fifteen F_1 trees were generated from this cross. Each F_1 was selfed to produce a segregating F_2 population. The F_2-11 population was used in this study because of its large size (100 individuals). Replicate plantings of the F_2 population were used to collect phenotype data of PTSL survival ratings for five years (from 2004 through 2008). The objective was to identify the chromosomal genomic regions that were

associated with the response to PTSL via SSR markers. Markers found to be associated with the response to PTSL can be used in breeding and selection programs.

Materials and Methods

Plant Materials and Genomic DNA Isolation:

The PTSL tolerant rootstock, Guardian® selection 3-17-7 (the maternal parent), and the PTSL susceptible rootstock, Nemaguard (the parental parent), were crossed in 1998 to produce 15 F_1s. Each F_1 was selfed to create a segregating F_2 population, and F_2 population sizes varied from 1(F_1-12) to 100 (F_1-11) (Table 2.1). All of these trees were planted at Musser Fruit Research Center near Seneca, South Carolina. Fresh leaf tissue from the parents, Guardian® selection 3-17-7 and Nemaguard, the 15 F_1s and the hundred F_2-11s were collected in the Summer 2005.

For each genotype, five grams of leaf tissue were placed into 5 packages with 1 gram each. Tissue packages were frozen in liquid nitrogen, and then stored at -80º C. For F_2-11, 50 genotypes were collected in 2005. The other 50 were unavailable: twenty-four trees were dead (dead trunk present/death from unknown cause) and twenty-six were missing (either the seedlings were never planted for unknown reasons or the seedlings were planted and died of causes unrelated to PTSL, and there was no visible plant in the location where the genotype should have been planted) when assessed in 2005 (Table 2.2). Leaf tissue from those genotypes was collected from seedlings in 2001 and 2002 and stored frozen at -80º C.

Genomic DNA was isolated using a modified sodium dodecyl sulfate (SDS) miniprep protocol (Dellaporta et al., 1983). DNA concentration was checked with picogreen dye (Invitrogen, Carlsbad, CA) on a TBS-380 fluorometer (Turner BioSystems, Sunnyvale, CA). Each DNA sample was diluted to 10ng/µl in de-ionized and distilled water for the subsequent SSR analysis.

Table 2.1. F_2 populations of 15 F_1s at Musser Fruit Research Center, Seneca, SC.

F_1[z]	Number of F_2 trees
1 (2001)	8
2 (2001)	37
3 (2001)	23
4 (2001)	9
5 (2001)	4
6 (2001)	20
7 (2001)	31
8 (2001)	3
9 (2001)	15
10 (2001)	18
11 (2001)	**59**
11 (2002)	**41**
12 (2001)	1
13 (2001)	17
14 (2001)	6
15 (2001)	14

[z]Year in parenthesis indicates seed harvest time. F_2-11 population (bold) was selected for this study because of the large number of progeny.

Table 2.2. Summary of F_2-11 genotypes at Musser Fruit Research Center (Seneca, SC) and the replicates planted at the Sandhill Research and Education Center (Pontiac, SC) in Summer 2005.

F_2-11 genotypes	F_2 at Musser[z]	F_2 at Sandhill
No.1	1 (D)	4
No.2	1 (S)	5
No.3	1 (D)	3
No.4	1 (S)	4
No.5	1 (S)	3
No.6	1 (D)	3
No.7	1 (D)	0
No.8	1 (D)	4
No.9	1 (M)	4
No.10	1 (S)	4
No.11	1 (M)	3
No.12	1 (S)	0
No.13	1 (S)	4
No.14	1 (D)	4
No.15	1 (S)	4
No.16	0 (T)	0
No.17	1 (D)	5
No.18	1 (M)	4
No.19	1 (S)	4
No.20	1 (D)	4
No.21	1 (D)	4
No.22	1 (S)	0
No.23	1 (M)	4
No.24	1 (S)	0
No.25	1 (S)	0
No.26	1 (S)	0
No.27	1 (M)	0
No.28	1 (D)	4
No.29	1 (S)	3
No.30	1 (M)	4
No.31	1 (D)	4
No.32	1 (S)	4
No.33	1 (S)	4
No.34	1 (D)	4
No.35	1 (D)	0
No.36	1 (S)	4
No.37	1 (S)	4
No.38	1 (M)	0
No.39	1 (S)	4
No.40	1 (D)	4
No.41	1 (M)	4
No.42	1 (S)	0
No.43	1 (D)	0
No.44	1 (S)	4
No.45	1 (S)	0

F_2-11 genotypes	F_2 at Musser	F_2 at Sandhill
No.46	1 (S)	4
No.47	1 (D)	4
No.48	1 (S)	4
No.49	1 (M)	4
No.50	1 (S)	4
No.51	1 (M)	0
No.52	1 (D)	0
No.53	1 (D)	0
No.54	1 (D)	0
No.55	1 (M)	0
No.56	1 (S)	4
No.57	1 (S)	0
No.58	1 (D)	0
No.59	1 (D)	0
No.60	1 (S)	0
No.61	1 (S)	0
No.62	0 (T)	0
No.63	1 (S)	0
No.64	0 (T)	0
No.65	1 (S)	0
No.66	1 (M)	0
No.67	1 (S)	0
No.68	1 (S)	0
No.69	1 (S)	0
No.70	1 (D)	0
No.71	1 (S)	0
No.72	0 (T)	0
No.73	0 (T)	0
No.74	0 (T)	0
No.75	1 (D)	0
No.76	1 (D)	0
No.77	1 (S)	0
No.78	1 (S)	0
No.79	1 (S)	0
No.80	0 (T)	0
No.81	1 (S)	0
No.82	1 (S)	0
No.83	1 (S)	0
No.84	1 (S)	0
No.85	0 (T)	0
No.86	1 (S)	0
No.87	1 (S)	0
No.88	0 (T)	0
No.89	0 (T)	0
No.90	0 (T)	0
No.91	1 (S)	0
No.92	1 (S)	0
No.93	0 (T)	0
No.94	1 (S)	0

F_2-11 genotypes	F_2 at Musser	F_2 at Sandhill
No.95	0 (T)	0
No.96	1 (S)	0
No.97	1 (S)	0
No.98	0 (T)	0
No.99	1 (S)	0
No.100	1 (S)	0

[z]D = dead. Trees without new shoots and leaves were scored as dead; M = missing. The map of distribution of F_2 population at Musser Fruit Research Center indicates that a tree was planted at that location but there was nothing there (no trunk, no stem, no shoot); S = survival. T = tissues only. Trees with leaf tissues collected from seedlings in the greenhouse were scored as tissues only.

SSR Markers:

Based on the unique positions of these markers in the *Prunus* genome, 170 SSRs, (evenly distributed across all the eight chromosomes), were used in this study. All of the markers were mapped to individual loci, except for five SSR markers that were multiple-loci (Table 2.3). Seventy SSRs were screened against the parents and F_1-11 while the remaining 100 SSRs were screened against the parents and four F_1s (i.e., F_1-2, F_1-6, F_1-7 and F_1-11) to identify the potential polymorphic SSRs for use in the corresponding F_2 populations. All of the SSR primer sequences were obtained from Genome Database for Rosaceae (GDR) (http://www.bioinfo.wsu.edu/gdr/) (Appendix I) and were synthesized by Integrated DNA Technologies (IDT, Coralville, IA) (http://www.idtdna.com/Scitools/Scitools.aspx).

Primer Labeling and PCR:

SSR primers were diluted to 10 pmol/µl in de-ionized and distilled water for Polymerase Chain Reaction (PCR) amplification. The forward primer of each primer-pair was radiolabelled with [γ -^{33}P] ATP using a 5'-end labeling protocol (Promega technical bulletin #519). Each 0.7µl labeling reaction contained 1.7 pmol of forward primer, 0.5µCi [γ -^{33}P] ATP (PerkinElmer, Waltham, MA), and 0.3 U T4 Polynucleotide Kinase (Promega, Madison, WI). For size reference, a DNA ladder (1kb Plus; Invitrogen, Carlsbad, CA) was similarly labeled.

The DNA sequence lying between each primer-pair was amplified by PCR. Amplifications were prepared as a 10µl reaction using the Go-Taq kit from Promega

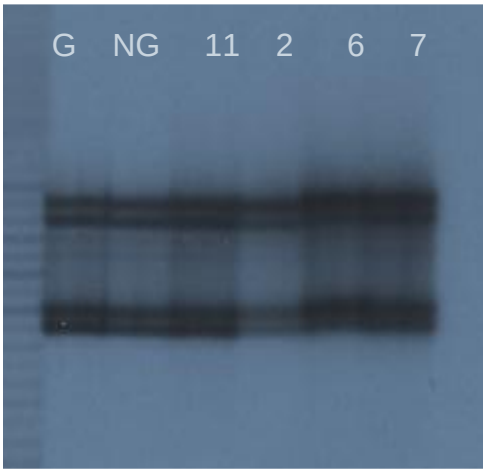
(Cat# PAM8295). Each reaction contained 30ng genomic DNA template, 0.5U Go-Taq polymerase, dNTPs (0.5mM each dNTP final), and $MgCl_2$ (1.5mM final). The entire 0.7µl radiolabeled forward primer reaction from the previous step was added along with 1.7 pmol of the reverse primer. The PCR cycling protocol was 95˚C for 5 minutes for 1 cycle; 94˚C for 45 seconds, annealing (from 46˚C to 62˚C) for 45 seconds and 72˚C for 45 seconds for 35 cycles; 72˚C for 8 minutes and then kept at 4˚C. The annealing temperature for each primer-pair was determined based on the primer sequences using IDT oligo design and analysis tool (http://www.idtdna.com/Scitools/Scitools.aspx).

Amplified DNA fragments were size fractionated through a denaturing polyacrylamide gel. Each 6% acrylamide gel (70ml) was prepared by adding 20:1 acrylamide: bisacrylamide and 7.5M urea in 1X TBE buffer. After denaturing the amplified DNA fragments at 95ºC for 5 minutes, samples were placed on ice until loaded. DNA fragments were fractionated at 80 watts for two hours in a vertical gel rig. Then, the gels were transferred to 3MM Whatman filter paper and dried for 90 minutes in a FB-GD-45 gel drier vacuum system (FisherBiotech, Wembley, West Australia, Australia). The dried gels were exposed to Kodak BioMax MR film (Eastman Kodak, Rochester, NY) in cassettes. The exposure time varied from 1 to 5 days at room temperature, depending on the strength of the radioactivity.

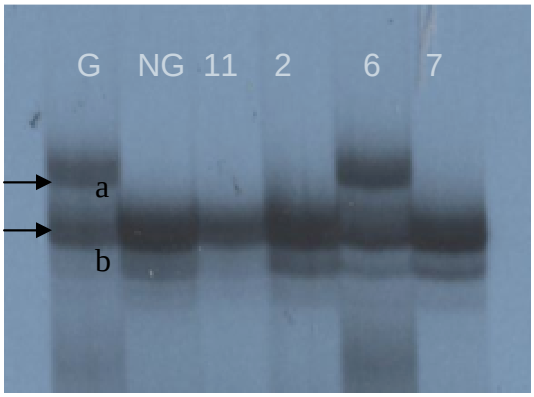
SSR Scoring:

SSR primers amplifying the same DNA band patterns between parents and F_1s in the gel were scored as monomorphic markers. SSR primers amplifying different patterns

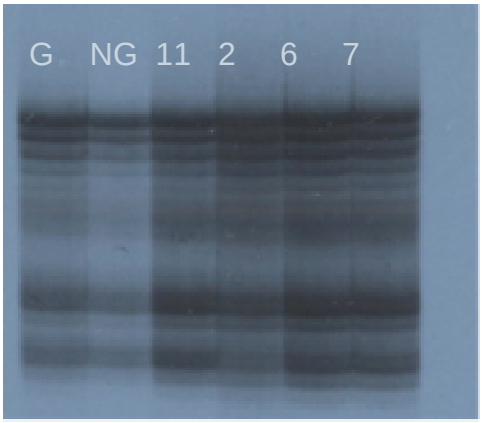
between parents and F_1s were scored as polymorphic markers. Generally, a simple pattern contains one to two bands while a complex pattern contains multiple bands (Figure 2.1). SSR markers that amplified fragments with faint or difficult to read bands were scored as inconclusive markers, which were not used in the study. Polymorphic markers falling into one of three categories were screened on the F_2 population. The first category occurs when each parent presents a distinct homozygous pattern, for example, "a" and "b", and the F_1 presents a heterozygous pattern "h". Pattern "a" represents the upper larger band group in the gel, pattern "b" represents the lower shorter band group and pattern "h" represents both pattern "a" and pattern "b" (Figure 2.2-1). The second category occurs when one parent presents homozygous pattern "a" or "b" and the other parent and F_1 present heterozygous pattern "h" (Figure 2.2-2). The third category occurs when there is band presence/absence; one parent and the F_1 have bands, resulting in "presence" pattern "P" while the other parent lacks bands, resulting in "absence" pattern "A" (Figure 2.2-3).



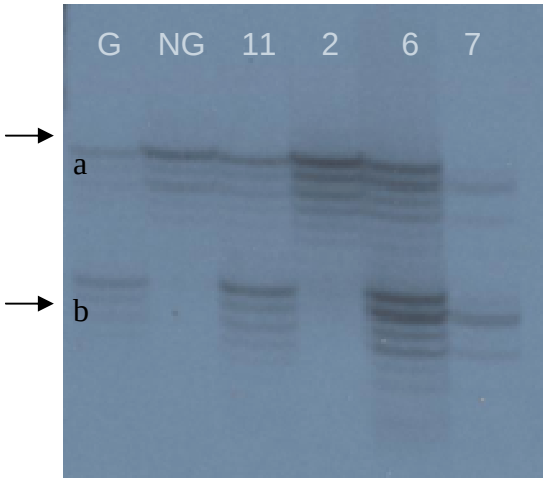
1-1. Simple monomorphic pattern amplified by Pacita4.



1-2. Simple polymorphic pattern amplified by M06a.



1-3. Multi-band monomorphic pattern amplified by CPPCT017.



1-4. Multi-band polymorphic pattern amplified by UDA011.

Figure 2.1. Examples of monomorphic and polymorphic markers. Image 1-1: Simple monomorphic pattern amplified by Pacita4; Image 1-2: Simple polymorphic pattern amplified by M06a; Image 1-3: Multi-band monomorphic pattern amplified by CPPCT017; Image 1-4: Multi-band polymorphic pattern amplified by UDA011. G= 3-17-7; NG=Nemaguard; and F_1-11, F_1-2, F_1-6 and F_1-7 are abbreviated as 11, 2, 6 and 7, respectively.

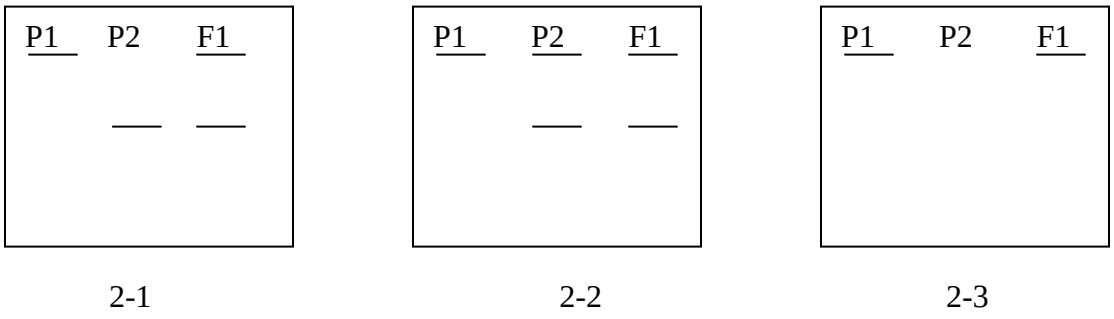


Figure 2.2. Three categories of polymorphic markers. P1 and P2 represent two parents, F1 represents F1 hybrid.

The Chi Square Test on Mendelian Ratios of SSRs:

The inheritance patterns of all the polymorphic markers in the F_2-11 population were statistically analyzed using a Chi square test by a SAS program. The target was to see whether the observed pattern segregation ratio fit the expected Mendelian ratio (i.e., 3:1 or 1:2:1) or not. Chi square value of each polymorphic marker can be calculated by the equation:

$$\chi^2 = \sum (O-E)^2/E \qquad \text{for all patterns}$$

O = observed number in one pattern

E = expected number in one pattern

χ^2 value of each marker has a corresponding p value under its specific degree of freedom, which equals to simply the number of genotypic patterns minus one. The SSR markers with p values greater than 0.05 were assumed not to deviate from Mendelian

inheritance. The SSR markers with p values less than 0.05 were assumed to deviate from Mendelian inheritance.

Phenotype Evaluation:

To rate PTSL presence and severity in this population, 38 F_2 genotypes were vegetatively replicated using nodal stem cutting propagation (Okie 1984). Each genotype was replicated no fewer than three and as many as five times resulting in 146 clones. All the replicates were planted at the Sandhill Research and Education Center (REC), Pontiac, SC in 2003. All F_2 trees were planted based on a completely randomized design (CRD).

Every spring from 2004 through 2008, all replicates trees were evaluated on the basis of the appearance of PTSL symptoms. Each tree was rated from 0 to 5 on the presence and severity of PTSL (Appendix II). A rating of "0" represents a healthy, symptomless tree; scores of "1" – "4" show increasing degree of symptoms; a rating of "5" represents death caused by PTSL (Figure 2.3). For each investigated year, the phenotype rating for each F_2 genotype is equal to the average rating obtained for its replicates.

3-1

3-2

Figure 2.3. Pictures of a healthy peach tree and a tree dead from PTSL. Picture 3-1 presents a healthy tree with PTSL rating 0 at the tree age of five; Picture 3-2 presents a dead tree with PTSL rating 5 at the tree age of five.

Nematode Population Count from Selected F_2 Replicates:

The phenotype was scored year by year. Not all of the F_2 replicates from the same genotype were rated the same in a given year. To address the effect of nematode population density variation on PTSL rating, in October 2008 soil samples from the sites of 32 F_2 replicates of 11 genotypes were collected for nematode population counts. Replicates of 11-05, 11-08 and 11-46 were rated as "0"; replicates of 11-31 were rated as "5"; individual replicates within each of the following genotypes 11-06, 11-23, 11-29, 11-31, 11-36, 11-44, 11-49 were rated either "0" or "5". For example, 11-44 had three replicates rated "5" and one replicate rated "0" in spring 2008. For each replicate, 0.45 kg of soil below the surface to 30 cm in depth, was collected by a cone tube soil sampler. Soil samples were kept below 25ºC overnight and sent to the Nematode Assay Laboratory at Clemson University to determine the number of nematodes in each sample.

Identification of SSR Markers Associated with the Response to PTSL:

The PTSL rating data of the F_2-11 population from the Sandhill REC was combined with the SSR marker inheritance data to identify the SSR loci associated with tolerance or susceptibility to PTSL. A Yearly Genotypic Mean of the patterns for each polymorphic marker was calculated by dividing the sum of the phenotype ratings of all F_2 trees (i.e., all the replicates of each F_2 genotype) that share the same inheritance pattern by the total number of those replicate trees. Analysis of Variance conducted by SAS software 9.1 (Glimmix procedure) was used to determine the level of variance among Yearly Genotypic Means. SSR markers whose Yearly Genotypic Means of PTSL rating show significant differences (at $p = 0.05$) indicate loci that might associate with the response to PTSL.

Map Construction and QTL Analysis:

To detect the PTSL QTLs in the peach genome, a genetic map with SSR markers segregating in the F_2-11 population was generated using Joinmap® 3.0 (Van Ooijen and Voorrips, 2001). Linkage groups were established at an LOD of 6.0. Genetic distance was calculated using the Kosambi function (Kosambi 1944). The PTSL phenotypic data of F_2-11 population spanning 5 years were combined with the SSR molecular marker inheritance data. QTL analysis was conducted using PLABQTL 1.2 software by implementing the composite interval mapping based on the stepwise multiple regression approach (Utz and Melchinger, 2006). QTL(s) were detected at an LOD of 2.2, which

was determined by a permutation test. The R^2 value was the percent of phenotypic variance explained by each QTL.

Results and Discussion

Identification of Polymorphic/Monomorphic Markers:

One hundred and seventy SSR markers developed from different *Prunus* species—peach (Aranzana et al., 2002; Dirlewanger et al., 2002; Yamamoto and Hayashi, 2002), apricot (Lopes et al., 2002), almond (Testolin et al., 2004), plum (Mnejja et al., 2004) and cherry (Cantini et al., 2001) were screened against the two parents (Guardian® 'BY520-9' selection 3-17-7 and Nemaguard) and the four F_1s (F_1-11, F_1-2, F_1-6 and F_1-7). One hundred and fifty markers amplified DNA fragments. This demonstrated that SSR markers with primer pairs developed from different *Prunus* species were highly transferable.

Seventy-six SSRs amplified monomorphic patterns among the parents and F_1s. The remaining 74 SSR markers (44%) amplified polymorphic patterns in the parents (Table 2.3). The percentage was lower than what was found in a previous study, using the same population (65%) (Blenda et al., 2007). This might be because only 40% of SSRs investigated in this study were developed from peach while all SSRs used in Blenda's study were developed from peach.

Table 2.3. Summary of 170 SSR markers and their corresponding amplification pattern in parents and F_1s.

Markers[z]	Location (cM) (Reference)[y]	Parents[x]	F_1-11	F_1-2	F_1-6	F_1-7
Linkage group 1						
CPPCT016	1.3 (T x E)	A, P	A	-	-	-
EPDCU3122	2.5 (T x E)	M	M	M	M	M
CPPCT010	3.8 (J x F)	S	S	S	S	S
CPSCT008	9.0 (T x E)	M	M	-	-	-
CPPCT024A	10.8 (T x E)	N	N	N	N	N
CPPCT004A	11.6 (T x E)	M	M	M	M	M
Pchcm4	13.6 (T x E)	A, P	A	-	-	-
UDA026	14.5 (*Prunus* bin map)	M	M	M	M	M
EPDCU5100	14.5 (T x E)	h, a	h	a	h	a
EPPCU5516	16-25 (*Prunus* bin map)	M	M	M	M	M
CPPCT027	23.1 (T x E)	M	M	-	-	-
CPDCT038	25.8 (T x E)	a/c, b/c	b/c	b/c	a/b	b/c
CPDCT019	31.2 (T x E)	b, h	h	-	-	-
CPSCT024	36.6 (T x E)	M	M	M	M	M
CPDCT024	37.2 (T x E)	N	N	N	N	N
Pchgms3	37.5 (T x E)	h, b	h	b	h	h
CPPCT034	40.5 (T x E)	b, h	b	-	-	-
CPDCT017	40.5 (T x E)	S	S	S	S	S
CPPCT034	40.5 (T x E)	M	M	-	-	-
pacita5	44.8 (P2175 x GN)	M	M	M	M	M
PMS67	45.9 (T x E)	A, P	P	A	P	A
EPDCU3489	46.7 (T x E)	S	S	S	S	S
BPPCT027	47.3 (T x E)	M	M	-	-	-
BPPCT016	55.2 (T x E)	b, h	h	-	-	-
CPPCT029	65.1 (T x E)	P, A	A	-	-	-
CPPCT019A	65.1 (T x E)	M	M	-	-	-
EPDCU2862	66.5 (T x E)	M	M	-	-	-
UDA031	75-78 (*Prunus* bin map)	A, P	P	P	A	A
UDA006	75-78 (*Prunus* bin map)	M	M	M	M	M
UDA009	75-78 (*Prunus* bin map)	S	S	S	S	S
BPPCT028	77.4 (T x E)	b. h	h	-	-	-
Linkage group 2						
pacita27	1.4 (P2175 x GN)	b, h	h	b	b	h
UDA008	0-8 (*Prunus* bin map)	A, P	P	A	A	P
UDA010	0-8 (*Prunus* bin map)	M	M	M	M	M
UDA029	0-8 (*Prunus* bin map)	a, h	h	a	a	h
CPPCT024B	8.4 (T x E)	M	M	M	M	M
MA024a	9.6-12.5 (*Prunus* bin map)	P, A	A	A	P	P
MA069a	9.6-12.5 (*Prunus* bin map)	S	S	S	S	S
CPDCT044	12.5 (T x E)	M	M	-	-	-
UDA051	19-20 (*Prunus* bin map)	M	M	M	M	M

Markers	Location (cM) (Reference)	Parents	F_1-11	F_1-2	F_1-6	F_1-7
BPPCT004	20.2 (T x E)	M	M	-	-	-
BPPCT001	20.9 (T x E)	a/b, a/c	a/a	a/b	a/b	a/c
BPPCT013	25 (T x E)	M	M	-	-	-
CPSCT019A	26 (*Prunus* bin map)	P, A	P	A	P	P
UDP98-411	27.8 (T x E)	M	M	M	M	M
UDP97-402	29.3 (T x E)	A, P	P	-	-	-
pchgms1	35.1 (T x E)	M	M	-	-	-
UDP98-406	81.6 (P2175 x GN)	b, a	h	-	-	-
BPPCT024	36.3 (T x E)	a/b	h	h	h	fail
BPPCT030	38 (T x E)	h, b	h	h	h	h
CPSCT021	39.4 (T x E)	b, a	h	-	-	-
CPSCT031	43.2 (T x E)	A, P	P	P	P	P
PceGA034	43.9 (T x E)	b, a	h	-	-	-
UDAp462	47-50 (*Prunus* bin map)	M	M	M	M	M
UDA020	47.1 (P x F)	h, b	b	b	h	h
CPSCT034	48.6 (T x E)	h, a	a	-	-	-
Linkage group 3						
MA034a	4-6 (*Prunus* bin map)	A,P	A	A	P	A
EPPCU5990	0-4 (*Prunus* bin map)	M	M	-	-	-
BPPCT007	11.2 (T x E)	M	M	-	-	-
UDP97-403	11.9 (T x E)	S	S	-	-	-
UDA011A	13.5 (T x E)	h, a	h	a	h	h
BPPCT039	18 (T x E)	M	M	M	M	M
CPPCT018	18 (T x E)	M	M	-	-	-
UDA033	18-22 (*Prunus* bin map)	P, A	P	-	-	-
EPDCU3083	19.8 (T x E)	S	S	S	S	S
CPSCT017	24-26 (*Prunus* bin map)	M	M	M	M	M
UDA022	24-36 (*Prunus* bin map)	P, A	P	P	A	P
CPDCT013A	28.2 (T x E)	N	N	N	N	N
CPDCT008	28.4 (T x E)	M	M	-	-	-
CPDCT025	36.4	S	S	S	S	S
Pacita4	57.8 (P2175 x GN)	M	M	M	M	M
CPDCT025	36.4 (T x E)	M	M	-	-	-
UDP96-008	36.4 (T x E)	h, b	h	h	b	h
CPDCT027	46.4 (T x E)	b, h	b	-	-	-
Linkage group 4						
CPSCT039	1.8 (T x E)	h, b	b	-	-	-
EPDCU5060	1.8 (T x E)	M	M	M	M	M
EPDC3822	6.7 (T x E)	b, h	h	b	b	h
pacita6	8.5 (J x F)	S	S	-	-	-
UDP98-024	11.3 (T x E)	h, b	h	-	-	-
CPDCT045	16.8 (T x E)	h, a	h	-	-	-
pacita25	16.9 (P2175 x GN)	M	M	M	M	M
BPPCT040	18.4 (T x E)	b, h	h	b	b	h
MA059a	22 (*Prunus* bin map)	M	M	M	M	M

Markers	Location (cM) (Reference)	Parents	F_1-11	F_1-2	F_1-6	F_1-7
Pchgms5	24.1 (T x E)	b, a	h	-	-	-
MA053a	28.3 (*Prunus* bin map)	M	M	M	M	M
UDP96-003	28.3 (T x E)	M	M	-	-	-
EPPCU1106	34-46 (*Prunus* bin map)	P, A	P	P	A	A
CPPCT003A	34.1 (T x E)	h, b	h	-	-	-
Pchgms31	38 (G x N)	b, h	h	-	-	-
BPPCT023	45.4 (T x E)	h, b	h	-	-	-
UDA027	49-62 (*Prunus* bin map)	P, A	P	P	A	A
BPPCT036	49.9 (T x E)	M	M	M	M	M
BPPCT035	50.9 (T x E)	a, b	h	-	-	-
PS12a2	78, 99 (P2175 x GN)	h, b	h	h	b	b
Linkage group 5						
UDA042	0 (*Prunus* bin map)	M	M	M	M	M
CPPCT004B	3.1 (T x E)	M	M	M	M	M
UDA048	3-8 (*Prunus* bin map)	M	M	M	M	M
BPPCT026	5.2 (T x E)	P, A	A	-	-	-
BPPCT042	5.2 (T x E)	M	M	-	-	-
CPSCT011	5.2 (T x E)	P, A	A	A	P	A
UDP97-401	11 (T x E)	P, A	P	-	-	-
pacita21	19.9 (P2175 x GN)	a, h	h	a	a	h
UDA043	15-21 (*Prunus* bin map)	c/c, a/b	a/c	b/c	b/c	a/c
BPPCT017	20.1 (T x E)	a, b	h	-	-	-
BPPCT037	25.6 (T x E)	M	M	-	-	-
Pchgms4	26.7 (T x E)	b, h	b	h	h	b
PceGA25	28.4 (T x E)	M	M	M	M	M
CPPCT013	29.2 (T x E)	M	M	M	M	M
CPDCT016	30.7 (T x E)	M	M	-	-	-
EPDCU5183	35.2 (T x E)	a, b	h	-	-	-
CPSCT022	40.7 (T x E)	M	M	-	-	-
BPPCT014	44 (T x E)	M	M	-	-	-
Linkage group 6						
EPPCU1198	0 (*Prunus* bin map)	N	N	N	N	N
UDA035	4-24 (*Prunus* bin map)	A, P	A	A	P	A
PS7a2	7 (J x F)	M	M	M	M	M
CPPCT008	8.7 (T x E)	M	M	-	-	-
UDP96-001	17.5 (T x E)	h, b	h	-	-	-
BPPCT008	30.1 (T x E)	a, b	h	-	-	-
CPPCT015	35.8 (T x E)	b/c, a/c	a/b	a/b	b/c	b/c
EPDCU2584	39.3 (T x E)	M	M	-	-	-
CPPCT023	41.5 (T x E)	M	M	M	M	M
Pchcm5	44.7 (T x E)	h, b	h	-	-	-
BPPCT025	56.4 (T x E)	b, a	h	-	-	-
EPPCU3090	58-65 (*Prunus* bin map)	P, P	A	-	-	-
UDP98-412	72 (T x E)	S	S	-	-	-
CPPCT030	80.2 (T x E)	M	M	-	-	-

Markers	Location (cM) (Reference)	Parents	F_1-11	F_1-2	F_1-6	F_1-7
CPPCT021	83.7 (T x E)	M	M	M	M	M
Linkage group 7						
EPPCU0445	0-24 (*Prunus* bin map)	M	M	M	M	M
UDA036	0-24 (*Prunus* bin map)	P, A	A	P	P	P
CPSCT004	9.5 (T x E)	M	M	-	-	-
CPPCT022	18.6 (T x E)	P, A	A	-	-	-
CPSCT026	22.3 (T x E)	M	M	-	-	-
UDP98-405	22.3 (T x E)	M	M	M	M	M
UDP98-408	23.7 (T x E)	M	M	M	M	M
CPSCT033	28.4 (T x E)	M	M	-	-	-
BPPCT029	29.6 (T x E)	h, h	h	b	a	b
Pchgms44	31.2 (P x F)	M	M	-	-	-
Pchgms46	31.2 (P x F)	M	M	-	-	-
UDAp-460	36-41 (*Prunus* bin map)	M	M	M	M	M
CPPCT033	38.9 (T x E)	M	M	-	-	-
CPSCT042	41.3 (T x E)	M	M	-	-	-
UCD-CH39	42-47 (*Prunus* bin map)	h, h	h	h	a	b
UDAp-426	42-47 (*Prunus* bin map)	a, b	h	a	b	b
MA021a	42-48 (*Prunus* bin map)	M	M	M	M	M
PS8e8	49 (T x E)	M	M	-	-	-
Pchcm2	51.4 (T x E)	h, a	h	a	a	a
CPDCT013B	56.1 (T x E)	N	N	N	N	N
MA061a	60-70 (*Prunus* bin map)	a, h	a	h	h	h
EPPCU6522	60-70 (*Prunus* bin map)	a, b	h	h	h	h
CPPCT017	61.8 (T x E)	M	M	-	-	-
EPDCU3392	64.7 (T x E)	M	M	-	-	-
PS5c3	70.6 (T x E)	A, P	A	-	-	-
Linkage group 8						
CPSCT018	0 (T x E)	P, A	A	-	-	-
UDP96-015	6 (P x F)	b, h	h	-	-	-
CPPCT019B	7.8 (T x E)	h, b	h	b	h	h
BPPCT006	14.1 (T x E)	M	M	-	-	
CPDCT020	15.2 (T x E)	N	N	N	N	N
BPPCT019	16.8 (T x E)	b, h	h	-	-	-
CPDCT034	16.8 (T x E)	S	S	S	S	S
BPPCT033	18.8 (T x E)	S	S	S	S	S
UDP96-019	20.8 (T x E)	M	M	-	-	-
EPDCU3516	22.8 (T x E)	M	M	M	M	M
BPPCT012	24.1 (T x E)	M	M	M	M	M
CPPCT006	24.8 (T x E)	M	M	-	-	-
PS1h3	31.6 (T x E)	A, P	P	-	-	-
M6a	30-40 (*Prunus* bin map)	M	M	M	M	M
CPDCT023	42.6 (T x E)	M	M	-	-	-
UDA038	42-59 (*Prunus* bin map)	N	N	-	-	-
EPPB 4233	42-59 (*Prunus* bin map)	M	M	M	M	M

Markers	Location (cM) (Reference)	Parents	F_1-11	F_1-2	F_1-6	F_1-7
EPDCU4205	43-60 (*Prunus* bin map)	N	N	-	-	-
UDP98-409	44.5 (T x E)	M	M	M	M	M
EPDCU3454	46.7 (T x E)	M	M	-	-	-
EPDCU3117	54.7 (T x E)	M	M	-	-	-

[z]Markers with a capitalized letter afterwards are mapped in multiple loci in the *Prunus* reference map.
[y]T x E: Joobeur et al., 1998; Aranzana et al., 2003; Dirlewanger et al., 2004a; *Prunus* bin map: Howad et al., 2005; J x F: Dirlewanger et al., 1998; P x F: Dettori et al., 2001; Verde et al., 2005; P2175 x GN: Dirlewanger et al., 2004b; G xN: Blenda et al., 2007.
[x]a represents homozygous banding patterns of high molecular weight DNA fragment(s); b/c represents homozygous banding patterns of low molecular weight DNA fragment(s); h represents heterozygous banding patterns. A represents pattern absent; P represents pattern present; M represents monomorphic pattern; N represents no amplification products. S represents the amplified patterns are difficult to score; - indicates F_1s that were not tested by the designated markers.

In this study, 53 SSR primer pairs amplified heterozygous patterns in F_1-11. Based on the DNA fragment patterns displayed on gel images, these 53 can be divided into three groups. In the first, 10 SSR primer pairs amplified bands scored as present (P) in one parent and absent or null bands scored in the other parent (A). F_1-11 was also scored as present (P). In the second, 13 SSR primer pairs amplified different homozygous patterns between the parents and a heterozygous pattern in F_1-11. In the last group, 30 SSR primer pairs amplified a homozygous pattern in one parent, but a heterozygous pattern in the other parent and in F_1-11. Pattern segregation for each of these markers was observed by screening them on the F_2-11 population. Based on the band clarity obtained from the F_2 population data, 47 out of the 53 markers were used in this study for further analysis. Examples of gel images of segregating patterns amplified by marker EPDCU5100 in F_2-11 population are shown in Figure 2.4.

SSR Pattern Inheritance-Chi Square Test:

The patterns of inheritance for 47 polymorphic markers was statistically analyzed using a Chi square test by a SAS program (Table 2.4). Nine markers (20%) had "p" values less than 0.05 under their specific degrees of freedom, indicating that their segregation pattern deviated from the expected Mendelian ratio (i.e., 3:1 or 1:2:1).

Phenotype Rating:

The replicated trees of population F_2-11 (38 genotypes) were evaluated annually for five years in late spring (2004-2008) for the presence and severity of PTSL (Table

2.5). Due to the nature of the disease syndrome progression of PTSL—in early years, PTSL symptoms appear only in the highly susceptible trees. Thus, in 2004 most trees were rated healthy. Since 2005, symptoms in susceptible trees have been observed, and continued in the following years (2006, 2007 and 2008). Because PTSL occurs commonly from the 3rd to the 6th year after planting, the phenotype data in 2008 were considered the most reliable.

Table 2.5 summarizes the distribution of PTSL ratings of F_2 genotypes year by year. PTSL ratings of the F_2-11 population vary continuously along a phenotypic gradient. It supports the hypothesis that PTSL tolerance or susceptibility is likely to be a quantitative trait rather than a simple Mendelian trait. Therefore, the inheritance of PTSL tolerance may be attributed to more than one gene and their interactions with the environment.

Effect of Nematode Population Density on PTSL Severity in F_2 Replicates:

For each year, there were replicates of the same F_2 genotype displaying distinctly different susceptibility to PTSL. For example, 11-44 had three replicates rated "5" and one replicate rated "0" in spring 2008. Ring nematode parasitism is considered the primary factor leading to PTSL syndrome. Variation in the density of ring nematode population might lead to replicates presenting different level of severity to PTSL. For example, a susceptible tree subjected to a low nematode population might exhibit a false tolerance. Nematode population density from the sites of 32 replicates is presented in Table 2.6. There was a trend observed for genotypes 11-23, 11-31 and 11-44 that the

replicates exposed to a low nematode population density (less than the South Carolina threshold of 50 nematodes per 100 cc soil) (Dickerson et al., 2000) were rated “0” and the replicates with high nematode population density were rated “5”. However, statistically, only genotype 11-23 showed that ring nematode density from replicates rated “0” was less than the replicate rated “5” at p value equal to 0.05. For genotypes 11-06, 11-29 and 11-49, the soil sample results showed that the replicates with low nematode population density were rated “5” while the replicates with high nematode population density were rated “0”. The replicates with scions rated “5” died years ago (2005), and the rootstock might be weak or dead also. Thus, ring nematodes had few host roots to develop high populations at the time when the soil was sampled.

Small sample size was an important limitation for statistical analysis of ring nematode population density within genotypes in this study. In addition, nematode distribution can vary dramatically even at the same sample site, and time of sampling (Nyczepir et al., 2004; Okie et al., 1994). Thus, checking nematode population density periodically instead of a one-time sampling and collecting soil samples from multiple places at each planting site will provide more information on nematode population density and its effect on severity of PTSL within replicates.

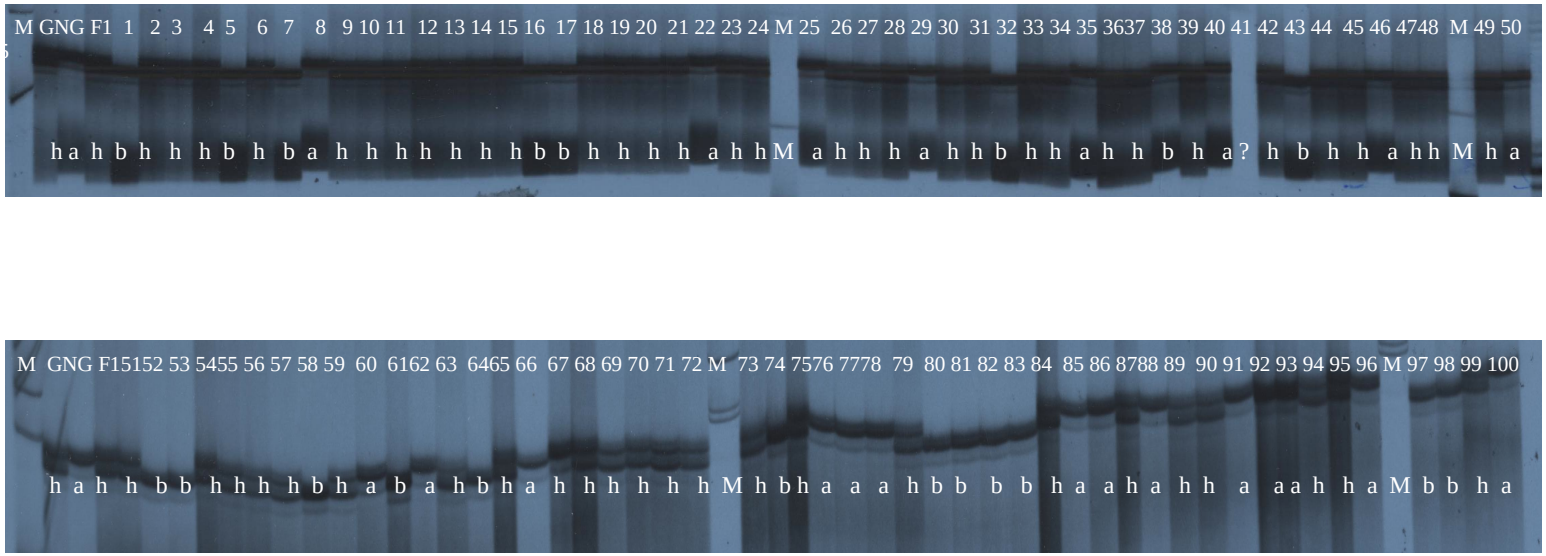


Figure 2.4. Gel images of EPDCU5100 patterns segregating in F_2 population. G: 3-1-7-7; NG: Nemaguard; F_1: F_1-11 numbers from 1 to 100 represent 100 F_2 genotypes. M: DNA ladder. “a”: Homozygous patterns of upper (larger) band(s). “b”: Homozygous patterns of lower (shorter) band(s). “h”: Heterozygous patterns.

Table 2.4. Segregation analysis of microsatellite loci in F_2-11 population.

SSR locus	Reference Locations (cM)[z]	Expected ratio	Observed ratio	χ^2	p value[y]
LG1					
EPDCU5100	14.5	1:2:1	24:56:19	2.12	0.33
CPDCT038	25.8	3:1	79:19	1.64	0.19
CPDCT019	31.2	1:2:1	13:48:39	13.6	0.001
Pchgms3	37.5	1:2:1	30:40:25	2.89	0.23
PMS67	45.9	3:1	75:24	0.03	0.86
BPPCT016	55.2	1:2:1	19:54:26	1.80	0.40
CPPCT019	65.1	1:2:1	23:46:26	0.28	0.86
UDA031	75-78	3:1	81:18	2.45	0.11
BPPCT028	77.4	1:2:1	16:63:20	7.68	0.02
Total: 9					
LG2					
pacita27	1.8	1:2:1	24:53:23	0.38	0.83
UDA008	0-8	1:2:1	20:54:22	1.58	0.45
UDA029	0-8	1:2:1	21:54:24	1	0.6
CPSCT019	26	1:2:1	28:50:17	2.81	0.24
BPPCT024	36.3	1:2:1	28:39:30	3.8	0.15
BPPCT030	38	1:2:1	32:40:28	4.32	0.11
CPSCT021	39.4	1:2:1	30:33:34	10.2	0.006
CPSCT031	43	1:2:1	29:38:33	6.08	0.04
PceGA034	43.9	1:2:1	29:36:33	7.22	0.02
UDP98-406	36	1:2:1	28:38:33	5.84	0.05
Total: 10					
LG3					
UDA011	13.5	1:2:1	24:40:35	6.09	0.04
UDA022	24-36	3:1	72:23	0.03	0.86
UDP96-008	36.4	1:2:1	27:52:20	1.24	0.53
Total: 3					
LG4					
EPDC3822	6.7	3:1	66:22	0	1
CPDCT045	16.8	1:2:1	21:52:27	0.88	0.64
BPPCT040	18.4	1:2:1	25:54:21	0.96	0.61
EPPCU1106	34-46	1:2:1	23:53:23	0.49	0.78
CPPCT003	34.1	1:2:1	23:54:23	0.64	0.72
Pchgms31	38	1:2:1	25:49:26	0.06	0.97
BPPCT023	45.4	1:2:1	20:50:29	1.64	0.44
UDA027	49-62	3:1	67:30	1.81	0.17
BPPCT035	50.9	1:2:1	21:47:31	2.27	0.32
PS12a2	78, 99	1:2:1	21:44:35	5.36	0.07
Total: 10					
LG5					

SSR locus	Reference Locations (cM)	Expected ratio	Observed ratio	χ^2	p value
BPPCT017	20.1	1:2:1	14:53:33	7.58	0.02
EPDCU5183	35.2	1:2:1	29:48:21	1.34	0.51
Pacita21	48.9	1:2:1	31:48:16	4.85	0.08
Total: 4					
LG6					
UDP96-001	17.5	1:2:1	20:45:34	4.77	0.09
BPPCT008	30.1	1:2:1	18:55:27	2.62	0.27
CPPCT015	35.8	1:2:1	24:55:18	2.48	0.28
Pchcm5	44.7	1:2:1	17:47:31	4.13	0.12
BPPCT025	56.4	1:2:1	23:56:21	1.52	0.46
Total: 5					
LG7					
UDAp-426	42-47	3:1	79:18	2.14	0.14
Pchcm2	51.4	1:2:1	25:59:14	6.55	0.03
Total: 2					
LG8					
UDP96-015	6	1:2:1	17:61:21	5.67	0.58
CPPCT019	7.8	1:2:1	23:46:26	0.28	0.86
BPPCT019	16.8	1:2:1	17:60:19	6.08	0.04
BPPCT033	18.8	1:2:1	18: 60: 22	4.32	0.11
Total: 4					

[z]Reference locations are referred to Table 2.3.
[y]$p < 0.05$ means that the observed pattern segregation ratio deviates from the Mendelian inheritance segregation ratio (1:2:1 or 3:1).

Table 2.5. Statistics of average PTSL rating of F_2 genotypes from 2004 to 2008.

Tested year	Number of F_2 genotypes classified by the average PTSL rating				
	0-1	1-2	2-3	3-4	4-5
2004	29	7	2	0	0
2005	7	8	9	10	4
2006	8	8	7	10	5
2007	7	7	8	6	10
2008	4	7	3	11	13

Identification of SSR Markers Associated with the Response to PTSL:

--Yearly Genotypic Means:

To identify whether the SSR markers screened on the F_2-11 population cosegregate or not with PTSL for any given year, the phenotype data of each replicate were combined with its corresponding genotype data. For each investigated marker, the genotypic data will remain fixed, but the phenotypic rating may change from year to year. Each F_2 genotype was scored for its amplified pattern (A, P or a, b, h), and all the replicates of a F_2 genotype were assigned the same banding pattern designation. For example, Marker EPDCU5100 amplified pattern "b" in genotype F_2-1 (Figure 2.4). F_2-1 has 4 replicates (Appendix II), so each replicate was assigned the genotypic data pattern "b". For every investigated year, each of the 4 replicates of F_2-1 had its own phenotypic rating (from 0 to 5).

Phenotype rating mean of a given genotype for a polymorphic marker was calculated on a year by year basis to determine the effect of the genotype to PTSL syndrome. Phenotype rating mean was termed as Yearly Genotypic Mean in this study. It can be calculated by dividing the sum of the phenotype ratings of all F_2 trees (i.e., all the replicates of each genotype) that share the same inheritance pattern by the total number of those replicated trees. In this study, only replicates planted at Sandhill REC were scored for phenotype rating. The original genotype, planted at Musser Fruit Research Center was not evaluated. Yearly Genotypic Means for each of the 47 SSR markers were calculated in the same way (Table 2.7).

--Analysis of Variance for SSRs Yearly Genotypic Means:

For each SSR locus, when there is a significant difference ($p < 0.05$) among the Genotypic Means of the inheritance patterns, this indicates that the SSR likely cosegregates with the response to PTSL in that year. When there is no statistical difference ($p \geq 0.05$) between the Genotypic Means of the inheritance patterns, this indicates that the SSR marker does not cosegregate with the PTSL response in that year.

Table 2.7 also gives an output of Analysis of Variance of each polymorphic marker from 2004 through 2008. In this table, variance levels among Yearly Genotypic Means are denoted as A – no significant difference between each pair of compared Genotypic Means; or B/C – significant difference between each two-compared Genotypic Means. For example, for marker EPDCU5100 in 2004, the Genotypic Means of pattern "a", "b" and "h" are 0.26, 0.31 and 0.57, respectively. Each Genotypic Mean was rated with the same letter A, meaning that there was no statistically significant difference among the Genotypic Means for each of the three inheritance patterns. Therefore, in 2004, marker EPDCU5100 did not associate with PTSL response. In 2005, the Genotypic Means of pattern "a", "b" and "h" were 0.59, 2.7 and 2.8, respectively. Genotypic Mean for pattern "a" was rated as B while the other two Genotypic Means were rated as A. This indicates that the Genotypic Means for pattern "a" was significantly different from pattern "b" and "h". In 2005, marker EPDCU5100 might associate with a PTSL response. Using the same method to interpret the data for 2006, 2007 and 2008, marker EPDCU5100 might associate with PTSL in these last years also. Beginning in 2005, marker EPDCU5100 started to show its association with PTSL and kept this trend in the

following years. It is likely that the genomic region where marker EPDCU5100 resides could be linked with genes controlling the PTSL response. The Analysis of Variance for all of the 47 SSR markers was calculated using the same method.

Nineteen markers did not cosegregate with PTSL response in any of the five years. Another eighteen markers showed cosegregation with PTSL response in the early years only or in random, non-consecutive years. These markers are unlikely to be fundamentally important in the genotypes' response to PTSL. In contrast, markers that may or may not indicate cosegregation in early years (e.g., 2004, 2005) but that do indicate cosegregation in subsequent consecutive years (e.g., 2007, 2008) are likely associated with the response to PTSL. There are nine markers that showed the trend of cosegregation with PTSL response for the last four or five consecutive years. They are assumed to be strongly associated with PTSL response. They are distributed on four linkage groups of the *Prunus* reference map — Linkage Group 1 (LG1), 2, 4 and 6 (Figure 2.5).

EPDCU5100, developed from almond (Howad et al., 2005), was the only SSR on LG1 showing association with the response to PTSL in the latter 4 years. Interestingly, EPDCU5100 was reported to be useful for MAS for breeding for *plum pox virus* resistance in apricot through a genetic study of a backcross population of 'Stark Early Orange' and 'Vester' (Lalli et al., 2008).

On LG2, three SSRs (pacita27, UDA029, UDA008) located within 10 cM from the top exhibited association with the response to PTSL for all five-year data. It indicated that this region of LG2 should associate for the response to PTSL syndrome. Previously,

pacita 27 developed from apricot (Lopes et al., 2002), was mapped to the root-knot nematode resistance trait in peach (Dirlewanger et al., 2004b). In the *Prunus* resistance map, several resistance gene analogs (RGAs) (AC33A, AC37A, AC31 and AG43A) were reported being detected in the same region of LG2 (Lalli et al., 2005). The top region of LG2 might be very interesting and have significant meaning for disease resistance in *Prunus*.

Also, on LG2, UDP98-406, developed from peach (Testolin et al., 2000), CPSCT031 developed from plum (Mnejja et al., 2004) and PceGA034 developed from sour cherry (Cantini et al., 2001) showed association with the population's response to PTSL in the latter 4 years. Interestingly, in Blenda et al. (2007) using the same population, UDP98-406 was reported to be associated with the response to PTSL. A powdery mildew resistance locus and a RGA (PC32B) are reported to be located within 10 cM of CPSCT031 and PceGA034 (Dettori et al., 2001; Lalli et al., 2005).

Ps12a2 on LG4 that was developed from sour cherry (Joobeur et al., 2000; Cantini et al., 2001) was found to associate with the response to PTSL in the latter 4 years. However, no RGAs or other resistance traits were reported to be located in this region.

Likewise, Pchcm5 on LG6 developed from peach (Sosinski et al., 2000) was found to associate with the response to PTSL in the latter 4 years. In Blenda et al. (2007), Pchcm5 was identified to be associated with the response to PTSL syndrome as well. In addition, this locus is where the major QTL associated with a powdery mildew resides (Dirlewanger et al., 1996).

Genetic Contribution of SSR Loci to the PTSL Syndrome:

To analyze the contribution/importance of each SSR locus to the PTSL syndrome, the difference between two extreme Genotypic Means of SSRs (EPDCU5100, pacita 27, UDA008, UDA029 UDP98-406, CPSCT031 and PceGA034, Ps12a2 and Pchcm5) was calculated using phenotypic data from 2008. The larger the difference, the more the marker contributes to PTSL.

Markers with a larger Genotypic Mean difference may be linked to the regions of chromosomes that are more responsive to the PTSL response than markers with a smaller Genotypic Mean difference. Figure 2.6 demonstrates that markers EPDCU5100, pacita 27, UDA008 and UDA029 generated Genotypic Mean differences ranging from 2.5 to 3.3, which are larger than the other 5 markers with Genotypic Mean differences ranging from 1.1 to 2. This suggested that these four SSR loci contribute more to the response to PTSL. In addition, future studies could focus on screening additional markers flanking loci EPDCU5100, pacita 27, UDA008 and UDA029.

Table 2.6. Nematode population density from the selected replicates of F_2 genotypes.

F_2 genotypes	Location[z]	PTSL rating[y]	Year of tree died	Nematodes /100 cc soil
11-05	3W	0		179
	5E	0		179
11-08	1E	0		54
	3E	0		50
	5W	0		227
	7W	0		31
11-46	2E	0		52
	3W	0		454
	4E	0		140
	6W	0		53
11-32	1E	5	2007	413
	3W	5	2005	61
11-06	1E	0		153
	3E	5	2007	30
	5E	0		252
11-29	1E	5	2005	12
	3W	0		57
	5E	0		194
11-49	2W	5	2005	26
	3W	0		52
	4E	0		130
11-23	1E	0		25
	3W	0		27
	4E	5	2008	48
11-31	1E	0		24
	3E	5	2004	83
11-36	1E	0		28
	3W	0		209
	4E	5	2008	242
11-44	2E	5	2005	96
	3W	0		37
	4E	5	2004	119

[z]The replicates were planted in seven double rows. A tree site is represented by a row number and the side of the row. W: West side of a row: E: East side of a row.
[y]PTSL rating of each tree is evaluated using a 0 to 5 system; 0 represents healthy, no symptoms and 5 represents scion death caused by PTSL.

Table 2.7. PTSL rating means of SSR pattern of 47 SSR markers.

EPDCU5100 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.26 (A)	0.59 (B)	0.52 (B)	0.69 (B)	0.63 (B)
b	0.31 (A)	2.70 (A)	3.00 (A)	3 04 (A)	3.16 (A)
h	0.57 (A)	2.80 (A)	2.70 (A)	3.38 (A)	3.76 (A)

pacita27 Patterns	Genotypic Mean on a yearly basis				
	2004	2005	2006	2007	2008
a	0.92 (A)	3.41 (A)	3.63 (A)	3.77 (A)	3.87 (A)
b	0.24 (B)	1.16 (C)	1.07 (C)	1.01 (C)	1.36 (B)
h	0.27 (B)	2.41 (B)	2.13 (B)	2.69 (B)	3.45 (A)

UDA008 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.08 (B)	0.71 (B)	0.58 (C)	0.65 (B)	0.76 (B)
b	0.86 (A)	3.41 (A)	3.55 (A)	3.78 (A)	3.78 (A)
h	0.39 (B)	2.77 (A)	2.55 (B)	3.03 (A)	4.07 (A)

UDA029 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.08 (B)	0.72 (C)	0.58 (C)	0.65 (C)	0.75 (B)
b	0.83 (A)	3.56 (A)	2.46 (B)	3.95 (A)	4.00 (A)
h	0.45 (B)	2.68 (B)	2.70 (A)	2.93 (B)	3.88 (A)

UDP98-406 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.67 (A)	2.61 (B)	2.65 (B)	2.90 (B)	3.09 (B)
b	0.37 (A)	2.02 (B)	3.00 (A)	2.04 (B)	2.76 (B)
h	0.55 (A)	3.10 (A)	2.70 (A)	3.45 (A)	3.87 (A)

CPSCT031 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.60 (A)	2.34 (B)	2.40 (B)	2.62 (B)	2.77 (B)
b	0.33 (A)	2.07 (B)	1.79 B)	2.08 (B)	2.84 (B)
h	0.60 (A)	3.36 (A)	3.47 (A)	3.85 (A)	4.27 (A)

PceGA034 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.60 (A)	2.34 (B)	2.40 (B)	2.61 (B)	2.70 (B)
b	0.36 (A)	1.94 (B)	1.66 (B)	1.87 (B)	2.67 (B)
h	0.67 (A)	3.29 (A)	3.44 (A)	3.83 (A)	4.32 (A)

Ps12a2 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.50 (A)	1.40 (B)	1.30 (B)	1.33 (B)	1.59 (B)
b	0.36 (A)	2.50 (B)	2.52 (B)	2.80 (A)	3.34 (A)
h	0.60 (A)	2.84 (A)	2.72 (A)	3.08 (A)	3.60 (A)

pchcm5 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.60 (A)	2.09 (B)	2.14 (B)	2.28 (B)	2.86 (B)
b	0.33 (A)	1.70 (B)	1.74 (B)	1.96 (B)	2.33 (B)
h	0.57 (A)	3.23 (A)	3.06 (A)	3.48 (A)	4.18 (A)

BPPCT008 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.56 (A)	2.28 (A)	2.55 (A)	2.79 (A)	2.97 (B)
b	0.42 (A)	2.32 (A)	2.21 (A)	2.29 (A)	2.74 (B)
h	0.49 (A)	2.80 (A)	2.53 (A)	3.00 (A)	3.76 (A)

BPPCT016 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.00 (A)	2.11 (A)	1.70 (A)	1.86 (A)	2.82 (A)
b	0.47 (A)	2.45 (A)	2.30 (A)	2.70 (A)	3.17 (A)
h	0.61 (A)	2.64 (A)	2.69 (A)	2. 96 (A)	3. 38 (A)

BPPCT017 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	1.25 (A)	3.87 (A)	3.75 (A)	4.37 (A)	4.37 (A)
b	0.46 (A)	2.69 (A)	2.50 (A)	2.72 (B)	2.84 (A)
h	0.44 (A)	2.29 (A)	2.30 (A)	2.60 (B)	3.36 (A)

BPPCT019 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.61 (A)	2.76 (A)	2.74 (A)	2.98 (A)	3.72 (A)
b	0.48 (A)	2.35 (A)	2.12 (A)	2.31 (A)	2.60 (A)
h	0.44 (A)	2.45 (A)	2.41 (A)	2.76 (A)	3.23 (A)

BPPCT023 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.38 (B)	1.37 (B)	1.38 (A)	1.50 (A)	1.69 (B)
b	0.24 (B)	2.30 (B)	2.46 (A)	2.85 (A)	3.10 (A)
h	0.72 (A)	2.90 (A)	2.63 (A)	2.86 (A)	3.68 (A)

BPPCT024 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.74 (A)	2.86 (A)	2.94 (A)	3.18 (A)	3.48 (A)
b	0.37 (A)	2.12 (A)	1.83 (B)	2.08 (B)	2.95 (A)
h	0.50 (A)	2.76 (A)	2.71 (B)	2.94 (B)	3.38 (A)

BPPCT025 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.34 (A)	2.36 (A)	2.79 (A)	2.52 (A)	3.26 (A)
b	0.43 (A)	2.21 (A)	1.79 (B)	2.31 (A)	3.20 (A)
h	0.55 (A)	2.70 (A)	2.81 (A)	2.99 (A)	3.27 (A)

BPPCT028 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.33 (A)	1.86 (A)	1.69 (A)	1.86 (A)	2.80 (A)
b	0.80 (A)	2.90 (A)	2.68 (A)	2.95 (A)	3.48 (A)
h	0.41 (A)	2.53 (A)	2.55 (A)	2.86 (A)	3.26 (A)

BPPCT030 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.52 (A)	2.18 (A)	2.00 (A)	2.35 (A)	3.08 (A)
b	0.73 (A)	2.87 (A)	2.94 (A)	3.16 (A)	3.25 (A)
h	0.32 (A)	2.61 (A)	2.57 (A)	2.85 (A)	3.40 (A)

BPPCT033 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.61 (A)	2.76 (A)	2.74 (A)	2.98 (A)	3.72 (A)
b	0.48 (A)	2.35 (A)	2.12 (A)	2.31 (A)	2.60 (A)
h	0.44 (A)	2.45 (A)	2.41 (A)	2.76 (A)	3.22 (A)

BPPCT035 Patterns	Genotypic Mean on a yearly basis				
	2004	2005	2006	2007	2008
a	0.58 (B)	2.11 (B)	2.23 (A)	2.33 (A)	1.69 (B)
b	0.21 (B)	2.11(B)	2.21 (A)	2.57 (A)	3 20 (A)
h	0.73 (A)	3.00 (A)	2.73 (A)	3.00 (A)	3.63 (A)

BPPCT040 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.67 (A)	2.75 (A)	2.61 (A)	3.01 (A)	3.47 (A)
b	0.31 (A)	2. 09 (A)	2.35 (A)	2.68 (A)	3.41 (A)
h	0.52 (A)	2.69 (A)	2.43 (A)	2.65 (A)	3.00 (A)

CPDCT019 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.41 (A)	3.16 (A)	2.25 (A)	2.58 (A)	3.58 (A)
b	0.63 (A)	2.57 (A)	2.67 (A)	2.96 (A)	3.45 (A)
h	0.38 (A)	2.35 (A)	2.28 (A)	2.56 (A)	2.96 (A)

CPDCT038 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
A	0.00 (A)	2.00 (A)	1.66 (A)	1.95 (A)	2.47 (A)
P	0.57 (A)	2.61 (A)	2.58 (A)	2.88 (A)	3.39 (A)

CPPCT003 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.57 (A)	2.80 (A)	2.76 (A)	3.25 (A)	3.61 (A)
b	0.28 (A)	2.17 (A)	2.32 (A)	2.79 (A)	3.21 (A)
h	0.62 (A)	2.69 (A)	2.43 (A)	2.52 (A)	3.12 (A)

CPPCT015 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.46 (A)	2.02 (A)	2.10 (A)	2.34 (A)	2.50 (B)
b	0.54 (A)	2.23 (A)	2.41 (A)	2.79 (A)	3.04 (B)
h	0.47 (A)	2.92 (A)	2.62 (A)	2.88(A)	3.74 (A)

CPPCT019 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.76 (A)	2.36 (A)	2.10 (A)	2.55 (A)	3.41 (A)
b	0.00 (A)	2.33 (A)	2.21 (A)	2.30 (A)	2.85 (A)
h	0.43 (B)	2.56 (A)	2.56 (A)	2.86 (A)	3.28 (A)

CPSCT019 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.36 (A)	1.99 (B)	2.23 (A)	2.54 (A)	2.49 (B)
b	0.54 (A)	2.45 (B)	2.12 (A)	2.32 (A)	3.21 (B)
h	0.56 (A)	2.90 (A)	2.71 (A)	3.06 (A)	3.78 (A)

CPSCT021 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.48 (A)	2.64 (A)	2.79 (A)	3.21(A)	3.21 (A)
b	0.35 (A)	2.07 (A)	1.79 (B)	2.07 (A)	2.90 (A)
h	0.57 (A)	2.80 (A)	2.81 (A)	3.03 (B)	3.55 (A)

EPDC3822 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
A	0.57 (A)	3.09 (A)	2.88 (A)	3.25 (A)	3.63 (A)
P	0.39 (A)	2.25 (A)	2.23 (A)	2.54 (A)	3.12 (A)

CPDCT045 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.29 (A)	2.00 (A)	2.27 (A)	2.60 (A)	3.30 (A)
b	0.67 (A)	2.75 (A)	2.61 (A)	3 01 (A)	3.47 (A)
h	0.54 (A)	2.77 (A)	2.50 (A)	2.70 (A)	3.07 (A)

EPDCU5183 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.41 (A)	2.38 (A)	2.33 (A)	2 52 (A)	2.79 (B)
b	0.66 (A)	2.54 (A)	2.41 (A)	2.66 (A)	3.95 (A)
h	0.48 (A)	2.60 (A)	2.53 (A)	2.91 (A)	3.29 (B)

EPPCU1106 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.57 (A)	2.80 (A)	2.76 (A)	3.25 (A)	3.61 (A)
b	0.28 (A)	2.17 (A)	2.32 (A)	2.79 (A)	3.21 (A)
h	0.62 (A)	2.69 (A)	2.43 (A)	2.52 (A)	3.12 (A)

pacita21 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.46 (A)	2.69 (A)	2.50 (A)	2.72 (B)	2.84 (A)
b	1.25 (A)	3.87 (A)	3.75 (A)	4.37 (A)	4.38 (A)
h	0.44 (A)	2.29 (A)	2.31 (A)	2.60 (B)	3.36 (A)

pchcm2 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.14 (A)	1.96 (A)	1.65 (B)	1.95 (B)	2.64 (A)
b	0.57 (A)	1.90 (A)	1.86 (B)	2.18 (B)	3.00 (A)
h	0.62 (A)	2.78 (A)	2.81 (A)	3.09 (A)	3.46 (A)

pchgms3 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.75 (A)	2.57 (A)	2.67 (A)	2.87 (A)	3.35 (A)
b	0.45 (B)	2.87 (A)	2.45 (A)	2.93 (A)	3.52 (A)
h	0.20 (B)	2.15 (A)	2.12 (A)	2.33 (A)	2.83 (A)

pchgms31 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.34 (A)	2.82 (A)	2.27 (A)	2.86 (A)	3.51 (A)
b	0.64 (A)	2.42 (A)	2.37 (A)	2.74 (A)	3.30 (A)
h	0.45 (A)	2.45 (A)	2.56 (A)	2.69 (A)	3.09 (A)

PMS67 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
A	0.64 (A)	2.50 (A)	2.33 (A)	2.51 (A)	3.26 (A)
P	0.40 (A)	2.52 (A)	2.47 (A)	2.85 (A)	3.18 (A)

UDA011 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.41 (A)	2.40 (B)	2.41 (A)	2.91 (A)	3.31 (A)
b	0.32 (A)	2.04 (B)	2.07 (A)	2.36 (A)	2.88 (A)
h	0.70 (A)	3.04 (A)	2.82 (A)	2.97 (A)	3.58 (A)

UDA022 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
A	0.80 (A)	2.52 (A)	2.48 (A)	2.95 (A)	3.36 (A)
P	0.45 (A)	2.42 (A)	2.33 (A)	2.60 (A)	3.15 (A)

UDA027 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
A	0.27 (A)	2.25 (A)	2.38 (A)	2.72 (A)	3.20 (A)
P	0.71 (A)	2.66 (A)	2.45 (A)	2.64 (A)	3.18 (A)

UDA031 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
A (G)	1.06 (A)	3.69 (A)	3.35 (A)	3.62 (A)	3.81 (A)
P (NG)	0.35 (B)	2.36 (B)	2.29 (B)	2.61 (B)	3.16 (A)

UDA043 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	1.41 (A)	3.50 (A)	3.58 (A)	3.83 (A)	3.75 (A)
b	0.34 (B)	2.68 (A)	2.43 (A)	2.72 (A)	2.87 (A)
h	0.44 (B)	2.29 (A)	2.31 (A)	2.60 (A)	3.36 (A)

UDAp426 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
A	0.62 (A)	2.74 (A)	2.00 (A)	2.30 (A)	3.00 (A)
P	0.48 (A)	2.56 (A)	2.58 (A)	2.88 (A)	3.40 (A)

UDP96-001 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.61 (A)	2.40 (A)	2.66 (A)	2.98 (A)	3.17 (A)
b	0.39 (A)	2.29 (A)	2.13 (A)	2.24 (A)	2.79 (A)
h	0.47 (A)	2.79 (A)	2.54 (A)	2.95 (A)	3.71 (A)

UDP96-015 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.52 (A)	2.57 (A)	2.55 (A)	2.81 (A)	3.61 (A)
b	0.57 (A)	1.76 (A)	1.80 (A)	1.81 (B)	2.00 (B)
h	0.46 (A)	2.68 (A)	2.56 (A)	2.94 (A)	3.42 (A)

UDP96-008 Patterns	Genotypic Mean on a yearly basis[z]				
	2004	2005	2006	2007	2008
a	0.21 (A)	2.00 (A)	2.00 (A)	2.13 (B)	2.34 (A)
b	0.71 (A)	2.26 (A)	2.21 (A)	2.65 (B)	3.00 (A)
h	0.54 (A)	2.84 (A)	2.73 (A)	3.05(A)	3.68 (A)

[z](A) indicates no significant difference between Genotypic Means ($p \geq 0.05$) in a given year; (B)/(C) indicates a significant difference between Genotypic Means ($p < 0.05$). The nine SSRs identified to be associated with the response to PTSL are listed first in the table. The remaining SSRs of the 47 are listed in an alphabetical order.

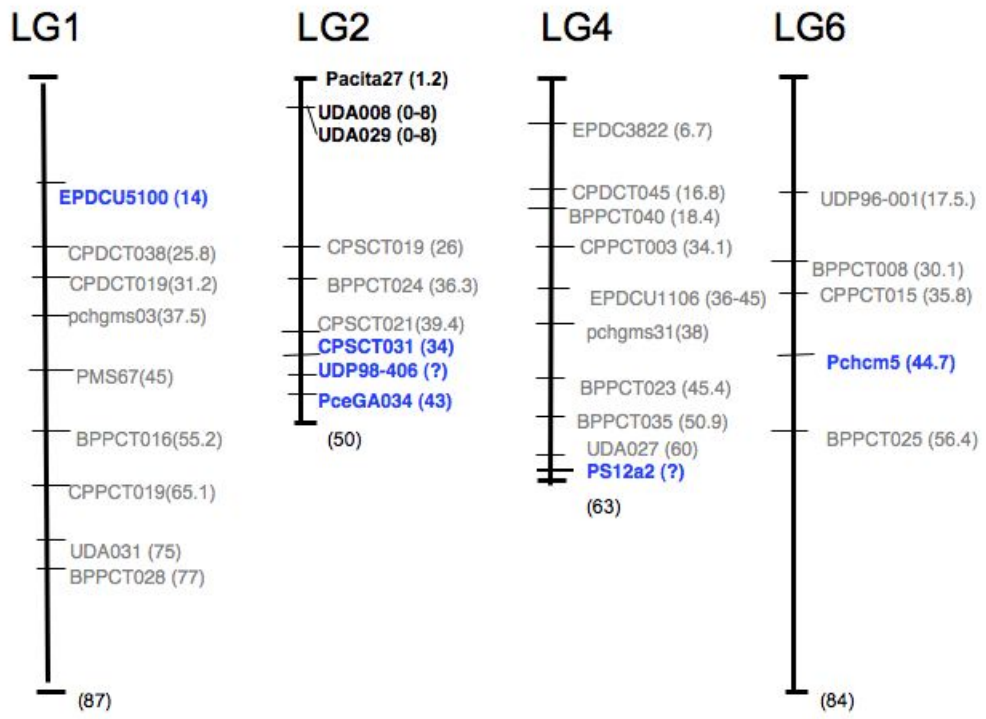


Figure 2.5. Distribution of SSRs and identification of those associated with the response to PTSL. Markers co-segregate with PTSL for all 5 years (from 2004 to 2008) are in black. Markers co-segregate with PTSL for consecutive 4 years (from 2005 to 2008) are in blue. Markers represent the subset of the 47 polymorphic markers distributed on these 4 linkage groups are in grey. ? indicates that SSR markers UDP98-406 (LG2) and Ps12a2 (LG4) were not mapped on the *Prunus* reference map. Their location are referred from map of Guardian® x Nemaguard constructed in this study.

Map Construction and QTL Analysis:

To detect QTLs associated with PTSL loci, a total of 47 SSR markers were analyzed to construct a linkage map from the F_2-11 population segregating for PTSL. Thirty SSRs were assigned to seven linkage groups (Figure 2.7). This was named the

"Guardian® x Nemaguard" (G x N) map. The map coverage was estimated at 217.5 cM with an average marker interval of 7.25 cM. It is shorter than other published peach genomes, probably because 47 markers is a low number to cover the entire peach nuclear genome. For example, in previous studies Yamamoto et al. (2005) used 178 markers to construct a genetic map with the coverage of 571 cM of the genome, and Blenda et al. (2007) used 158 markers to construct a genetic map with the coverage of 737 cM. The orientation of seven linkage groups was verified according to the *Prunus* reference map (Joobeur et al., 1998) and *Prunus* bin map (Howad et al., 2005). LG2 was represented by two groups, LG2a and LG2b. LG7 and LG8 could not be mapped because of the small number of markers segregating in the G x N population located on these linkage groups and also the spacing between the markers. Marker order shown in this map agreed with the *Prunus* reference map and *Prunus* bin map, except for marker UDAp-426 (Table 2.8). UDAp-426 was mapped on LG1 in the G x N map but was reported being mapped on LG7 in the *Prunus* bin map. It is possibly a misplacement in the bin mapping because of its low mapping precision.

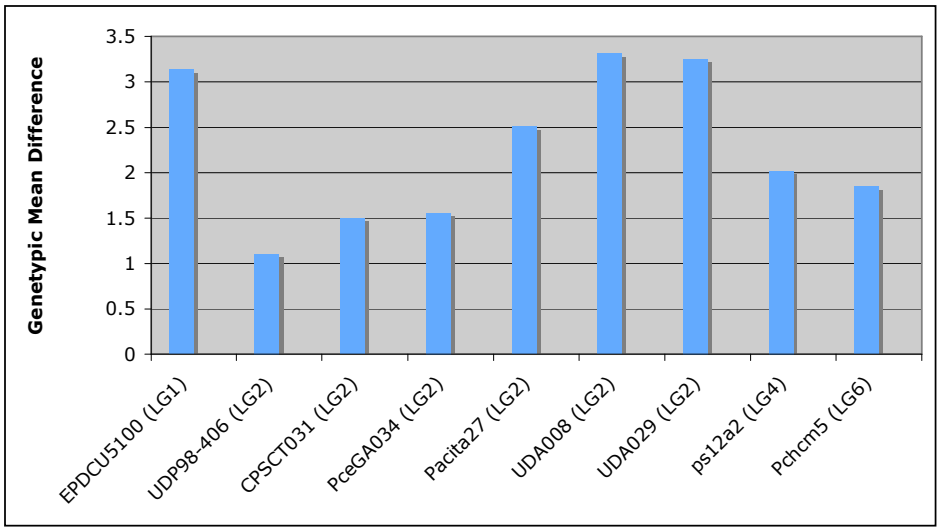


Figure 2.6. Comparison of differences of Genotypic Means in 2008.

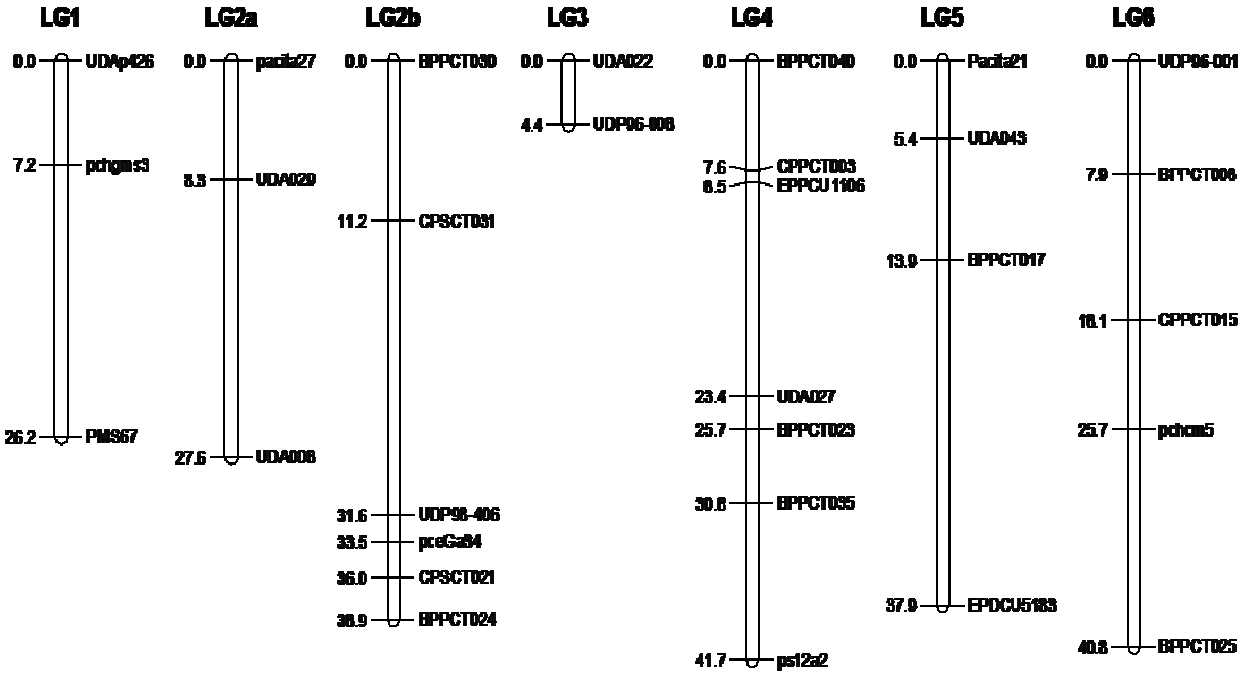


Figure 2.7. A genetic map (Guardian x Nemaguard) generated based on the F_2-11 population.

Table 2.8. Marker order comparison between the genetic map (Guardian® x Nemaguard) and the reference maps.

SSRs	Location[z]	
	G x N	Reference location
LG1		
UDAp426	0.0	42-47 (LG7) (*Prunus* bin map)
pchgms03	7.2	37.5 (T x E)
PMS67	26.2	45.9 (T x E)
LG2a		
pacita27	0.0	1.8 (P2175 x GN)
UDA029	8.27	0-8 (*Prunus* bin map)
UDA008	27.6	0-8 (*Prunus* bin map)
LG2b		
BPPCT030	0.0	38 (T x E)
CPSCT031	11. 2	43 (T x E)
UDP98-406	31.6	81.6 (P2175 x GN)
PceGA034	33.5	43.9 (T x E)
CPSCT021	35.9	39.4 (T x E)
BPPCT024	38.8	36.3 (T x E)
LG3		
UDA022	0.0	24-36 (*Prunus* bin map)
UDP96-008	4.3	36.4 (T x E)
LG4		
BPPCT040	0.0	18.4 (T x E)
CPPCT003	7.6	34.1 (T x E)
EPPCU1106	8.4	34-46 (*Prunus* bin map)
UDA027	23.3	49-62 (*Prunus* bin map)
BPPCT023	25.7	45.4 (T x E)
BPPCT035	30.8	50.9 (T x E)
PS12a2	41.7	78, 99 (P2175 x GN)
LG5		
Pacita21	0.0	19 (P2175 x GN)
UDA043	5.4	15-21 (*Prunus* bin map)
BPPCT017	13.8	20.1 (T x E)
EPDCU5183	37.8	35.2 (T x E)
LG6		
UDP96-001	0.0	17.5 (T x E)

BPPCT008	7.9	30.1 (T x E)
CPPCT015	18.1	35.8 (T x E)
Pchcm5	25.7	44.7 (T x E)
BPPCT025	40.8	56.4 (T x E)

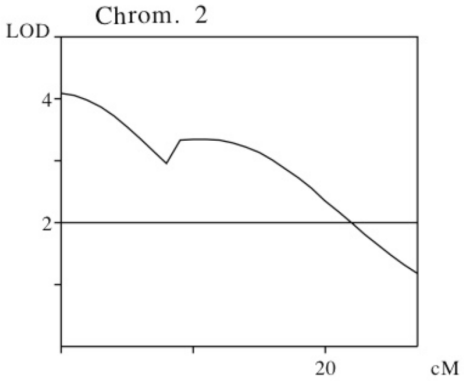
[z]T x E: Joobeur et al., 1998; Aranzana et al., 2003; and Dirlewanger et al., 2004a; *Prunus* bin map: Howad et al., 2005; P2175 x GN: Dirlewanger et al., 2004b.

A QTL analysis using PLABQTL 1.2 version was run based on the genetic map described above. Compared with the single marker analysis, the QTL analysis was possible to distinguish the relative position of the trait to the marker(s) and the size of the QTL. The data indicated that a QTL was detected on LG2a for the phenotype data from 2004 through 2008 in total (Fig. 2.8; Table 2.8). The QTL explained as much as 31.2% (2004) and as little as 14.3% (2007) of the phenotypic variance. Additional QTLs associated with PTSL might exist. Two SSRs, pacita 27 and UDA029, were detected at the peak of the corresponding QTL. Overall, the mapped interval for the QTL remained the same from year to year. This analysis agreed with the genomic region detected through the single SSR analysis described previously.

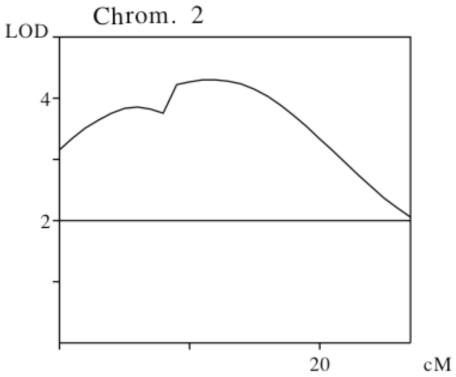
The QTL detected within 10 cM from the top of LG2a could include gene(s) controlling PTSL susceptibility and tolerance (Figure 2.7). In the future, developing more SSR markers to saturate this region will further define the specific region and alternately lead to the identification of the target genes.

Table 2.9. QTLs associated with PTSL.

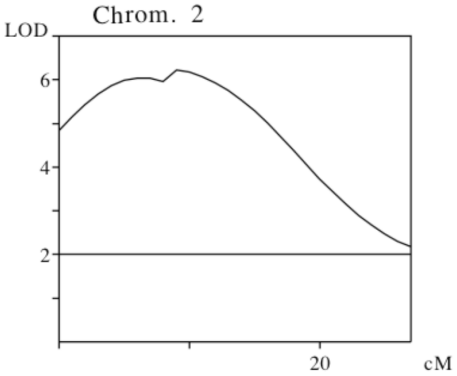
2004					
QTL	Linkage Group	SSR(s)	Interval (cM)	LOD value at peak	R^2 (%)
PTSL1	LG2a	Pacita27	0-8	4.09	17.2
PTSL2		UDA029	0-21	3.35	14.9
2005					
QTL	Linkage Group	SSR(s)	Interval (cM)	LOD value at peak	R^2 (%)
PTSL2	LG2a	UDA029	0-21	4.3	19.2
2006					
QTL	Linkage Group	SSR(s)	Interval (cM)	LOD value at peak	R^2 (%)
PTSL2	LG2a	UDA029	0-16	6.23	25.8
2007					
QTL	Linkage Group	SSR(s)	Interval (cM)	LOD value at peak	R^2 (%)
PTSL1	LG2a	Pacita27	0-9	3.21	14.3
2008					
QTL	Linkage Group	SSR(s)	Interval (cM)	LOD value at peak	R^2 (%)
PTSL2	LG2a	UDA029	2-18	3.6	16.3



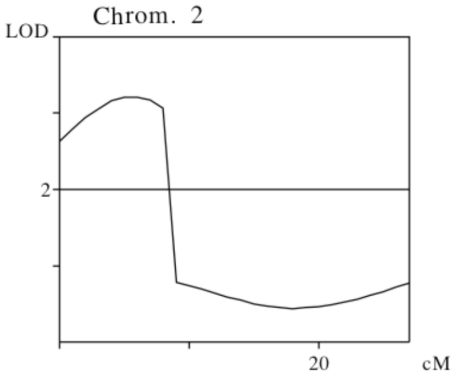
QTL(s) detected for 2004



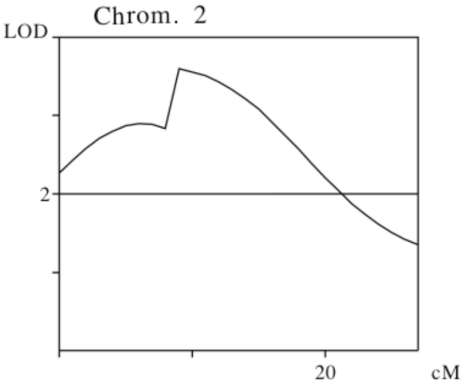
QTL(s) detected for 2005



QTL(s) detected for 2006



QTL(s) detected for 2007



QTL(s) detected for 2008

Figure 2.8. PTSL QTLs detected for phenotypic data from 2004 through 2008.

Conclusion

This study explored the genetic basis for the tolerance trait of Guardian® 'BY520-9' selection 3-17-7 to the PTSL disease syndrome. PTSL tolerance is a complex trait that may very well be controlled by polygenes. By using a population segregating for the tolerance to PTSL, nine SSRs out of 47, distributed on 4 linkage groups were identified to be associated with the response to PTSL. A QTL was identified to be associated with the response to PTSL as well. The upper terminus of LG2 where markers pacita 27 and UDA029 are located was important to the genetic basis study of PTSL, because both analyses (single SSR analysis and QTL analysis) identified this region. Additional markers flanking or near the interesting loci will be used to continue further isolating peach chromosomal genomic regions in the future with the ultimate goal of cloning the gene(s) responsible for tolerance to PTSL. Additionally, the identified SSR markers will be useful to find more PTSL-tolerant rootstocks in *Prunus* selection programs. Breeders, who are interested in introducing this trait into their breeding lines, could use these markers to identify the progeny with this trait.

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CHAPTER THREE

PEACH ROOTSTOCK IDENTIFICATION BY DNA-FINGERPRINTING WITH MICROSATELLITE /SSR MARKERS

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Abstract

Peach rootstocks are usually propagated from seeds. Seedlings are difficult to distinguish morphologically, and once grafted, no above ground material is available for visual identification. To avoid misidentification and to protect plant varieties and patents, DNA fingerprinting was investigated as a robust rootstock identification tool. The objective of this study was to distinguish progeny from among seven peach seedling rootstocks: Bailey, Halford, Lovell, Nemaguard, Nemared, Guardian® (selection 3-17-7) and S-37. We initially screened 102 *Prunus* microsatellite (SSR) markers on Lovell, Nemaguard, Nemared and selection 3-17-7. Seventy-five markers showed polymorphism among these rootstocks. The polymorphic markers were then used to screen Bailey, Halford and S-37. Based on the patterns of amplified DNA fragments (two seedlings from each rootstock were tested), eight SSR-markers reproducibly divided the seven rootstocks into as many as five groups. It was necessary to use a multiplex approach to uniquely identify each rootstock because no single SSR locus evaluated thus far was able to differentiate all seven genotypes. To confirm the identity of the SSR markers, we

cloned the polymorphic DNA fragments amplified by one of the eight polymorphic SSR primers, which was developed for an AC-enriched sequence isolated from almond. DNA sequence analysis showed that the amplified fragments shared a common AC-enriched repeat with copy number ranging from 5 to 14. Taken together, these results demonstrate that this microsatellite-based DNA fingerprint system has great potential for peach rootstock identification.

Introduction

Peach [*Prunus persica* (L.) Batsch] is an economically important fruit tree species in the *Rosaceae*. The annual world peach production is approximately 10 million metric tons (Fideghelli et al., 1998), with 1.3 metric tons produced in the United States alone. In commercial production, peach trees are actually composed of two genotypes, the scion and the rootstock. Scion cultivars are selected and released for their agronomic traits such as fruit size, taste and skin color. In contrast, rootstocks are selected and released for traits such as biotic or abiotic stress resistance or tree vigor in specific environments.

There are five or six peach seedling rootstocks commonly used in the United States. These are Lovell, Halford, Nemaguard, Nemared, Bailey and Guardian® (selection 3-17-7). Another former peach rootstock that is distant parent of Guardian® is S-37. All of these rootstocks have compatibility with many scion cultivars and some possess specific pest or disease resistance to nematodes and/or peach tree short life. Our research efforts focused on these seven rootstocks.

Clearly, rootstocks play an important role in commercial peach production. Unfortunately, peach rootstock seedlings are very difficult to identify using morphological traits. Also, once grafted, any characteristic leaf, floral or fruit traits of the rootstock phenotype will not be visible. However, DNA fingerprinting could provide evidence to demonstrate that apparently identical rootstocks are in fact genetically distinct. Rootstock identification is important for peach breeders and growers. It provides evidence to protect plant variety protection (PVP), patents for breeders and growers can be more confident in their purchases since they have a method to identify and confirm rootstocks in their orchards.

Many DNA-based marker systems can be used for fingerprinting. Restriction fragment length polymorphism **(**RFLP) has been used for cultivar identification in rose (Hubbard et al., 1992) and tall fescue (Busti et al., 2004). Amplified fragment length polymorphism (AFLP) has been used successfully to identify apricot (Geuna et al., 2003) and mango (Kashkush et al., 2001) cultivars. Randomly amplified polymorphic DNA (RAPD) has been used to identify strawberry (Gidoni et al., 1994) and calla lily (Hamada and Hagimori, 1996) cultivars.

Microsatellites (Simple Sequence Repeats, SSRs), another PCR-based system like RAPDs and AFLPs, have been used frequently in recent years for linkage map construction and DNA fingerprinting. SSRs are DNA fragments consisting of 1 to 6 nucleotide repeats distributed throughout the genome. SSRs show variation in fragment length based on the repeat copy numbers in one genotype compared to another. This variation can be used for molecular characterization. In contrast with the other marker

systems described above, SSRs have high reproducibility and are easily detectable. Hundreds of SSR markers have been developed in the Rosaceae and used widely to characterize *Prunus* species such as apricot (Romero et al., 2003) and almond (Amirbakhtiar et al., 1989).

In this study, we used SSR markers to identify seedlings from seven peach rootstock genotypes. Our results demonstrated that this SSR marker system had the potential to unambiguously identify peach seedling rootstocks at the molecular level.

Materials and Methods

Peach Rootstock Accessions and Genomic DNA Isolation:

Leaf tissue from seven peach rootstock cultivars (Lovell, 3-17-7, Nemaguard, Nemared, S-37, Halford and Bailey), and from two additional seedlings of each rootstock was collected during the summers of 2005 and 2006. All samples were collected from Musser Fruit Research Center near Clemson University. Five grams of young leaf tissue of each rootstock accession were frozen in liquid nitrogen and stored at the -80°C.

Genomic DNA was isolated from frozen leaf tissue (1g fresh weight) using a modified Sodium Dodecyl Sulfate (SDS) method (Dellaporta et al., 1983). DNA concentrations were measured using picogreen dye (Invitrogen, Carlsbad, CA) on a TBS-380 fluorometer (Turner BioSystems, Sunnyvale, CA). For each sample, the genomic DNA was then diluted to 10ng/µl.

SSR Markers and PCR-amplification:

The SSR markers (102) investigated were developed from four *Prunus spp,* (e.g., almond, apricot, cherry and peach) (Testolin et al., 2004; Lopes et al., 2002; Vaughan and Russell, 2004; Dirlewanger et al., 2002). The primer sequences were obtained from the Genome Database for Rosaceae (GDR) (http://www.bioinfo.wsu.edu/gdr/). The forward primer of each marker pair was radiolabeled with [γ-P^{33}] ATP by 5'-end labeling reaction using a modified version of the process found in Promega technical bulletin # 519 (www.promega.com/tbs/tb519/tb519.pdf).

Denaturing Polyacrylamide Gel Electrophoresis:

Samples were size fractionated in a 6% denaturing polyacrylamide gel on a vertical gel electrophoresis rig. After 2 hours at 80 watts, the gel was transferred to 3MM Whatman filter paper and dried for 90 minutes using a FB-GD-45 gel dryer vacuum system (FisherBiotech, Wembley, West Australia, Australia). The dried gel was exposed to Kodak BioMax MR film (Eastman Kodak, Rochester, NY) at room temperature with the exposure time varying from 1 to 5 days.

Sequencing PCR-amplified Polymorphic Fragments:

Polymorphic DNA fragments amplified by one SSR marker, UDA014, were cloned and sequenced to confirm their identity as SSRs. The amplified DNA fragments were separated in 3% Nusieve (Cambrex, Rockland, ME) agarose gel and stained with ethidium bromide. The polymorphic fragments were cut from the gel, and purified using a rapid gel extraction system (Marligen Biosciences, Ijamsville, MD).

The fragments were ligated into a TA cloning vector, pGEM-Teasy (Promega, Madison, WI), following the manufacturer's instruction. Ligated plasmids were transformed into *Escherichia coli* strain DH5αMCR by a heat shock protocol (Hanahan 1983; Jessee and Bloom, 1988). Plasmid DNA from putative transformants was isolated using an alkaline lysis plasmid miniprep protocol (Sambrook et al., 1989).

Sequencing reactions were set up using a SequiTherm Excel™ II DNA sequencing kit (Epicentre® Biotechnologies, Madison, WI). Sequencing products were analyzed using a LiCor 4200 automated sequencer (LI-COR, Lincoln, NE).

Results and Discussion

One hundred and two SSR markers were initially screened against four rootstocks—Lovell, 3-17-7, Nemaguard and Nemared. Nineteen markers amplified monomorphic patterns and thus, these markers did not differentiate among these rootstocks. Eight markers did not amplify any products. Seventy-five markers showed polymorphisms among the four rootstocks and divided the four rootstocks into two to four groups. Based on the number of amplified patterns and ease of scoring, twenty of the seventy-five polymorphic markers were screened against all seven rootstocks. Seven of the twenty SSR markers were less informative because they did not amplify a new pattern from the three additional rootstock genotypes tested. The remaining thirteen polymorphic SSR markers divided the seven rootstocks into groups as many as seven. Figure 3.1 shows the polymorphic pattern amplified by SSR marker EMPAS02. Nemaguard, Nemared and Bailey each had unique patterns. In addition, Lovell and

Halford had a common, but unique pattern, and 3-17-7 and S-37 shared a pattern but it differed from that of all the others. Thus, EMPAS02 divided the seven rootstocks into five groups.

The reproducibility of the patterns amplified by the thirteen polymorphic SSR markers was tested. Two additional seedling accessions of each rootstock were screened with the thirteen SSR primer pairs. Five of the thirteen markers did not produce consistent patterns between the seedlings of each rootstock and each original accession. Thus, these five markers were not helpful to this study and were no longer used. The other eight markers showed consistent patterns between some of the original rootstocks and their corresponding seedlings. The results are summarized in Table 3.1.

Four of the eight SSR markers (i.e., pacita16, Ps12a2, UDA011 and UDA036) amplified consistent patterns between the original and its additional two accessions among all seven rootstocks. These four markers can be used to subgroup all seven rootstocks. For example, marker pacita16 amplified five patterns among all seven rootstocks. Lovell, Bailey and Halford share the same pattern, and therefore, group together. On the other hand, Nemared, Nemaguard, 3-17-7 and S-37 each have their own unique patterns, and thus, group separately.

The other four markers (i.e., EMPAS02, EMPAS11, EPPISF12 and UDA014) amplified consistent patterns among the original accession and its seedlings for four or five of the rootstocks, but produced inconsistent patterns for the remaining rootstock accessions. Thus, these four markers can be used only to subgroup the rootstocks with consistent patterns. For example, EMPAS02 showed consistent patterns only among the

accessions of Nemared, Bailey, Halford and S-37, but inconsistent patterns among the Lovell, 3-17-7 and Nemaguard accessions. Furthermore, Nemared, Bailey, Halford and S-37 each had a unique pattern and could be grouped separately. Based on our overall results, the eight selected markers could divide the seven rootstocks into as many as five groups.

At the present time, the seven rootstocks could not be uniquely identified by a single SSR marker. Single markers (i.e., EMPAS02 and pacita16) could identify as many as four rootstocks. Combinations of SSR markers could be used to differentiate the seven rootstocks. At least two markers must be selected in a combination. For example, pacita16 identifies 3-17-7, Nemaguard, Nemared and S-37 because each of these rootstocks has a unique pattern for this SSR marker. Then UDA036 could be used to identify Bailey by its own unique pattern. Unfortunately, no SSR markers can identify Lovell from Halford. In addition to SSR combination pacita16/UDA036, other marker combinations can be used to confirm the results (e.g., Ps12a2/UDA036).

These eight selected markers were developed from almond, cherry and apricot. Although these markers amplify polymorphic fragments in peach rootstocks, an additional SSR marker developed from peach might be the single perfect marker. Furthermore, an additional 10 seedlings of each rootstock from independent sources will be used to corroborate the results obtained in the initial study.

To confirm that the amplified polymorphic DNA fragments originated from microsatellites, we cloned DNA fragments amplified by SSR marker UDA014, (an AC-enriched sequence, approximately 160bp in length that was initially developed from an

almond genomic library). Two DNA fragments from 3-17-7 and Nemaguard and one DNA fragment from each of the other five rootstocks were sequenced. The results are shown in Table 3.2.

Sequencing results showed that these 9 cloned fragments varied in length from 133 bp to 157 bp. All 9 clones contained the AC-repeat. The large 157 bp fragment cloned from 3-17-7 had the greatest number of AC repeats (copy number = 14). Thus, as expected, the 134 bp fragment cloned from Nemaguard had the least number of AC repeats (copy number = 5). These results confirm that the amplified DNA fragments are in fact SSRs, and the amplified fragments showed variation in fragment length based on difference in the number of repeat copies, which can be used to help identify the different rootstocks.

Conclusion

With the exception of Nemared, which bears red leaves, the other six peach rootstocks are difficult to identify morphologically. Each of the eight selected markers can divide the seven rootstocks into subgroups. Up to this point, no single SSR could uniquely distinguish all seven rootstocks. However, choosing marker combinations based on the alleles they detect can distinguish each rootstock from the other, except Lovell and Halford. Our initial study demonstrates that the SSR marker system used here has the capability to differentiate mislabeled rootstock seedlings, identify unknown rootstocks and to provide evidence for plant variety protection or patent protection.

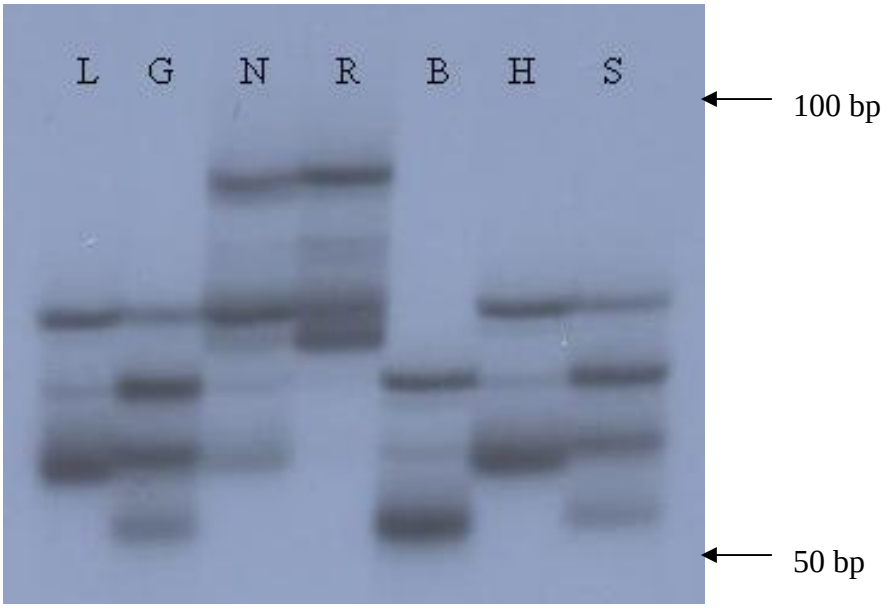

Figure 3.1. Polymorphic pattern amplified by SSR EMPAS02.
L: Lovell; G: 3-17-7; N: Nemaguard; R: Nemared; B: Bailey; H: Halford; S: S-37.

Table 3.1. Summary of confirmed polymorphic markers.

Markers		Amplification Patterns[z]					Inconsistent patterns between original and new accessions[y]
	Group No.	a	b	c	d	e	
EMPAS02	4	R	B	H	S		L^{H}, G^{S}, N
EMPAS11	3	R	B, H	S			$L^{B/H}$, G, N
EPPISF12	4	L, H	G	B	S		N, R^{G}
Pacita16	5	L, B, H	G	N	R	S	
Ps12a2	4	L, H	G, S	N, R	B		
UDA011	4	L, R, H	G, S	N	B		
UDA014	3	L, H	R, B	S			G, N
UDA036	4	L, N, H	G, R	B	S		

[z]Amplification patterns of rootstocks Lovell (L), 3-17-7 (G), Nemaguard (NG), Nemared(NR), Bailey (B), Halford (H) and S-37 (S). Pattern "a" amplified from one marker is different from pattern "a" amplified from any other markers (similarly for patterns b, c, d or e and each marker).

[y]Rootstocks with a superscript(s) share a common pattern with the corresponding rootstock(s) for the specific SSR marker.

Table 3.2. Sequencing results of DNA fragments amplified by UDA014.

DNA fragment Origin	Fragment length (bp)	Number of AC repeats
Bailey	133	6
Halford	135	7
Lovell	137	7
Nemaguard-1	143	11
Nemaguard-2	134	5
Nemared	133	6
S-37	133	6
3-17-7-1	157	14
3-17-7-2	143	11

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CHAPTER FOUR

IDENTIFICATION OF PEACH [*PRUNUS PERSICA* (L.) BATSCH] ROOTSTOCK SEEDLINGS USING DNA-FINGERPRINTING WITH MICROSATELLITE (SSR) MARKERS

Introduction

A commercial peach tree is actually composed of two genotypes, the upper part or scion and the below ground part, the rootstock. Rootstocks interact with soil and provide nutrients to the whole plant, playing an important role in water and nutrient transportation, vegetative growth and tree survival.

Peach rootstocks with specific characteristics such as pathogen resistance or environmental adaptability are normally developed through years of breeding selection and field evaluation. Once a new rootstock is released, plant variety protection (PVP) or plant patents can be granted to the breeders, which gives them exclusive marketing rights to the rootstock in the United States (Strachan 1992). However, some growers may buy a few patented trees and propagate them without paying royalties to breeders (Warner 2004). Thus, peach rootstock identification is essential to support a PVP or patent, settle infringement disputes and protect agriculture business from unfair competition (Janick et al., 1983). Moreover, mislabeled, misrepresented rootstocks can lead to huge income losses through orchard replacement and yield loss (Harper and Kime, 2001). Rootstock identification would allow tree nurseries to certify the true-to-type rootstocks they market. Growers could then purchase certified rootstocks with confidence.

Traditional identification evaluations are made based on the observed morphological traits/phenotypes of the mature tree such as fruit or flower characters (Arulsekar et al., 1986). Many characters take a long time to be observable and can be affected by environmental conditions, developmental stage of the trees or human judgment (Janick et al., 1983). In the peach industry, rootstocks are mostly seed propagated. Peach rootstock seedlings are very difficult to identify using morphological traits (Figure 3.1). DNA fingerprinting could provide evidence at the molecular level to demonstrate that rootstocks that look morphologically identical are genetically different.

Many DNA-based marker systems can be used for fingerprinting. Restriction fragment length polymorphism **(**RFLP) has been used for cultivar identification in many crops such as avocado (Lavi et al., 1991), soybean (Smith and Smith, 1992), tomato (Vosman et al., 1992), grape (Bowers et al., 1993), wheat (Vaccino et al., 1993) and persimmon (Maki et al., 2001). Similarly, randomly amplified polymorphic DNA (RAPD) has been used to identify cultivars from apple (Koller et al., 1993), red raspberry (Graham et al., 1994), strawberry (Gidoni et al., 1994), mango (Schnell et al., 1995), olive (Fabbri et al., 1995), peach (Lu et al., 1996), *Prunus* rootstocks (Casas et al., 1999) and barley (Fernandez et al., 2002). Amplified fragment length polymorphism (AFLP) has been used successfully to identify bermudagrass (Zhang et al., 1999), mango (Kashkush et al., 2001), apple (Tignon et al., 2000), apricot (Geuna et al., 2003), sesame (Laurentin and Karlovsky, 2007) and yellow bean (*Phaseolus vulgaris* L.) cultivars (Pallottini et al., 2004).

Microsatellites (Simple Sequence Repeats, SSRs), a polymerase chain reaction (PCR)-based molecular marker system, show variation in fragment length based on the repeat copy number in one genotype compared to another. This variation can be used for molecular characterization. Compared with other molecular markers, SSRs are highly reproducible and easily detected (Powell et al., 1995). In *Prunus*, a large number of SSR markers have been developed from different species (Sosinski et al., 2000; Testolin et al., 2000; Lopes et al., 2002; Vaughan and Russell, 2004; Dirlewanger et al., 2002, Aranzana et al., 2002) and have been used widely to characterize *Prunus* species such as apricot (Romero et al., 2003; Zhebentyayeva et al., 2003) and peach (Aranzana et al, 2003; Bouhadida et al., 2007).

There are six peach rootstocks commonly used in the United States – Lovell, Halford (Philip and Davis, 1936), Nemaguard (Brooks and Olmo, 1997; Okie 1998) Nemared (Ramming and Tanner, 1983), Bailey (Putensen 1988) and the Guardian® 'BY520-9' (Okie et al., 1994). S-37 is a former peach rootstock (Okie et al., 1994). These rootstocks have compatibility with many scion cultivars and some possess specific resistance to different pathogens, nematodes and/or peach tree short life (Beckman et al., 1997; Reighard et al., 1997; Reighard and Loreti, 2008). Identification of these peach rootstocks had been conducted via *Prunus* SSR marker combination (Liu et al., 2007).

This chapter is an extension of studies reported in Chapter Three, including the search of a single perfect marker that can differentiate peach rootstocks commonly used in this country, as well as a South African rootstock, Kakamas (Lotze 1997). Twenty SSR markers developed from peach and those identified to be valuable in peach rootstock

identification in Liu et al. (2007) were investigated on rootstocks Lovell, Halford, Nemaguard, Nemared, Guardian ® 'BY520-9' selection 3-17-7, Bailey, S-37, Kakamas and a number of their seedlings. The results demonstrated that SSR markers behave as precise, and non-disputable tools and can be used in rootstock identification. Application of this technique would be of considerable benefit to the peach industry.

Lovell Bailey Nemaguard

Figure 4.1. Peach rootstock seedlings of three cultivars.

Materials and Methods

Peach Rootstock Accessions and Genomic DNA Isolation:

Leaf tissue was collected from rootstocks Lovell, Guardian® 3-17-7, Nemaguard, Nemared, Bailey, Halford, S-37, Kakamas, and their seedlings at the Musser Fruit Research Center near Clemson University (Table 4.1). Five grams of young leaf tissue of each rootstock accession were frozen in liquid nitrogen and stored at -80°C.

Table 4.1. Sources and statistics of rootstock clones and their seeds.

Genotypes	Sample Quantity	Rootstock Source	Year planted
Lovell	1	Musser Fruit Research Center	2002
Nemaguard	1	Musser Fruit Research Center	2002
3-17-7	1	Musser Fruit Research Center	1993
Nemared	1	W. Howell, NRSP5, Prosser, WA	2006
Halford	1	W. Howell, NRSP5, Prosser, WA	2005
S-37	1	Musser Fruit Research Center	2004
Bailey	1	W.Howell, NRSP5, Prosser, WA	2005
Kakamas	1	T. Gradziel, U.C. Davis	2007
		Seed Source	
Lovell	14	Musser Fruit Research Center	2007
Nemaguard	14	Musser Fruit Research Center	2007
3-17-7	100	Musser Fruit Research Center	2007
Nemared	14	Burchell Nursery, Modesto, CA	2007
Halford	10	T. Gradziel, U.C. Davis	2007
Bailey	14	P. Baugher, Adams County Nursery, Aspers, PA	2007
S-37	14	Musser Fruit Research Center	2007
Kakamas	14	T. Gradziel, U.C. Davis	2007

Genomic DNA was isolated from frozen leaf tissue (1g fresh weight) using a modified sodium dodecyl sulfate (SDS) method (Dellaporta et al., 1983). DNA

concentrations were measured using picogreen dye (Invitrogen, Carlsbad, CA) on a TBS-380 fluorometer (Turner BioSystems, Sunnyvale, CA). For each sample, the genomic DNA was then diluted to 10ng/µl in de-ionized and distilled water.

SSR Markers:

Twenty SSR markers developed from peach (Cipriani et al., 1999; Testolin et al., 2000; Dirlewanger et al., 2002, Aranzana, et al., 2002) and eight SSRs identified to be valuable for rootstock identification (Liu et al., 2007) were investigated in this study (Table 4.2). The primer sequences were obtained from the Genome Database for Rosaceae (GDR) (http://www.bioinfo.wsu.edu/gdr/). Primer pairs were synthesized by Integrated DNA Technologies (IDT, Coralville, IA) (http://www.idtdna.com/Scitools/Scitools.aspx).

Primer Labeling and PCR:

SSR primers were diluted to 10 pmol/µl for PCR amplification. The forward primer of each primer pair was radiolabelled using a 5' end labeling protocol (Promega technical bulletin #519). Each 0.7µl labeling reaction contained 1.7 pmol of forward primer, 0.5µCi [γ -^{33}P]-ATP (PerkinElmer, Waltham, MA), and 0.3 units T4 polynucleotide kinase (Promega, Madison, WI). For size reference, a DNA ladder (1kb Plus; Invitrogen, Carlsbad, CA) was similarly labeled.

Table 4.2. SSRs markers investigated and their references.

Markers	Species	Sequences (5' to 3')	Annealing Temp	References
BPPCT001	*P. persica*	F: AATTCCCAAAGGATGTGTATGAG R: CAGGTGAATGAGCCAAAGC	57˚C	Dirlewanger et al. (2002)
BPPCT007	*P. persica*	F: TCATTGCTCGTCATCAGC R: CAGATTTCTGAAGTTAGCGGTA	57˚C	Dirlewanger et al. (2002)
BPPCT008	*P. persica*	F: ATGGTGTGTATGGACATGATGA R: CCTCAACCTAAGACACCTTCACT	57˚C	Dirlewanger et al. (2002)
BPPCT015	*P. persica*	F: ATGGAAGGGAAGAGAAATCG R: GTCATCTCAGTCAACTTTTCCG	57˚C	Dirlewanger et al. (2002)
BPPCT017	*P. persica*	F: TTAAGAGTTTGTGATGGGAACC R:AAGCATAATTTAGCATAACCAAGC	57˚C	Dirlewanger et al. (2002)
BPPCT038	*P. persica*	F: TATATTGTTGGCTTCTTGCATG R: TGAAAGTGAAACAATGGAAGC	57˚C	Dirlewanger et al. (2002)
CPPCT001	*P. persica*	F: TGCTTTCCACGCACACTG R: GCCAAGCATTGCGTCGTT	52˚C	Aranzana et al. (2002)
CPPCT002	*P. persica*	F: GGAGCTGCAATATTGCTG R: GTTAGGGAAGCATCTCAC	52˚C	Aranzana et al. (2002)
CPPCT004	*P. persica*	F: TCATTCGAAGACGACCGT R: GTCTAGGCACGTTGCTAG	52˚C	Aranzana et al. (2002)
CPPCT005	*P. persica*	F: CATGAACTCTACTCTCCA R: TGGTATGGACTCACCAAC	52˚C	Aranzana et al. (2002)
CPPCT006	*P. persica*	F: AATTAACTCCAACAGCTCCA R: ATGGTTGCTTAATTCAATGG	59˚C	Aranzana et al. (2002)
CPPCT017	*P. persica*	F: TGACATGCATGCACTAAACAA R:TGCAAATGCAATTTCATAAAGG	60˚C	Aranzana et al. (2002)
CPPCT022	*P. persica*	F: CAATTAGCTAGAGAGAATTATTG R: GACAAGAAGCAAGTAGTTTG	50˚C	Aranzana et al. (2002)
CPPCT028	*P. persica*	F: ACATATGCCTTATCAGCTT R: ATTGAAGAGAAAGCAGTGT	50˚C	Aranzana et al. (2002)
CPPCT029	*P. persica*	F: CCAAATTCCAAATCTCCTAACA R: TGATCAACTTTGAGATTTGTTGAA	55˚C	Aranzana et al. (2002)
CPPCT030	*P. persica*	F: TGAATATTGTTCCTCAATTC R: CTCTAGGCAAGAGATGAGA	50˚C	Aranzana et al. (2002)
UDP98-022	*P. persica*	F: CTAGTTGTGCACACTCACGC R: GTCGCAGGAACAGTAAGCCT	56˚C	Testolin et al. (2000)
UDP98-025	*P. persica*	F: GGGAGGTTACTATGCCATGAAG R: CGCAGACATGTAGTAGGACCTC	56˚C	Testolin et al. (2000)
UDP98-407	*P. persica*	F: AGCGGCAGGCTAAATATCAA R: AATCGCCGATCAAAGCAAC	54˚C	Cipriani et al. (1999)
UDP98-408	*P. persica*	F: ACAGGCTTGTTGAGCATGTG R: CCCTCGTGGGAAAATTTGA	54˚C	Cipriani et al. (1999)
EMPAS02	*P. avium*	F: CTACTTCCATGTTGCCTCAC R: AACATCCAGAACATCAACACAC	53˚C	Vaughan et al. (2004)
EMPAS11	*P. avium*	F: ACCACTTTGAGGAACTTGGG	54˚C	Vaughan et

		R: CTGCCTGGAAGAGCAATAAC		al. (2004)
EMPAS12	*P. avium*	F: TGTGCTAATGCCAAAATACC R: ACATGCATTTCAACCCACTC	55˚C	Vaughan et al. (2004)
pacita16	*P. armeniaca*	F: TGACGTCTCTCTCCCTCCCCTTCCT R: CCCTCTCTTTTTCTCTAGCCCCACC	50˚C	Lopes et al. (2002)
ps12a2	*P. avium*	F: GCCACCAATGGTTCTTCC R: AGCACCAGATGCACCTGA	55˚C	Joobeur et al. (2000) Cantini et al. (2001)
UDA011	*P. dulcis*	F: TGGATTGTTTTCCCCTGGTA R: TGGATTGTTTTCCCCTGGTA	56˚C	Testolin et al. (2004)
UDA014	*P. dulcis*	F: TAAAATACACACGCGCACAC R: ACCAAGCATCGTCACTAGCC	56˚C	Testolin et al. (2004)
UDA036	*P. dulcis*	F: AATTCACATATATACCCGTACACAC R: TGTTGGATTGTTTCCTCTGG	52˚C	Testolin et al. (2004)

DNA fragments between each primer pair annealing sites were amplified by PCR. PCR amplifications were prepared as a 10µl volume reaction using the Go-Taq kit from Promega (Cat# PAM8295). Each reaction contained 30ng of DNA template, 0.5U Go-Taq polymerase, dNTPs (0.5mM each dNTP final), $MgCl_2$ (1.5mM final) and 1.7 pmol of the reverse primer.

For ease of handling, a radiolabeling reaction premix (containing forward primers, [γ -^{33}P]-ATP, T4 polynucleotide kinase and kinase buffer) of 7µl for ten reactions was prepared and mixed with 80µl of PCR premix (containing the reverse primer, Go-Taq polymerse, dNTP, $MgCl_2$ and polymerse buffer) for ten reactions. The 87µl reaction premix was aliquoted equally to eight reactions of 8.5µl for each.

The PCR cycling protocol was 95˚C for 5 minutes; 35 cycles of 94˚C for 45 seconds, annealing (from 46˚C to 62˚C) for 45 seconds and 72˚C for 45 seconds; 72˚C for 8 minutes, and then kept at 4˚C. The annealing temperature for each primer pair was determined based on the primer sequences and was calculated using Integrated DNA Technologies (IDT) online oligo design and analysis tools (http://www.idtdna.com/Scitools/Scitools.aspx).

Denaturing Polyacrylamide Gel Electrophoresis:

Samples were size-fractionated in a 6% denaturing polyacrylamide gel on a vertical gel electrophoresis rig. Each 6% acrylamide gel (70ml) was prepared by adding 20:1 acrylamide: bisacrylamide and 7.5M urea in 1X TBE buffer. After 2 hours at 80 watts, the gel was transferred to 3MM Whatman filter paper and dried for 90 minutes

using a FB-GD-45 gel dryer vacuum system (FisherBiotech, Wembley, West Australia, Australia). The dried gel was exposed to BioMax MR film (Eastman Kodak, Rochester, NY) at room temperature with the exposure time varying from 1 to 5 days.

SSR Scoring:

SSR primers amplifying the same DNA band patterns among rootstock genotypes were scored as monomorphic markers. SSR primers amplifying different patterns among rootstock genotypes were scored as polymorphic markers. Only polymorphic markers were then screened on rootstock seedlings for examining pattern reproducibility/consistency between the original rootstock clone and the rootstock seedlings.

Results and Discussion

Polymorphic and Monomorphic Markers:

Twenty-eight SSR markers were screened against eight rootstocks, Lovell, Guardian® selection 3-17-7, Nemaguard, Nemared, Bailey, Halford, S-37 and Kakamas. All the SSR markers amplified DNA fragments. Five SSR markers (BPPCT038, CPPCT001, CPPCT002, CPPCT005 and CPPCT030) showed monomorphism, and could not differentiate any rootstocks. Twenty-two markers (81%) were polymorphic, higher than the percentage in two previous studies, 69% (Aranzana et al., 2002) and 59% (Blenda et al., 2006). All the SSR markers used in this study were selected from the

polymorphic markers being used in previous peach cultivar fingerprinting and pedigree studies (Casas et al., 1999; Cipriani et al., 1999; Testolin et al., 2000; Aranzana et al., 2002; Aranzana et al., 2003; Liu et al., 2007; Bouhadida et al., 2009). The number of alleles detected at each locus by polymorphic markers ranged from two to seven (Table 4.3).

Identification of Rootstock Clones:

Within the polymorphic markers, ten markers – BPPCT001, BPPCT008, BPPCT015, BPPCT017, CPPCT022, CPPCT029, UDP98-025, EMPAS11, pacita16 and UDA011 – were more informative because they amplified at least four patterns among the eight tested rootstocks. Thus, rootstock identification was focused on these markers. The patterns amplified by each of the selected ten polymorphic markers were summarized in Table 4.4. As an example, BPPCT001 amplified six patterns among the eight tested rootstocks. Lovell (L) and Halford (H) shared the same pattern; Bailey (B) and Kakamas (K) shared a second identical pattern. 3-17-7 (G), Nemaguard (NG), Nemared (NR) and S-37 (S), each had their unique patterns that can be identified directly. Each rootstock can be discriminated by at least one single SSR marker (e.g., Lovell) and at most nine markers (e.g., S-37). Unfortunately, no single perfect marker was found to identify all rootstocks. Marker BPPCT008 had the most potential for identification because it amplified seven patterns and was capable of identifying as many as six rootstocks uniquely.

Table 4.3. Number of alleles detected by each of the 23 polymorphic SSRs among eight rootstocks.

Markers	Number of alleles
BPPCT001	7
BPPCT008	6
BPPCT015	3
BPPCT017	4
BPPCT038	3
CPPCT004	2
CPPCT006	3
CPPCT017	3
CPPCT022	4
CPPCT028	2
CPPCT029	3
UDP98-022	3
UDP98-025	3
UDP98-407	3
UDP98-408	4
EMPAS02	4
EMPAS11	5
EMPAS12	3
pacita16	6
ps12a2	3
UDA011	3
UDA014	4
UDA036	4

Table 4.4. SSR amplification patterns in rootstock identification.

SSR markers	Location (cM)[z]	Pattern quantity	Amplification Patterns[y]						
			a	b	c	d	e	f	g
BPPCT001	LG2 (20.9)	6	**L** ^{MO}, **H** ^{MO}	G ^{PO}	NG ^{PO}	NR ^{MO}	B ^{MO}, K ^{MO}	**S** ^{PO}	
BPPCT008	LG6 (30.1)	7	L ^{PO}	G ^{MO}, B ^{MO}	NG ^{PO}	NR ^{MO}	H ^{MO}	S ^{PO}	**K** ^{MO}
BPPCT015	LG4 (44.0)	4	L ^{PO}, H ^{PO}	G ^{MO}, NG ^{MO}, NR ^{MO}	B ^{MO}, K ^{MO}	S ^{PO}			
BPPCT017	LG5 (20.1)	4	L ^{MO}, G ^{MO}, B ^{MO}, H ^{MO}, K ^{MO}	**NG** ^{MO}	**NR** ^{MO}	S ^{PO}			
CPPCT022	LG7 (18.6)	5	L ^{MO}, NG ^{MO}, NR ^{MO}, H ^{MO}	G ^{PO}	**B** ^{MO}	S ^{PO}	K ^{MO}		
CPPCT029	LG1 (65.1)	5	L ^{MO}, B ^{MO}, H ^{MO}	G ^{PO}, NR ^{PO}	NG ^{MO}	S ^{PO}	K ^{PO}		
UDP98-025	LG2 (9.6)	5	L ^{MO}, B ^{MO}, H ^{MO}, K ^{MO}	**G** ^{PO}	NG ^{MO}	NR ^{MO}	**S** ^{PO}		
EMPAS11	Not mapped	5	L ^{MO}, B ^{MO}, H ^{MO}	G ^{PO}	NG ^{PO}	NR ^{MO}	S ^{PO}		
pacita16	LG2 (25.5)	6	L ^{MO}, B ^{MO}, H ^{MO}	G ^{MO}	NG ^{PO}	NR ^{MO}	S ^{MO}	K ^{MO}	
UDA011	LG3, 4 (13.5) (49.0-62.0)	6	L ^{PO}, NR ^{PO}, H ^{PO}	G ^{PO}, S ^{PO}	NG ^{PO}	B ^{MO}	K ^{PO}		

[z]Map location of SSRs of BPPCT, CPPCT and UDP Series are referred from *Prunus* reference map (Joobeur et al., 1998; Aranzana et al., 2003; Dirlewanger et al., 2004). Map location of SSR pacita16 is referred from "JxF" map (Dirlewanger et al., 1998). Map location of UDA011 is referred from *Prunus* bin map (Howad et al., 2005).

[y]Amplification patterns of rootstocks Lovell (L), 3-17-7 (G), Nemaguard (NG), Nemared (NR), Bailey (B), Halford (H), S-37 (S) and Kakamas (K). Pattern "a" amplified from one marker is different from pattern "a" amplified from any other markers (similarly for patterns b, c, d or e and each marker). Bold indicated the SSR marker amplified the characteristic allele only existing in the corresponding rootstock(s). MO represents monomorphic pattern, PO represents polymorphic pattern.

Previous peach rootstock identification (Lu et al., 1996) was attempted only on rootstock clones or a small number (2) of rootstock seedlings (Liu et al., 2007). However, for the ease of handling and low cost, open-pollinated seeds that are harvested from rootstock clonal cultivars, usually are propagated as commercial rootstocks. Thus, peach rootstock seedling identification has significant meaning to commercial peach growers, and would be necessary to support PVP patents and other disputes of rootstock identity and ownership. To achieve the goal of seedling identification, 10 seedlings of Halford, and 14 seedlings each of Nemaguard, Nemared, Bailey, Lovell, S-37 and Kakamas were screened by the 10 SSR markers for testing the reproducibility of the patterns amplified in rootstock clones. The rootstock Guardian® 'BY520-9' is used extensively in the southeastern part of U.S. for its tolerance to the PTSL syndrome (Okie 1998). Plant Variety Protection (PVP) was applied for Guardian® rootstock seedlings (PVP patent No. 9400013). One of the Guardian® selections 3-17-7 has superior horticultural characteristic such as high seed germination and vigorous uniform growth. Application of fingerprinting 3-17-7 seedlings would benefit peach growers in the southeastern U.S. and the breeders who developed this rootstock. Thus, more effort in this study was put on the identification of 3-17-7 seedlings. SSR markers with promising potential for identifying the 3-17-7 genotypes were screened on 100 3-17-7 seedlings. The remaining polymorphic markers were only tested on fourteen 3-17-7 seedlings.

Rootstock Seedling Identification of 3-17-7:

SSR markers BPPCT001, CPPCT022, EMPAS11, UDP98-025 amplified unique but heterozygous patterns in the 3-17-7 genotype (Table 4.4). They were used to screen 100 3-17-7 seedlings. As expected, the pattern amplified in the original 3-17-7 clone segregated in the seedlings. Figure 4.2 shows a partial gel image of DNA band patterns amplified by marker EMPAS11 in 20 3-17-7 seedlings. Six seedlings (No. 1, 6, 12, 13, 18 and 19) displayed only the lower pattern of the 3-17-7 genotype, which is the same as the pattern amplified in the Lovell, Bailey, Halford and Kakamas genotypes and the upper band pattern of the S-37 clone. Seven seedlings (No. 5, 7, 8, 9, 15, 16 and 17) displayed the characteristic same heterozygous pattern as the 3-17-7 genotype. The remaining seedlings (No. 2, 3, 4, 10, 11, 14 and 20) amplified only the upper band pattern of 3-17-7, which was the same as the band pattern amplified in the original Nemared clone. Hence, the segregated patterns amplified by EMPAS11 made putative 3-17-7 seedlings difficult to be identified from other rootstock clones or their seedlings. This was true for markers BPPCT001, CPPCT022 and UDP98-025 as well. For marker UDP98-025, one of the two alleles forming the heterozygous pattern was characteristic to 3-17-7. However, there was a limitation that only the seedlings with this characteristic allele could uniquely be identified as 3-17-7 seedlings. In addition, marker pacita16 amplified a unique homozygous pattern in the 3-17-7 genotype. All the tested 3-17-7 seedlings could reproduce the parental pattern. However, this allele is not characteristic to 3-17-7 since it can exist in Nemaguard seedlings as well.

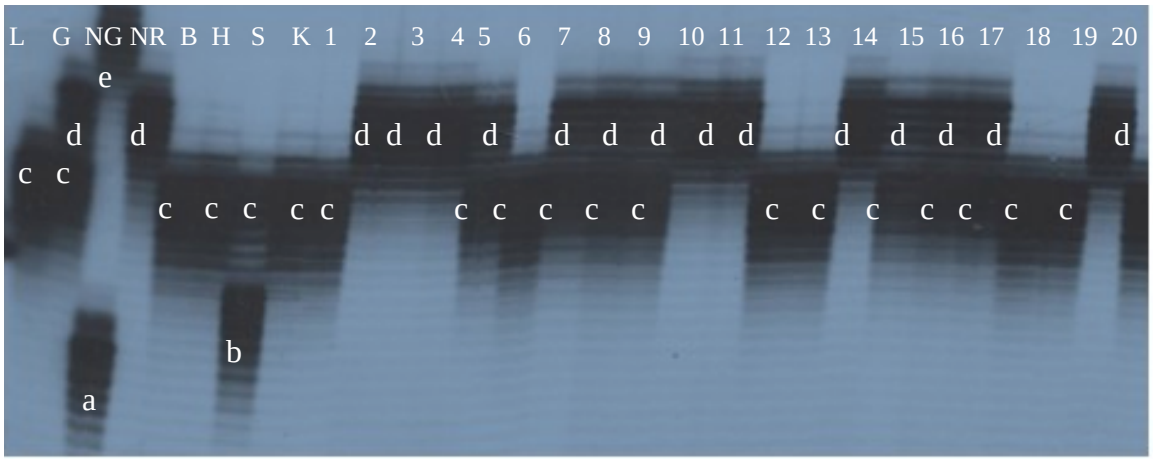


Figure 4.2. DNA band patterns amplified by marker EMPAS11 in 20 3-17-7 seedlings. L: Lovell; G: 3-17-7; N: Nemaguard; R: Nemared; B: Bailey; H: Halford; S: S-37; K: Kakamas. Each allele was labeled with a lower case letter.

No single SSR marker could uniquely identify 3-17-7 seedlings from those of the other rootstocks. Thus, marker combinations selected from BPPCT001, CPPCT022, EMPAS11, UDP98-025 and pacita16 was necessary. At least two markers were selected in one combination (e.g., EMPAS11/pacita16) (Figure 4.3). The DNA band pattern amplified by EMPAS11 in rootstock genotypes indicated that this marker was only able to identify 3-17-7 seedlings from the Nemaguard clone and its seedlings. It is because that the pattern amplified in 3-17-7 did not exist in Nemaguard, but was found in other rootstocks. Marker pacita16 was capable of differentiating seedlings of 3-17-7 from S-37, because there were no common alleles at this locus for 3-17-7 and S-37. As a result of using this marker combination, 3-17-7 seedlings could be identified from all the other rootstocks.

Seedling Identification of Rootstocks Lovell, Nemaguard, Nemared, Bailey, Halford, S-37 and Kakamas:

Based on the results obtained by screening SSR markers on the eight rootstock cultivars single SSR markers with the potential to identify seedlings of rootstocks Nemaguard, Nemared, Bailey and Kakamas were found. Marker BPPCT017 amplified a homozygous pattern in an allele that only existed in Nemaguard. EMPAS11 amplified highest and lowest molecular weight DNA fragments that could distinguish Nemaguard from the other rootstocks. Each of the two alleles forming the heterozygous pattern could be detected in Nemaguard only (Figure 4.3-1). Thus, markers BPPCT017 and EMPAS11 were able to identify Nemaguard seedlings from the other rootstocks. Fourteen seedlings were screened by BPPCT017 and EMPAS11. Twelve of them reproduced the pattern amplified in Nemaguard (Table 4.5). The other two "off-type" seedlings were discussed in a later section.

Marker BPPCT017 amplified a unique homozygous pattern from an allele characteristic to the Nemared genotype. Marker CPPCT022 amplified a unique homozygous pattern from an allele characteristic to the Bailey genotype and marker BPPCT008 amplified a unique homozygous pattern from an allele characteristic to the Kakamas genotype (Table 4.4). Thus, these three markers were able to identify Nemared, Bailey and Kakamas seedlings from the other rootstocks. Seedlings from Nemared, Bailey and Kakamas were screened by BPPCT001, CPPCT022 and BPPCT008. The characteristic patterns amplified in the original rootstock genotypes were reproducible in their seedlings (Table 5).

In the S-37 genotype, all the polymorphic markers amplified heterozygous patterns, and nine of them were unique. However, only two alleles detected at two different loci, where one was detected by BPPCT001 and the other one was detected by UDP98-025 (Table 4.4) were characteristic for S-37. Thus, there was a limitation that only the seedlings with a characteristic allele could uniquely be identified as S-37 seedlings. The putative S-37 seedlings not having a characteristic allele, could be differentiated from all the other rootstocks through marker combinations (e.g., EMPAS11/pacita16).

However, no single SSRs or even marker combinations were found capable of identifying Lovell and Halford seedlings from the other rootstock cultivars or seedlings. Nine of the ten SSRs amplified the same patterns in Lovell and Halford (Table 4.4). The remaining marker BPPCT008 amplified a heterozygous unique pattern in Lovell, but the segregated patterns could be found in Halford as well. Thus, BPPCT008 could not identify Lovell seedlings from Halford. Since marker BPPCT001 amplified a homozygous pattern in an allele characteristic to both Halford and Lovell, this marker was capable to identify seedlings of Lovell and Halford altogether from the other rootstocks. In fact, Halford may be a seedling from Lovell (Philip and Davis, 1936). This could explain why the seedlings of Halford and Lovell are difficult to differentiate.

All the results of rootstock seedling identification indicated that seedling identification is more difficult than the parent genotype identification. It was because heterozygous patterns obtained in a rootstock cultivar segregated in its seedlings. The same segregated patterns might exist in seedlings of other rootstock cultivars. Overall,

for rootstock seedling identification in the future, SSRs amplifying a unique homozygous pattern in an allele characteristic to the rootstock would be the best marker candidate.

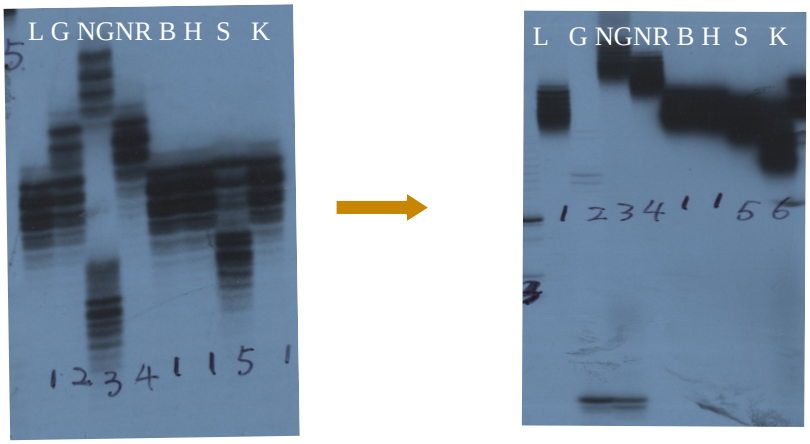
Reproducibility of Patterns Amplified in Rootstock Clones and their Seedlings:

Ten polymorphic SSRs were screened in the seedlings of the eight rootstocks to test the degree of reproducibility of patterns amplified in the rootstock genotypes. All the seedlings of rootstocks 3-17-7 (n= 100), Bailey (n= 14) and Kakamas (n= 14) reproduced the patterns amplified in their rootstock parents (Table 4.5). This result supported that the seeds were trueness to type.

All Nemared seedlings reproduced the parental alleles observed in the Nemared genotype except in seedling No. 7 at one locus where marker pacita16 is located. The parental allele and a second allele matching what was found in the Lovell, Bailey and Halford cultivars were detected. There might be a polymorphism caused by primer mispairing at this locus. The result also agreed and confirmed the quality of certified Nemared seeds provided by Burchell Nursery (Modesto, CA).

For Lovell seedling No. 8, patterns amplified by markers BPPCT001, CPPCT022, CPPCT029, EMPAS11 and pacita16 did not exist in the Lovell genotype. These “new” patterns amplified by BPPCT001, CPPCT022, CPPCT029 and pacita16 existed in the Kakamas, and the “new” pattern amplified by EMPAS11 was found in the Nemaguard. The remaining tested Lovell seedlings (n= 13) reproduced the expected marker patterns amplified in the Lovell genotype.

Similarly, patterns in Nemaguard seedlings No. 3 and No. 6 amplified by BPPCT001, BPPCT008, BPPCT017, CPPCT022 and EMPAS11 were not found in Nemaguard but were revealed in 3-17-7. Patterns amplified by BPPCT001, BPPCT008, CPPCT029 and pacita16 in Halford seedlings No. 5 and No. 6 did not exist in the Halford parent (Table 4.5). Patterns amplified by BPPCT001, BPPCT008, BPPCT017, CPPCT022 in S-37 seedlings No. 1 and No. 2 did not exist in the S-37 parent (Table 5). The off-type seedlings were double-checked through a separate DNA isolation and PCR reaction and the off-type patterns were reproducible. Because rootstock seeds were harvested from open-pollinated rootstock cultivars, there was a chance for outcrossing, resulting in the genetic "contamination" in some seeds.



EMPAS11 pacita16

Figure 4.3. Marker combination (EMPAS11/pacita16) used to identify Guardian® 'BY520-9' selection 3-17-7. For each gel image, DNA banding patterns labeled with the same number indicate that the designated SSR amplified the same patterns between/among the rootstocks. The total number represents the total number of patterns amplified by this SSR among the eight rootstocks. L: Lovell; G: 3-17-7; N: Nemaguard; R: Nemared; B: Bailey; H: Halford; S: S-37; K: Kakamas.

Table 4.5. Summary of pattern reproducibility in rootstock seedlings.

SSR markers	Lovell[z]	3-17-7	Nemaguard	Nemared	Bailey	Halford	S-37[y]	Kakamas
BPPCT001	No. 8^{K}	√	No. 3^{G}	√	√	No. $5^{NR, B}$ $6^{NR, B}$	No. 1^{B}, 2^{B}	√
BPPCT008	√	√	No. 3^{G}, 6^{G}	√	√	No. 5^{L}, 6^{L}	√	√
BPPCT015	√	√	√	√	√	√	√	√
BPPCT017	√	√	No. 3^{G}, 6^{G}	√	√	√	No. 1^{L}, 2^{L}	√
CPPCT022	No. 8^{K}	√	No. 3^{G}	√	√	√	No. 1^{L}, 2^{L},	√
CPPCT029	No. 8^{K}	√	√	√	√	No. 5^{S}, 6^{S}	√	√
UDP98-025	√	√	√	√	√	√	√	√
EMPAS11	No. 8^{NG}	√	No. 3^{G}, 6^{G}	√	√	√	√	√
Pacita16	No. 8^{K}	√	√	No. $7^{L, B,}_{H}$	√	No. 5^{S}, 6^{S}	No. $1^{?}$, $2^{?}$	√
UDA011	√	√	No. 3^{G}, 6^{G}	√	√	√	No. 1^{NG}, 2^{NG}	√
Summary:	No. 8	N/A	No. 3^{G}, 6^{G}	No. 7	N/A	No. 5, 6	No. 1, 2	N/A

[z]√ represents that all the seedlings of a rootstock produced the pattern characteristic of the parent rootstock cultivar by the designated marker. Seedling numbers mean that the amplified patterns of the seedlings did not exist in their parent genotype. The superscripted rootstock abbreviation means that the amplified pattern of the seedlings by the designated marker can be found in that rootstock.

[y]? represents the patterns that are not same to any rootstock tested.

Conclusion

Twenty-eight *Prunus* SSR markers were used to evaluate polymorphism in peach rootstocks Lovell, Nemaguard, Nemared, Guardian® 'BY520-9' selection 3-17-7, Bailey, Halford, S-37 and Kakamas for fingerprinting. Twenty-three showed polymorphism. Ten SSR markers were found to amplify as least four patterns among the eight rootstocks. Each rootstock can be discriminated by at least one single SSR marker (e.g., Lovell) and at most nine markers (e.g., S-37). No single perfect marker was found to identify all rootstocks. Marker BPPCT008 had the most potential for identification because it amplified seven patterns and was capable of distinguishing as many as six rootstocks directly.

Rootstock seedling identification was conducted by screening open-pollinated seedlings. It turned out to be more difficult than parent genotype identification. This was because heterozygous patterns obtained in a rootstock clone segregated in its seedlings. The segregated patterns might exist in seedlings of other rootstock cultivars or seedlings. However, unique segregated patterns were found in the rootstock seedlings. Seedlings of several rootstocks were identified by single SSR markers such as Nemared (marker BPPCT017), Bailey (marker CPPCT022), Kakamas (marker BPPCT008) and Nemaguard (markers BPPCT017 and EMPAS11). Seedlings of 3-17-7 and S-37 were identified by marker combinations (e.g., EMPAS11/pacita16). Seedlings of Lovell and Halford were identified by single SSRs (e.g., BPPCT001) from the other rootstocks. However, there were no SSRs or marker combination to differentiate Lovell and Halford seedlings. This

SSR system was sensitive such that any off-type seedlings could be identified; therefore, genetic quality of seedlings could be evaluated through pattern reproducibility.

In summary, the SSR markers presented in this study were used as a practical fingerprinting system for rootstock seedling identification. Applying this study to the peach industry will allow peach growers to test rootstocks they purchased and also will be helpful to protect seed propagated proprietary rights (i.e., PVP) for peach breeders and fruit tree nurseries.

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Appendix I: SSR Primer pair sequences used in Chapter Two that were retrieved from GDR database in 2007.

SSRs	Origin	Sequences (5' to 3')	Annealing Tem
BPPCT001	*P. persica*	F: AATTCCCAAAGGATGTGTATGAG R: CAGGTGAATGAGCCAAAGC	57˚C
BPPCT004	*P. persica*	F: CTGAGTGATCCATTTGCAGG R: AGGGCATCTAGACCTCATTGTT	57˚C
BPPCT006	*P. persica*	F: GCTTGTGGCATGGAAGC R:CCCTGTTTCTCATAGAACTCACAT	57˚C
BPPCT007	*P. persica*	F: TCATTGCTCGTCATCAGC R: CAGATTTCTGAAGTTAGCGGTA	57˚C
BPPCT008	*P. persica*	F: ATGGTGTGTATGGACATGATGA R: CCTCAACCTAAGACACCTTCACT	57˚C
BPPCT012	*P. persica*	F: ACTTCCATTGTCAGGCATCA R: GGAGCAACGATGGAGTGC	57˚C
BPPCT013	*P. persica*	F: ACCCACAAATCAAGCATATCC R: AGCTTCAGCCACCAAGC	57˚C
BPPCT014	*P. persica*	F: TTGTCTGCCTCTCATCTTAACC R: CATCGCAGAGAACTGAGAGC	57˚C
BPPCT015	*P. persica*	F: ATGGAAGGGAAGAGAAATCG R: GTCATCTCAGTCAACTTTTCCG	57˚C
BPPCT016	*P. persica*	F: GATTGAGAGATTGGGCTGC R: GAGGATTCTCATGATTTGTGC	57˚C
BPPCT017	*P. persica*	F: TTAAGAGTTTGTGATGGGAACC R:AAGCATAATTTAGCATAACCAAGC	57˚C
BPPCT019	*P. persica*	F: TGATACCACCATCCAATCTAGC R: TTGCTGGGACATGGTCAG	57˚C
BPPCT023	*P. persica*	F: TGCAGCTCATTACCTTTTGC R: AGATGTGCTCGTAGTTCGGAC	57˚C
BPPCT024	*P. persica*	F: GAGGAATGTGCCTCTTCTGG R: CTCCCGTACGCGTTTACC	57˚C
BPPCT025	*P. persica*	F: TCCTGCGTAGAAGAAGGTAGC R: CGACATAAAGTCCAAATGGC	57˚C
BPPCT026	*P. persica*	F: ATACCTTTGCCACTTGCG R: TGAGTTGGAAGAAAACGTAACA	57˚C
BPPCT027	*P. persica*	F: CTCTCAAGCATCATGGGC R: TGTTGCCCGGTTGTAATATC	57˚C
BPPCT028	*P. persica*	F: TCAAGTTAGCTGAGGATCGC R: GAGCTTGCCTATGAGAAGACC	57˚C
BPPCT029	*P. persica*	F: GGACGGACAGAAATGAAGGT R: CCTTAACCCACGCAACTCC	57˚C
BPPCT030	*P. persica*	F: AATTGTACTTGCCAATGCTATGA R: CTGCCTTCTGCTCACACC	57˚C
BPPCT033	*P. persica*	F: GTAGCCGGAGCCGTGTAT	57˚C

		R: CTAGAACCCTATAAACACATGGC	
BPPCT035	*P. persica*	F: TGAAGGATGGCTCTGATACC R:AATTCATCTACTTCTTCCTCAAGC	57˚C
BPPCT036	*P. persica*	F: AAGCAAAGTCCATAAAAACGC R: GGACGAAGACGCTCCATT	57˚C
BPPCT037	*P. persica*	F: CATGGAAGAGGATCAAGTGC R: CTTGAAGGTAGTGCCAAAGC	57˚C
BPPCT039	*P. persica*	F: ATTACGTACCCTAAAGCTTCTGC R: GATGTCATGAAGATTGGAGAGG	57˚C
BPPCT040	*P. persica*	F: ATGAGGACGTGTCTGAATGG R: AGCCAAACCCCTCTTATACG	57˚C
BPPCT042	*P. persica*	F: AACCCTACTGGTTCCTCAGC R: GACCAGTCCTTTAGTTGGAGC	57˚C
CPDCT008	*P. amygdalus*	F: GAAGCAGCCATTCCTAGTGC R:TGTTTATGGACCTTAGTAGTCTGG	55˚C
CPDCT013	*P. amygdalus*	F:GTTTTAGAAACCTCATTCCAACTT R: AATTCTAACACTGGGGTATTGT	62˚C
CPDCT016	*P. amygdalus*	F: GGAAACCTGATTAGGGCACTT R:GGTCTGCTATACTGACCTAGGATT	62˚C
CPDCT017	*P. amygdalus*	F: CGTGCCACGAGAATGAGAAT R: CCAGGACTTAGGAGGTGTCG	62˚C
CPDCT019	*P. amygdalus*	F: AAAACTCCTCTCCTTTTCCCTTT R: TCTTCCTCACCACCTCAAGC	56˚C
CPDCT020	*P. amygdalus*	F: TTGAATCGGAGTTGGAAAGAA R: CGGTGCTGGGAGAATCGT	55˚C
CPDCT023	*P. amygdalus*	F: GTGGCAAATGTTGGCAAAG R: AACACAAAGCAGCACCAAGA	62˚C
CPDCT024	*P. amygdalus*	F: TGAAATCTTTAAATCACCCGACT R: CTTGCTTGCTTGCTTCACCT	54˚C
CPDCT025	*P. amygdalus*	F: GACCTCATCAGCATCACCAA R: TTCCCTAACGTCCCTGACAC	55˚C
CPDCT027	*P. amygdalus*	F: TGAGGAGAGCACTGGAGGAG R: CAACCGATCCCTCTAGACCA	62˚C
CPDCT034	*P. amygdalus*	F: GAGAACCTTTTGTTTGGCCTTA R: CGTCGTATTTAGTGCCGTTG	53˚C
CPDCT038	*P. amygdalus*	F: ATCACAGGTGAAGGCTGTGG R: CAGATTCATTGGCCCATCTT	55˚C
CPDCT044	*P. amygdalus*	F: ACATGCCGGGTAATTAGCAA R: AAAATGCACGTTTCGTCTCC	62˚C
CPDCT045	*P. amygdalus*	F: TGTGGATCAAGAAAGAGAACCA R: AGGTGTGCTTGCACATGTTT	62˚C
CPPCT003	*P. persica*	F: GTAACGAAGAAGTTACGGG R: AACTGTCGCTGCTGGGTT	52˚C
CPPCT004	*P. persica*	F: TCATTCGAAGACGACCGT R: GTCTAGGCACGTTGCTAG	52˚C
CPPCT006	*P. persica*	F: AATTAACTCCAACAGCTCCA R: ATGGTTGCTTAATTCAATGG	59˚C

CPPCT008	*P. persica*	F: GAGCTCTCACGCATTAGTTT R: TTTGACTGCATAACAAAACG	59˚C
CPPCT010	*P. persica*	F:GAATATTTGGATTGCAAAGG R: GGAATATAAGCTCTGCTGCT	59˚C
CPPCT013	*P. persica*	F: GCATTTCGAGAGCTGTATTT R: GTCTTACGTGCAGCTTCATT	59˚C
CPPCT015	*P. persica*	F:TGGAGTGCCAATACTATTTA R:CATATGCATGGTTATGGT	50˚C
CPPCT016	*P. persica*	F: AATTCCCTATGGAAATTAGA R: CGCATATTATAGGTAGGAAA	50˚C
CPPCT017	*P. persica*	F: TGACATGCATGCACTAAACAA R:TGCAAATGCAATTTCATAAAGG	60˚C
CPPCT018	*P. persica*	F: TACGTGCACCCTACTGCTTG R: TTCCAAAGTTAGTCAATTTCTTTC	60˚C
CPPCT019	*P. persica*	F: AATTCAATGTCAAGACACA R: TCATCAAAATAAATATCCAGT	60˚C
CPPCT021	*P. persica*	F: CGGATCCCAGTTGTATTAAATG R: GAGGAACTGGTTATCACCTTGG	60˚C
CPPCT022	*P. persica*	F: CAATTAGCTAGAGAGAATTATTG R: GACAAGAAGCAAGTAGTTTG	50˚C
CPPCT023	*P. persica*	F: CATGGTTTGCAACTGTCTTCA R: GACACAGGTGTGTAGATCATTGG	55˚C
CPPCT024	*P. persica*	F: TTCTCCCAAAAACCAAAACC R: TCATTGGCTGCTAAGTGTCCT	50˚C
CPPCT027	*P. persica*	F: GAGCAGTTCATAAGTTGGAACAA R: CGATAAAGATTTTGACTGCATGA	55˚C
CPPCT029	*P. persica*	F: CCAAATTCCAAATCTCCTAACA R: TGATCAACTTTGAGATTTGTTGAA	55˚C
CPPCT030	*P. persica*	F: TGAATATTGTTCCTCAATTC R: CTCTAGGCAAGAGATGAGA	50˚C
CPPCT033	*P. persica*	F: TCAGCAAACTAGAAACAAACC R: TTGCAATCTGGTTGATGTT	50˚C
CPPCT034	*P. persica*	F:TCGGTTTTTAAAATTCCAAAAGTT R: ACCCTTATTTGCACCCAACA	60˚C
CPSCT008	*P. salicina*	F: TGGATCCAATCCAAGAGTCTG R: GCAGCAAGTTGTTCTTGGTTC	55˚C
CPSCT011	*P. salicina*	F: ATTTGGGTTTGCGACTCAAG R: ACTCATCCCTTGCCCTTTCT	55˚C
CPSCT017	*P. salicina*	F: CAACTCCAAGCTCTGCTCCT R: AGAGCTACACCAGCCAAAGG	57˚C
CPSCT018	*P. salicina*	F: AGGACATGTGGTCCAACCTC R: GGGTTCCCCGTTACTTTCAT	52˚C
CPSCT019	*P. salicina*	F: CCACACATCCCACCACTCTT R: AATTCTAACACTTGGGTATTGTT	53˚C
CPSCT021	*P. salicina*	F: GCCACTTCGGCTAAAAGAGA R: TCCATATCTCCTCCTGCTTGA	46˚C
CPSCT022	*P. salicina*	F: TGTCTGCCTCTCATCTTAACCA	62˚C

		R: TTCTTGAGCAGCCCATCTTCT	
CPSCT024	*P. salicina*	F: TGGGTCGTCTTCTTTATCGTG R: CCTCACCAAAACGGTAGTCAG	54˚C
CPSCT026	*P. salicina*	F: TCTCACACGCTTTCGTCAAC R: AAAAAGCCAAAAGGGGTTGT	54˚C
CPSCT031	*P. salicina*	F: TTCAGATGAAAAAGAAAAAGAAAGT R: AAAGAAACGCTTGTCTTGCAC	52˚C
CPSCT033	*P. salicina*	F: TCCTCATTTGAGTGTTGTGGA R: TGCCCAATTTGAAAACTTTGT	52˚C
CPSCT034	*P. salicina*	F: AGGTGGACAATAGCCGTGAT R: TTTCCAGACCCTGAGAAAGC	62˚C
CPSCT039	*P. salicina*	F: GCCGCAACTCGTAAGGAATA R: TCCACCGTTGATTACCCTTC	55˚C
CPSCT042	*P. salicina*	F: TGGCTCAAAAGCTCGTAGTG R: CCAACCTTTCGTTTCGTCTC	62˚C
EPDC3822	*P. amygdalus*	F: TGGGTTGATGTCATGTCAGG R: ATCACTGCTTCGCCTTCATT	54˚C
EPDCU2584	*P. amygdalus*	F: TTCAGCTCATCTAGTTTCATCACC R: CACGGTTCGAACAACATCTG	54˚C
EPDCU2862	*P. amygdalus*	F: GTGGAAAAACCTGCTCCAGA R: TCATTCTCTTCCCCAGATGC	57˚C
EPDCU3083	*P. amygdalus*	F: TCTTCTCCCTCTCCCTCAGC R: CCCATGACCCTCTTCTTCAA	56˚C
EPDCU3117	*P. amygdalus*	F: CAGAGGGAACAGTGTGAGCA R: TGTTGTTGTCGACCCTGAAA	57˚C
EPDCU3122	*P. amygdalus*	F: AGCGGAGTGTACAGCAAGGT R: AGCGGAGTGTACAGCAAGGT	58˚C
EPDCU3392	*P. amygdalus*	F: CTTTTCATGGGTTCCTCACC R: ATCAACCAGTTCACGCACAA	57˚C
EPDCU3454	*P. amygdalus*	F: GAGGCGGAGGAAGAAGAGGAT R: TGCTGCTGATGAAGGAGATG	57˚C
EPDCU3489	*P. amygdalus*	F: AAATCAGCTCCCATCACTCC R: AGCTGAGTGGAACCAGAGGA	56˚C
EPDCU3516	*P. amygdalus*	F: ACCGTTAACGAGGCTCAGTC R: ACCTCCACTGCCATATCCAC	57˚C
EPDCU4205	*P. amygdalus*	F: CAGCCCTCACTCTCTGATCG R: ATTGCCTCCTCCTTCCATTT	55˚C
EPDCU5060	*P. amygdalus*	F: ACCAAATTGGACATGCAACC R: CGGTCGAGAAGACTGAGGAG	55˚C
EPDCU5100	*P. amygdalus*	F: CTCTTCTCGCCTCCCAATTT R: TGCTTAGCCCTGGGTACAAG	56˚C
EPDCU5183	*P. amygdalus*	F: AGCAGTCTTTGCCAAATCAA R: TACAGGGTCCACATGATCCA	54˚C
EPPB 4233	*P. persica*	F: CGATTAAAAAGCCTCTGGC R: TCATGGTCATGGCTGAGTT	52˚C
EPPCU044	*P. persica*	F: CCAAAAGTCTCAGCCCGAAA	56˚C

5		R: ATACCACCAGCTTCGGCTCC	
EPPCU1106	*P. persica*	F: CGAAGCTGAATCGAGATTATGA R: CCGAAACACAATACTCTTGCAT	52˚C
EPPCU1198	*P. persica*	F: TTGCCAGTTCATCATTGTTTG R: ACCATTATGCCTTGGTCACAGG	54˚C
EPPCU3090	*P. persica*	F: AGACAGAGGGGACAGAGCAA R: CGCGCGGAGAGATAATAGAC	56˚C
EPPCU5516	*P. persica*	F: TCCTTCTGCCAGCTCAATAC R: GAATGGAGAGAATGGGTGTG	53˚C
EPPCU5990	*P. persica*	F: AAACCAGATCAACCCTACCC R: ATGAGGAAAACCCACATCCA	54˚C
EPPCU6522	*P. persica*	F: GACAGACAGACGGACAGACG R: ACCCTCTCCCTGACTTCCTC	57˚C
M6a	*P. persica*	F: AGAAGGGCAAGCCCAAGTGC R: TGCAAAGCCAGAGCCCACAA	60˚C
MA021a	*P. persica*	F: TGAGCTCCGATCATTATAGA R: CACAGGATGGGCGTATCTTT	52˚C
MA024a	*P. persica*	F: AACCCAATCCAATATCAACC R: GGGGGATCTCTCAACTCAA	52˚C
MA034a	*P. persica*	F: GACCATTGCCTCGTAATCTT R: AGCGCTAGCTATCACCTACC	54˚C
MA053a	*P. persica*	F: TCACTCTCCAGTAAACACTATG R: AGCCACTACAATGATAGCAA	55˚C
MA059a	*P. persica*	F: TCAACTTTCAACTGCCATTAGA R: GAGTGGGAGAGTTTGGACC	55˚C
MA061a	*P. persica*	F: ACCAAAAAGCCAAGTCGAACA R: CGTTTTCTTCTAGGGCAGTTCA	55˚C
MA069a	*P. persica*	F: GGAAATGAACACATCTCGTCAGTAA R: AACAGCCAAAAGGAGACAACC	55˚C
MA075a	*P. persica*	F: TACTAGTCTACCAGTACTGTGACTC R: GCGTCTCCGTACTCTCTT	55˚C
Pacita4	*P. armeniaca*	F: GTGAAAATGAAAGAATCGCTACC R: TGTCCCTTGACGCCCAGATTTCTCC	50˚C
Pacita5	*P. armeniaca*	F: GTTGTGTTTACTTTTTTCTTAACGG R: GTATCACAAGTGAGAACATAAGAGG	50˚C
Pacita6	*P. armeniaca*	F: TGGATGGATGAACATGAGCGGTGGT R:TTCATGCATTAGTTTACTTTTCATG	50˚C
Pacita21	*P. armeniaca*	F: GATTATATAAGTTGGTTTTTGTAAG R:GTATTCTATAATGTATAAATGTACG	50˚C
Pacita25	*P. armeniaca*	F: CTCTACAATTTTGGGTTCTTCTTGG R: CCTTAAACAAAAAGATGAACAAATG	46˚C
Pacita27	*P. armeniaca*	F: GATCCCTCAACTGAATCTCTC R: CGTCACAACAATAGATGCGAAGG	46˚C

PceGA25	*P. cerasus*	F: GCAATTCGAGCTGTATTTCAGATG R: CAGTTGGCGGCTATCATGCTTAC	56˚C
PceGA34	*P. cerasus*	F: GAACATGTGGTGTGCTGGTT R: TCCACTAGGAGGTGCAAATG	55˚C
Pchcm2	*P. persica*	F: GTCAATGAGTTCAGTGTCTACACTC R: AATCATAACATCATTCAGCCACTGC	55˚C
Pchcm4	*P. persica*	F: ATCTTCACAACCCTAATGTC R: GTTGAGGCAAAAGACTTCAAT	51˚C
Pchcm5	*P. persica*	F: CCAGTAGATTTCAACGTCATC TACA R: GGTTCACTCTCACATACACTCGGAG	50˚C
Pchgms1	*P. persica*	F:GGGTAAATATGCCCATTGTGCAATC R: GGATCATTGAACTACGTCAAT CCTC	56˚C
Pchgms2	*P. persica*	F: GTCAATGAGTTCAGTGTCTACACTC R:AATCATAACATCATTCAGCCACTGC	55˚C
Pchgms3	*P. persica*	F: ACGGTATGTCCGTACACTCTCCAT G R: CAACCTGTGATTGCTCCTATTAAA C	57˚C
Pchgms4	*P. persica*	F: ATCTTCACAACCCTAATGTC R: GTTGAGGCAAAAGACTTCAAT	50˚C
Pchgms5	*P. persica*	F: CCAGTAGATTTCAACGTCATCTACA R: GGTTCACTCTCACATACACTCGGAG	56˚C
Pchgms31	*P. persica*	F: TATCAGGTAAGGACCACTG R: GCTGCCGACGCTGTCAATTTC	52˚C
Pchgms44	*P. persica*	F: GTTCAGCGAGCCCAGACTCA R: CAAGTCATCTGCCCAGACGGTA	58˚C
Pchgms46	*P. persica*	F: ACACCAAAAGCCACTCAAGTCTC R: CGTCTCTGGCTATTGGCTATTGCT	58˚C
PMS67	*P. avium*	F: AGTCGCTCACAGTCAGTTTCTC R: TTAACTTAACCCCTCTCCCTCC	55˚C
PS12a2	*P. avium*	F: GCCACCAATGGTTCTTCC R: AGCACCAGATGCACCTGA	55˚C
PS1h3	*P. avium*	F: TGAGGAGCATAATGACAGT R: TCACCATGTGTCATACT	48˚C
PS5c3	*P. avium*	F: AGATCTCAAAGAAGCTGA R: AGCTTATGCATATACCTG	46˚C
PS7a2	*P. avium*	F: CAGGGAAATAGATAAGATG R: TCTAATGGTGGTGTTCATT	46˚C
PS8e8	*P. avium*	F: CCCAATGAACAACTGCAT R: CCCAATGAACAACTGCAT	48˚C
UCD-	*P. avium*	F: CACTGTCTCCCAGGTTAAACTC	55˚C

CH39		R: CCTGAGCTTTTGACACATGC	
UDA006	*P. amygdalus*	F: ATTCTCCAAGGCGATAAGCA R: TTAGGCACCTGTCCCCTACA	56˚C
UDA008	*P. amygdalus*	F: AGACGCTTTGCATACATACAAGT R: TGCAGGAACTGGGATTAGAGA	55˚C
UDA009	*P. amygdalus*	F: AAAACATCTCTCTCCTCCATGC R: AGTTCTCTGGCAGCACAAGC	56˚C
UDA010	*P. amygdalus*	F: GACTCACATACACGTGGGTTTC R: GGTGTGATTTGTGTGTGTGC	55˚C
UDA011	*P. amygdalus*	F: TGGATTGTTTTCCCCTGGTA R: TGGATTGTTTTCCCCTGGTA	56˚C
UDA020	*P. amygdalus*	F: TGTGCACCAAACACAACTGA R: GCAGTGTTGCCAATGTTGAT	55˚C
UDA022	*P. amygdalus*	F: GCCGTCTCATTTTCCCATTA R: GTGCGATGGAGGAGCACT	55˚C
UDA026	*P. amygdalus*	F: AAAAACCTGAAAACACACACAC R: GCTCGGCTGTTCAAAAATAG	52˚C
UDA027	*P. amygdalus*	F: GGAGACAGACGGAGCAACAT R: CTTCAATCTCGCTCCCAAAG	55˚C
UDA029	*P. amygdalus*	F: GAATCTCATATTCTGCACCACA R: TTGGCATTCCGTAAGGTACA	53˚C
UDA031	*P. amygdalus*	F: AACAACATCCTACAGGTCTCTCTC R: GTCCATCTCTGCACACCAGA	56˚C
UDA033	*P. amygdalus*	F: AATTCACTTCATCTCCTCTCTCTC R: TTGTTCAGAGCTGAAATCCAGA	54˚C
UDA035	*P. amygdalus*	F: GGTGGATGAGGGTTTCACAC R: GCCATCTCAAAGCCCATAAC	55˚C
UDA036	*P. amygdalus*	F: AATTCACATATATACCCGTACACAC R: TGTTGGATTGTTTCCTCTGG	52˚C
UDA038	*P. amygdalus*	F: CCATCCATGTATATCTTATGTCTAAGT R: TCTTGACAACCCAAAGTGGA	53˚C
UDA042	*P. amygdalus*	F: CCAGAGCTCGTCCACTTAAC R: AGAGCTAGAGATGTAAATACACACAC	54˚C
UDA043	*P. amygdalus*	F: GAATACATAAATGGGATACCAAGGA R: TTTGGACTCATACCATTTTGTG	51˚C
UDA048	*P. amygdalus*	F: AGACGCAGGATAGCAACAGG R: CCAAACCACTGTATATACTTTAACTGC	55˚C
UDA051	*P. amygdalus*	F: AGTGCAAGATGTTCGGCATA R: TGCCCCAAGAGCTTAACGTA	55˚C
UDAp-426	*P.*	F: GAAGTGCAATACCCCAAAGC	54˚C

	amygdalus	R: GGGGAGACTTGCAAGAAAGA	
UDAp-460	*P. amygdalus*	F: TCATCAGTCAGGTGGTGCTC R: TGACAGCCTAATCAGCCATTT	56˚C
UDAp-462	*P. amygdalus*	F: CTGTGTGTAATACATGGGGAGAG R: CTTCAAGCCCTTCTTCGTTG	54˚C
UDP96-001	*P. persica*	F: AGTTTGATTTTCTGATGCATCC R: TGCCATAAGGACCGGTATGT	57˚C
UDP96-003	*P. persica*	F: TTGCTCAAAAGTGTCGTTGC R: ACACGTAGTGCAACACTGGC	56˚C
UDP96-008	*P. persica*	F: TTGTACACACCCTCAGCCTG R: TGCTGAGGTTCAGGTGAGTG	57˚C
UDP96-015	*P. persica*	F: CCTTGACCTATTTGTTCGTCA R: ACTAGTCAAACAATCCCCCG	56˚C
UDP96-019	*P. persica*	F: TTGGTCATGAGCTAAGAAAACA R: TAGTGGCACAGAGCAACACC	56˚C
UDP97-401	*P. persica*	F: TAAGAGGATCATTTTTGCCTTG R: CCCTGGAGGACTGAGGGT	57˚C
UDP97-402	*P. persica*	F: TCCCATAACCAAAAAAAACACC R: TGGAGAAGGGTGGGTACTTG	57˚C
UDP97-403	*P. persica*	F: CTGGCTTACAACTCGCAAGC R: CGTCGACCAACTGAGACTCA	57˚C
UDP98-024	*P. persica*	F: CCTTGATGCATAATCAAACAGC R: GGACACACTGGCATGTGAAG	57˚C
UDP98-025	*P. persica*	F: GGGAGGTTACTATGCCATGAAG R: CGCAGACATGTAGTAGGACCTC	56˚C
UDP98-405	*P. persica*	F: ACGTGATGAACTGACACCCA R: GAGTCTTTGCTCTGCCATCC	56˚C
UDP98-406	*P. persica*	F: TCGGAAACTGGTAGTATGAACAGA R: ATGGGTCGTATGCACAGTCA	55˚C
UDP98-408	*P. persica*	F: ACAGGCTTGTTGAGCATGTG R: CCCTCGTGGGAAAATTTGA	54˚C
UDP98-409	*P. persica*	F: GCTGATGGGTTTTATGGTTTTC R: CGGACTCTTATCCTCTATCAACA	52˚C
UDP98-411	*P. persica*	F: AAGCCATCCACTCAGCACTC R: CCAAAAACCAAAACCAAAGG	53˚C
UDP98-412	*P. persica*	F: AGGGAAAGTTTCTGCTGCAC R: GCTGAAGACGACGATGATGA	57˚C

Appendix II: Phenotype rating of the replicates of F_2-11 population from 2004 through 2008.

Location[z]			PTSL rating[y]				
		F_2	2004	2005	2006	2007	2008
1	E	11-01	0	5	5	5	5
4	E	11-01	5	5	5	5	5
4	E	11-01	0	3	5	5	5
7	W	11-01	0	5	5	5	5
1	E	11-02	0	5	5	5	5
1	E	11-02	0	5	5	5	5
4	E	11-02	0	5	5	5	5
5	W	11-02	0	0	0	1	0
7	W	11-02	0	0	0	0	3
3	E	11-03	0	5	5	5	5
5	E	11-03	0	5	5	5	5
7	W	11-03	5	5	5	5	5
1	E	11-04	0	5	5	5	5
4	W	11-04	0	5	5	5	5
5	W	11-04	0	5	5	5	5
7	W	11-04	0	3	0	5	5
2	W	11-05	0	0	0	0	0
3	W	11-05	0	0	0	0	0
5	E	11-05	0	0	0	0	0
1	E	11-06	0	1	0	0	0
3	E	11-06	0	1	3	5	5
5	W	11-06	0	0	0	0	0
1	E	11-08	0	0	0	0	0
3	E	11-08	0	0	0	0	0
5	W	11-08	0	0	0	0	0
7	W	11-08	0	0	0	1	0
1	E	11-09	0	5	5	5	5
3	E	11-09	0	0	5	5	5
5	E	11-09	0	0	3	2	0
7	W	11-09	0	5	5	5	5
1	E	11-10	0	5	5	5	5
3	W	11-10	0	5	5	5	5
4	W	11-10	0	0	1	5	5
7	E	11-10	0	5	5	5	5
1	E	11-11	0	0	0	1	5
3	E	11-11	0	4	3	0	0

5	W	11-11	0	0	0	0	0
1	E	11-13	3	5	5	5	5
3	W	11-13	0	0	0	0	0
5	E	11-13	0	0	0	1	4
6	E	11-13	0	5	5	5	5
1	E	11-14	0	2	4	5	5
3	W	11-14	0	0	3	5	5
5	W	11-14	0	0	0	5	5
6	E	11-14	0	5	5	5	5
1	E	11-15	0	1	0	0	5
3	W	11-15	0	0	0	0	0
4	E	11-15	0	5	5	5	5
6	E	11-15	0	5	5	5	5
1	E	11-17	0	1	0	1	3
1	E	11-17	0	3	4	5	5
3	W	11-17	0	0	0	1	0
5	E	11-17	0	0	0	2	5
7	W	11-17	0	5	5	5	5
2	E	11-18	0	5	5	5	5
3	W	11-18	0	0	0	0	3
4	E	11-18	0	1	0	1	5
6	W	11-18	0	0	0	0	3
1	E	11-19	0	1	0	1	0
3	E	11-19	0	5	5	5	5
5	E	11-19	0	5	5	5	5
7	W	11-19	0	5	5	5	5
1	E	11-20	0	0	0	0	0
3	W	11-20	0	0	0	0	5
5	E	11-20	0	0	0	0	0
7	W	11-20	0	0	0	0	0
1	E	11-21	0	0	0	0	3
3	W	11-21	0	0	3	0	5
4	W	11-21	4	5	5	5	5
6	E	11-21	0	5	5	5	5
1	E	11-23	0	1	0	0	0
3	W	11-23	0	0	0	0	0
4	E	11-23	0	2	0	0	5
6	E	11-23	0	1	0	0	0
2	W	11-28	0	5	5	5	5
3	W	11-28	0	0	0	5	5
4	E	11-28	0	5	5	5	5
6	E	11-28	0	5	5	5	5

1	E	11-29	0	5	5	5	5
3	W	11-29	0	0	0	0	0
5	E	11-29	0	0	0	1	0
1	E	11-30	0	0	0	0	0
3	W	11-30	0	0	0	0	4
5	E	11-30	5	5	5	5	5
6	E	11-30	0	5	5	5	5
1	E	11-31	0	1	0	0	0
3	E	11-31	5	5	5	5	5
4	E	11-31	0	5	5	5	5
6	E	11-31	5	5	5	5	5
1	E	11-32	0	1	4	5	5
3	W	11-32	0	5	5	5	5
4	E	11-32	0	5	5	5	5
6	E	11-32	0	5	5	5	5
1	E	11-33	0	3	0	1	5
3	W	11-33	0	0	0	0	5
4	E	11-33	0	5	5	5	5
6	E	11-33	3	5	5	5	5
1	E	11-34	0	2	0	0	3
3	W	11-34	0	2	3	5	5
5	W	11-34	5	5	5	5	5
6	E	11-34	0	5	5	5	5
1	E	11-36	0	4	2	1	0
3	W	11-36	0	0	0	0	0
4	E	11-36	0	4	0	0	5
6	W	11-36	0	5	5	5	5
1	E	11-37	0	0	0	0	0
3	E	11-37	0	5	5	5	5
4	W	11-37	0	0	0	0	0
7	W	11-37	0	0	0	0	2
1	E	11-39	0	5	5	5	5
3	E	11-39	0	5	5	5	5
4	W	11-39	0	5	5	5	5
6	W	11-39	4	5	5	5	5
2	E	11-40	0	0	0	0	0
3	W	11-40	0	0	0	0	0
4	E	11-40	0	0	0	0	0
6	E	11-40	0	0	0	0	2
1	E	11-41	0	1	0	0	3
3	W	11-41	0	0	0	0	0
5	W	11-41	0	5	5	5	5

7	E	11-41	0	2	0	2	5
2	E	11-44	3	5	5	5	5
3	W	11-44	0	0	0	0	0
4	E	11-44	5	5	5	5	5
6	W	11-44	0	5	5	5	5
2	E	11-46	0	0	0	0	0
3	W	11-46	0	0	0	0	0
4	E	11-46	0	0	0	0	0
6	W	11-46	0	0	0	0	0
3	E	11-47	0	3	0	0	4
4	W	11-47	0	0	0	1	5
6	E	11-47	0	5	5	5	5
1	E	11-48	0	1	0	1	4
3	W	11-48	4	5	5	5	5
5	E	11-48	0	0	0	0	2
6	E	11-48	0	5	5	5	5
2	W	11-49	4	5	5	5	5
3	W	11-49	0	0	0	0	0
4	E	11-49	0	1	3	1	0
6	E	11-49	3	5	5	5	5
1	E	11-50	0	1	0	0	0
3	E	11-50	5	5	5	5	5
5	W	11-50	0	0	0	0	0
7	W	11-50	0	0	0	1	0
2	W	11-56	0	5	5	5	5
3	E	11-56	0	5	5	5	5
4	W	11-56	0	0	0	5	5
6	W	11-56	5	5	5	5	5

[z]The replicates were planted in seven double rows. Each tree site is represented by a row number and the side of the row. W: West side of a row: E: East side of a row.
[y]PTSL rating of each tree is evaluated using a 0 to 5 system; 0 represents healthy, no symptoms; 1-4 scores increasing degree of symptoms; and 5 represents scion death caused by PTSL.