Characterisation and mapping of bacterial wilt (*Ralstonia solanacearum*) resistance in the tomato (*Solanum lycopersicum*) cultivar Hawaii 7996 and wild tomato germplasm

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ABBREVIATIONS

a	additive effect
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of variance
ATP	Adenosine 5´-Triphosphate
AVRDC	Asian Vegetable Research and Development Center
AUDPC	Area Under Disease Progress Curve
AUDPCPWP	Area Under Disease Progress Curve calculated from Percentage of Wilted Plants
AUDPCDI	Area Under Disease Progress Curve calculated from Disease Index
BC	Backcross
Bv	Biovar
CA	Citric Acid
CAPS	Cleaved Amplified Polymorphic Sequence
Cfu	Colony forming units
^{o}C	degree celsius
CIRAD	The Centre International de Recherche en Agriculture pour le Développement
cM	Centi Morgan
cm	centimeter
CORR	Correlation
CTAB	Cetyl Trimethyl Ammonium Bromide
cv.	cultivar
DAI	Days After Inoculation
DArT	Diversity Arrays Technology
ddH_2O	Double distilled water
DI	Disease Index
DNA	Deoxyribonucleic acid
dNTP	Nucleotides
EDTA	Ethylenediaminetetraacetic Acid
et al.	*et alii* (and others)
EW	East-West
FC	Fruit Color
FW	Fruit Weight
ID	Indonesia
IN	India
Inc.	Incorporated
IPB-UPLB	Institi nstitute of Plant Breeding of the University of the Philippines at Los Banos
JM	JoinMap
g	gram
GMI	Global Medical Instrumentation

GRSU	Genetic Resource and Seed Unit
kb	kilobase
kg	kilogram
K_2O	Potassium oxide
LB-A medium	Luria-Bertani medium containing Ampicillin
LG	Linkage Group
LOD	Logarithm of Odds
µl	microliter
M	Mole
m	meter
MAS	Marker-Assited Selection
MilliQ water	Deionized water purified in a Milli-Q system
MJ PT-200	MJ PCR machine PT200 gradient
MgO	Magnesium oxide
$MgCl_2$	Magnesium chloride
mg	miligram
ml	mililiter
mM	milimolar
MP	MultiPoint
N	Nitrogen
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NEB	New England Biolabs
ng	nanogram
O.D.	Optimal Density
QTL	Quantitative Trait Loci
RAMP	Randomly Amplified Microsatellite Polymorphism
RAPD	Random Amplification of Polymorphic DNA
RAUDPC	Relative Area Under Disease Progress Curve
RCBD	Randomized Complete Block Design
RIL	Recombinant Inbred Line
RFLP	Restriction Fragment Length Polymorphism
RGA	Resistant Gene Analog
rpm	rotations per minutes
R. solanacearum	*Ralstonia solanacearum*
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PCP	Percentage of Colonized Plants
pH	Potential of hydrogen
PH	Philippines
pmol	picomole
P/L	Pty Limited
P_2O_5	Phosphorus pentoxide
PVP	Polyvinyl Pyrrolidone
PWP	Percentage of Wilted Plants
R2	Phenotypic variation explained
RN	Reunion

S.	*Solanum*
SGN	The SOL Genomics Network
SM1	Semi-selective Medium 1
SNP	Single Nucleotide Polymorphism
SPI	Sympodial Index
spp.	species (plural)
SSC	Soluble Solid Content
SSCP	Single-Stranded Conformation Polymorphism
SSD	Single Seed Descent
SSLP	Simple Sequence Length Polymorphism
SSR	Simple Sequence Repeat
STMS	Sequence-Tagged Microsatellite Site
STR	Short Tandem Repeat
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TE	Tris EDTA
TEMED	Tetramethylethylenediamine
TGRC	Tomato Genetic Resource Center
TH	Thailand
Tm	melting temperature
Tris-HCl	Tris Hydrochloride
TTC	Tetrazolium Medium
TW	Taiwan
WAI	Weeks After Inoculation
W	Watt
w/v	weight per volume
WVa700	West Virginia 700
V	Volt
v/v	volume per volume
U	Unit
UV	Ultraviolet

ABSTRACT

Bacterial wilt caused by race 1 strains of *Ralstonia solanacearum* is one of the most important and widely distributed plant diseases in the tropics and subtropics, particularly on tomato. Planting resistant material is the most suitable measure for the control of tomato bacterial wilt. To elucidate genetic control of resistance in Hawaii 7996, a stable resistance source, a population of 188 F_9 recombinant inbred lines (RILs) derived from a cross between *S. lycopersicum* Hawaii 7996 (resistance parent) and *S. pimpinellifolium* West Virginia 700 (susceptible parent) was used for this study. First, the genetic map was improved, which contained a total of 362 markers with 74 AFLP, 260 DArT, 5 RFLP, 1 SNP, and 22 SSR markers. These markers were split into ten major and two minor linkage groups, spanning 2131.7 cM. However, a framework map of 106 loci (32 AFLP, 59 DArT, 6 RFLP, 11 SSR) distributed over 15 linkage groups covering 1089.1 cM was used for quantitative trait loci (QTL) mapping using composite interval mapping. In addition, association of 13 markers belonging to certain chromosomes with disease resistance were determined separately by single marker analysis. The phenotypic data used for the QTL analysis included a total of 22 datasets: 16 for disease evaluations and 6 for morphological traits. Disease reactions of the RIL population were evaluated in 16 trials against race 1 and race 3 strains in six countries both in the field or at seedling stage.

A total of 37 QTLs were identified. Out of these 37 QTLs detected, 31 QTLs were identified for bacterial wilt resistance, one for sympodial index, two for citric acid, two for soluble solid content and one for fruit color (a/b). They explained between 5.0% and 34.7% of the phenotypic variation, depending on the traits. QTLs located on chromosome 6, LGA and LGB showed significant linkages with disease reactions against several pathogen strains and in several locations and should be targeted for fine mapping. Resistance mechanism in Hawaii 7996 appeared to be related to the suppression of the pathogen colonization, as similar QTLs were found for visual symptom data as well as colonization data. Possible linkages between fruit size, critic acid, and fruit color with bacterial wilt resistance were observed. Several SNPs have been found that would be useful in fine mapping of QTL to develop closely linked markers for marker-assisted selection and gene cloning. In order to find more diverse resistance sources to overcome the highly variable pathogen strains, a total of 252 wild *Solanum* accessions and one

population of forty-nine introgression lines (ILs) of LA716 were screened for resistance to a race 1 biovar 4 strain Pss186 of *Ralstonia solanacearum*. Most wild tomato accessions were highly susceptible. However, five wild tomato accessions of *S. pennellii*, i.e. LA1943, LA716, LA1656, LA1732 and TL01845 were resistant to strains Pss186 and Pss190 but susceptible to Pss4. Only IL6-2, which has an introgression segment on chromosome 6, was found to be resistant to Pss186 among screened ILs. These new resistant sources will provide breeders more resources to breed for durable resistance to bacterial wilt of tomato.

Keywords: *Ralstonia solanacearum*, quantitative trait loci, resistance.

ZUSAMMENFASSUNG

Bakterielle Welke verursacht durch Rasse 1 Stämme von *Ralstonia solanacearum* ist eine der bedeutendsten und weitverbreitetsten Pflanzenkrankheiten in den Tropen und Subtropen, insbesondere bei Tomate. Die geeignetste Maßnahme zur Kontrolle dieser Krankheit bei Tomate ist der Anbau resistenter Pflanzen. Um die genetische Kontrolle der Resistenz von Hawaii 7996, einer stabilen Resistenzquelle, aufzuklären, wurde in der vorliegenden Arbeit eine Population von 188 Rekombinanten Inzuchtlinien (RIL) in der F_9 Generation aus der Kreuzung zwischen *S. lycopersicum* Hawaii 7996 (resistenter Elter) und *S. pimpinellifolium* West Virginia 700 (anfälliger Elter) untersucht. Zunächst wurde die genetische Karte auf insgesamt 362 Marker, davon 74 AFLPs, 260 DArTs, 5 RFLP, 1 SNP und 22 SSR Marker, erweitert. Diese Marker verteilten sich auf zehn große und zwei kleinere Kopplungsgruppen mit insgesamt 2.131,7 cM. Für die QTL (quantitative trait loci) Kartierung mit Hilfe von „composite interval mapping“ wurde eine Framework-Karte mit 106 Loci (32 AFLP, 59 DArT, 6 RFLP, 11 SSR) verteilt auf 15 Kopplungsgruppen mit 1.089,1 cM benutzt. Zusätzlich dazu wurden 13 Marker, die verschiedenen Chromosomen zugeordnet waren, auf ihre Assoziation mit der Resistenz in einer „single marker analysis“ untersucht. Die für die QTL Analyse verwendeten phänotypischen Daten setzten sich aus 22 Datensätzen zusammen: 16 Datensätze aus Resistenzevaluierungen und 6 morphologische Merkmale. Die Resistenzreaktion der RIL Population gegenüber Rasse 1 und Rasse 3 Stämmen wurde in 16 Versuchen in sechs Ländern sowohl im Feld als auch im Sämlingsstadium untersucht.

Insgesamt wurden 37 QTLs identifiziert. Davon wurden 31 QTLs für Resistenz gegen *Ralstonia*, einer für sympodialen Index, zwei für Säuregehalt, zwei für Gehalt an löslichen Feststoffen und einer für Fruchtfarbe (a/b) entdeckt. Die QTLs erklärten abhängig vom Merkmal zwischen 5.0% und 34.7% der phänotypischen Variation. QTLs auf Chromosom 6, LGA und LGB zeigten eine signifikante Kopplung zur Resistenz gegen mehrere Pathogenstämme an mehreren Orten und sollten das Ziel einer Feinkartierung sein. Der Resistenzmechanismus in Hawaii 7996 scheint mit der Pathogenbesiedelung zusammenzuhängen, da ähnliche QTLs für visuelle Symptome und Daten aus Colonisierungsexperimenten gefunden wurden. Mögliche Kopplungen zwischen Fruchtgröße, Säuregehalt, Fruchtfarbe und *Ralstonia*-Resistenz wurden beobachtet. Mehrere SNPs, die für eine Feinkartierung der QTLs zur Entwicklung von eng

gekoppelten Markern für eine Marker-gestützte Selektion oder eine Genklonierung genutzt werden können, wurden identifiziert. Mit dem Ziel weitere Resistenzquellen gegen das hoch variable Pathogen zu finden, wurden insgesamt 252 Accessionen von *Solanum* Wildarten sowie eine Population mit 49 Introgressionslinien (ILs) aus LA716 auf Resistenz gegen den Rasse 1 Biovar 4 Stamm Pss186 von *Ralstonia solanacearum* untersucht. Die meisten Tomaten Wildarten waren stark anfällig. Allerdings zeigten fünf Accessionen von *S. pennellii*, LA1943, LA716, LA1656, LA1732 und TL01845, Resistenz gegenüber den Stämmen Pss186 und Pss190, waren aber anfällig gegenüber Pss4. Von den untersuchten ILs war nur die Linie IL6-2, die auf Chromosom 6 eine Introgression trägt, resistent gegen Pss186. Mit dieser neue Resistenzquelle steht der Züchtung eine weitere Resource für die Entwicklung dauerhafter Resistenz gegenüber bakterieller Welke bei Tomate zur Verfügung.

Keywords: *Ralstonia solanacearum*, quantitative trait loci, Resistenz.

GENERAL INTRODUCTION

Tomato (*Solanum lycopersicum*) is one of the most important vegetables worldwide because of the versatility of its use in both fresh and processed foods. However, tomato production is beset by many production constraints, one of which is bacterial wilt. This disease caused by the soil-borne pathogen *Ralstonia solanacearum* (E. F. Smith), formerly called *Pseudomonas solanacearum* E. F. Smith (Yabuuchi *et al.* 1995), is one of the most important bacterial plant diseases in the world. Bacterial wilt affects hundreds of different species, mainly in tropical and subtropical climates, including many crops such as potato, tomato, eggplant, pepper, ground nut, and banana (Hayward, 1991). Several methods have been employed to control this disease; however, the introduction of resistant varieties is considered the most successful, practical, environmentally sound, and economical control strategy (Denny, 2006). However, breeding durable resistance to bacterial wilt is challenging because inheritance of resistance is complicated by interactions between the plant genotype and pathogen strains as well as the effect of the environment on resistance expression (Grimault and Prior, 1993).

In the genus *Solanum*, resistance to bacterial wilt was first reported in the wild tomato *S. pimpinellifolium*. It was described to be controlled by a small number of major genes and associated with fruit size (Acosta *et al*. 1964). In 1988, Opena *et al*. also found only a few different resistance genes appear to be involed in several different bacterial wilt resistance sources. Among a series of lines from Hawaii, Hawaii 7996 is the most stable resistance source (Wang *et al*. 1998). The decission on the most appropriate and efficient strategy to transfer the stable resistance from Hawaii 7996 depends on our knowledge of the genetic control.

Rapid advances in crop biotechnology have provieded new tools in plant breeding. DNA markers are a very useful tool because they can be used to construct high density molecular maps, making it possible to locate more precisely genes affecting either simple or complex traits (Paterson *et al*. 1991). DNA markers tightly associated or linked to a gene of interest can be used in marker-assisted selection, and thus, can increase the efficiency of selection particulary for traits that are strongly influenced or dependent on the environment for trait expression (Young, 1996).

In tomato, molecular mapping of bacterial wilt resistance genes has been initiated and important QTLs have been identified (Denesh *et al*. 1994; Thoquet *et al*. 1996a; b; Wang *et al*. 2000). Among these, several QTLs were mapped in Hawaii 7996 based on F_2 or F_3 populations derived from a cross with the susceptible parent line 'West Virginia 700' (WVa700) (Thoquet *et al*. 1996a; Thoquet *et al*. 1996b; Mangin *et al*. 1999; Wang *et al*. 2000). Mapping, however, relied on the use of F_2 or F_3 population and therefore the effect of different enviroments and strains or races of the pathogen could not be extensively evaluated. The use of recombinant inbred lines (RILs) can overcome such limitation since RILs can serve as a permanent mapping resource that will permit replicated tests in multiple environments using different strains of the pathogen. Carmielle *et al*. (2006) used F_8 RILs derived from the same cross Hawaii 7996 x WVa700 and demonstrated environmental factors influenced the expression of resistance against the race 3-phylotype II strain JT516.

The primary goals of this study were (1) to use of F_9 RILs to identify QTL general and specific to various environments directed towards development of PCR-based markers linked to important QTL for marker-assisted selection (MAS); (2) to evaluate wild tomato germplasm for resistance to race 1 strains of *R. Solanacearum* to find diverse resistance sources to possibly overcome the highly variable pathogen strains.

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Chapter 1

Construction of a genetic linkage map for mapping bacterial wilt resistance in the tomato cultivar Hawaii 7996

1.1 INTRODUCTION

In the genus *Solanum*, several accessions of cultivated tomato (*S. lycopersicum*) showed resistance to bacterial wilt (Wang *et al.* 1997). Results from various genetic analysis and inheritance studies demonstrated that the resistance is most likely polygenic (Mohamed *et al.* 1997; Prior *et al.* 1994; Thoquet *et al.* 1996b; Wang *et al.* 2000). Resistance has been difficult or impossible to transfer to desirable cultivars due to the number of Quantitative Trait Loci (QTL) and/or linkage of QTL to undesirable traits. In addition, the inheritance of resistance is further complicated by interactions between the plant genotype and pathogen strains, as well as environmental effects on resistance expression (Grimault and Prior, 1993; Hayward, 1991). All of these factors have made breeding for resistance very challenging. Breeding a resistant variety using un-adapted germplasm as a donor typically requires a series of backcrosses to the cultivated recurrent parent, alternating with progeny testing, to combine desirable characteristics. This procedure is time consuming and costly. The application of molecular markers to facilitate the introgression of disease resistance to crop cultivars helps to alleviate time and cost constraints (Zhang *et al.* 2002). Molecular markers have gained favor in plant breeding as a powerful approach permiting construction of high density genetic maps making it possible to locate genes more precisely (Stuber, 1992). The potential number of DNA markers for any plant species is potentially unlimited, which allows the development of linkage maps with a high degree of resolution (Helentjaris *et al.* 1986).

Amplified fragment length polymorphisms (AFLPs), combine the reproducibility of RFLP and the speed and convenience of PCR-based marker techniques. Reproducibility of AFLPs is assisted by the use of restriction enzymes that cut specific sites in the genome, use of primers specifically designed based on synthetic adaptor sequences, and stringent amplification conditions (Vos *et al.* 1995). AFLPs yield a large number of bands, and can

be used without prior knowledge of genome sequence information. One of the drawbacks is generating primarily dominant and anonymous markers. However, AFLPs have been shown to be useful in saturating genetic maps in species with large genomes. AFLP maps have been rapidly applied in many crop species, for example barley (Becker *et al.* 1995; Powell *et al.* 1997), potato (van der Voort *et al.* 1998), rice (Mackill *et al.* 1996) and tomato (Haanstra et al. 1999).

Microsatellites, also called simple sequence repeats (SSRs), short tandem repeats (STRs), simple sequence length polymorphism (SSLP), or sequence-tagged microsatellite sites (STMS) consist of short DNA sequences (usually 1-6bp in length) that are tandemly repeated from two to thousand times (Stallings *et al.* 1991). The DNA sequences flanking the SSRs were found to be unique and such conserved sequences have been exploited to design suitable primers for amplification of the SSR loci using PCR. SSR polymorphism results from variation in the number of repeat units at a particular SSR locus. Variation in the number of repeat units is postulated to be due to unequal crossing over or slippage of DNA polymerase during replication of repeat tracts (Coggins and O'Prey, 1989). Microsatellites are considered useful for construction of high-density maps due to their high polymorphism level, co-dominant character, abundance, and wide distribution over the genome. It is technically simple as it relies on PCR technology; the technique is sensitive, since only a small quality of DNA is required. SSR markers are inherited in Mendelian fashion. In addition, SSR markers in some cases display good transferability from one species to another within the same genus (Rajora *et al.* 2001; Shepherd *et al.* 2002) and can be thus used as convenient anchor points in the construction of intra-specific and inter-specific consensus maps. The technology is also readily transferable since information can be communicated as simple sequences of primer pairs. The major limitation of the SSR marker technology, however, is the initial investment and the technical expertise to clone and sequence the loci. Nonetheless, the application of SSR marker technology in many plant species has dramatically increased over the years and continuing efforts are underway to design more primers based on available sequence information in the plant genome databases.

Single nucleotide polymorphisms (SNPs) are an alteration of a single nucleotide in a DNA sequence and can be detected and used as markers. Sequence variation consists of single-base differences or small insertions and deletions (indels) at specific nucleotide positions. Their frequent occurrence provides a large source of genetic markers that are more likely to be located close to target genes of interest. Sequence variants of SNPs are the markers of

choice for genotyping and mapping because of their abundance and amenability to high-throughput screening. In addition, SNPs can contribute directly to a phenotype or they can associate with a phenotype as a result of linkage disequilibrium (Daly *et al.* 2001; Kim *et al.* 2004; Thornsberry *et al.* 2001). Because of availability of high throughput detection systems, SNPs are suited for automation (Landegren *et al.* 1998). Many SNP methodologies have been described (Landegren *et al.* 1998). It may involve target sequence amplification and then distinction of DNA sequence variants by short hybridization probes or by restriction endonuclease. In combination with a PCR assay, the corresponding SNP can be analyzed as a cleaved amplified polymorphic sequence (CAPS) marker or as single-stranded conformation polymorphism (SSCP) technique.

Cleaved amplified polymorphic sequence (CAPS) markers have proven to be a powerful tool for molecular genetic analysis. CAPS markers rely on differences in restriction enzyme digestion patterns of PCR fragments caused by nucleotide polymorphism generating a simple type of data coded as heterozygote or homozygote (Konieczny and Ausubel, 1993; Michaels and Amasino, 1998). The costs of a CAPS assay is generally low, especially when it relies on commonly used restriction enzymes. It requires minimum amounts of genomic DNA and simple electrophoresis systems to reveal polymorphism; however, the only drawback is that sequence information is needed to tag the desired DNA fragments.

Diversity arrays technology (DArT) involves using microarrays that does not require sequence knowledge, and thus may become very useful for crop researchers. A single DArT assay simultaneously types hundreds to thousands of SNPs and insertion/deletion polymorphisms spread across the genome. It is sequence-independent and can be processed in a cost-effective and speedy manner of hundreds to thousands of individual samples by using a proper setup and software (Wenzl *et al.* 2004). DArT offers a rapid and DNA sequence-independent shortcut to medium-density genome scans of any plant species (Yang *et al.* 2006). Hence, since the whole genome was first profiled using DArT markers in barley, approximately 2.3 million data points for 4,000 lines have been generated for barley breeders and researchers (Wenzl *et al.* 2006) and it has been rapidly applied in many other crops such as sugarcane (Lakshmanan *et al.* 2005), wheat (Semagn *et al.* 2006), cassava (Xia *et al.* 2005), and pigeon pea (Yang *et al.* 2006).

Genetic mapping of tomato using restriction fragment length polymorphism (RFLP) was first published in 1986 (Bernatzky and Tanksley, 1986). Since then, more markers, mainly RFLP, were added onto the existing molecular linkage map. More than 1000 markers are

available for tomato covering 1,276 map units and their localizations on the molecular linkage maps correspond to both random genomic clones and cDNA clones (Tanksley *et al.* 1992). After that, simple sequence repeats in tomato genome were characterized and placed in this high-density map (Broun and Tanksley, 1996; Grandillo and Tanksley, 1996b; Suliman-Pollatschek *et al.* 2002, Frary *et al*. 2005) as well as SNP and AFLP (Haanstra et al.1999; Suliman-Pollatschek et al. 2002).

Two hundred-ninety RFLP markers have been utilized to construct a linkage map to identify markers associated with bacterial wilt resistance from an F_2 population derived from a cross between L286, a bacterial wilt susceptible cultivar and C285, a resistant wild tomato relative (*S. lycopersicum* var. *cerasiforme*) (Danesh *et al.* 1994). However, only 69 markers were polymorphic and useful for segregation analysis. Of the polymorphic RFLP markers analyzed, 59 markers mapped to 11 linkage groups on the tomato genetic map by using the software MAPMAKER II (Lander *et al*. 1987). A follow-up study was conducted using an F_2 population derived from a cross between a bacterial wilt susceptible line *S. pimpinellifolium*, West Virgina 700 (WVa700), and a highly resistant cultivar Hawaii 7996 (H7996). A genetic map with 60 RFLP markers constructed using the software JOINMAP and the Kosambi mapping function (Thoquet *et al.* 1996a). RFLP markers require appreciable amounts of relatively pure DNA, are time consuming, costly and technically demanding. Therefore, Balatero (2002) constructed a linkage map consisting of 80 markers, which included 70 AFLPs, 7 RGAs (resistant gene analogs), and 1 SSR based on a F_6 recombinant inbred line population derived from a cross of H7996 x WVa700.

The study presented here was conducted at AVRDC with the overall primary goal of improving the efficiency of breeding programs in tomato through the application of molecular markers and to broaden the genetic base of tomato for improvement of durable resistance to *Ralstonia solanacearum*. In particular, the study aimed to: 1) Construct a genetic linkage map of H7996 x WVa700 using F_9 recombinant inbred lines, and 2) use this map to identify DNA markers associated with resistance to bacterial wilt in H7996.

1.2 MATERIALS AND METHODS

1.2.1 Plant materials

A population of 188 F_9 recombinant inbred lines (RILs), series number: 1-200 (except RIL number 7, 19, 34, 61, 99, 110, 123, 133, 174, 180, 181, 190) derived from a cross between H7996 (*S. lycopersicum*, resistant*)* and WVa700 (*S. pimpinellifolium*, susceptible) (Thoquet *et al*. 1996a) provided by Bacteriology Unit, AVRDC-The World Vegetable Center (AVRDC), were used in this study. This cross was made in France and advanced upto F_3 using single seed descent (SSD) method (Tigchelaar and Casali, 1976). Seeds of F_3 lines were then sent to the Institute of Plant Breeding of the University of the Philippines, Los Banos for generation advance to produce the F_5 recombinant inbred lines. Generation advance of H7996 x WVa700 mapping population from F_6 to F_9 generation was made at AVRDC.

1.2.2 DNA preparation and quantification

1.2.2.1 DNA preparation

DNA of two single plants of each of all 188 F_9 RILs and the two parental lines were extracted using two methods as described by Diversity Arrays Technology (DArT P/L, Yarralumla, ACT 2600, Australia) (DArT method) and by Murray *et al*. (1980) and has been modified by Fulton *et al*. (1995) (Fulton method). In the Fulton method, a 50-100mg sample (approximately 4-8 new leaflets, up to 1.5cm long) of young leaf tissue was harvested and placed in a 1.5ml microcentrifuge tube. To each tube, 200µl of freshly prepared buffer (2.5 parts of extraction buffer (0.35M sorbitol, 0.1M Tris pH 7.5, 5mM EDTA) + 2.5 parts of lysisbuffer (0.2M Tris, 0.05M EDTA, 2M NaCl, 2% CTAB) + 1 part of sarcosyl (5%)) was added to the leaf tissue and was ground using plastic pestle with power drill. An additional 550µl of fresh microprep buffer was added, and the tube vortexed gently before the sample was incubated at 65^{o}C for 30-120 minutes. An equal volume of chloroform:isoamyl alcohol (24:1) was added and the content was mixed well by sandwiching the tubes between two racks and inverting 100 times. Samples were then centrifuged for 5 minutes at 10,000rpm. The upper aqueous phase was transferred into a 1.5ml-sterile microcentrifuge tube and precipitated by mixing 1 volume of supernatant with

0.75 volume of ice-cold isopropanol. The precipitated samples were centrifuged at 10,000rpm for 5 minutes and the supernatant was poured off, and approximately 50µl of 75% ethanol was then used to wash the pellet and left over night at -20^{o}C. After centrifugation, the supernatant was removed; the pellet was dried at room temperature for 1-2 days and dissolved in 50µl of sterile TE (Tris-EDTA) buffer.

In the DArT method, one young tomato leaflet was collected and cut into 5-6 pieces and put in a 1.5ml microcentrifuge tube and stored at -80^{o}C. The young tomato leaflet pieces were ground to fine powder with plastic pestle in liquid nitrogen, then 500µl of fresh working buffer (2.5 parts of extraction buffer (0.35M sorbitol, 0.1M Tris pH 7.5, 5mM EDTA) + 2.5 parts of lysisbuffer ((0.2M Tris, 0.05M EDTA, 2M NaCl, 2% CTAB) + 1 part of sarcosyl (5%) + 2% PVP)) was added and the content was mixed well after incubating at 65^{o}C for 5 minutes. The tubes were further incubated at 65^{o}C for 30 minutes. Five-hundred microliters of chloroform:isoamyl (24:1) was added, and the tubes were gently inverted to mix. Then, the tubes were centrifuged at 6,000rpm for 10 minutes. The supernatant was transferred to a new sterile centrifuge tube and a 0.8 volume of cold isopropanol was added to each tube of aqueous supernatant to precipitate DNA. The mixture was centrifuged for 15 minutes at 6000rpm, and the supernatant was discarded. A volume of 500µl of 70% ethanol was added, and then tubes again centrifuged at 6,000rpm for 30 minutes, and the ethanol discarded. The DNA was dried at room temperature for 1-2 days. The DNA precipitate was suspended in 50µl of sterile TE buffer by incubating at 65^{o}C for 20 minutes. RNA was eliminated through DNA incubation with 3.0µg/ml RNAse at 37^{o}C for 45 minutes.

1.2.2.2 DNA quantification

An agarose gel method was used to quantify and identify the quality of DNA samples. The concentration of genomic DNA was estimated by comparing the size and intensity of each sample band with those of sizing standard, DNA ladder. DNA was diluted into two different ratios for each of the two methods: 1:50 for the Fulton method and 1:10 for the DArT method. Seven microliters of diluted DNA from the DArT method and 14µl of diluted DNA from the Fulton method were run on a 1% agarose gel in 1% TAE (Tris-Acetate-EDTA) buffer at 50V for 2.5 hours. The gel was stained with ethidium bromide for 10 minutes then washed in sterile distilled water for 15 minutes and then visualized under UV light for DNA detection. A 1kb ladder (Bertec Enterprise, Taiwan) was used as a standard for calculation of DNA concentration. The presence of DNA was recorded when a band appeared on the

gel; and the concentration of DNA was calculated by comparing band intensity and amounts of ladder run on the same gel. The final dilution was done by adjusting concentration to 100ng/µl for the DArT method and 5-10ng/µl for the Fulton method.

1.2.3 DNA marker analysis

1.2.3.1 AFLP analysis

AFLP analysis was performed using a slightly modified procedure of Vos *et al*. (1995). The restriction enzymes, adaptors and primers used in AFLP analysis are listed in Table 1.1 (Balatero, 2000). The following describes the detailed procedure of the AFLP analysis.

Restriction digestion

From each sample, 250ng/15µl of genomic DNA was digested with 15µl cocktail including 10µl sterile MilliQ water, 3.0µl 10X buffer 2 (NEB) (500mM NaCl, 100mM Tris-HCl, 100mM $MgCl_2$, 10mM dithiothreitol, pH 7.9), 0.8µl *Eco*RI (20U/µl) (New England Biolabs) (NEB) and 1.2µl *Mse*I (10U/µl) (NEB) at 37^{o}C for two hours by using a MJ PT-200 thermocycler (MJ Research, GMI, Inc., Minnesota, USA). Then, 5µl of digestion product was loaded on 1% agarose gel and 1kb ladder (Bertec Enterprise, Taiwan) was used as molecular weight marker. Digestion products would display a smear of about 100-1000bp indicating that digestion was completed. The mixture was incubated for 15 minutes at 70^{o}C to inactivate the restriction enzymes.

Ligation of adapter sequence

Twenty microliters of digestion were ligated with 1.0µl *Eco*RI adaptor (5pmol/µl) (NEB), 1.0µl *Mse*I adaptor (50pmol/µl) (NEB), 1.0µl 10X Ligase buffer (500mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 100mM DTT, 10mM ATP) (NEB), 6.6µl sterile MilliQ water, and 0.4µl T4 DNA ligase (400U/µl) (NEB). The ligated mixture was gently mixed, centrifuged briefly and incubated at 16^{o}C overnight using the MJ PT-200 thermocycler (MJ Research, GMI, Inc., Minnesota, USA). The ligations were then performed a 1:10 dilution using sterile MilliQ water. Diluted and undiluted ligation mixtures were stored at -20^{o}C.

Table 1.1 List of adaptors and primers used for AFLP analysis

Name	Enzyme	Type	Sequence (5'-3')
EcoRIadpI	*Eco*RI	Adaptor	CTGGTAGACTGCGTACC
EcoRIadpII	*Eco*RI	Adaptor	CTGACGCATGGTTAA
MseIadpI	*Mse*I	Adaptor	GACGATGAGTCCTGAG
MseIadpII	*Mse*I	Adaptor	TACTCAGGACTCAT
E-A	*Eco*RI	Primer + 1	AGACTGCGTACCAATTCA
E-AAC (E1)	*Eco*RI	Primer + 3	AGACTGCGTACCAATTCAAC
E-AAG (E2)	*Eco*RI	Primer + 3	AGACTGCGTACCAATTCAAG
E-ACA (E3)	*Eco*RI	Primer + 3	AGACTGCGTACCAATTCACA
E-ACC (E4)	*Eco*RI	Primer + 3	AGACTGCGTACCAATTCACC
E-ACG (E5)	*Eco*RI	Primer + 3	AGACTGCGTACCAATTCACG
E-AGG (E8)	*Eco*RI	Primer + 3	AGACTGCGTACCAATTCAGG
M-C	*Mse*I	Primer + 1	GATGAGTCCTGAGTAAC
M-CAA (M1)	*Mse*I	Primer + 3	GATGAGTCCTGAGTAACAA
M-CAC (M2)	*Mse*I	Primer + 3	GATGAGTCCTGAGTAACAC
M-CAG (M3)	*Mse*I	Primer + 3	GATGAGTCCTGAGTAACAG
M-CAT (M4)	*Mse*I	Primer + 3	GATGAGTCCTGAGTAACAT
M-CTA (M5)	*Mse*I	Primer + 3	GATGAGTCCTGAGTAACTA
M-CTC (M6)	*Mse*I	Primer + 3	GATGAGTCCTGAGTAACTC
M-CTG (M7)	*Mse*I	Primer + 3	GATGAGTCCTGAGTAACTG
M-CTT (M8)	*Mse*I	Primer + 3	GATGAGTCCTGAGTAACTT

Pre-amplification

A pre-amplification was carried out to amplify the ligated DNA fragments. The first PCR (pre-amplification) was performed in a 96-well micro-titer plate. Each reaction consisted of 0.6µl E primer (10µM), 0.6µl M primer (10µM), 1.6µl dNTPs (2.5mM) (PROtech), 2µl 10X PCR buffer with 15mM Mg^{2+} (Violet, Taiwan), 10µl sterile MilliQ water, 0.2µl Tag DNA polymerase (2U) (Violet, Taiwan) with 5µl of diluted ligation. The components were mixed gently, centrifuged briefly and then covered with a PCR plastic plate cover. The PCR program was 28 cycles of 30 seconds at 94°C (denaturation), 30 seconds at 56°C (annealing), and 1 minute at 72°C (extension) using a MJ PT-200 thermocycler (MJ Research, GMI, Inc., Minnesota, USA). The quality and quantity of pre-amplification products were checked by running 10µl of each pre-amplified product in 1.0% agarose and using 1kb ladder (Bertec Enterprise, Taiwan) as molecular weight marker. The product that looked like a smear lying within 50bp to 500bp would indicate successful pre-amplification. Pre-amplification solutions were diluted with a 1:25 dilution by taking 5µl

of the pre-amplification DNA mixture into a 96-well micro-titer plate and adding 120µl sterile MilliQ water. This was used as template DNA for selective AFLP amplification. Unused portion of the pre-amplification template mixture was stored at –20^{o}C for long-term use.

Selective amplification

Selective amplification was also performed in a 96-well micro-titer plate. Three selective nucleotides (+3) on the *Mse*I primer combined with three selective nucleotides (+3) on the *Eco*RI primer were selected for use. The sequences of the primers used are shown in Table 1.1. Twenty microliter mix contained 9.1µl sterile MilliQ water, 1.0µl E primer (E-ANN) (5µM), 1.2µl M primer (E-CNN) (5µM), 1.6µl dNTPs (2.5mM) (PROtech, Taiwan), 2µl 10X PCR buffer with 15Mm Mg^{2+} (Violet, Taiwan), and 0.1µl Tag DNA polymerase (2U) (Violet, Taiwan) with 5µl template DNA. After covering the 96-well micro-titer plate by the plastic cover, the samples were amplified following one cycle of 94^{o}C for 30 seconds, 65^{o}C for 30 seconds and 72^{o}C for 1 minute; 12 cycles of subsequently lowering the annealing temperature (65^{o}C) by 0.7^{o}C per cycle while keeping 94^{o}C for 30 seconds and 72^{o}C for 1 minute; twenty-eight cycles of 94^{o}C for 30 seconds, 56^{o}C for 30 seconds, and 72^{o}C for 1 minute and soak at 10^{o}C using a MJ PT-200 thermocycler (MJ Research, GMI, Inc., Minnesota, USA).

Detection of amplified bands using silver staining

Following amplification, reaction products were mixed with 10µl tracking dye (95% formamide, 5M NaOH, bromophenol blue, xylene cyanol FF) then denatured at 94^{o}C for 4 minutes. PCR products were electrophoresed in a 6% denaturing polyacrylamide gel (19:1 acrylamide-bisacrylamide, 7.5M urea) in 0.5X TBE buffer (25mM Tris, 25mM boric acid, 0.5mM EDTA, pH 8.0) using Aluminum Backed Sequencing system (Model #: S3S from Owl Scientific, Inc). Electrophoresis was performed at constant power of 75W for 3.5 hours including 1 hour pre-run to warm the gel to 45-50°C. Each gel included a lane of the low molecular weight DNA ladder (NEB). Following electrophoresis, DNA bands were visualized based on a silver staining procedure developed by Promega (Madison, Wisconsin). Gels were fixed in 10% glacial acetic acid solution tray for 20-30 minutes (until the tracking dyes were no longer visible). The gels were then washed three times for 2 minutes using distilled water and transferred to a staining solution tray consisted of 0.1% (w/v) silver nitrate and 0.15% (v/v) of 37% formaldehyde solution for 30 minutes. The

gels were dipped briefly into the tray containing distilled water, drained and placed immediately into a tray of chilled developing solution consisting of 3% (w/v) sodium carbonate, 0.15% (v/v) of 37% formaldehyde and 0.02% sodium thiosulfate (400µl of 10mg/ml per 2 liters of solution) for an additional 2-3 minutes or until all bands became visible. The time taken to dip the gels in distilled water and transfer into developing solution was no longer than 5-10 seconds. The developing reaction was terminated, fixed (10% glacial acetic acid) and gels then washed twice with distilled water. All steps above were done with constant shaking, and the volumes of the solutions used in each step were typically two litters. Gels were air-dried at least overnight and then scored. After scoring, the gels were scanned to document the image.

Scoring AFLP markers

AFLP is dominant type of marker, thus scoring is based on the presence (+) or absence (-) of band. Once the gels were dried, they were scored manually for the presence or absence of polymorphic bands across genotypes and individual scores were converted to either "1" (band present) or "0" (band absent). In cases where bands were not clear to score, they were treated automatically as missing data.

1.2.3.2 Microsatellite or SSR analysis

Eighty-five SSRs selected based on Tomato-EXPEN 2000 map (http://www.sgn.cornell.edu) and four unmapped SSRs (Smulders *et al.* 1997) were surveyed for polymorphism using the two parental lines, H7996 and WVa700 on 1% agarose gels. All but one unmapped SSR (SSR3) showed polymorphism. Hence, SSR markers were re-run on 5% polyacrylamide gel and 24 more SSR markers showed polymorphism between the two parents. Twenty five SSR markers were then mapped on the 188 F_9 RILs. Primer sequences and repeat motifs for polymorphic SSR markers are listed in Table 1.2. Each PCR reaction (25µl final volume) contained 15-20ng of genomic DNA, 10X PCR buffer (10mM Tris-HCl, pH 9.0; 50mM KCl; 15mM MgCl2), 20mM dNTPs, and 20µM of each forward and reverse primer and 2U of *Taq* DNA polymerase (Violet, Taiwan). PCR reactions were performed in a MJ PT-200 thermocycler (MJ Research, GMI, Inc., Minnesota, USA). The amplification profile consisted of an initial denaturation for 5 minutes at 94°C followed by 35 cycles of 30 seconds at 94°C, 45 seconds at the annealing temperature 50–60°C (depending on the Tm of the primers), 45 seconds elongation at 72°C, and a final extension step of 7 minutes at 72°C.

Table 1.2 List of polymorphic SSR primers used for mapping population

No.	Primer code	Marker code	Chromosome	Forward primer	Reverse primer	Annealing Tm. (^{o}C)
1	01-138.0	s01138.0	1	AATTCACCTTTCTTCCGTCG	GCCCTCGAATCTGGTAGCTT	50
2	02-022.0	s02022.0	2	TGCAGGTATGTCTCACACCA	TTGCAAGAACACCTCCCTTT	50
3	02-036.6	s02036.6	2	GGGTTATCAATGATGCAATGG	CCTTTATGTCAGCCGGTGTT	50
4	03-074.1	s03074.1	3	TGCCAATCCACTCAGACAAA	TGGATTCACCAAGGCTTCTT	50
5	03-099.0	s03099.0	3	GATCGGCAGTAGGTGCTCTC	CAAGAAACACCCATATCCGC	50
6	04-015.0	s04015.0	4	TGGCATGAACAACAACCAAT	AGGAAGTTGCATTAGGCCAT	55
7	04-037.0	s04037.0	4	GAAGGGACAATTCACAGAGTTTG	CCTTCAACTTCACCACCACC	50
8	04-054.5	s04054.5	4	AATGAAGAACCATTCCGCAC	ACATGAGCCCAATGAACCTC	55
9	04-056.0	s04056.0	4	ACATGAGCCCAATGAACCTC	AACCATTCCGCACGTACATA	55
10	04-058.0	s04058.0	4	GCGATGAGGATGACATTGAG	TTTACAGGCTGTCGCTTCCT	60
11	04-058.1	s04058.1	4	TGTTGGTTGGAGAAACTCCC	AGGCATTTAAACCAATAGGTAGC	60
12	06-006.1	s06006.1	6	TCCTCAAGAAATGAAGCTCTGA	CCTTGGAGATAACAACCACAA	50
13	06-099.8	s06099.8	6	GGAATAACCTCTAACTGCGGG	CGATGCCTTCATTTGGACTT	55
14	07-002.0	s07002.0	7	AGTGGCTCTCACCTACTGCG	CAATTCTCAGGCATGAAACG	55
15	08-001.0	s08001.0	8	TGTTGCTCGAACTCTCCAAA	CATAGGAGAGGTAACCCGCA	60
16	08-055.0	s08055.0	8	GTTTCTATAGCTGAAACTCAACCTG	GGGTTCATCAAATCTACCATCA	50
17	08-055.1	s08055.1	8	TTCGTTGAAGAAGATGATGGTC	CAAAGAGAACAAGCATCCAAGA	55
18	09-051.0	s09051.0	9	CCGTTACCTTGGTCCATCAC	GGGAGATGCCACATCACATA	50
19	09-058.0	s09058.0	9	ATTGTACAAAGACCCGTGGC	GTTGCACACTGGATCAATGC	55
20	10-033.1	s10033.1	10	AGGGTCCTTCGTTTGGAACT	GCATTCCACTTGTGAAGCAT	60
21	10-033.2	s10033.2	10	TTTCCACCTCAAACCACTCC	CCCTTTGACCTGTGCCA	55
22	10-034.5	s10034.5	10	GCAGAGGATATTGCATTCGC	CAAACCGAACTCATCAAGGG	55
23	10-075.0	s10075.0	10	TGGCTGCCTCTTCTCTGTTT	TTTCTTGAAGGGTCTTTCCC	55
24	11-040.0	s11040.0	11	CCGAGGCGAATCTTGAATAC	GCACCATCTCTTGTGCCTCT	50
25	SSR3	SSR3	unmapped	CTCGTCTTTAGGTATCAATGGAGAT	TCAATGCTACTCAATGGCTCA	50

The reaction products were denatured by heating for 4 minutes at 84°C with 1/3 volume of tracking dye (98% formamide, 10mM of EDTA, 0.25% each of bromphenol blue and xylene cyanol), then run on a polyacrylamide gel electrophoresis system. Condition of electrophoresis and staining were similar to the one used for AFLP analysis. Each gel included lanes of 25bp molecular size marker (Promega, Madison, WI). SSRs are co-dominant markers; hence, residual heterozygosity in the F_9 RILs can be detected. Symbols H (for H7996) and W (for WVa700) were used to score the entire RIL population.

1.2.3.3 SNP analysis

Eleven SNP markers selected from Tomato Mapping Resource Database (http://www.tomatomap.net/) were screened in the H7996 and WVa700 (Table 1.3). PCR amplification reactions were prepared in a total volume of 25µl containing 10X PCR buffer (100mM Tris-HCl, pH 9.0; 500mM KCl; 15mM MgCl2), 20mM dNTPs, 20µM of each forward and reverse primer and 2U of *Taq* DNA polymerase (Violet, Taiwan), and 20ng genomic DNA as template for PCR. The amplification procedure consisted of an initial denaturation for 5 minutes at 94°C and 35 cycles of 30 seconds denaturation at 94ºC, 1 minute primer annealing at 50ºC or 55ºC depends on primers used, 2 minutes extension at 72ºC, followed by a final extension at 72ºC for 5 minutes. After amplification, 5µl of PCR product was digested in a 10µl cocktail including 7.3µl sterile ddH_2O, 2.5µl 10X buffer 2 (NEB) (500mM NaCl, 100mM Tris-HCl, 100mM $MgCl_2$, 10mM dithiothreitol, pH 7.9), 0.2µl restriction enzyme (20U/µl) (NEB) by using a MJ PT-200 thermocycler (MJ Research, GMI, Inc., Minnesota, USA). The digested products (15µl) were separated in 1% agarose gels and 1X TBE buffer (10.8g trizma base, 5.48g boric acid, and 4ml EDTA (0.5mM)/1l of distilled water) for 1.5 to 2 hours at 96V. A 100bp ladder was used as molecular weight marker. After electrophoresis, gels were stained with ethidium bromide (1.5µg/ml) for 10 minutes, de-stained in distilled water for 15 minutes and photographed under UV light. Scoring was similar to SSR analysis.

Table 1.3 List of SNP primers used for screening of the parents

No.	Primer code	Chromosome	Forward primer/Reserve primer	Restriction enzyme
1	LEOH8.1	9	TCAAATCACAAAATTAACCTATTCTTT GACCATTTTCCTAACTCTTCAGG	
2	LEOH10	4	TGCCAGATTGACTGTGAAGG GGAACCCTGCATTGTTCTTG	BsaJ I
3	LEOH16.1	5	TGAATTTTCTGTCATCGTTGG TTTCGGAATCTTTGTTGAATTG	
4	LEOH16.2	5	TCGACGCTGCACAGAAATAC TTCCTCCTCCTTATCTCCTTCA	BsaW I
5	LEOH17.1	Multiple	CAGACGAGAAGCAAGTTGAGG CTACCACTGCGTGCTTTGAC	
6	LEOH19	12	AAGGCTCAGAAAGGGTCCAT TGAGTTCATCAACACATCACACA	BsaB I
7	LEOH23.1	2	GAGAGAAAAAGGGCACAAGG ACCGACAAACGCATAGATCA	Msp I
8	LEOH31.3	9	TTGCAATGGCTTCTCTCCTC ACTTGTCCGTTTCTCGCTTG	Msp I/ Mse I
9	LEOH36	1	TCACAAAAATGGCGATGAGA CCACCTGTGGATCCTTGACT	Bcl I
10	LEOH37	4	TTGATATATTCCATGTGTGTCTC AACTACAAATTAACAAACTTAAATGG	NmuC I
11	LEOH40.1	7	TGAGTTGGTGAACCATGGAA CCAAAGTTGGGACCTTTTGA	NmuC I

1.2.3.4 Providing of DArT and RFLP markers

The F_8 RFLP marker genotype data were kindly provided by Dr. Pascale Besse, CIRAD, a collaborator of Dr. Jaw-Fen Wang, AVRDC.

DArT marker data were provided by AVRDC. The data were produced by Diversity Arrays Technology Pty Limited, Canberra, Australia, under a contract with AVRDC.

1.2.3.5 Marker codes

Each AFLP marker was assigned a three-part name consisting of 3 letters as "afh" and the primer combination number followed by the letter. Each SSR marker was numbered of chromosome followed by its position (cM) on the chromosome. DArT markers were named following by capital "D" and number of each clone on the 96-well plates.

1.2.3.6 Linkage analysis

The markers were coded as follows: an individual homozygous F_9 RIL like resistant parent H7996 = 'H', susceptible parent WVa700 = 'W' and missing data = '-'. The genotyping scores of 188 RILs were analyzed using the MultiPoint mapping software package (http://www.multiqtl.com). The approach of multilocus ordering implemented in MultiPoint employs evolutionary algorithms of discrete optimization, which uses the minimization of the total map length as the mapping criterion (Mester *et al*. 2003, 2004). The population type "RIL-selfing" was used and the initial clustering of all markers into 37 linkage groups was based on a preset threshold recombination rate (RR) of 0.27. Initial linkage groups could be further merged into 12 linkage groups/chromosomes where markers were reordered. Map distances were calculated using the Kosambi mapping function, which assumes positive interference between crossovers. Linkage groups then were compared with an evaluation version of Joinmap 4.0 (Van Ooijen and Voorrips, 2006). With JoinMap 4.0, the regression mapping algorithm and Kosambi cM units were used for genetic linkage analysis.

1.3 RESULTS

1.3.1 Polymorphism screening between H7996 and WVa700

The polymorphism screening between H7996 and WVa700 is summarized in Table 1.4. A total of 121 primers of AFLP, SNP and SSR was screened, and amplified with 1008 bands. A total of 913 distinct bands were yielded from 21 *Eco*RI/*Mse*I selective primers. Out of 913 AFLP bands, 76 bands showed polymorphism. For SNP, 12 bands were generated from 11 primers. Of the twelve SNP bands, only one showed polymorphism and used for screening the F_9 RILs. Whereas, 83 bands were generated from 89 SSR primers. Twenty five SSRs out of the 83 amplified loci were polymorphic between H7996 and WVa700. In general, the rate of polymorphism was relatively low—8.3% for AFLP and SNP markers; however, polymorphic percentage was higher for SSR marker with 30.1%.

Table 1.4 Summary of polymorphism screened between the parental lines H7996 and WVa700 using AFLP, SNP, and SSR markers

Type of marker	No. of marker used	Total no. of band	No. of polymorphic bands	Percent polymorphism
AFLP	21	913	76	8.3
SNP	11	12	1	8.3
SSR	89	83	25	30.1
Total	**121**	**1008**	**102**	

The number of visible AFLP bands produced per primer combination ranged between 24 and 61 bands with a mean of 43.5 bands (Appendix Table 1.1). The polymorphic bands ranged from 1 to 9 with a mean of 3.7 polymorphic bands per primer combination. The highest percentage of polymorphism was obtained from primer combination E4&M5 with 20.9% and lowest was from E2&M8 with 1.7%. The siz of each polymorphic AFLP marker was estimated based on the mobility of low molecular weight DNA ladder (effective size range: 25 bp to 766 bp) (Appendix Table 1.2). The AFLP fragments amplified ranged in size from 81bp to 487bp. The number of polymorphic bands coming from H7996 was 40, whereas the remaining 36 bands came from WVa700.

Out of 11 SNP markers, eight SNP markers were digested with restriction enzymes after amplification and product gained from primer LEOH31.3 showed different product sizes

when different restriction enzymes used (Appendix Table 1.3). The size of amplified and digested products ranged from 180bp to 1200bp. Out of 11 primers, only one showed polymorphism between H7996 and WVa700. The size of the parental line H7996 was 1000bp, whereas 1200bp was for WVa700.

Of the eighty-nine SSR primers screened for polymorphism between the two parental lines, 83 primers gave amplification products. Out of 83, only one primer showed polymorphism on 1% agarose. Then amplification products were electrophoresised on 5% polyacrilamide gel and resulted 24 more primers revealed polymorphism (Figure 1.1). Using the 25bp marker (Promega), the molecular weight of each polymorphic marker was estimated based on the mobility of each band (Appendix Table 1.4). The size of amplified product ranged from 95bp to 454bp, meanwhile, products of polymorphic primers ranged from 95bp to 402bp. The size difference between the two parental lines was from 1bp to 42bp.

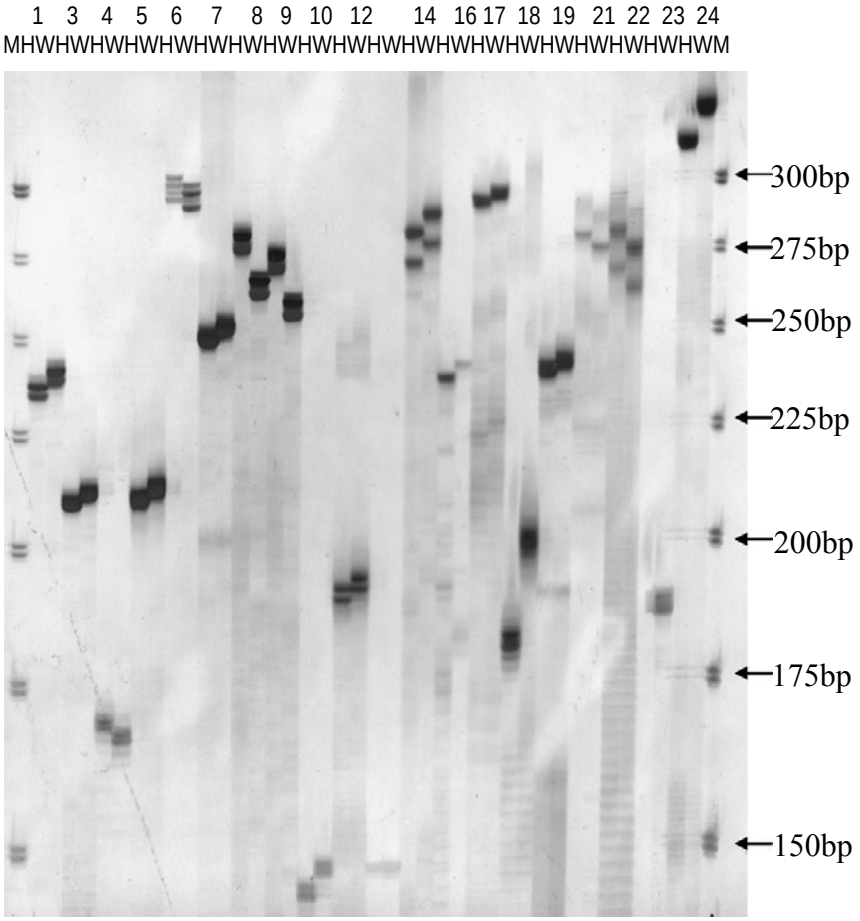


Figure 1.1 Polymorphic SSR primers screening between the resistant (H7996) and the susceptible parents (WVa700). Lanes H = H7996; W = WVa700; M = 25bp marker; 1, 2, 3, etc. = polymorphic SSR primers.

1.3.2 Segregation analysis of polymorphic markers

The twenty-one AFLP primer combinations, 25 SSR primers and 1 SNP primer were selected to screen on the mapping population. Generally, AFLP markers are scored as dominant marker, the band is either presence or absence as illustrated in Figure 1.2. Unlike AFLP, SSR and SNP markers are co-dominant marker. Segregation of polymorphic SNP and SSR markers is shown in Figure 1.3.

A total of 256 markers (60.8%) showed deviations from the expected segregation ratio of 1:1 for resistance:susceptible F_9 RILs (Table 1.5). Forty-nine of the 76 AFLP markers (64.5%) deviated from the expected segregation ratio. For SSR, of the 25 polymorphic markers, 9 (36.0%) deviated significantly from the expected 1:1 Mendelian segregation ratio. One SNP marker also deviated from the expected segregation ratio (100%). Out of 313 DArT and 6 RFLP markers, about 62.0% of DArT markers (Appendix table 1.5) and 50.0% for RFLP markers (Appendix table 1.6) deviated from expected segregation ratio.

Table 1.5 Summary of Chi-Square Goodness-of-Fit for 1:1 Mendelian segregation of markers used for construction of genetic linkage map

Marker type	Total number of markers	Goodness of fit	
		Fitted (P>0.05)	Distorted (P<0.05)
AFLP	76	27	49
SSR	25	16	9
SNP	1	0	1
DArT	313	119	194
RFLP	6	3	3
Total	**421**	**165**	**256**

Critical χ^2 values for 1 degree of freedom: 3.841 (P=0.05) and 6.635 (P=0.01);
P: Probability value.

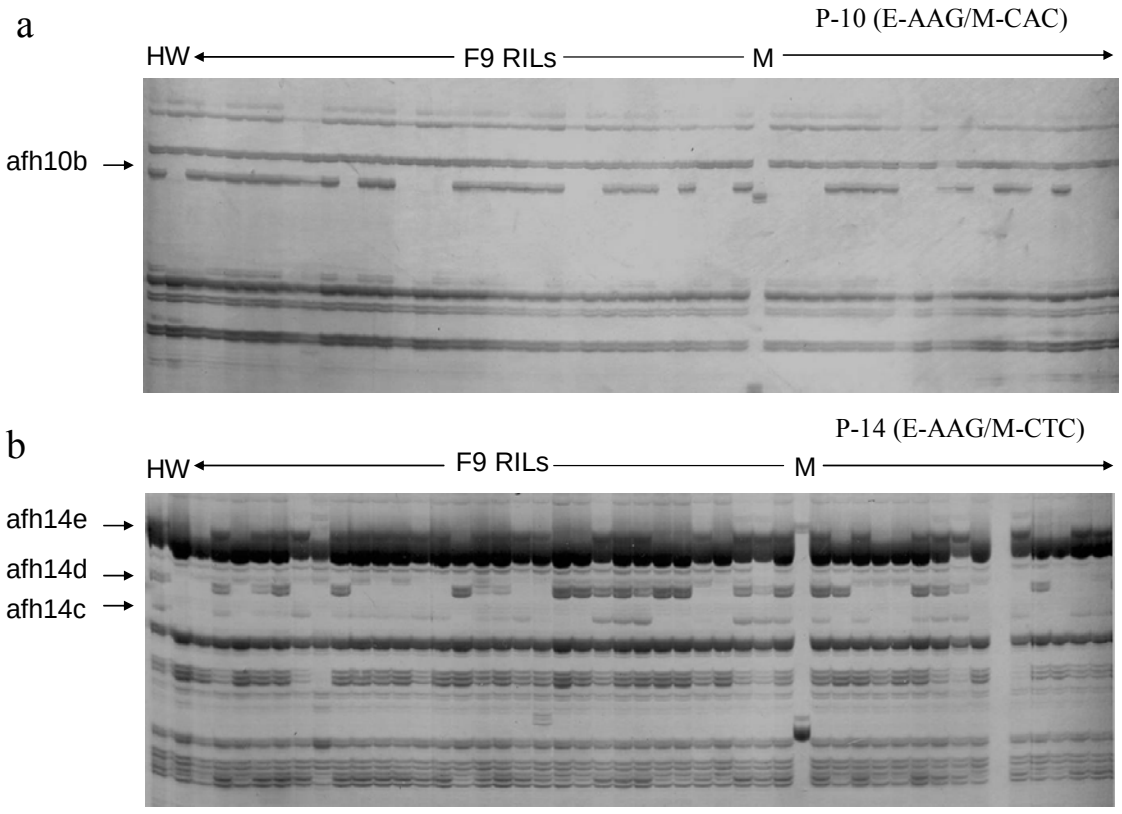
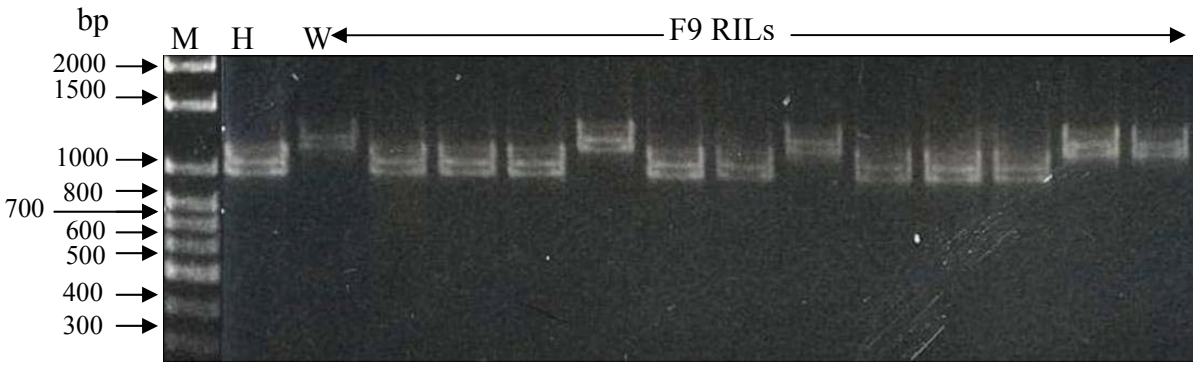
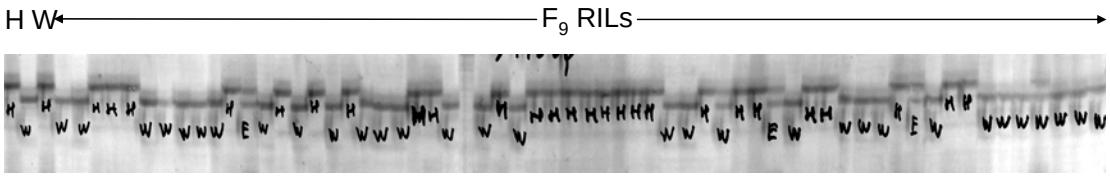


Figure 1.2 Segregation of AFLP markers using different *Eco*RI/*Mse*I primer combinations. a) an AFLP dominant type of markers from E-AAG/M-CAC; b) multiple AFLP markers (loci) in a single gel from E-AAG/M-CTC. Lanes H = H7996; W = WVa700; M = Low molecular weight marker (Promega).

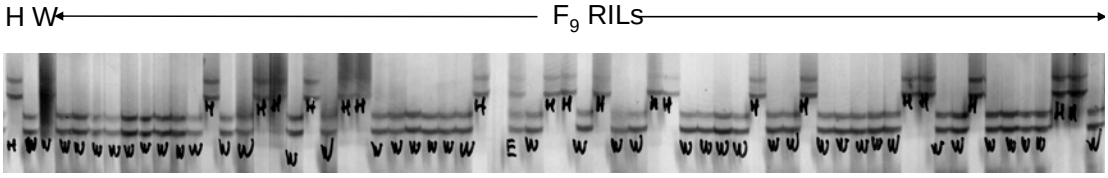
a) SNP marker



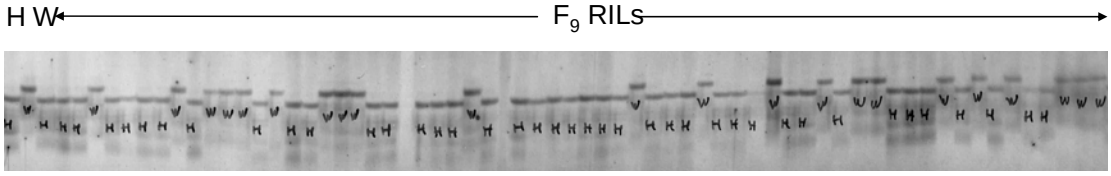
b) SSR markers



Primer code 03-074.1



Primer code 04-054.5



Primer code 06-006.1

Figure 1.3 Segregation of a) SNP primer LOH36 digested with enzyme Bcl I, and b) SSR primers 03-074.1, 04-054.5 and 04-045.5 in the F_9 RILs. Lanes H = H7996; W = WVa700; M = 100bp marker (Promega).

1.3.3 Genetic linkage map of H7996 x WVa700

A total of 421 markers, including 76 AFLP, 25 SSR, 1 SNP, 313 DArT and 6 RFLP markers were mapped into 37 linkage groups at a recombination rate (RR) of 0.27, each with 1-53 loci. This RR was chosen is that anchor markers of each chromosome were in one linkage group, excepted some anchor markers were not linked and located in one group itself; e.g. with RR = 0.25, some anchor markers in the same chromosome were located in different linkage groups; however, with RR = 0.3, some anchor markers belonging to different chromosomes were merged into one linkage group; and there were non-significant differences between RR of 0.25 and 0.26, 0.28 and 0.27, and 0.29 and 0.3.

In addition, an evaluation version of JOINMAP 4.0 (JM) was used to compare grouping of markers to confirm the marker localizations from MultiPoint (MP). Based on anchor markers and grouping information from JM and MP itself, the final mapping was performed by merging two or more linkage groups that belong to the same chromosome, e.g. five linkage groups for chromosome 1; two linkage groups for chromosome 4; two linkage groups for chromosome 3; two linkage groups for chromosome 7; three linkage groups for chromosome 8; two linkage groups for chromosome 9; three linkage groups for linkage group A (LGA), and two linkage groups for linkage group B (LGB). Number of markers in linkage groups belonging to chromosome 2, 6, 10, and 11 in MP were similar to JM at LOD of 8, 5, 8 and 10, respectively.

Thus, out of 421 markers, 362 markers including 74 AFLP, 260 DArT, 5 RFLP, 1 SNP, and 22 SSR markers from 25 linkage groups were split into ten major and two minor linkage groups with a total length of the linkage map of 2131.7 cM. Each major group contained at least 1 anchor marker to assign it to one of the ten tomato chromosomes. The ten major groups could be assigned to ten tomato chromosomes, while the minor linkage groups could be considered as chromosome 5 and 12. A total of 59 non-informative loci (14%) belonged to 12 remained linkage groups were excluded from mapping for the following reasons: (i) they did not meet the threshold of the selected recombination rate from MP or LOD from JM; (ii) big gap would be made when they merged with the selected linkage groups, and hence, the map length could be contributed negatively.

The distribution of markers between linkage groups was unequal (Table 1.6). Most of the AFLP markers were mainly distributed on chromosome 3, 4, 6 and 12; whereas, DArT markers were most frequent in chromosome 4 and followed by chromosome 11, 2, 1, 9, 3,

and 4. All RFLP markers used were located on chromosome 6, excluded marker TG564F8 was merged into LGA. SSR markers distributed into 10 assigned chromosomes. The number of markers positioned on chromosome 11 was numerousest and followed by chromosome 7 and 4. Chromosome 2 had the highest marker density (2.7 cM/interval), while LGA had the lowest (10.4 cM/interval). The number of markers per linkage group ranged from 10 to 53; the length of the linkage group ranged from 48.6 to 298 cM. Clear clustering of markers was observed in the genetic linkage map and co-segregating markers were presented in all 12 linkage groups (Figure 1.4). Most of co-segregating DArT markers located on chromosome 1 and 7 and followed by chromosome 2, 4 and 11.

Table 1.6 Comparison of the genetic length and numbers of AFLP, DArT, RFLP, SNP, SSR markers mapped per linkage group of the RIL mapping population

Chromosome/ linkage group	cM	AFLP	DArT	RFLP	SNP	SSR	Number of markers	Marker density (cM/interval)
1	261.6	2	27		1	1	31	8.4
2	91.1		32			2	34	2.7
3	278.1	10	22			1	33	8.4
4	209.8	15	22			7	44	4.8
6	263.3	15	9	4		1	29	9.1
7	172.4	3	46			1	50	3.4
8	174.4	3	15			3	21	8.3
9	131.6	1	24			2	27	4.9
10	88.4	5	11			3	19	4.7
11	298.0	19	33			1	53	5.6
LGA	114.4	1	9	1			11	10.4
LGB	48.6		10				10	4.9
Total	**2131.7**	**74**	**260**	**5**	**1**	**22**	**362**	**5.9**

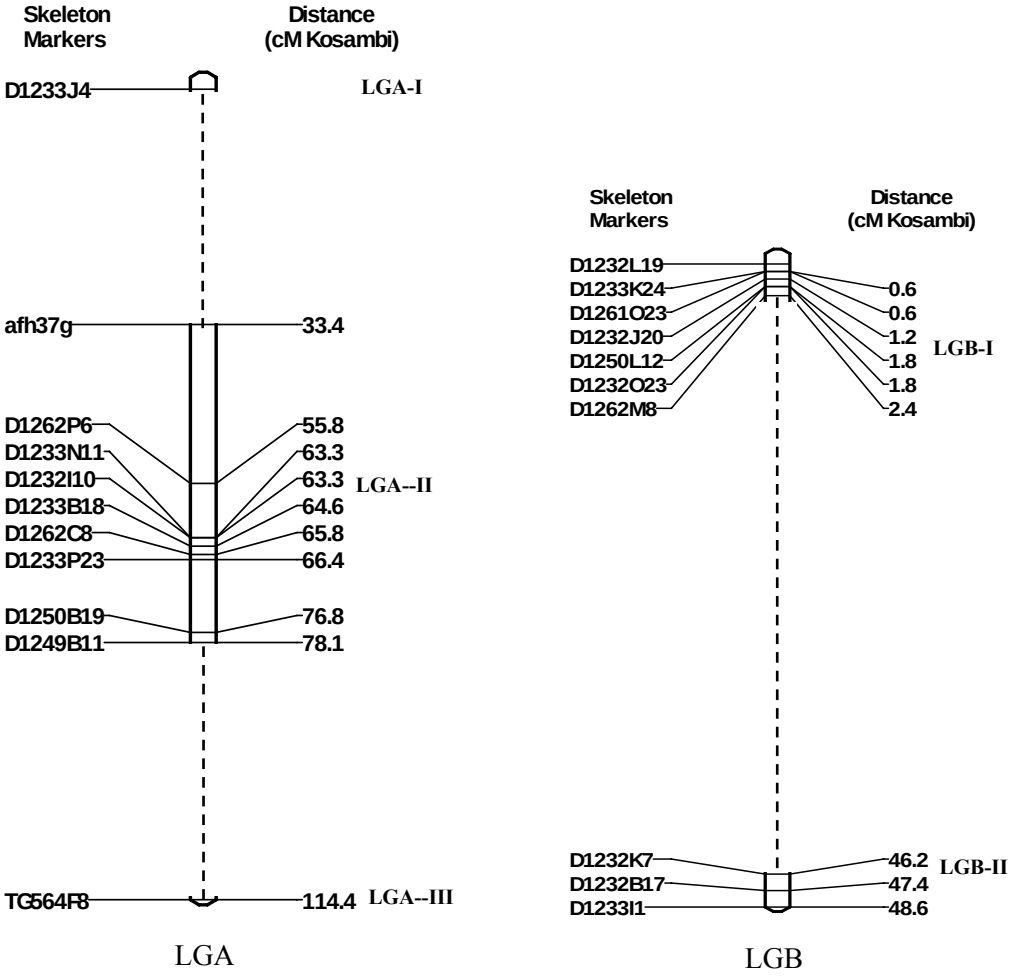


Figure 1.4 Genetic linkage map of H7996 x WVa700. The names of markers (termed "skeleton markers") are listed on the left and distances (cM, Kosambi mapping function) are listed in the right. The dashed lines are connections between linkage groups suggested by MultiPoint of the nearest clusters (i.e. C1-III closed to C1-IV; C3-I closed to C3-II; LGA-I closed to LGA-II, LGA-II closed to LGA-III) or by Joinmap 4.0 (i.e. markers in C1-I and C1-II were in one group of 5.0/5(9); C1-IV and C1-V: 6.0/4(20); C4-I and C4-II: 7.0/4 (39); C7-I and C7-II: 7.0/2 (50); C8-I, C8-II and C8-II: 4.0/3 (21); C9-I and C9-II: 7.0/6 (27); LGB-I and LGB-II: 3.0/3 (10) or based on anchor markers (i.e. anchor marker LEOH36 in C1-II and s0138.0 in C1-V).

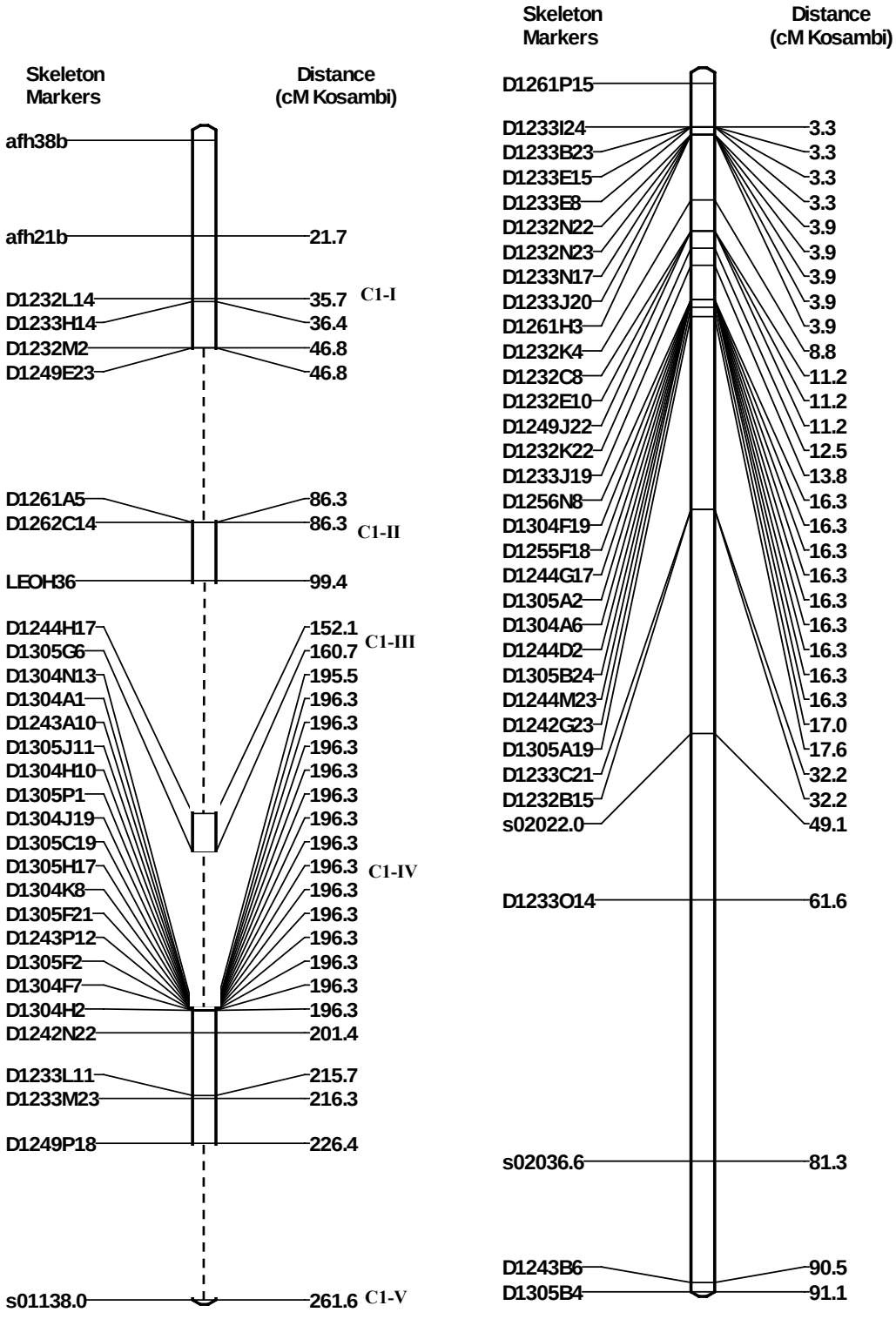


Figure 1.4 continued

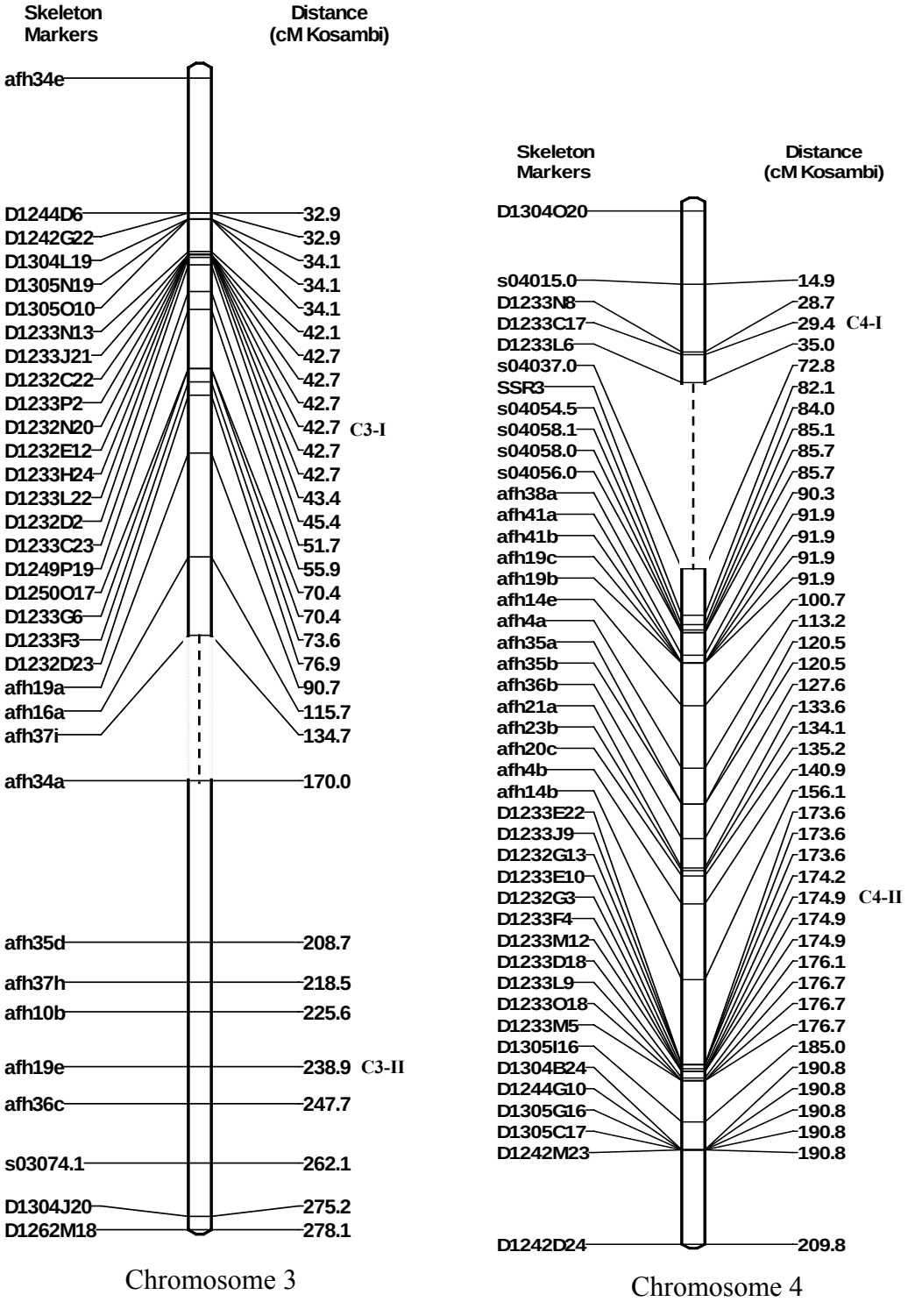


Figure 1.4 continued

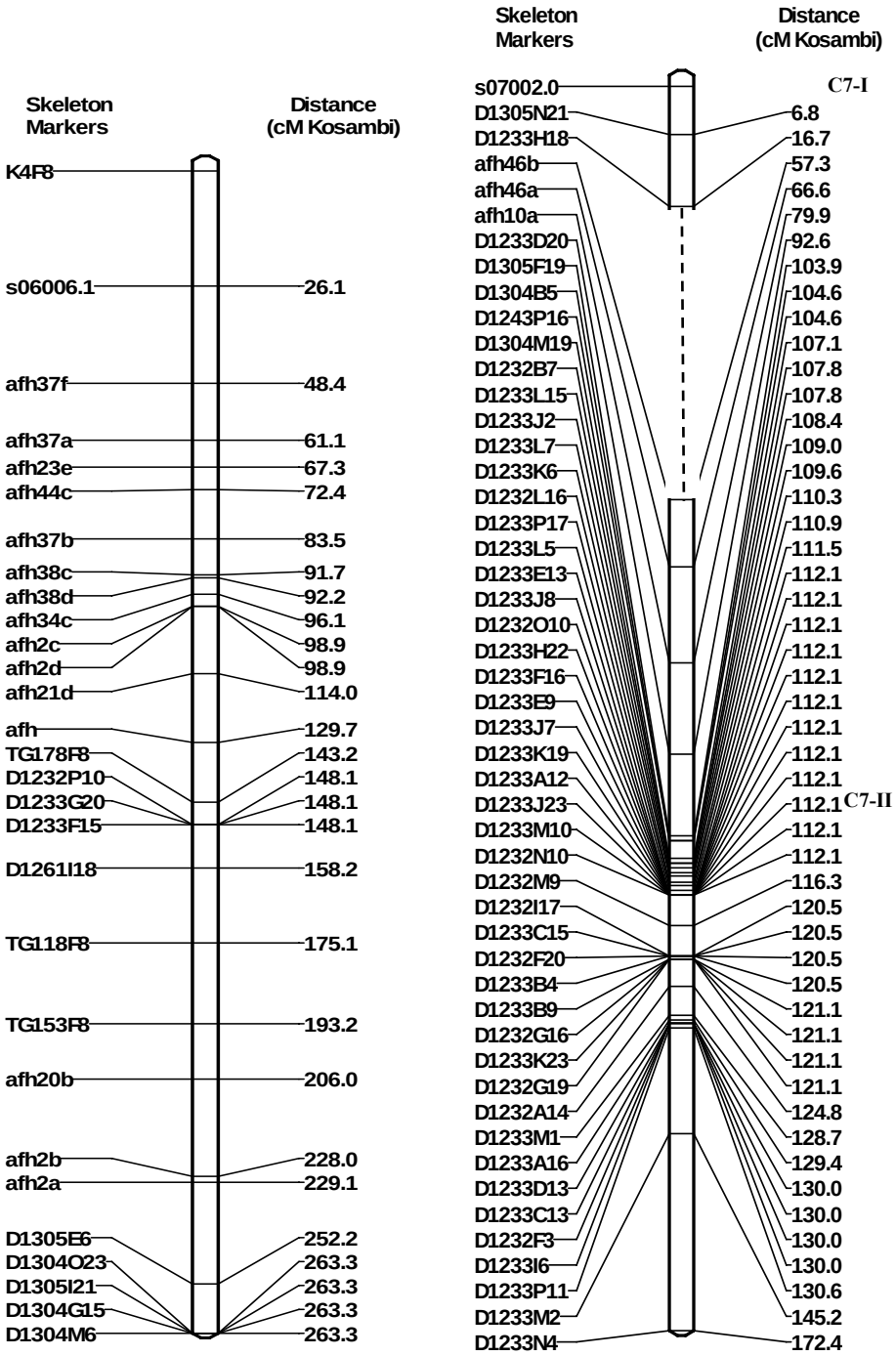


Figure 1.4 continued

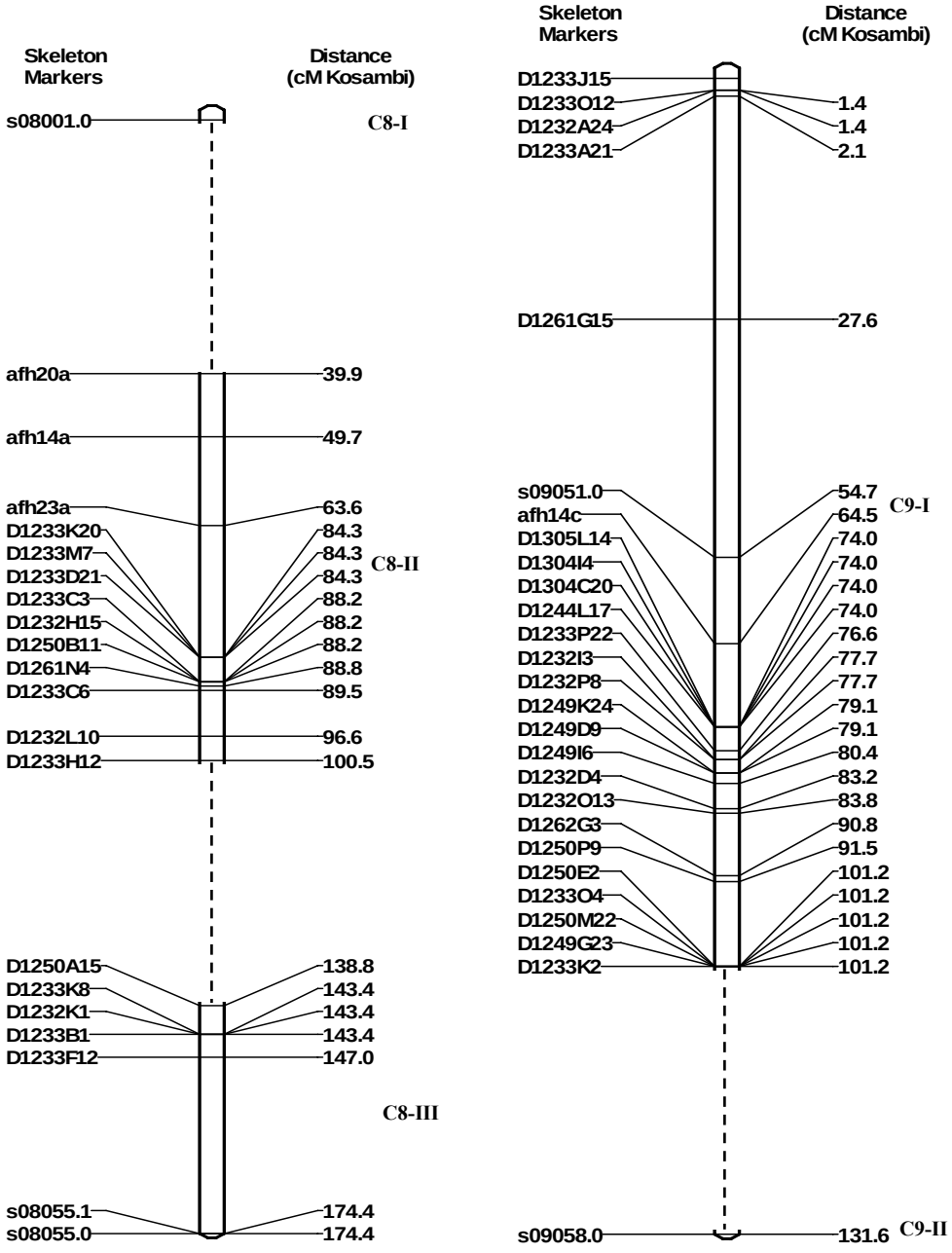


Figure 1.4 continued

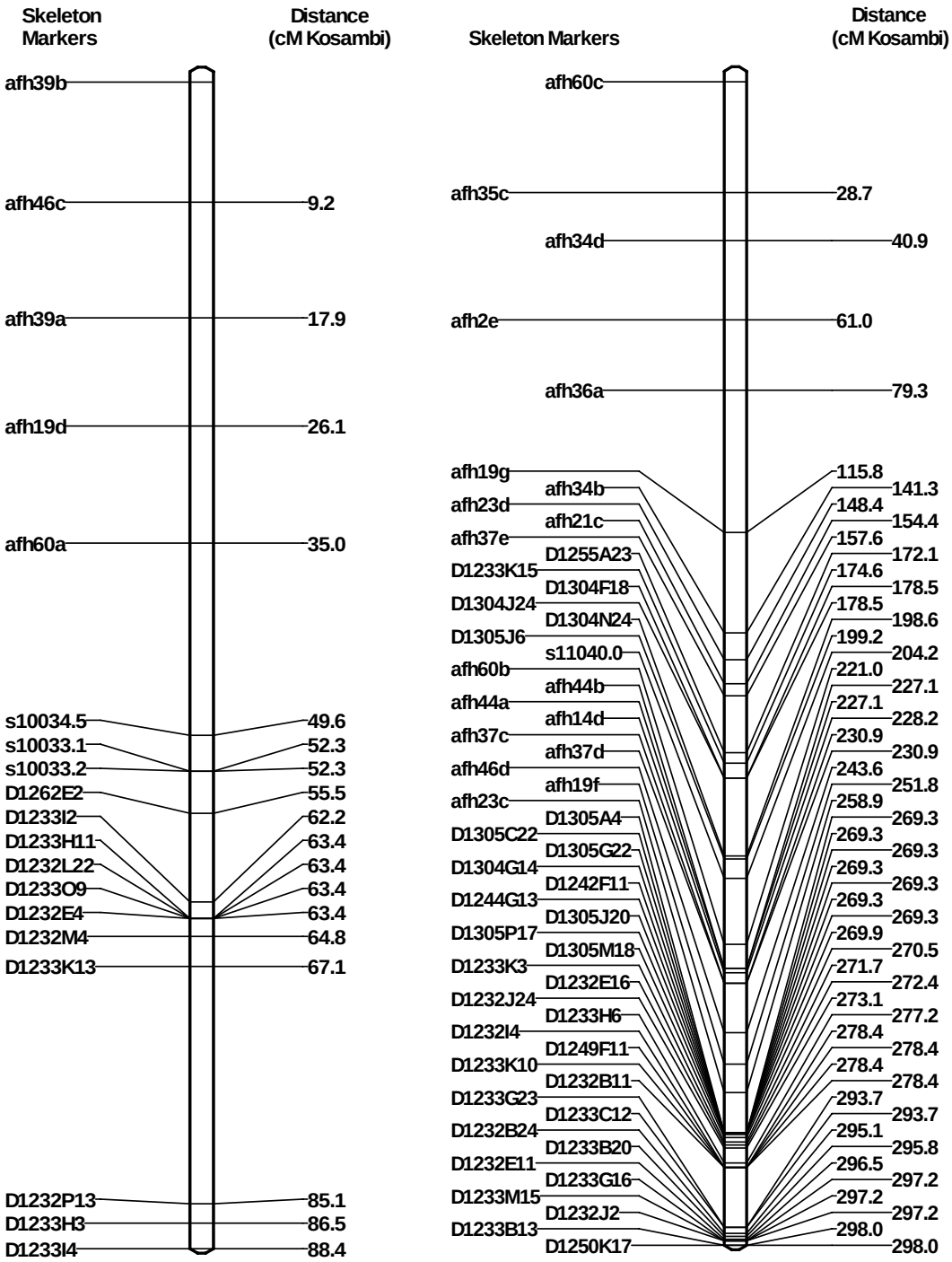


Figure 1.4 continued

1.4 DISCUSSION

1.4.1 Polymorphism between H7996 and WVa700

Eighty-five SSRs and four unmapped SSRs were surveyed on the two parental lines, *S. lycopersicum* H7996 and *S. pimpinellifolium* WVa700, with approximately 30% polymorphism was consistant with results of Liu *et al*. (2005), who found 23.58% polymorphic bands gained between *S. lycopersicum* Mill. XE 98-7 and *S. pimpinelifolium* LA2184. A lower level of polimophism (8%) was observed using 21 AFLP primer combinations and eleven SNP primers. Similar amount of the polymorphic AFLP bands was found in H7996 and WVa700 (31 and 35 respectively) in the present study. This could be due to the restriction sites in these two species were similar—*S. lycopersicum* is a cultivated species, whereas *S. pimpinellifolium* is phylogenetically similar to *S. lycopersicum*. Thus, DNA polymorphism between *S. lycopersicum* and *S. pimpinellifolium* is usually lower than between *S. lycopersicum* and either *S. pennelli* or *S. habrochaites* (Miller and Tanksley, 1990), which was demonstrated by Zhang *et al.* (2002), who found 98% RFLP markers, which selected from a high-density linkage map of *S. lycopersicum* x *S. pennelli*, showed polymorphism between *S. lycopersicum* Mill. Line NC84173 and *S. habrochaites* Humb. and Bonpl. accession PI126445.

1.4.2 Segregation distortion

Deviation from expected Mendelian segregation ratios has been reported previously in mapping populations (Lee *et al.* 2006; Lu *et al.* 2002; Pradhan *et al.* 2003; Törjék *et al.* 2006). Segregation distortion has been found in most plant pedigrees when large numbers of markers were mapped (Bradshaw and Stettler, 1994).

The cause of skewed segregation could be physiological and genetic factors (see Lu *et al.* 2002). In tomato, factors associated with the distorted segregation ratio are gametophytic selection, viability selection of segregating plants (Foolad, 1996) and spore function (Tanksley and Loaiza-Figueroa, 1985). Distored segregation in tomato has been reported in many interspecific crosses and proposed to be greater in wilder crosses compared with crosses between closely related species, and generally higher in filial than in backcross population. Several studies confirmed this proposition; e.g 8.3% distortions were observed

in *S. lycopersicum* x *S. pimpinellifolium* BC_1 population (Grandillo and Tanskley, 1996b), 9.9% in *S. lycopersicum* x *S. pimpinellifolium* BC_1 population (Chen and Foolad, 1999), 20% in a *S. lycopersicum* x *S. habrochaites* BC_1 population (Bernacchi and Tanksley, 1997). The while, 75% in *S. lycopersicum* × *S. cheesmanii* recombinant inbred line population (Paran, 1995). Thus, the overall level of distortion in the present study (60.8%) was consistent with previous studies.

A large number of AFLP and DArT markers exhibited segregation distortion in this study. The level of distortion of AFLP marker in the present study is quite high compared with other crops using the same marker technique (silver staining): 21.6% using double haploid population in rice (Maheswaran *et al.* 1997) and 6% in barley (Becker *et al.* 1995). This could be due to the differences of population types used. The segregation distortion in this study was in accordance with results of Carlos (1998), who found 50% segregation distortion for AFLP markers in a F_7 recombinant inbred line population of tomato.

Markers deviating from the expected segregation ratio are generally believed to be linked to genes that are subject to direct selection; for example: a lethal allele in *Populus* spp. affecting embryo development was the cause of segregation distortion of markers (Bradshaw and Stettler, 1994); markers cosegregating with the *Melampsora* resistance gene also showed a significant deviation (Cervera *et al.* 2001). Therefore, all markers in this study should be used in the mapping process to avoid missing of parts of linkage groups. However, including markers with segregation distortion will increase the chance of type I errors (*i.e.*, rejection of the null hypothesis) of false linkage; thus, leading inaccuracy of map distance between markers (Cloutier *et al.* 1997). Nevertheless, when searching for a stable order of markers for QTL analysis, we preferable deleted markers with higher missing data point and greater χ^2 values.

1.4.3 Map construction

The AFLP technique has been widely used for construction of linkage maps (Bratteler *et al.* 2006; Hawthorne, 2001; Mignouna *et al.* 2002; Travis *et al.* 1998). On contrary, DArT is a new technique and most commonly used in wheat and barley for construction of linkage maps (Semagn *et al.* 2006; Wenzl *et al.* 2006).

In tomato, many linkage maps have been constructed based on different populations and markers used (Tanksley *et al.* 1992; Danesh *et al.* 1994; Vanooijen *et al.* 1994; Thoquet *et*

al. 1996a; Bernacchi and Tanksley, 1997; Zhang *et al.* 2002; Liu *et al.* 2005); however, only two mapping populations involved two sources of bacterial wilt resistance and have been used for mapping QTL for bacterial wilt resistance in tomato. The first mapping population utilized 71 F_2 individuals from the cross between L285, a resistant *S. lycopersicum* var. *cerasiforme* line and CLN286, a susceptible tomato cultivar (Danesh *et al.* 1994). Of the 290 RFLP markers screened for polymorphism, 67 were polymorphic of which 59 mapped to 11 linkage groups covering 1220 cM of the total genome. Screening of additional 80 RAPD primers yielded only 12 primers that were useful for linkage mapping. Using 112 recombinant inbred lines of the same cross (L285 x CLN286), seventy-four out of the 242 polymorphic AFLP markers were placed on 7 linkage groups of the tomato genome (Carlos, 1998). Average AFLP marker polymorphism was 12.4%.

The second mapping population utilized 188 F_2 individuals from the cross H7996 (resistant *S. lycopersicum* line) x WVa700 (a susceptible *S. pimpinellifolium* line) (Thoquet *et al.* 1996a). Eighty-eight of the 462 RFLP probes tested showed polymorphism but only 60 were mapped. In addition, screening of 300 arbitrary RAPD primers resulted only 13 useful and mappable RAPD markers. Of the eight microsatellite sequences examined, only one sequence showed polymorphism. Using the same cross, Balatero *et al*. (2002) have used F_6 RILs to construct a genetic linkage map for bacterial wilt resistance utilizing AFLP, RGA, and SSR markers. Average marker polymorphism was 9.0% for AFLP, 10.4% for RGA, and 14.3% for SSR. The map was constructed using MAPMAKER software and consisted of 12 linkage groups and 80 markers (72 AFLP, 7 RGA and 1 SSR). The map length spaned a total of 378.1 cM. Thus, clearly, the level of marker saturation of the two mapping population is very low to allow marker assisted selection. There is a need to saturate the map and to identify markers that could be tightly linked to the resistance factor.

In the present study, we constructed a linkage map using MultiPoint software (MP) and compared with Joinmap software (JM) using the DNA profiles of 188 F_9 RILs derived from the same cross H7996 x WVa700. The map consisted of 362 out of 415 polymorphic markers including 74 AFLP, 260 DArT, 5 RFLP, 1 SNP and 22 SSR markers covering 2131.7 cM of the 12 linkage groups with an average marker distance of 5.9 cM. The map was about 1.6 times of the published RFLP-based map of tomato by Tanskley *et al.* (1992). This large coverage could be due to the large gaps between connections of the linkage groups. However, the alignment of the linkage groups calculated in MP was in accordance with JM. This is the first linkage map of tomato for mapping bacterial wilt

resistance that utilizes DArT marker technology. The DNA sequences of DArT clones could be used to convert DArT markers to single-marker assay formats for applications in breeding program.

An uneven distribution of recombinant events along chromosome led markers tending to cluster (Tanksley *et al.* 1992). In the present linkage map, we found both DArT and AFLP loci showed a tendency to clusters. This could be due to DArT markers may be indicative of gene-rich regions and representative of redundant clones in the whole genome (Semagn *et al.* 2006) and the use of *Eco*RI-*Mse*I combination in the present study showed higher level of polymorphism than other combinations (Barrett and Kidwell, 1998). DArT markers were clustered around both centromeres and distal regions over the 12 linkage groups. DArT markers showed the highest frequency of clustering and number of markers co-segregating (Figure 1.4). This result was in accordance with results of Semagn *et al*. (2006) that the frequency of clustering of DArT markers was higher than AFLP and SSR markers by 3 and 15 times, respectively.

Most AFLP markers clustered in the centromere of chromosome 4 and near centromere of chromosome 6. Similar results were observed in several studies in which *Eco*RI/*Mse*I AFLP markers were used to construct linkage maps (Castiglioni *et al.* 1999; Strommer *et al.* 2002). The reasons of clustering of AFLP markers near the centromeres could be repetitive elements characteristic of centromere may produce a number of AFLP markers representing variants of single repeats (Castiglioni *et al.* 1999; Vuylsteke *et al.* 1999) or the centromere regions are rich of noncoding DNA (Peters *et al.* 2001). The present map provides insights regarding the distribution of AFLP and DArT markers for linkage mapping. It also provides readily detectable markers for identifying novel QTLs linked to bacterial wilt resistance and morphological traits.

1.5 SUMMARY

A genetic linkage map for bacterial wilt resistance in H7996 was constructed using 188 F_9 RILs derived from a cross between *S. lycopersicum* H7996 (resistance parent) and *S. pimpinellifolium* WVa700 (susceptible parent) using amplified fragment length polymorphism (AFLP), diversity arrays technology (DArT), restriction fragment length polymorphism (RFLP), single nucleotide polymorphism (SNP) and simple sequence repeat (SSR) markers. The map contained a total of 362 markers including 74 AFLP, 260 DArT, 5 RFLP, 1 SNP, and 22 SSR markers, which were split into ten major and two minor linkage groups, spanning 2131.7 cM. All five marker types showed significant ($P<0.05$) segregation distortion, but it was highest for SNP (100%) followed AFLPs (64.5%), DArTs (62.0%), RFLPs (50.0%) and SSRs (36.0%). The overall percentage of markers with segregation distortion was 60.8%. The distribution of markers between linkage groups was unequal. AFLP markers were mainly distributed in chromosome 3, 4, 6 and 12; whereas, DArT markers were most frequent in chromosome 4 followed by chromosome 11, 2, 1, 9, 3, and 4. Chromosome 2 showed the highest density of markers (2.7 cM/interval), while LGA showed the lowest (10.4 cM/interval). The length of the linkage groups ranged from 48.6 to 298 cM. There was no mixing of AFLP and DArT markers in the same cluster and clear clustering of AFLP and DArT markers was observed in the genetic linkage map. This could be due to (1) DArT markers may be indicative for gene-rich regions and (or) the result of inclusion of redundant clones in the genomic representations, and (2) the use of *Eco*RI-*Mse*I combination in the present study showed higher level of polymorphism than other combinations. The present study is the first report of the use of AFLP, DArT, RFLP, SNP, and SSR markers for construction of linkage map for bacterial wilt resistance in H7996.

Chapter 2

Detection of QTLs for bacterial wilt resistance in Hawaii 7996 and its relationship with morphological traits

2.1 INTRODUCTION

Bacterial wilt caused by *Ralstonia solanacearum* is one of the most widespread and destructive disease on tomato worldwide. *R. solanacearum* strains can be grouped into five races and five biovars (Buddenhagen, 1962; Hayward, 1991). Large genotype and phenotype variations have been observed among strains of *R. solanacearum* (Denny, 2006). It is a soil-born pathogen, and the pathogen enters plant roots through wounds or natural openings where lateral roots emerge. It first colonizes the host's root cortex, then infects the vascular parenchyma, and finally invades the xylem elements (Vasse *et al.* 1995). Once inside the xylem, the bacteria multiply and spread rapidly throughout the plant's vascular system. Susceptible tomato plants respond to these high bacterial populations by wilting and dying. Various methods to control bacterial wilt have been reported (Guo *et al.* 2004; Ji *et al.* 2005; Nonomura *et al.* 2001; Trigalet and Trigalet-Demery, 1990); however, none is as simple and effective as the use of resistant varieties.

Tomato varieties with resistance to bacterial wilt have been selected or developed by researchers in many locations around the world (Atabug, 1981; AVRDC, 1994; Barnes and Vawdrey, 1992; Opena, 1990; Peter *et al.* 1992; Scott *et al.* 2003; Scott, 1992; Scott *et al.* 2005; Vudhivanich, 1995; Wang *et al.* 1997). However, commercial cultivars with good stable resistance under different environments against diverse strains of the pathogen across regions are still lacking.

Among the known resistance sources to bacterial wilt in tomato, genetic control was only studied on a few sources. Hayward (1991) has indicated that the inheritance of resistance to bacterial wilt in tomato can be complicated by interaction between the plant genotype and pathogen strains as well as the effects of the environment on resistance expression. Monma *et al*. (1997) studied inheritance of bacterial wilt resistance in tomato in two crosses D-9 (resistant parent) x TPL-5 (susceptible parent) and TPL-5 x Hawaii 7998

(resistant parent) and found that mean resistance indices of the F_1 generation of the two crosses were lower than the resistant parent suggestting bacterial wilt resistance is partially recessive as there was incomplete dominance toward susceptibility. In addition, genetic nature of bacterial wilt resistance in tomoto accession LA 1421 (*S. lycopersicum* var. *cerasiforme*) was studied in two crosses LA 1421 x Cascade and LA 1421 x Caraibo, and significant differences were observed between generation means in the two crosses indicating resistance identified in LA 1421 may be different when different susceptible parents used. Genetic mechanism seems to be complex with a duplicate form of epistasis and variation observed in the F_2 and BC_2 progeny of the Caraibo cross sugested genetic combination between the two resistance sources can lead to higher level of resistance (Mohamed *et al*. 1997).

Resistance to bacterial wilt in tomato cultivar H7996 has been studied and reported to be a stable resistance source (Wang *et al.* 1998). In order to decide the most appropriate and efficient strategy in transferring the stable resistance in H7996 could depend on our knowledge of the genetic control. The mode of inheritance of resistance in H7996 could vary depending on strains and inoculation methods used according to previous studies, e.g when bacterial strain 8217 (race 1, biovar 1), a spontaneous mutant from strain GT1, was used to inoculate F_2 plants that rerived from cross H7996 (resistant parent) x Froradel (susceptible parents) in the field, the resistance in H7996 was reported to be controlled by a single gene (Grimault, 1995). The while, a major locus controlled resistance to bacterial strain Pss4 (race 1, biovar 3) was reported as well when a soil drenching without root wounding method was used to inoculate F_2 plants that rerived from cross H7996 (resistant parent) x WVa700 (susceptible parents) (Wang *et al.* 2000). In addition, the use of the same cross as in Wang *et al*. (2000), the resistance to strain GMI8217 was reported to be polygenic, when a drenching method and a scale of 1 to 9 was used in disease evaluation (Thoquet *et al.* 1996a, b; Mangin *et al.* 1999).

Quantitative Trait Locus (QTL) mapping is an effective approach for studying complex and polygenic forms of disease resistance, which is based on the use of DNA markers (Tanksley, 1993). With QTL mapping, the roles of individual loci in genetically complex traits can be described and fundamental questions that have vexed researchers in the field of plant pathology for decades can be addressed (Young, 1996).

Progenies derived from a cross between the resistant cultivar H7996 and the susceptible WVa700 have been used for studying the genetic control of the resistance in H7996 (Thoquet *et al.* 1996a, b; Mangin *et al.* 1999; Wang *et al.* 2000). Several QTLs were mapped in F_2 or F_3 populations evaluated at different geographic locations against different strains. And evidences of strain specific resistance have been demonstrated. Common QTLs associated with TG73 or TG118 were detected on chromosome 6 in all studies used the same cross (Thoquet *et al.* 1996a, b; Wang *et al.* 2000; Cameille *et al.* 2006). And there were another six QTLs detected. Among them, one QTL is specific to tests using F_2 cutting (Thoquet *et al.* 1996a), and another QTLs was detected on chromosome 4 in the F_3 population (Thoquet *et al.* 1996b). All the resistant loci were carried from the resistance parent H7996, where it explained from 30% to 50% of the phenotypic variation. Meanwhile, in F_3 population, two putative new QTLs were found on chromosome 3 and 8. In addition, a weak putative QTL previously detected on chromosome 10 was again observed using data from a field trial and one QTL on chromosome 11 was found to be specific to F_2 cutting (Thoquet *et al.* 1996b). Furthermore, a QTL which controlled about 70% variation on chromosome 12 was suggested to be specific to strain Pss4 (Wang *et al.* 2000). However, in the previous studies, the use of F_2 or F_3 mapping population does not allow extensive evaluation of the environment and strain effects.

Resistance to bacterial wilt was difficult to combine in certain breeding programs with the resistance to root knot nematodes conferred by the *Mi* gene (Acosta *et al.* 1964), suggesting that one important locus may reside on chromosome 6. This observation was confirmed in other lines of tomato (Prior *et al.* 1994) and by mapping with molecular markers (Aarons *et al.* 1992; Thoquet *et al.* 1992; Danesh *et al.* 1994; Wang *et al.* 2000; Balatero *et al.* 2002). In addition, QTL on chromosome 7 and 10 were identified as being association with resistance in L285, a resistant accession (*S. lycopersicum* var. *cerasiforme*) beside the important QTL close to CT184 around 40 cM on chromosome 6 (Danesh *et al.* 1994); and Danesh and Young (1994) demonstrated that the resistance controlled by this locus could be strain-specific.

Recombinant inbred line (RIL) has many advantages over the other populations that are used for genetic mapping and quantitative trait locus analysis. RIL can serve as a permanent mapping resource that will permit replicated trials in multiple environments or evaluating with different strains of the pathogen. Using RIL is especially powerful for analyzing quantitative traits because replicated trials can be analyzed using identical

genetic materials (Burr *et al*. 1991). Carmielle *et al*. (2006) used F_8 RILs that derived from the cross H7996 x WVa700 to identify QTLs for resistance to *R. solanacearum* race 3-phylotype II strain JT516 in different seasons. A major QTL, *Bwr-6* and a minor one, *Bwr-3*, were detected in each season for all resistance criteria and both QTLs showed stronger effects in the hot season than in the cool one; however, QTLs *Bwr-4* and *Bwr-8* were only detected in the hot season, demonstrating that environmental factors may influence the expression of resistance against the race 3-phylotype II strain JT516. AVRDC has continued advanced the cross of H7996 and WVa700 to F_9 and have a RIL population ready for this study.

Resistance cultivars can only be accepted by farmers when desired fruit traits exist. If resistant trait linked with undesired fruit characteristics, special efforts would be required to break the linkage. Acosta *et al*. (1964) have found the association of bacterial wilt resistance with small fruit size; however other researchers did not find such an association (Danesh *et al.* 1994; Monma *et al.* 1997). Scott *et al*. (2003) have broken apparent linkage of a hypothetical fruit size gene with a bacterial wilt resistance gene by crossing breeding lines with medium-large fruit and intermediate resistance. The two lines Fla. 8109 and Fla. 8109B developed have a good level of bacterial wilt resistance and large fruit size. Difference in fruit and other morphological traits exists among H7996 and WVa700. Therefore, the RIL population can be used to exam the possible association between bacterial wilt resistance and morphological traits.

Therefore, the objectives of this study were: 1) to map QTLs for bacterial wilt resistance with special emphasis on identifying QTLs required for stable resistance; 2) to derimine association of QTLs for bacterial wilt resistance with morphological traits; and 3) to develop PCR-based markers for identifying molecular markers closer linked to detected QTLs.

2.2 MATERIALS AND METHODS

2.2.1 Plant materials

A RIL population consisting of 188 lines in the F_9 generation derived from the cross between H7996 and a susceptible tomato line WVa700 was used. The RIL population and the two parental lines H7996 and WVa700, as well as a susceptible control L390 were evaluated for resistance to *R. solanacearum* under screen-house conditions. Seeds of all tomato genotypes were obtained from the Bacteriology Unit, AVRDC. Before sowing, tomato seeds were soaked in 4X diluted Clorox for 5 minutes, rinsed under running water for 15 minutes, and then sown immediately in 2-inch pots. Seeds of WVa700 were sown 2 days earlier, and those of H7996 and L390 were sown 2 days later than the F_9 RILs because of different germination rates of the lines. The potting mixture used consisted of sand, soil, rice husk and compost in the ratio of 1:3:1:1 and has been steam-sterilized. Seedling were fertilized everyweek with 500X of foliar fertilizer (BASF Foliar Nitrophoska) after thinning and stop at least 4 days before inoculation.

2.2.2 Evaluation of resistance to bacterial wilt

2.2.2.1 Bacterial strains and inoculation

Strains of *R. solanacearum* Pss4 (race 1 biovar 3), Pss186 (race 1 biovar 4), Pss190 (race1 biovar 4) (Jaunet and Wang, 1999) were used to evaluate the resistance of the RIL population. Strains Pss4, Pss186 and Pss190 were isolated from tomato and provided by the Bacteriology Unit, AVRDC. Stored cultures at -80^{o}C of *R. solanacearum* were streaked on tetrazolium medium (TTC) (Kelman, 1954) and incubated at 30°C for 2 days. Several typical fluidal single colonies from TTC were transferred to 523 medium (Kado and Heskett, 1970) and cultured at 30^{o}C for 24 hours. A dense suspension of each strain was prepared by transferring bacterial mass from 523 medium plate into a tube with 5ml sterile distilled water. A total of 0.1ml of the bacterial suspension was spread on one fresh 523 plate and kept at 30^{o}C for 24 hours. Bacterial masses were harvested and suspended with distilled water until O.D. value reached 0.3 at the wavelength of 600nm (about 10^8 cfu/ml). Three-week old seedlings with four fully expanded true leaves were inoculated by pouring 20ml of the above suspension on the soil surface of each pot.

2.2.2.2 Evaluation based on visual symptoms

Experimental design

The experiments were conducted following a randomized complete block design (RCBD) with two replications in a screen-house at AVRDC. For each replication, 8 plants per line and 48 plants of each parental line and L390 were included. Each plant was assigned an identification number and arranged randomly within each replication to reduce the environment effect on disease development. Evaluations against strain Pss4 and Pss186 were conducted successively separated by one week in the same screen-house. The screenings were conducted from May 31, 2004 to September 18, 2004.

Data recording

Disease reactions were evaluated at 4, 7, 10, 14, 21, and 28 days after inoculation (DAI) using a 0-5 scale, where 0 means no symptom; 1 means one leaf wilted; 2 means two - three leaves wilted; 3 means four or more wilted leaves; 4 means all leaves wilted; 5 means death of the plant (Winstead, 1952) (Figure 2.1). Three methods of data collection and processing were used for analyses:

(i) Percentage of wilted plants (PWP): Plants were either scored as healthy (scale 0) or wilted (scale 1 to 5). PWP = (N_W/N_T) x 100, where N_T is number of total plants and N_W is number of wilted plants;

(ii) Disease index (DI): DI was calculated following the formula (Winstead and Kelman, 1952): DI = $[(N_0 \times 0 + N_1 \times 1 + N_2 \times 2 + N_3 \times 3 + N_4 \times 4 + N_5 \times 5)/(N_T \times 5)] \times 100$; where N_0 to N_5 are the number of plants at each scale, and N_T is number of total plants;

(iii) Relative area under disease progress curve (RAUDPC) (Fry, 1978): Firstly PWP or DI was used to calculate area under disease progress curve (AUDPC), which expresses the dynamics of disease development according to Shaner and Finney (1977). AUDPC was calculated following the formula of AUDPC = $\sum_{i=1 \text{ to } n-1} [(Y_{i+1} + Y_i)/2] \times [X_{i+1} - X_i]$, where Y_i is either percentage of wilted plants (AUPDCPWP) or disease index (AUPDCDI) at the i*th* observation (i = 1 being the first observation at time zero), and X_i is time at the i*th* observation, and n is total number of observation. Values of AUDPCPWP and AUDPCDI were divided by the number of days of the entire observation period to calculate RAUPDC.

Figure 2.1 Tomato plants showing different severity after inoculation of *R. solanacearum*. Numbers indicated beside plant were rating scale, where 0: no symptom, 1: one leaf wilted; 2: two -three leaves wilted, 3: four or more wilted leaves, 4: all leaves wilted, 5: dead.

2.2.2.3 Evaluation based on colonization degree

Protocol development

Three susceptible RILs (number 79, 121 and 141) and three resistant RILs (number 13, 41, and 200) were used. They were selected based on evaluation results over trials kindly provided by Dr. Jaw-Fen Wang, Bacteriology Unit, AVRDC (personal communication). The seedlings were inoculated as described above. Assay of colonization (method described below) was conducted at 3, 4, 5 and 6 days after inoculation for strain Pss190 and at 4, 5, 6 and 7 days after inoculation for strain Pss4. At each sampling time, 8 plants were harvested randomly. Percentage of colonized plants were calculated and differences between the resistant group and the susceptible group and values of standard deviation were evalauted. Since the difference between the two groups was the largest and the standard error was the lowest at 6 days after inoculation when inoculating with strain

Pss4, it was decided that the sampling time would be at 6 DAI for detemining the percentage of colonized plants of the entire RIL population.

Assay for colonization

Symptomless plants were harvested, roots were cut off, and all leaves were removed. The remaining stems were washed with tap water, rinsed in distilled water, sprayed with 70% alcohol, and blotted dry on paper towels. Each plant was sectioned at the mid-point of the stem with a sterilized razor blade. The cut surface of the upper stem portion was pressed tightly on the surface of a semi-selective medium 1 (SM1) plate (Tsai *et al*, 1985) for 5 seconds per print and five prints were made continuously for each cut surface. The SM1 plates were incubated at 30^{o}C for 3 days. When fluidal bacterial mass was observed on at least one out of five prints, the plant was scored as positive for pathogen colonization as illustrated in Figure 2.2. Percentage of colonized plants (PCP) was then calculated following the formula of PCP = $((N_C + N_W)/N_T) \times 100$, where N_T is number of total plants, N_W is number of wilted plants, and N_C is number of plants shown positive colonization.

A B

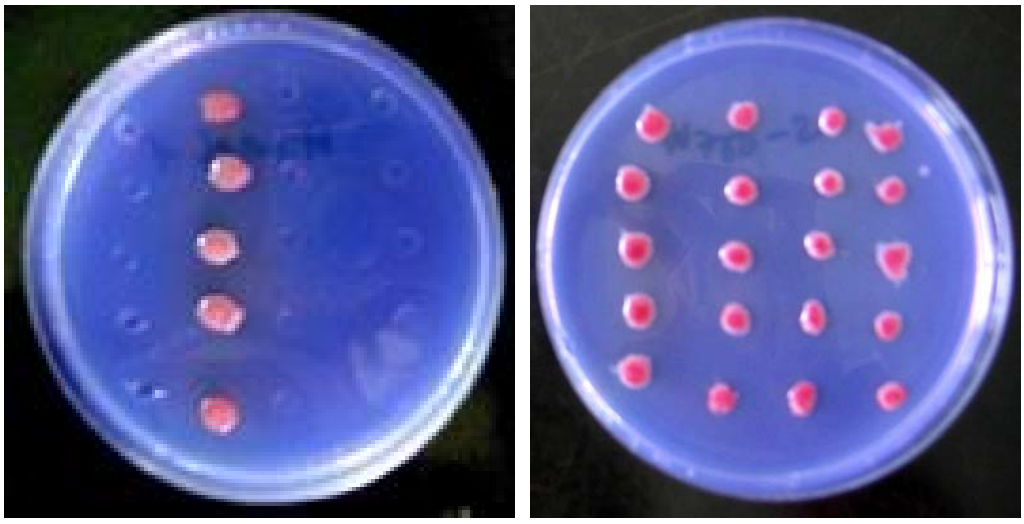


Figure 2.2 Colonization by Pss4 scored after inocubation at 30^{o}C for 3 days. A plate with H7996 samples shown one out of four plants was colonized (A); and WVa700 samples shown all four plants were colonized (B).

Experimental design

Experiments were laid out as a randomized complete block design (RCBD). When more than one strain was used in one experiment, split-plot design was followed with main-plots consisted of strains and subplots of RILs. All experiments consisted of three replications.

For developing the protocol, each RIL comprised of 32 plants per replication. Each plant was assigned a number and arranged randomly. The protocol was developed from July 5, 2005 to September 16, 2006. Due to limited space in greenhouse, 3 replications for phenotyping all RILs were conducted one week apart in the same greenhouse and consisted of 8 plants per line. The plants of each line were divided into 4 groups and each group was assigned an identification number and completely randomized. The experiments were conducted from September 23, 2005 to November 10, 2006.

2.2.3 Evaluation of morphological traits

2.2.3.1 Experimental design

The experiment was laid out in a non-replicated plot trial with five plants per RIL. One week before transplanting, the seedlings were hardened by slightly reducing water and exposing them directly to sunlight. About 12 hours before transplanting to the field, the seedlings were watered thoroughly. Plants were transplanted into the AVRDC farm with spacing of 60 x 40cm in a plot size of 1.2 x 1m, which has been mulched with polyethylene plastic sheets. Fertilizer was applied in strips along the the field before mulching with a 15-15-15 fertilizer (15% N, 15 % P_2O_5, 15% K_2O, and 4% MgO) of 120kg per hectare. In addition, 40kg per hectare were applied every 30days as side-dressing during the trial. Plants were staked and irrigated by seepage from ditches. Pesticide was spayed every week to control major insect pests and foliar diseases. The experiment was conducted from 20 November 2004 to 15 May 2005 at AVRDC farm.

2.2.3.2 Sampling and data collection

Plant growth habit

Plant growth habit was recorded as sympodial index (SPI), the number of leaf nodes per sympodium (i.e., between successive inflorescences) (Rick, 1986). The character was scored by counting the number of leaf nodes in 5-7 sympodia and deriving the mean per plant. Sympodia were examined from three different branches of each plant. The sympodial index was calculated following: SPI = LN/S, where LN is number of leaf nodes of each branch, S is number of sympodiums of each branch.

Fruit characters

Tomato fruits at the fully red-ripe stage, approximately 110 days after transplanting, were harveseted once. Sampling dates varied among RILs depending on their maturity.

Fruit weight and skin color

Five fruits from each of the five plants per RIL were harvested and scaled for fruit weight. The skin of each fruit from individual plants was peel, put on a light box to evaluate whether skin color was clear or yellow.

Fruit quality analyses

The fruit quality assays were conducted by the Nutrition Unit of AVRDC, and the methods were described below in brief. Each sample consisted of 10-100 fully ripened fruits harvested from 5 plants of each RIL. Fruit were cut, blended with a homogenizer, and filtered through gauze to remove seeds, skin, and membranes. From each sample, a plastic cup and a 40ml tube were prepared containing 30-50g of tomato slurry. The cup was used to measure color and pH. The slurry was centrifuged at the same day with a Himac CR 21 centrifuge at 8000rpm for 10 minutes, and the supernatants were used to measure soluble solids concentration and citric acid.

The pH value was measured using an Orion Model 420A pH Meter. Soluble solid concent was measured as $^{\circ}$Brix with a digital refractometer (PR-101; Atago, Tokyo, Japan). Color was measured by a colorimeter (Nippon Denshoku Kogyo Co., Ltd. Osaka, Japan) on three scales represented as a, b, and L. Color values of fresh tomato slurry were calculated as a/b. As chromaticity increases, a color becomes more intense; as it decreases, a color becomes duller. When a/b is greater than 1, the color is redder, whereas a/b is smaller than 1, the color is more yellow. The citric acid was measured by pipetting 5ml of the supernatants into an 80ml beaker and filling up to 60ml with ddH_2O. Then, 0.05N NaOH solution was pipetted slowly into the beaker until the pH reached 8.1. Thus, the amount of NaOH added was determined. Citric acid (CA) was calculated based on the formula (Nutrition Unit's protocol):

CA (%) = [(N x V x 192/3) x 5ml x 100]/1000, where N is the concentration of 0.05 N NaOH prepared, V is the amount of 0.05N NaOH solution used. "192" is the molecular weight of NaOH, 3 is three titratable protons from the carboxyl groups (3 -COO-); 5ml is the amount of tomato juice.

2.2.4 Data analysis

Transformation with $\log_{10}(x+1)$ was performed for RAUDPC, while arcsine square root was used for disease index and percentage of wilted plants to improve the normality of the data. These transformed data were used for analysis of variance (ANOVA). ANOVA was performed with the SAS's GLM procedure (SAS 8.2, SAS Institute, Cary, NC, USA). These analyses were carried out in order to determine the effect of genotype and strain as well as the interaction between genotype and strain on disease development. Correlation among visual symptoms, colonization and morphological traits was conducted with the SAS's CORR procedure.

2.2.5 QTL analysis

For QTL mapping, co-segregating markers in the genetic map, which has been described in Chapter 1, were omitted. However, some markers caused unstable order in the map were not included for QTL analysis. Hence, the stability of the marker order was performed via MultiPoint package (http://www.multiqtl.com) by keeping one marker form each cluster. The stability obtained for each linkage group was tested using 100 resampling (jackknife) runs, allowing those markers that caused local neighborhood instability in the map to be detected and removed. This procedure was iteratively used with final verification based on 1000 jackknife runs until a stable ordering of markers (termed "skeleton" markers) was obtained. A total of 15 linkage groups as described in Chapter 1 (Figure 1.4) were used for QTL analysis. Thus, a subset of 106 loci (32 AFLP, 59 DArT, 6 RFLP, and 11 SSR markers) distributed over 15 linkage groups was selected. The map covered 1089.1 Kosambi cM that corresponded to about 51.1% of the linkage map (Chapter 1) and about 85% of the genome, when compared to the saturated genome map (Tanksley *et al.* 1992). In addition, 13 markers that hinted belonging to certain chromosome but not sufficient to include them in the framework for QTL mapping was determined association with resistance separately by single marker analysis. They are D1262C14, LEOH36, D1244H17, s01138.0 (chromosome 1), s07002.0 (chromosome 7), s08001.0 (chromosome 8), s09058.0 (chromosome 9), D1233J4, TG564F8 (LGA), D1232L19, D1262M8, D1232K7, and D1233I1 (LGB).

QTL detection was performed using composite interval mapping analysis (Zeng, 1994) using QTL Cartographer (Bastern *et al.* 2005). A 1,000-permutation test was performed with QTL Cartographer to estimate the appropriate significance threshold for analysis.

LOD threshold from 2.5 to 3.2 corresponding to a genome-wide significance level of 0.05 was chosen. Mapchart software (Voorrips, 2001) was then used to draw QTLs on the linkage groups. Single marker analysis was then performed by QTL Cartographer (Bastern *et al.* 2005) for the 13 markers indicated above to identify significant marker locus-trait associations. When *F* values less than 0.05, QTLs were considered significant.

The phenotypic data used for the QTL analysis include 22 datasets as described in Table 2.5. They included three datasets on disease evaluation and six datasets on morphological traits that were produced by the author for this study. The other 13 datasets on disease evaluation were produced or collected by Jaw-Fen Wang, AVRDC, from various collaborators in different countries.

2.2.6 Fine mapping

2.2.6.1 Bulk segregant analysis

Bulked segregant analysis (BSA) (Michelmore *et al.* 1991) was used to identify markers linked to bacterial wilt resistance. Bulks were prepared by combining 100ng of DNA (total DNA per reaction = 500ng) from seven resistant RILs (number 13, 41, 46, 70, 95, 130, and 200) and seven susceptible RILs (number 6, 69, 79, 80, 124, 141, and 158). The resistant and susceptible bulks were analyzed together with the parental lines H7996 (resistant parent) and WVa700 (susceptible parent) using twenty-one AFLP primer combinations (Appendix Table 1.1) to identify potential linked markers.

2.2.6.2 Conversion of AFLP, DArT and RFLP markers into PCR-based markers

Fragment isolation

The AFLP markers that showed polymorphism between the resistant and susceptible pools and the two parents presented band either in H7996 or WVa700 were excised from the polyacrylamide gel with a razor blade. The DNA-containing gel was transferred into an Eppendorf tube, mixed with 50µl sterile MiliQ water, and kept at 4^{o}C overnight to release the DNA fragment from the gel. After the gel was spun down, the DNA-containing supernatant was transferred into a new Eppendorf tube and diluted with sterile MiliQ water in a 2 to 1 ratio and used as template for the subsequent amplification. The *Eco*RI+3 and *Mse*I+3 primers that revealed the polymorphic bands, were used to re-amplify the isolated DNA with the same reaction conditions as for the AFLPs. The re-amplification products

were loaded onto 1% agarose gel in 1X TAE buffer at 90V for one hour, stained in ethidium bromide (1.5µg/ml) solution for 5 minutes, de-stained in distilled water for 15 minutes and visualized under UV light. The correct-size bands were then excised from the gel under UV light and transferred into an Eppendorf tube. DNA template in agarose gel was melted at 85^{o}C for 15 minutes. The DNA/agarose mixture was diluted with warm, sterile MilliQ water to 0.1-1.0 ng DNA/µl. The diluted-melted in-gel DNA was again amplified by using the corresponding primers and the same conditions as for the main amplification for the AFLPs. The PCR products were used for sequencing.

DArT and RFLP markers were excised from the 1% agarose gel and extracted using Wizard SV gel and PCR clean-up system kit (Promega, Madison, Wisconsin). Primers of DArT and RFLP markers used for fine mapping are listed in Table 2.1.

Cloning fragments

The PCR reactions were used for ligation of the fragment into a plasmid using the Pgem T-easy vector system from Promega (Madison, Wisconsin). The ligation reaction contained 5.0µl 2X Rapid ligation buffer, 0.5µl pGEM-T easy vector (50ng), 3.5µl PCR product, 1.0µl T4 DNA ligase (400U/µl) and 1.0µl sterile deionized water in a total volume of 10µl. The reaction was incubated overnight at 4^{o}C.

Transformation

The ligated reactions were then transformed into *E.coli* competent cells (Invitrogen Taiwan, Ltd.) by adding 50µl recently thawed competent cells to a sterile-1.5µl microcentrifuge tube containing 2µl of each ligation reaction on ice. Colonies were grown on agar LB-A medium and incubated for 12 hours at 37^{o}C. Five white colonies were isolated and transferred into fresh LB-A medium and incubated overnight at 37^{o}C. Colonies were also transferred into 0.5ml of liquid LB-A medium and incubated over night at 37^{o}C with an agitation at 150rpm for extraction of plasmid DNA. Among the eight cloned fragments, seven resulted in good colonies and good growth in liquid LB-A medium.

Table 2.1 DArT and RFLP primers used for fine mapping

No.	Marker code	Marker	Reference	Forward primer (5'-3')	Reverse primer (5'-3')	Size (bp)	Tm (^{o}C)[1]
1	2.1[2]	TG31	SGN	TGGTGCCCTACCTTTTATGG	TGGATTCCAACCACACTCAA	~2400	
2	2.2	D1261P15 (IB5)	Present study	CCAGAACGGTCCGTAAGATT	TCGTAGACTGCGTATCCGTAAA	109	
3	2.3	D1232K4 (IC3)	Present study	GTAGTGCCAGAACGGTCCAT	GCGTATCCGGATCTCTCTCA	435	
4	2.4	D1305B24 (IIF5)	Present study	GTAGTGCCAGAACGGTCGAT	GGATCCAGTGCAGGGATAGA	609	
5	2.5	TG1	SGN	CTTCACGAATCTTCGCTTCC	GAACAAACGGATAGGCAGAAAC	~700	
6	2.6	D1233C21 (IVC3)	Present study	GTAGTGCCAGAACGGTCGAT	GACTGCGTATCCGGATCATAA	124	
7	2.7	cLEC7P21	Frary *et a*l. 2005	TGAACAGAAAGCACGAGTGG	GACAGTTCTTCGAAGCGTTTG	350	55
8	2.8	T1616	Frary *et a*l. 2005	TTGGAAGAAGAGGAGGACGA	CCCAGAAAGATCCAAGTCCA	1500	55
9	3.1	TG324	Frary *et a*l. 2005	CACTTGGTTGATGGATAGTG	CTTCTAGTAGTCCAACAGCAACTG	1300	55+
10	3.2	cLPT5E7	SGN	ACAGCCAAGTCGAAGTCCTC	CCCAAGGGGGTTTAAGAGAA	1800	55
11	3.3	TG130	Frary *et a*l. 2005	CGGAACCCACTTTGTTTTTC	ATCAACCCTGCAAGCTCAAC	1500	55
12	3.4	TG585	Frary *et a*l. 2005	TGGAAAGCCAGACACACAGA	CAGGGGTATCAGTAGGCAGTG	491	55
13	3.5	T1388	Frary *et a*l. 2005	GCGATTTGGCTATCTGGGTA	AACCGAAAGGCTTTTCCAAG	1000	55+
14	3.6	cLPT2E21	Frary *et a*l. 2005	CGAAGATGTTGCTTGATTGC	AAGCAGGAGCTGGACACAAT	1250	55
15	3.7	TG74	Frary *et a*l. 2005	CATGCTTGAAAAGCAGTGGA	GATTATACGAGGCCTCAAGGA	2300	55
16	4.1	D1233O18 (IIIE11)	Present study	GTAGTGCCAGAACGGTCGAT	GCAGCTCTTGGGTAGCAAAT	107	
17	4.2	D1233J4 (IID9)	Present study	GTAGTGCCAGAACGGTCCAT	TTTCTTTGGGGTTCATGGTG	301	
18	4.3	D1249B11 (IIA3)	Present study	GCAGACCACCAAATCCAACT	TGGGCAATTCAAGTCAACAA	369	
19	4.4	T707	Frary *et a*l. 2005	TCGTGGATTATGGGCTTCTT	GGTAAGGCTGCAACACATCA	458	50
20	4.5	T1405	Frary *et a*l. 2005	CACCAACAACTAGCCCTTGA	AAGCAATTCCTCCAGCTTCA	535	55
21	4.6	cLEC7B23	Frary *et a*l. 2005	GGAGAACACGGCTACCTCAG	AGCTGGAAATGAGGTTTTGC	600	55
22	6.1	TG178	Frary *et a*l. 2005	AGCTTTGGACTTGGATGGTG	AGCTTTGGACTTGGATGGTG	~1630	
23	6.2[3]	D1261I18 (IB2)	Present study			78	
24	6.3	TG118	Frary *et a*l. 2005	AAACTCTCGCATGGAAGCTTAG	ACAGCTTTCCTTGACAGAATCC	~2500	
25	6.4	D1304O23 (IIIF12)	Present study	GTAGTGCCAGAACGGTCCAT	TTTGCACTGTTTGAATTTGGA	128	
26	6.5	T892	Frary *et a*l. 2005	TGGCTCTTCGGACTTTAGTGA	AGCACCTTCTGCGTTCATCT	1200	55
27	6.6	T507	Frary *et a*l. 2005	CCTTTTATCTCCTCCGGTGT	TCTGTCCACTCACATGGATCA	800	55

[1]Annealling temperature.
[2]First number is chromosome number and second number is number of marker of each chromosome selected.
[3]Primer has not designed from this DArT marker; blank: not screened yet.
SGN: SOL Genomics Network (http://www.sgn.cornell.edu/).

Plasmid sequencing and sequence analysis

Plasmid DNA was then extracted by using Plasmid DNA extraction kit (Promega, Madison, Wisconsin) and sent for sequencing (Mission Biotech, Taiwan). After sequencing, the Sequence Manipulation Suite (http://bioinformatics.org/sms/index.html) was used to analyze the clone sequences. ClustalW (http://www.ebi.ac.uk/clustalw/) was used to align the sequences of the two parental lines H7996 and WVa700 and SNP sites then were found.

Primer design and PCR amplification

Oligonucleotide primer pairs were designed by using the computer program Primer3 (Web software provided by Steve Rozen and Whitehead Institute for Biomedical Research). Traditional allele-specific primers were designed with 20 nucleotides and 3'end nucleotide of primer corresponds to the single nucleotide polymorphism (SNP) site. To obtain primers specific to the identified SNP, the fragment sequences containing the SNP site were also entered into the Web-available SNAPER program (http://ausubellab.mgh.harvard.edu/). Primers having a G+C content of approximately 50% and 18-20 nucleotides long were selected. The Tm was limited by 55 and 60°C, and primers were synthesized by MB (Mission Biotech, Taiwan). Seven primer pairs were designed from AFLP fragment sequences, three from DArT marker sequences. Six forward and two reverse primers were designed from RFLP sequences, where the 3'end nucleotide corresponds to the site of SNPs, and another 3 forwards primers were designed based on the SNAPER program (Table 2.2).

Table 2.2 List of primers designed from AFLP, DArT and RFLP clones

No.	Primer code	Primer sequence (5'-3')	Tm (°C)[1]
1	3.1-400aF[2]	AATGGTTAAACCTGCATGAG	50.7
2	3.1-400bF[2]	ATTGAATGGTTAAACCTGCATGAG	53.9
3	3.4-85aF[2]	ACAAATCAAGAGCTTCTACC	49.8
4	3.4-85bF[2]	TAGTACAAATCAAGAGCTTCTACC	52.0
5	3.6-106aR[2]	ATTTTGACAGTGGACTGCAG	53.2
6	3.6-106bR[2]	CAAATTTTGACAGTGGACTGCAG	54.9
7	4.4-426aF[3]	GCTTCTTAGACGTCATTGAT	49.9
8	4.4-426bF[3]	GGGCTTCTTAGACGTCATTGAT	54.7
9	3.1-400W1[3]	AATGGTTAAACCTGCATGTG	51.2
10	3.1-400W2[3]	AATGGTTAAACCTGCACGAG	53.2
11	3.6-106W1[3]	CAAATTTTGACAGTGGACTGTAG	52.1
12	3.6-106W2[3]	ATTTTGACAGTGGACTGCTG	53.2
13	TAFLP001.afh20b[4]	GACTGCGTACCAATTCACAGG TGGTCTTTCGAGGACTAGGTTG	61.3 62.1
14	TAFLP004.afh2a[4]	GAGTCCTGAGTAACACTAAG GTGCCCTCAGTTTGTACTTGC	56.3 61.3
15	TAFLP005.afh2b[4]	TACCAATTCAACGCATGGAG TACCAATTCAACGCATGGAG	57.4 56.3
16	TAFLP007.afh37b[4]	ATGGTTTCCAGTACGGTGGA TGCGTACCAATTCACCAAAA	58.4 54.3
17	TAFLP008.afh37c[4]	TCACCTATGGAGCCATTTCC TTGAAAGGAACCTTTGAGATTATG	58.4 58.4
18	TAFLP009.afh37h[4]	TGCGTACCAATTCACCAAAA CCTCGTGTGACGAGCATAGA	54.3 60.5
19	TAFLP011.afh23c[4]	GCGTACCAATTCACATGAGC GGTCCACATGTTGGGTGAAT	58.4 58.4
20	D1233O18[5]	GTAGTGCCAGAACGGTCGAT GCAGCTCTTGGGTAGCAAAT	55.6 56.7
21	D1233J4[5]	GTAGTGCCAGAACGGTCCAT TTTCTTTGGGGTTCATGGTG	53.2 57.1
22	D1249B11[5]	GCAGACCACCAAATCCAACT TGGGCAATTCAAGTCAACAA	52.9 55.6

[1]Melting temperature; [2]Forward primers designed from RFLP clones; [3]Reverse primers designed from RFLP clones; [4]Primers designed from AFLP clones; [5]Primer designed from DArT clones. Underline letters are SNP sites.

General PCR reactions were performed in a volume of 15µl contained 15-20ng of genomic DNA, 10X PCR buffer (10mM Tris-HCl, pH 9.0; 50mM KCl; 15mM MgCl2), 20mM dNTPs, 20µM of each forward and reverse primer and 2U of *Taq* DNA polymerase (Violet, Taiwan). The amplifications were following a general AFLP program, as previous

described in details (Chapter 1), and a touch-down program. In the general program, the amplification profile consisted of an initial denaturation for 5 minutes at 94 °C followed by 35 cycles of 45 seconds at 94°C, 45 seconds at the annealing temperature with a gradient of 45–70°C, 1 minute elongation at 72°C, and a final extension step of 10 minutes at 72°C. The amplification profile of the touch-down program consisted of an initial denaturation for 5 minutes at 94°C, twenty cycles of subsequently lowering the annealing temperature (65^{o}C) by 0.5^{o}C per cycle while keeping 94^{o}C for 30 seconds and 72^{o}C for 30 seconds; 26 cycles of 94^{o}C for 30 seconds, 50^{o}C for 30 seconds, and 72^{o}C for 30 seconds and a final extension step of 10 minutes at 72°C. The amplifications were performed with a MJ PT-200 thermocycler (MJ Research, GMI, Inc., Minnesota, USA). The PCR products were mixed with 3µl of loading buffer (5mg/ml Blue dextran in deionized formamide), and 5µl of loading buffer which contained 1µl of 100bp or 1kb ladder were loaded onto 6% of NuSieve Agarose gel and 1% general agarose gel. Gels were run in 1X TBE (0.09M Tris-borate, 0.002M EDTA) buffer with 94V for 2.5 hours. After electrophoresis, gel was stained with ethidium bromide (1.5µg/ml) for 10 minutes, de-stained in distilled water for 15 minutes and photographed under UV light.

2.2.6.3 Inverse PCR

Inverse PCR was performed as described by Bowtell's Labotary (http://www.protocol-online.org/prot/Molecular_Biology/PCR/Inverse_PCR/) with slight modifications. Approximately 1µg of genomic DNA of each parent H7996 and WVa700 was completely digested with 10µl cocktail including 4µl of 4-base cutters (20U/µl) (NEB), and 6.0µl 10X buffer (500mM NaCl, 100mM Tris-HCl, 100mM $MgCl_2$, 10mM dithiothreitol, pH 7.9) and incubated at 37^{o}C or 65^{o}C for 2 hours using a MJ PT-200 thermocycler (MJ Research, GMI, Inc., Minnesota, USA). Each 4-base cuter was treated separately as different reaction. Digestion reactions were heated to stop enzyme reaction at 65°C for 20 minutes then diluted to 50µl sterile MiliQ water and self-ligated with 50µl 10X Ligase buffer (500mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 100mM DTT, 10mM ATP) (NEB), 10µl T4 DNA ligase (NEB) (400U/µl) and 390µl sterile MiliQ water at 16^{o}C overnight. A 0.8 volume of cold isopropanol was added to each tube of ligation product to precipitate DNA. The mixture was placed at -20^{o}C for 2 hours and then centrifuged for 15 minutes at 6000rpm, and the supernatant was discarded. A volume of 500µl of 70% ethanol was then added, and again centrifuged at 6,000rpm for 30 minutes, and the ethanol discarded. The DNA was dried at room temperature. The DNA precipitate was suspended in 10µl of

sterile MiliQ water and used as template DNA to digest with 1µl of 6-base cuter, 1µl of 10X restriction digest buffer and incubate for 2 hours at 37^{o}C or 65^{o}C (depends on restriction enzymes) by using a MJ PT-200 thermocycler (MJ Research, GMI, Inc., Minnesota, USA).

The digestion was then used as DNA template for PCR to amplify unknown flanking sequence using primers covering the cloned AFLP fragment. The amplification procedure consisted of an initial denaturation for 5 minutes at 94°C and 35 cycles of 45 seconds for denaturation at 94ºC, 30 seconds for annealing at 50ºC (or 55ºC), 30 seconds for extension at 72ºC, followed by a final extension at 72ºC for 5 minutes. The amplification was separated in 1% agarose gels and 1X TBE buffer (10.8g trizma base, 5.48g boric acid, and 4ml EDTA (0.5mM)/1L of distilled water) for 1.5 to 2 hours at 96V. After electrophoresis, gels were stained with ethidium bromide (1.5µg/ml) for 10 minutes, de-stained in distilled water for 15 minutes and photographed under UV light.

2.2.6.4 Randomly amplified microsatellite polymorphism (RAMP)

The RAMP PCR was performed as described by Wu *et al*. (1994) with modifications. Sixteen primers with GA and CA repeats were synthesized by MB (Mission Biotech, Taiwan) (Table 2.3) and tested for their potential for repeat polymorphism. The PCR solution was composed of 15-20ng of genomic DNA, 10X PCR buffer (10mM Tris-HCl, pH 9.0; 50mM KCl; 15mM MgCl2), 20mM dNTPs, 20µM RAMP primer and 20µM covering-AFLP-marker primer and 2U of *Taq* DNA polymerase (Violet, Taiwan). PCR reactions were performed in a MJ PT-200 thermocycler (MJ Research, GMI, Inc., Minnesota, USA). The amplification profile consisted of an initial denaturation for 5 minutes at 94°C followed by 35 cycles of 45 seconds at 94°C, 30 seconds at the annealing temperature 40–60°C (using temperature gradient), 30 seconds elongation at 72°C, and a final extension step of 7 minutes at 72°C.

Table 2.3 Randomly amplified microsatellite polymorphism primers

No.	Primer sequence	Tm (^{o}C)[1]
1	GT$(GA)_4$	17.9
2	GC$(GA)_4$	22
3	TC$(GA)_4$	17.9
4	TG$(GA)_4$	17.9
5	CG$(GA)_4$	22
6	AC$(GA)_4$	17.9
7	AG$(GA)_4$	17.9
8	CT$(GA)_4$	17.9
9	GT$(CA)_4$	17.9
10	GC$(CA)_4$	22
11	TC$(CA)_4$	17.9
12	TG$(CA)_4$	17.9
13	CG$(CA)_4$	22
14	AC$(CA)_4$	17.9
15	AG$(CA)_4$	17.9
16	CT$(CA)_4$	17.9

[1]Melting temperature

2.3 RESULTS

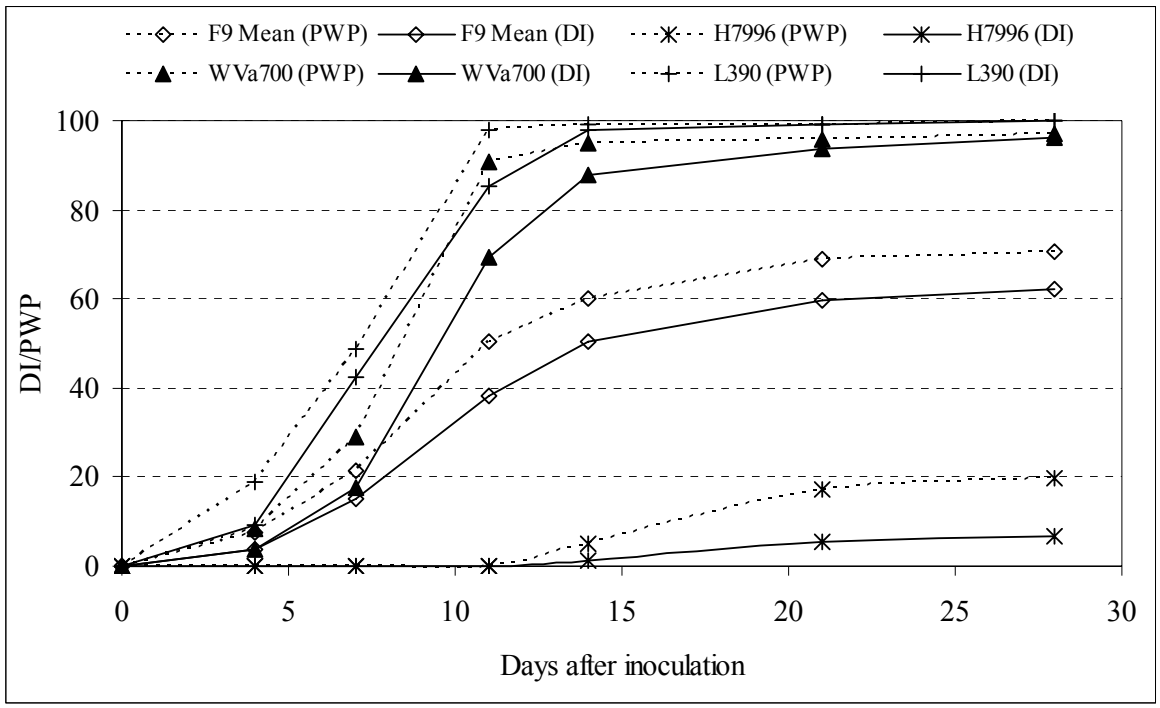
2.3.1 Resistance to strain Pss4 and Pss186 in F_9 RILs

The RIL population was evaluated at seedling stage on their variation of resistance to strains Pss4 and Pss186. Severe wilting of L390 plants, the susceptible control, indicated the environment and the inoculation were suitable for the disease development with both Pss4 and Pss186 (Figure 2.3). The severity caused by both strains on WVa700 was slightly lower than that on L390.

In general, disease progressed most rapidly between 4 and 14 days after inoculation. For example, 4 days after inoculation with Pss4, percentage of wilted plants ranged from 3.75 to 9.38 on WVa700 and L390, respectively. At 11 DAI, nearly 100% of two genotypes displayed wilting and disease index reached near maximum (Figure 2.3). About 80% (inoculated with the strain Pss4) and 95% (inoculated with the strain Pss186) of H7996 remained healthy at 28 DAI. On the contrary, the susceptible line WVa700 had only 3.1% survival when inoculated with Pss4 and 13.5% with Pss186, while almost L390 plants were wilted at 28 days after inoculation with Pss4 (0.2% survival) and only 6.2% remained healthy plants with Pss186. Pss4 caused faster and more severe disease development than Pss186. Resistance in H7996 was stable to strain Pss186 than Pss4 (Figure 2.3). By the end of the test, F_9 population showed an intermediate level of resistance by the strain Pss186 (53% of plants were healthy) and a lower level of resistance by the strain Pss4 (30% of plants were healthy). The two trials were conducted with only a week apart, and in the same net-house with similar temperature and relative humidity profile. Thus, differences in symptom development of the two strains suggest variation in their virulence. The higher virulence observed on Pss4 agreed with Jaunet and Wang (1999).

Significant effects of strain, entry and strain x entry were detected with all the 4 processed data (Table 2.4). The inconsistent entry rank between strains and the magnitude difference indicated the significant effect of strain x entry. Such significant effect of genotype x strain could complicate our further mapping analysis.

A; Pss4



B; Pss186

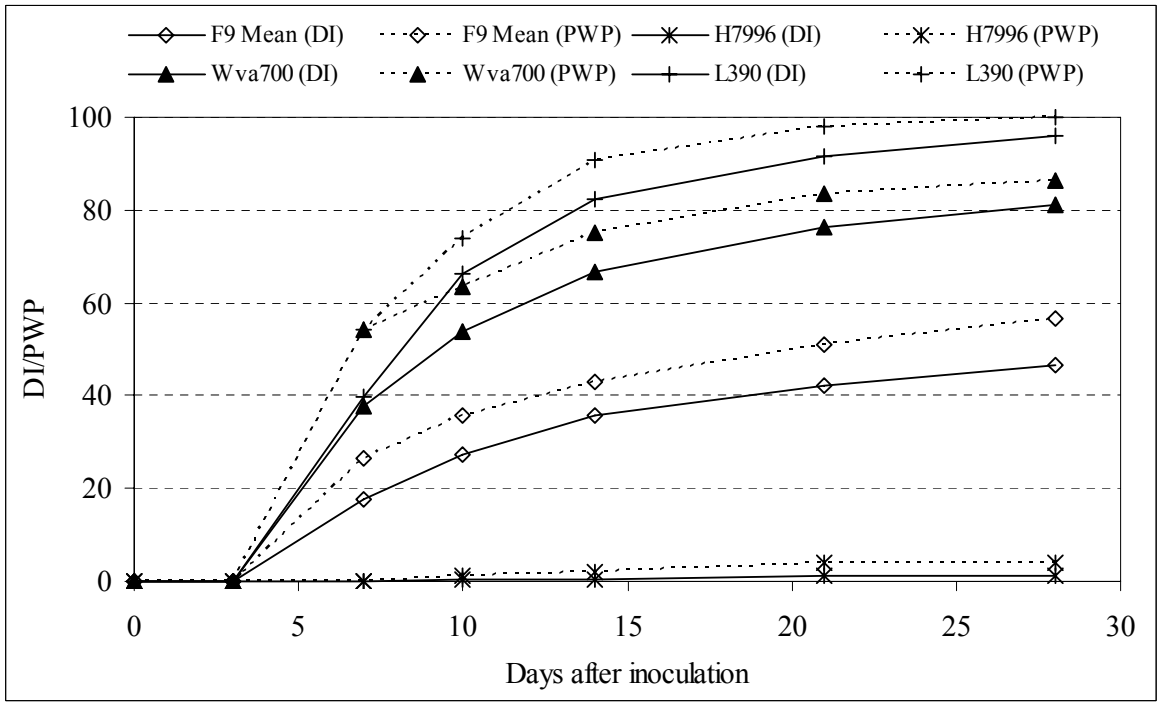


Figure 2.3 Severity of bacterial wilt expressed as diseased index (DI) (continuous lines) and percentage of wilted plants (PWP) (dashed lines) after inoculation with Pss4 (A) and Pss186 (B) in H7996 (resistant), WVa700 (susceptible), F_9 population mean and L390 (control check).

The frequency distribution of RILs with different severity appeared differently between the two strains in the F_9 population (Figure 2.4). A greater number of F_9 families was closer to the susceptible parent when inoculated with Pss4. RAUDPC-calculated from PWP, DI and PWP of F_9 population inoculated with Pss186 followed a normal distribution pattern, whereas RAUDPC calculated from disease index was skewed toward resistance. With strain Pss4, distribution of DI and PWP were skewed toward the susceptible side, while RAUDPC calculated from DI and PWP was distributed normally. Because of the different distributions observed with four kinds of processed data, different QTLs could be detected when using different processed data. Thus, the four data procession methods can be used in QTL mapping. Common QTL identified for the four methods could be more important.

Table 2.4 Combined analyses of variance of the effects of strain (S; Pss4 and Pss186), entry (E; 188 RILs and two parents) and S x E on percentage of wilted plants, disease index and RAUDPC

Source of variation	DF	MS			
		RAUDPC-DI	RAUDPC-PWP	DI	PWP
Strain (S)	1	2.9540**	2.9167**	9.3166**	8.1535**
Entry (E)	190	0.6090**	0.4238**	0.4654**	0.4362**
E x S	190	0.0864**	0.0717**	0.0699**	0.0704**

**: significant at $P < 0.01$.
RAUDPC-DI: relative area under disease progress curve calculated from DI; RAUDPC-PWP: relative area under disease progress curve calculated from PWP. Both were log-transformed; DI: disease index at 28 days after inoculation; PWP: percentage of wilted plants at 28 days after inoculation. Both were transformed to arcsine of the square root for analysis. MS: mean square; DF: degree of freedom.

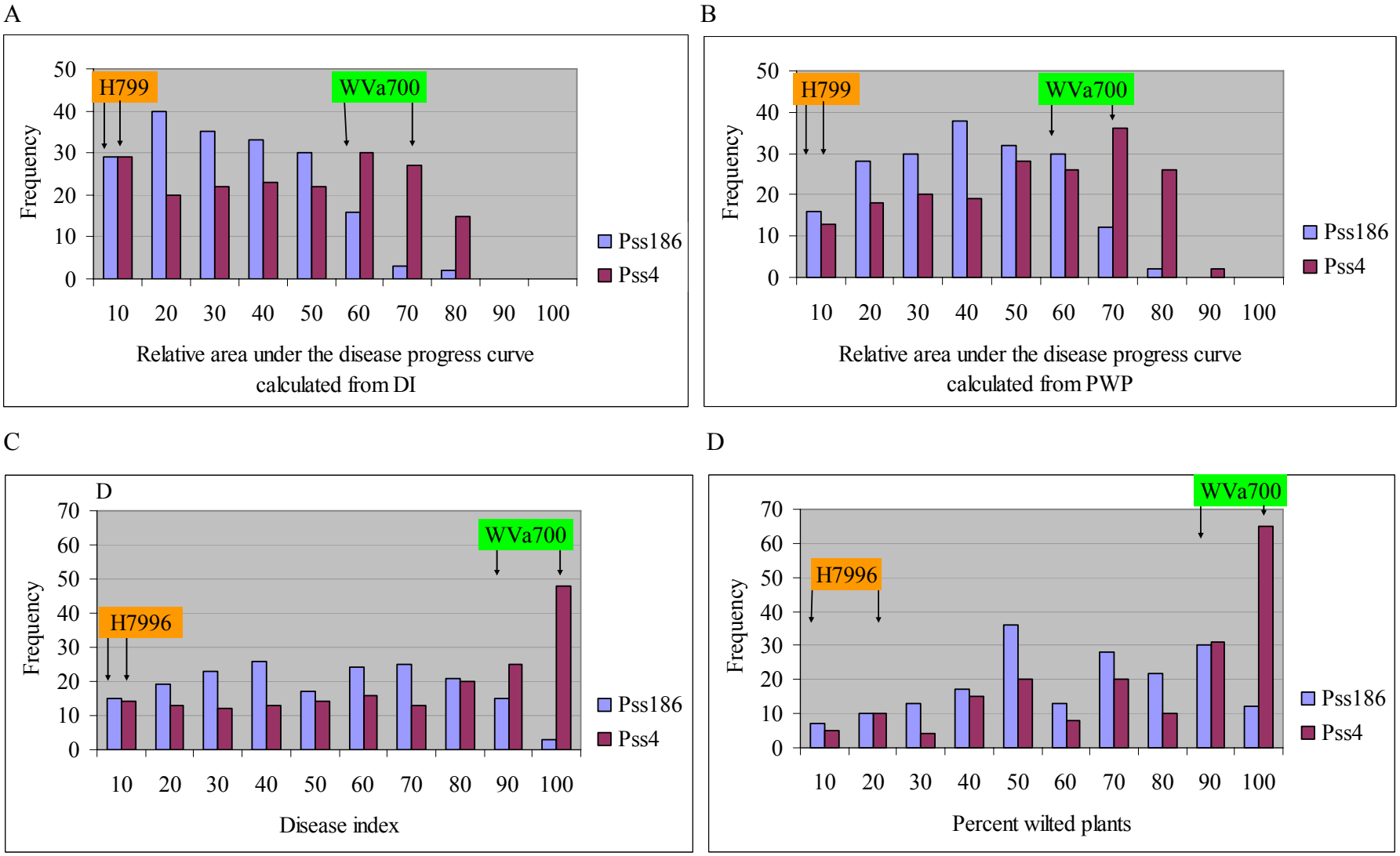


Figure 2.4 Frequency distribution of relative area under the disease progress curve (RAUDPC) calculated from disease index (RAUDPC-DI) (A); RAUDPC calculated from percentage of wilted plants (RAUDPC-PWP) (B); disease index (C); and percentage of wilted plants (D) in F_9 populations after inoculated with Pss4 and Pss186. Arrows indicate the locations of H7996 and WVa700.

2.3.2 Colonization by the pathogen in F_9 RILs

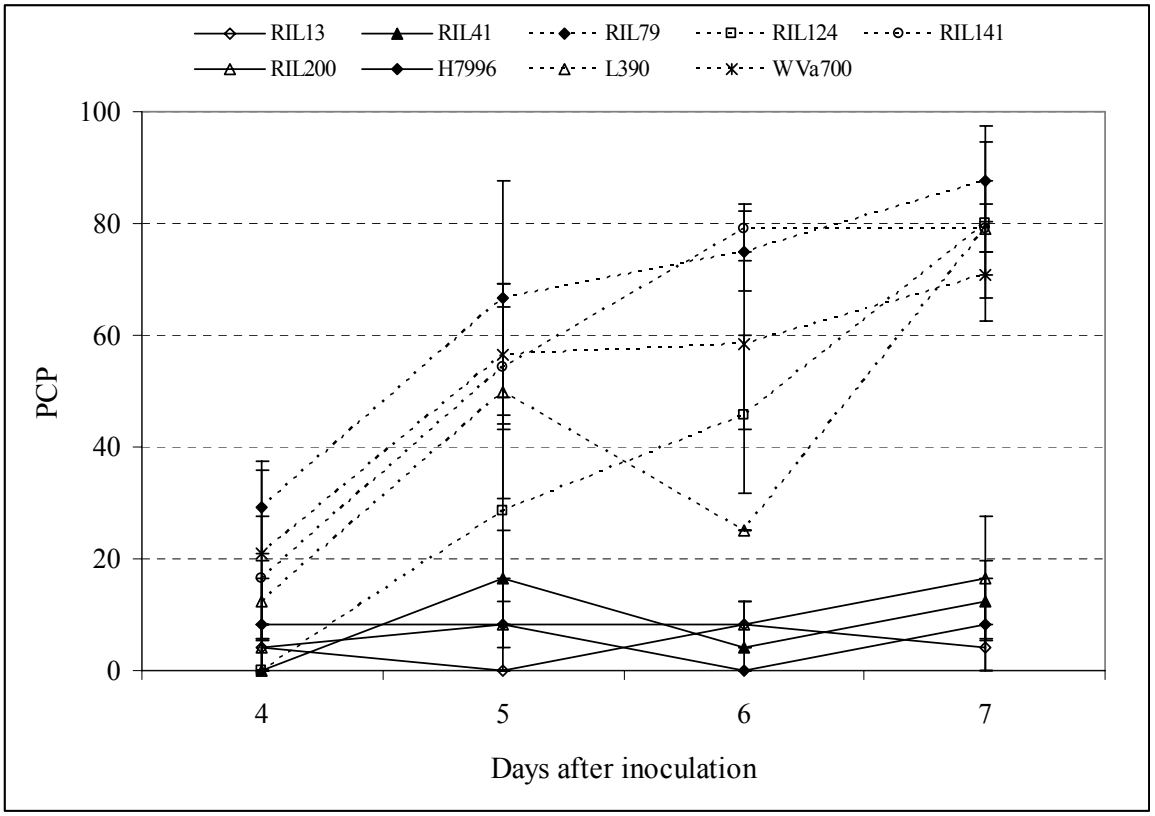
2.3.2.1 Protocol development

When inoculated with strain Pss4, differences on the percentage of colonized plants (PCP) between the resistant and susceptible plant genotypes were obvious at 5, 6, and 7 days after inoculation. Means of PCP on H7996, RIL13, 41, and 200 ranged from 0 to near 20% during the observation period, while that on L390, WVa700, RIL79, 124 and 141 increased from 12.5% to 88.7%. Since the difference between the two groups was the largest and the standard error was the lowest at 6 days after inoculation when inoculating with strain Pss4 (Figure 2.5 A), it was decided that the sampling time would be at 6 DAI for detemining the percentage of colonized plants of the entire RIL population.

A similar progress on pathogen colonization was observed in the tested genotypes, when inoculated with Pss190 (Figure 2.5 B). However, the degree of colonization was similar between the resistance and susceptible groups. At 6 DAI, the pathogen presence in resistant parent H7996 was similar to susceptible parent WVa700 and the susceptible check with 62.5% of colonized plants.

Colonization maximized after 6 days inoculated in the three susceptible lines, which ranged from 83.3% to 95.8%; and the two resistant RIL13 and 200 had high percentage of colonized plants 83.3% and 70.8%, respectively. RIL41 had the lowest percentage of colonized plants with 31.0%. The high degree of colonization by Pss190 on H7996 and other resistant RILs confirms the high aggressiveness of the strain Pss190 (Jaunet and Wang, 1999). And the idea of evaluating the entire RIL population on colonization by Pss190 was dropped.

A; Pss4



B; Pss190

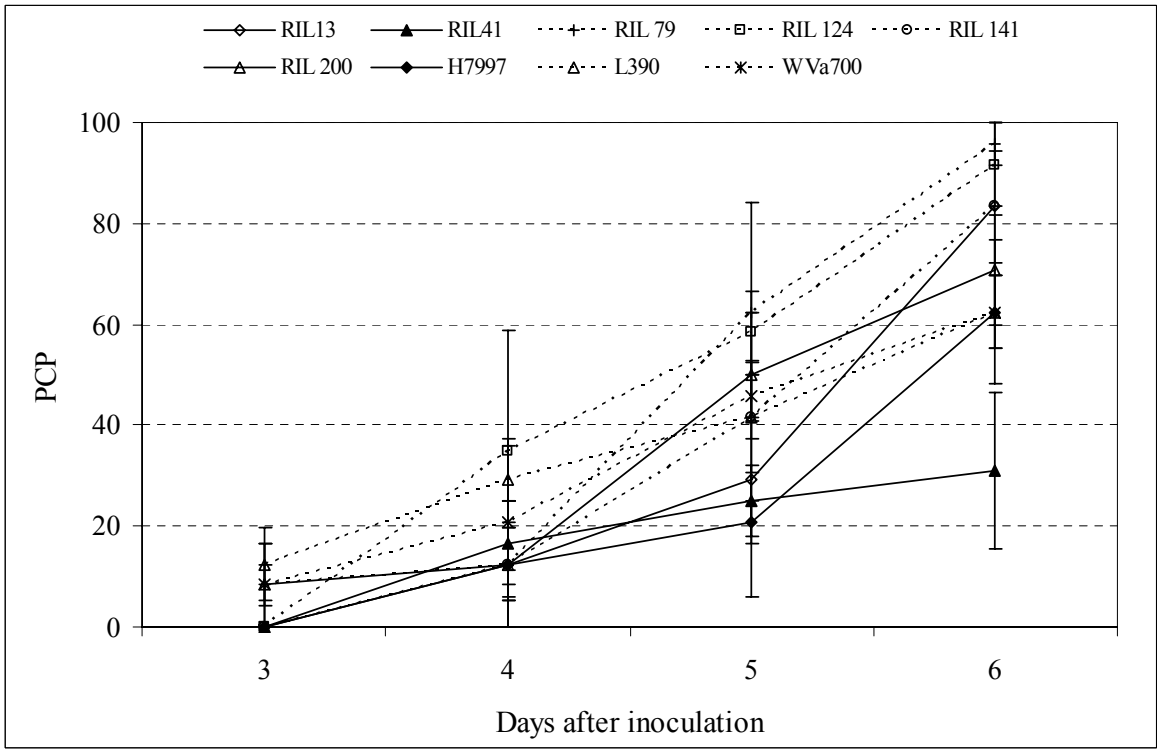


Figure 2.5 Changes of percentage of colonized plants (PCP) of selected RILs, H7996, WVa700 and L390 when inoculated with Pss4 (A) and Pss190 (B).

2.3.2.2 Colonization by strain Pss4 in F_9 RILs

Colonization by Pss4 in the F_9 RILs was evaluated at 6 DAI following the developed protocol. *R. solanacearum* was detected in 12.5% of symptomless H7996 plants, whereas it was detected in 56.3% of the symptomless WVa700 plants, and 81.3% of the symptomless L390 plants. Thus, the condition for the disease development was similar to the condition during the development of the protocol. The percentage of colonized plants of the RILs ranged between 0% and 100%. The pathogen was not detected in four RILs 26, 125, 160, and 162. The frequency distribution of the RILs was illustrated in Figure 2.6. Although a greater number of F_9 families were towards the susceptible parent side, the distribution resembles a bimodal with peaks at 20% and 90%.

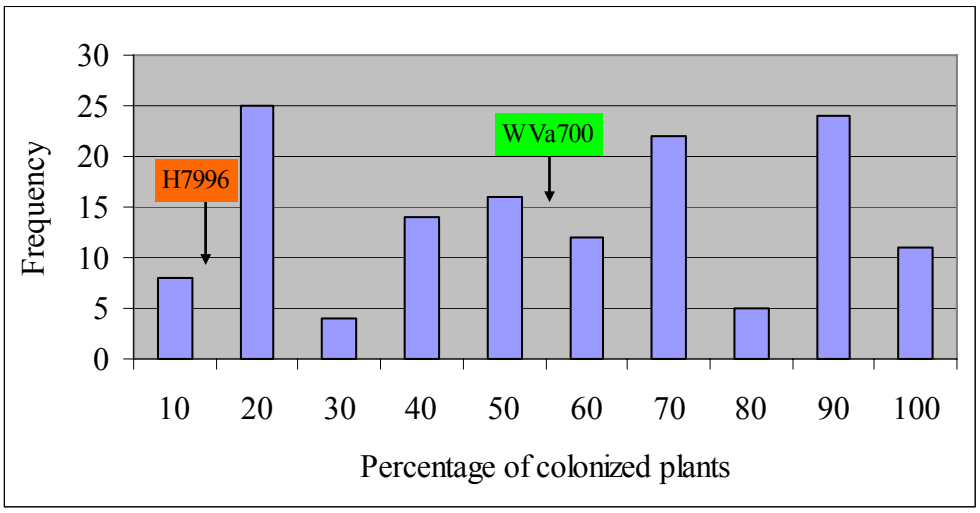


Figure 2.6 Frequency distribution of percentage of colonized plants in F_9 RILs population when inoculated with Pss4. Arrows indicate the locations of H7996 and WVa700.

2.3.3 Morphological trait distribution

2.3.3.1 Sympodial index (SPI)

The susceptible parent WVa700 had a SPI of 2.9 and intended to be indeterminate, whereas the resistant parental line H7996 was semi-determinate with a SPI of 2.5. In the F_9 RILs, SPIs ranged between 1.5 and 3.2 and the frequency distribution of SPI indicated a peak at 3 (Figure 2.7).

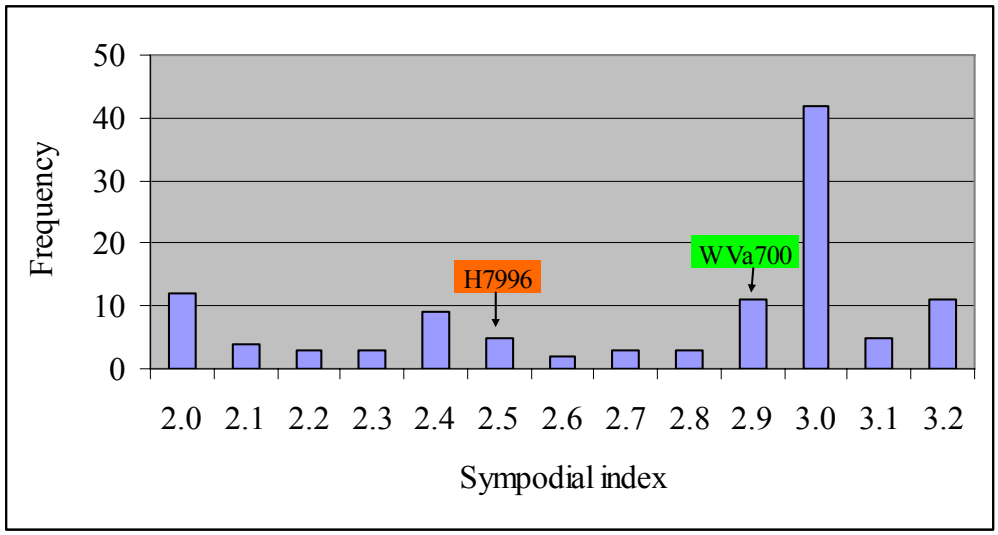


Figure 2.7 Frequency distribution of sympodial index. Arrows show locations of parents H7996 and WVa700.

2.3.3.2 Fruit weight

Large difference in fruit size was observed in the two parents in this study (Figure 2.9). An average of 33.8g fruit was obtained in the resistant parental line H7996, whereas 2.3g fruit in the susceptible parental line WVa700 as illustrated in Figure 2.8. Fruit weight of the RILs ranged from 3.46g to 28.30g with an average of 10.62g. Distribution of the fruit weight of mapping population was normal and skewed toward the small fruit parent.

Figure 2.8 Fruit size of the two parental lines H7996 (left) and WVa700 (right).

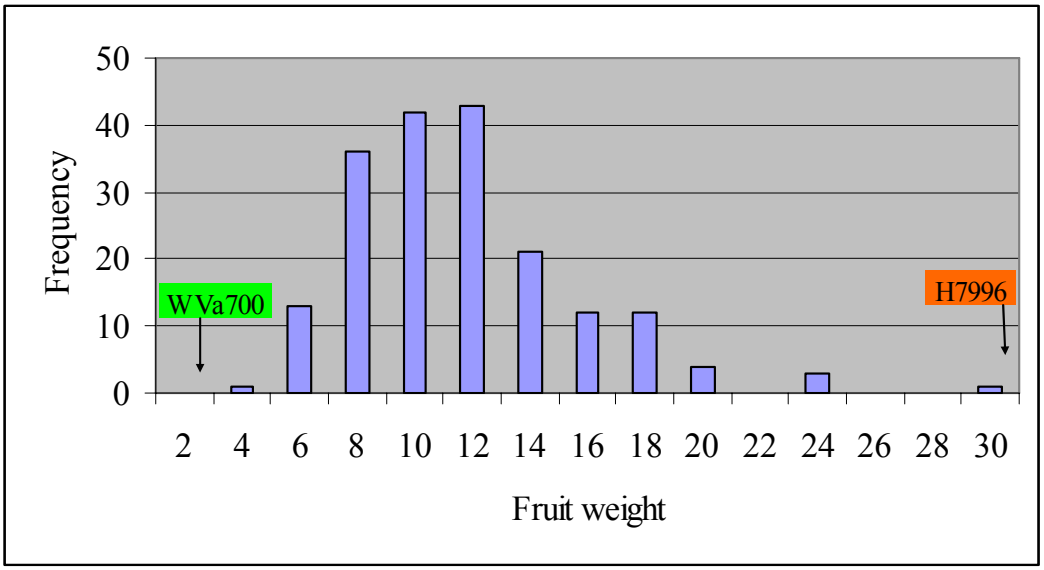


Figure 2.9 Frequency distribution of fruit weight. Arrows show locations of parents H7996 and WVa700.

2.3.3.3 Skin color

Tomato color is a combination of skin and flesh color. Red tomatoes have a yellow skin and red flesh and contain most carotenes in the form of lycopene. In the colorless, which are usually pink, precursors of phytoene and phytofluene, and minor pigment such as beta-carotene, zeta-carotene and neurosporene are present in lesser amount (Brandt *et al.* 2006; Sacks and Francis, 2001). Figure 2.10 illustrates skin color of the two parental lines observed in this study. Out of 188 RILs, 97 RILs had yellow skin as H7996, and 51 RILs had transparent skin as WVa700. The remaining RILs, plants had fruits with either yellow or transparent skin color.

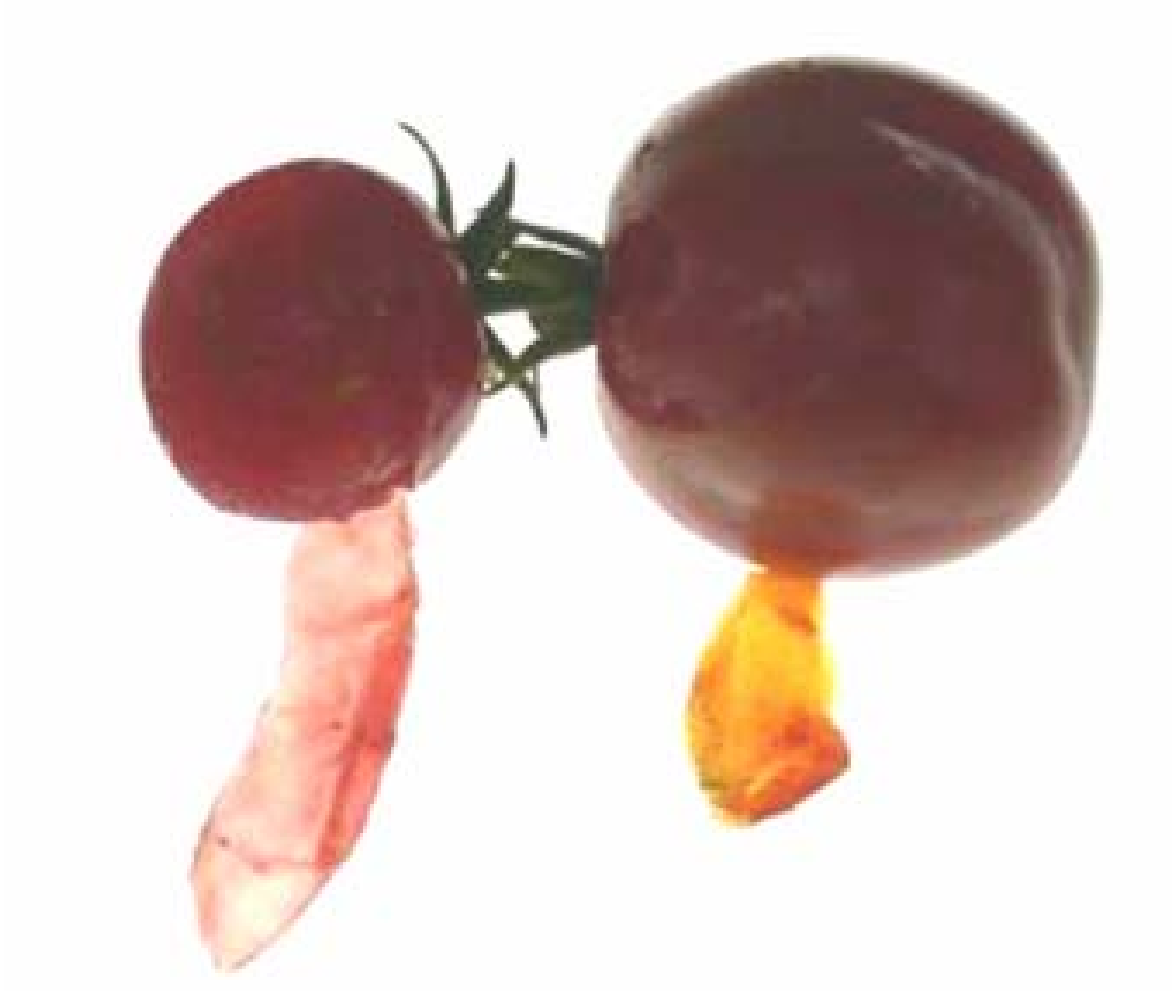


Figure 2.10 Skin colors of the two parental lines H7996 (right) and WVa700 (left).

2.3.3.4 Fruit quality

Citric acid

The citric acid value of 0.55 was obtained from H7996; meanwhile 0.45 was gained from WVa700. Citric acid of the RILs ranged from 0.24 to 0.77 with an average of 0.48 and followed a normal distribution (Figure. 2.11A).

Fruit pH value

Fruits of H7996 had a relatively lower pH (3.97) than fruit of WVa700 (4.47). Among the RILs, the fruit pH ranged between 3.94 and 4.91 with an average of 4.39, and exhibited a normal distribution (Figure 2.11B).

Soluble solid content

A higher soluble solid content was observed in the susceptible parent line WVa700 with 6.1^{o}Brix, whereas the resistant parent H7996 had 4.9^{o}Brix. In the F_9 RIL population, soluble solid content ranged from 3.8^{o}Brix to 7.9^{o}Brix with an average of 5.71^{o}Brix, and exhibited a continuous distribution (Figure 2.11C).

Color value (a/b)

Fruit color is one of the most important factors affecting fruit appearance in tomato. It is determined by the fruit flesh and skin color. The fruit color in this study was measured based on a/b ratio. The parent H7996 had higher color value (1.58) compared to WVa700 (1.19) indicating fruit color of H7996 is redder than WVa700. This is because H7996 had yellow skin, whereas WVa700 had colorless skin. The color of the F_9 RIL population ranged from 0 to 2.01 and the frequency distribution of the color shown in Figure 2.11D is nearly normal.

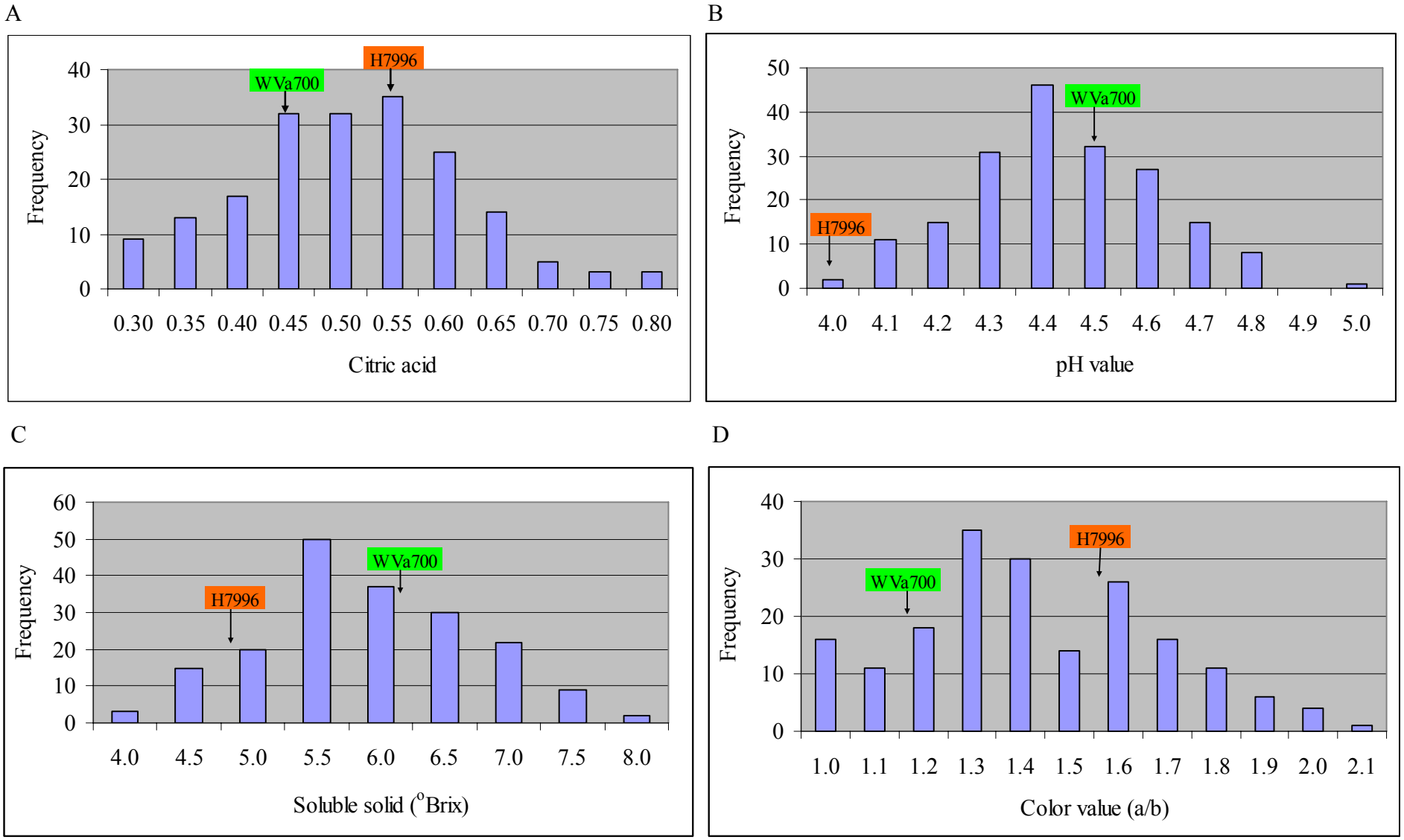


Figure 2.11 Frequency distribution of fruit quality: Citric acid (A); pH value (B); Soluble solid (C); Color value (D). Arrows indicate locations of parents H7996 and WVa700.

2.3.4 Correlation among traits

Beside the nine traits collected in this study, results from 13 additional resistance tests at different locations were kindly provided from Dr. Jaw-Fen Wang, AVRDC and Dr. Wang's collaborators. All the traits used are summarized in Table 2.5 and were used to analyse QTLs for bacterial wilt resistance and to evaluate whether the genetic control for internal pathogen multiplication and movement is similar to visual symptom severity.

Correlation coefficients between all traits (bacterial wilt resistance and morphological traits) are presented in Table 2.6. Visual symptoms of most datasets were significantly correlated in a positive manner with each other, excluded PH1. Colonization by Pss4 (TW3) observed in the mid-stem was significantly correlated with all visual symptom datasets, excluded RN2, which was evaluated with JT516, a race 3 strain isolated from potato, whereas Pss4 is a race 1 strain isolated from tomato. Strongest positive correlation between colonization and visual symptom was TW2 (r = 0.70). As both tests were evaluated with Pss4, this indicates that visual symptom development is highly depending on the colonization and later multiplication of the pathogen. Positive correlation between colonization and PH1 was poorest with r = 0.17 and no correlation between colonization and RN2.

Significant correlation between the disease reactions and morphological traits was not consistant among datasets. Symposial index was not significantly correlated with any disease reaction datasets. FW was significantly correlated with IN1 (r = 0.21), RN2 (r = 0.21), TH2 (r = 0.17) and TH4 (r = 0.14). Significant negative correlations were observed between CA and ID2 (r = -0.18) and RN2 (r = -0.27), whereas significant positive correlation was observed between SSC and PH2 (r = 0.16), pH and RN2 (r = 0.20), and FC and RN2 (r = 0.31), TW1 (r = 0.20), TW2 (r = 0.15), TH2 (r = 0.16), TH3 (r = 0.20).

Among the morphological traits, the strongest negative correlation between CA and pH value with r = -0.81. SPI was positively correlative with SSC (r = 0.34). A few correlations were significant between fruit weight and fruit quality. FW was negatively correlated with CA (r = -0.19) and SSC (r = -0.27), whereas positively correlated with FC (r = 0.20). CA was positively correlated with SSC with r = 0.19 while negatively correlated with pH (r = -0.81) and FC (r = -0.33). Negative correlation was observed between SSC and FC (r = -0.23). On the contrary, pH was positively correlated with FC.

Table 2.5 Trial summary and trait code of traits analysed in the recombinant inbred line population

	Code	Trait name	Country	Strain used and placed evaluated	Rating[1]	Generation
1	IN1	IIHR	India	natural population (race 1, Bv3?); Banglore (farm)	DI	F_9
2	IN2	IIHR2	India	natural population (race 1, Bv3?); Banglore (farm)	DI	F_9
3	ID1	INDO-EW	Indonesia	natural population and Ps18 (eggplant, race 1, Bv3); EW (farm)	DI	F_9
4	ID2	INDO-EW2	Indonesia	natural population and Ps18 (eggplant, race 1, Bv3 and Bv5); EW (farm)	DI	F_9
5	PH1	TM22-SH	Philippine	TM22 (race 1); IPB, UPLB (screenhouse)	SC	F_6
6	PH2	TM151-SH	Philippine	TM151(race 1); IPB, UPLB (screenhouse)	SC	F_6
7	RN1	JT519	Reunion	JT519 (*Pelargonium asperum*, race 1, Bv3); CIRAD (screenhouse)	DI	F_8
8	RN2	JT516	Reunion	JT516 (potato, race 3, Bv2); CIRAD (screenhouse)	DI	F_8
9	TW1	Pss186-SH	Taiwan	Pss186 (tomato, race 1, Bv4); AVRDC (screenhouse)	SC	F_9
10	TW2	Pss4-SH	Taiwan	Pss4 (tomato, race 1, Bv3); AVRDC (screenhouse)	SC	F_9
11	TW3	Colonization	Taiwan	Pss4 (tomato, race 1, Bv3); AVRDC (screenhouse)		F_9
12	TW4	TW-TSS	Taiwan	natural population (race 1, Bv3/4); TSS (farm)	DI	F_9
13	TH1	TH-MP	Thailand	(tomato, race 1, Kanchanaburi); Marco Polo Seeds farm	SC	F_9
14	TH2	SC6-2	Thailand	SC6 (race 1, Bv3); EW (farm)	DI	F_9
15	TH3	Syngnt-THAI	Thailand	Syngenta (farm)	DI	F_9
16	TH4	THAI-EW	Thailand	natural population (race 1), EW (farm)	DI	F_9
17	SPI	Sympodial index	Taiwan	AVRDC (farm)		F_9
18	FW	Fruit weight	Taiwan	AVRDC (farm)		F_9
19	CA	Citric acid	Taiwan	AVRDC (farm)		F_9
20	SSC	Soluble solid	Taiwan	AVRDC (farm)		F_9
21	pH	pH value	Taiwan	AVRDC (farm)		F_9
22	FC	Fruit color (a/b)	Taiwan	AVRDC (farm)		F_9

[1]DI: disease incidence; SC: severity score

Table 2.6 Correlation between the 22 traits used (bacterial wilt resistance and morphological traits). See Table 2.5 for trait abbreviation

	IN1	IN2	ID1	ID2	PH1	PH2	RN1	RN2	TW1	TW2	TW3	TW4	TH1	TH2	TH3	TH4	SPI	FW	CA	SSC	pH
IN2	.24*																				
ID1	.44**	.38**																			
ID2	.27**	.18*	.61**																		
PH1	-.05	.06	.13	.11																	
PH2	.24**	.21**	.58**	.25**	.20**																
RN1	.34**	.37**	.65**	.40**	.15	.53**															
RN2	.20	.16	.35**	.12	.07	.28**	.26**														
TW1	.34**	.27**	.71**	.39**	.09	.52**	.63**	.32**													
TW2	.44**	.39**	.84**	.47**	.13	.58**	.65**	.28**	.73**												
TW3	.35**	.38**	.64**	.42**	.17*	.41**	.54**	.19	.63**	.70**											
TW4	.49**	.31**	.74**	.49**	.09	.46**	.66**	.45**	.60**	.66**	.53**										
TH1	.36**	.36**	.65**	.35**	.08	.47**	.54**	.21*	.63**	.69**	.56**	.59**									
TH2	.39**	.30**	.57**	.25**	.11	.43**	.56**	.34**	.57**	.62**	.43**	.61**	.50**								
TH3	.44**	.33**	.81**	.43**	.15*	.52**	.63**	.32**	.67**	.78**	.60**	.64**	.61**	.59**							
TH4	.53**	.39**	.76**	.47**	.16*	.51**	.64**	.35**	.62**	.74**	.59**	.76**	.58**	.58**	.71**						
SPI	-.11	.14	.07	.08	.13	.13	.24	-.05	.15	.03	.03	.10	.07	.04	.12	.09					
FW	.21*	.03	.06	.01	-.05	.02	.11	.21*	.13	.13	.06	.14	-.01	.17*	.12	.14*	-.11				
CA	.057	.04	-.09	-.18*	-.03	.01	-.14	-.27**	-.06	-.03	-.03	-.13	-.08	-.04	-.12	-.12	-.14	-.19**			
SSC	-.16	.11	.03	.03	.04	.16*	.18	.01	-.03	.00	.03	.02	-.03	-.01	-.02	.07	.34**	-.27**	.19**		
pH	-.14	-.08	-.02	.12	.11	.00	.08	.20*	-.04	-.05	-.05	.03	.02	.01	.04	.09	.17	.04	-.81**	.08	
FC	.06	.11	.21	.07	.04	.03	.08	.31**	.20**	.15*	.09	.18	.10	.16*	.20**	.14	.11	.20**	-.33**	-.23**	.23**

*significant at $P < 0.05$; **significant at $P < 0.01$

2.3.5 QTL detection

Composite interval mapping identified a number of genomic regions significant for QTL associations for bacterial wilt resistance and morphological traits. The symbol used to identify the QTL for bacterial wilt resistance is "*bwr*" followed by linkage group code and sequential number of QTLs in the same linakge group. QTLs for morphological traits were name with trait code given in Table 2.5, followed by a number indicating linkage group code. Their map positions are shown in Figure 2.12. A total of 37 QTLs has been detected and are listed in Table 2.7. Out of 37 QTLs detected, 31 QTLs was identified for bacterial wilt resistance, one for sympodial index, two for citric acid, two for soluble solid content and one for fruit color (a/b). The phenotypic variation explained between 5.0% and 34.7%. The LOD ranged from 2.7 to 10.6.

2.3.5.1 QTLs linked to bacterial wilt resistance

The 31 QTLs detected were associated with BW resistance against race 1 strains present in Indonesia (6 QTLs), Philippine (1 QTL), Reunion (3 QTLs), Taiwan (11 QTLs) and Thailand (9 QTLs), as well as against race 3 strain present in Reunion (1 QTL). Alleles from the susceptible parent WVa700 of the QTLs located on chromosome 1 and 2 contributed to the resistant phenotype, whereas all QTLs on chromosome 3, 6, 8 and LGA, alleles from the resistant parent H7996 distributed to the resistant phenotype. Percentage of phenotypic variation explained by each of the QTL varied from 5.3% to 34.7%.

The presence of QTL *bwr1* located on chromosome 1 between the markers afh38b and afh21b was detected for resistance against a Reunion strain JT519 but not JT516 confirming strain-specific resistant QTLs presented in the F_9 RIL population. Similarly, QTL *bwr3* on chromosome 3 between markers afh34a and afh10b presented for resistance against a Filipino strain TM151 but not TM22. QTL *bwr6.8* in vicinity of RFLP marker TG153F8 on chromosome 6 has similar expression—resistance against a Taiwanese strain Pss4, but not Pss186. On contrary, the QTL on chromosome 8, *bwr8.1*, between markers afh23a and D1233H12 was detected for resistance against a Taiwanese strain Pss186, but not Pss4. These QTLs could be related to strain specificity of the resistance.

Table 2.7 QTLs detected in association with bacterial wilt resistance and morphological traits from composite interval mapping

No.	Trait code	QTL	Chromosome	Location (CI)[1]	LOD[2]	R^2 (%)[3]	a[4]
1	RN1	*bwr1*	1	0 (0-9.3)	3.1	8.3	-5.9
2	CA	*ca1*		22 (11-31)	2.6	5.0	0.0
3	ID1	*bwr2.1*	2	32 (23-34)	3.1	5.3	-6.6
4	TW2	*bwr2.2*		30 (21-44)	5.5	11.4	-7.7
5	TW4	*bwr2.3*		28 (17-51)	2.7	6.0	-6.4
6	PH2	*bwr3*	3	2 (0-16)	4.5	10.2	6.7
7	ID1	*bwr6.1*	6	115 (109-124)	10.6	23.9	13.8
8	ID1	*bwr6.2*		137 (127-150)	3.3	8.2	9.0
9	ID2	*bwr6.3*		121 (114-136)	7.0	23.0	5.3
10	RN1	*bwr6.4*		123 (115-143)	6.6	23.8	10.5
11	RN1	*bwr6.5*		135 (114-143)	7.0	34.7	12.7
12	RN2	*bwr6.6*		123 (111-138)	3.1	12.8	5.7
13	TW1	*bwr6.7*		117 (106-127)	5.8	15.5	7.1
14	TW2	*bwr6.8*		115 (101-125)	6.0	13.7	8.3
15	TW3	*bwr6.9*		121 (108-145)	3.2	9.0	4.5
16	TW4	*bwr6.10*		117 (107-126)	3.3	10.3	9.4
17	TW4	*bwr6.11*		139 (131-146)	8.5	25.8	13.3
18	TH2	*bwr6.12*		65 (53-74)	4.8	14.1	8.1
19	TH2	*bwr6.13*		117 (105-138)	3.2	8.8	6.4
20	TH3	*bwr6.14*		135 (127-149)	3.1	10.1	7.1
21	TH4	*bwr6.15*		117 (109-125)	8.3	22.7	13.7
22	TH4	*bwr6.16*		139 (135-150)	3.2	10.9	10.6
23	CA	*ca6*		119 (109-137)	3.5	11.2	0.0
24	FC	*fc6*		74 (63-87)	5.4	16.4	0.1
25	TW1	*bwr8.1*	8	44 (35-49)	3.0	5.7	4.3
26	TH1	*bwr8.2*		44 (35-48)	4.1	8.0	6.5
27	SSC	*ssc9*	9	39 (26-50)	5.6	18.7	0.4
28	SPI	*spi10*	10	0 (0-6)	5.8	19.4	-0.2
29	SSC	*ssc11*	11	157 (156-160)	4.1	9.7	-0.4
30	ID1	*bwra.1*	LGA	8 (3-9)	8.2	13.9	11.1
31	ID1	*bwra.2*		15 (10-17)	8.1	16.7	11.8
32	TW2	*bwra.3*		8 (5-9)	9.8	17.7	10.0
33	TW2	*bwra.4*		15 (10-17)	9.4	20.1	10.4
34	TW4	*bwra.5*		6 (0-9)	4.6	8.8	7.8
35	TH2	*bwra.6*		9 (1-17)	3.5	6.5	5.4
36	TH4	*bwra.7*		8 (2-9)	6.5	12.3	10.7
37	TH4	*bwra.8*		13 (9-17)	5.8	12.8	10.7

[1]The most likely location of the QTL (location) is indicated in cM from the top of the linkage group, followed by the confidence interval (CI) of this location.
[2]LOD: Maximum value of the log-likelihood in the marker interval.
[3]R^2 (%): Partial coefficient of determination, i.e., percentage of phenotypic variation explained by the QTL calculated by QTL CARTOGRAPHER.
[4]a: Additive effect; "-" signs indicate alleles effected the trait were carried in susceptible parent WVa700; the rest value without sign "-" indicate alleles were carried in resistant parent H7996.

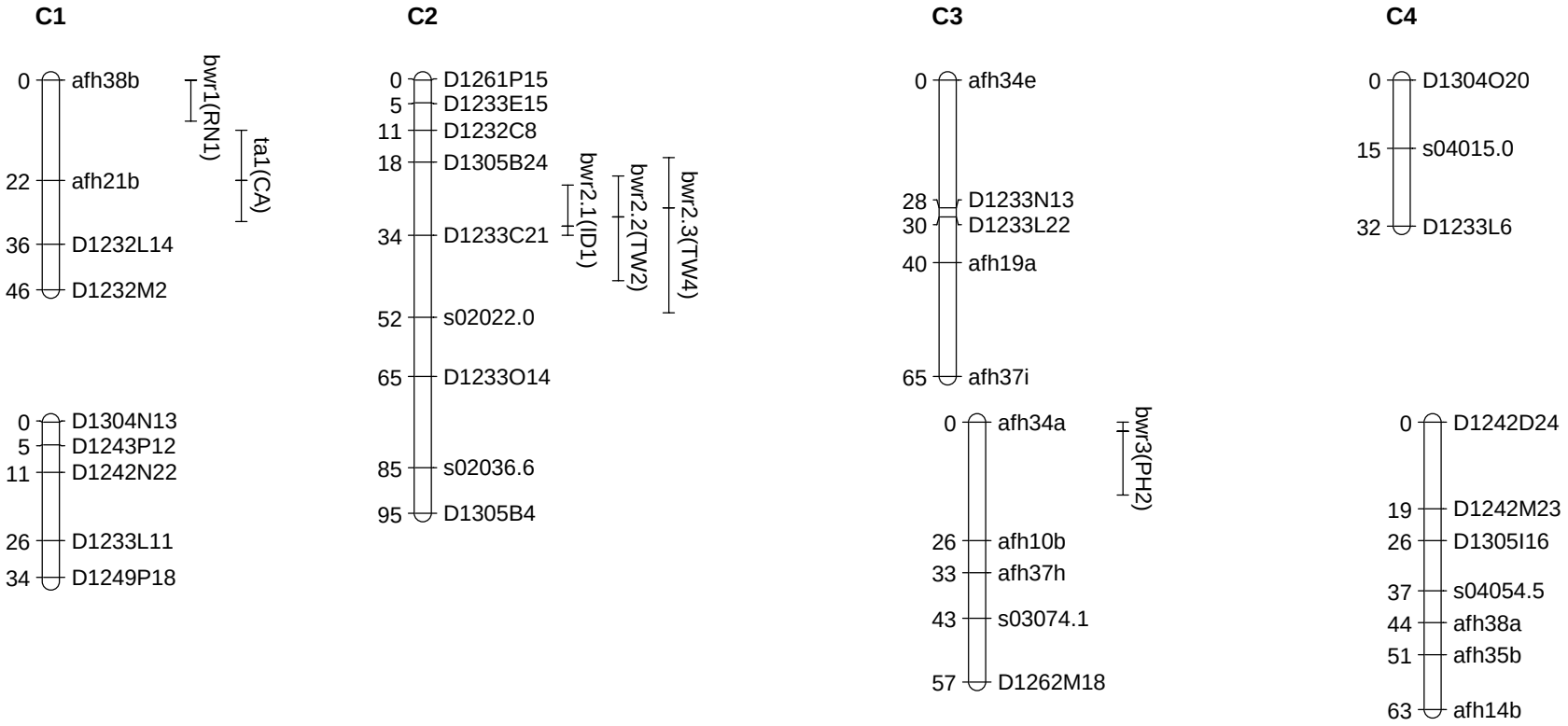


Figure 2.12 Map location of the QTLs associated with bacterial wilt resistance and morphological traits in the F_9 RIL population. The QTL position together with its confidence interval are presented in the right of linkage groups and indicated by horizontal lines. Trait codes are in brackets (see table 2.5 for trait abbreviation).

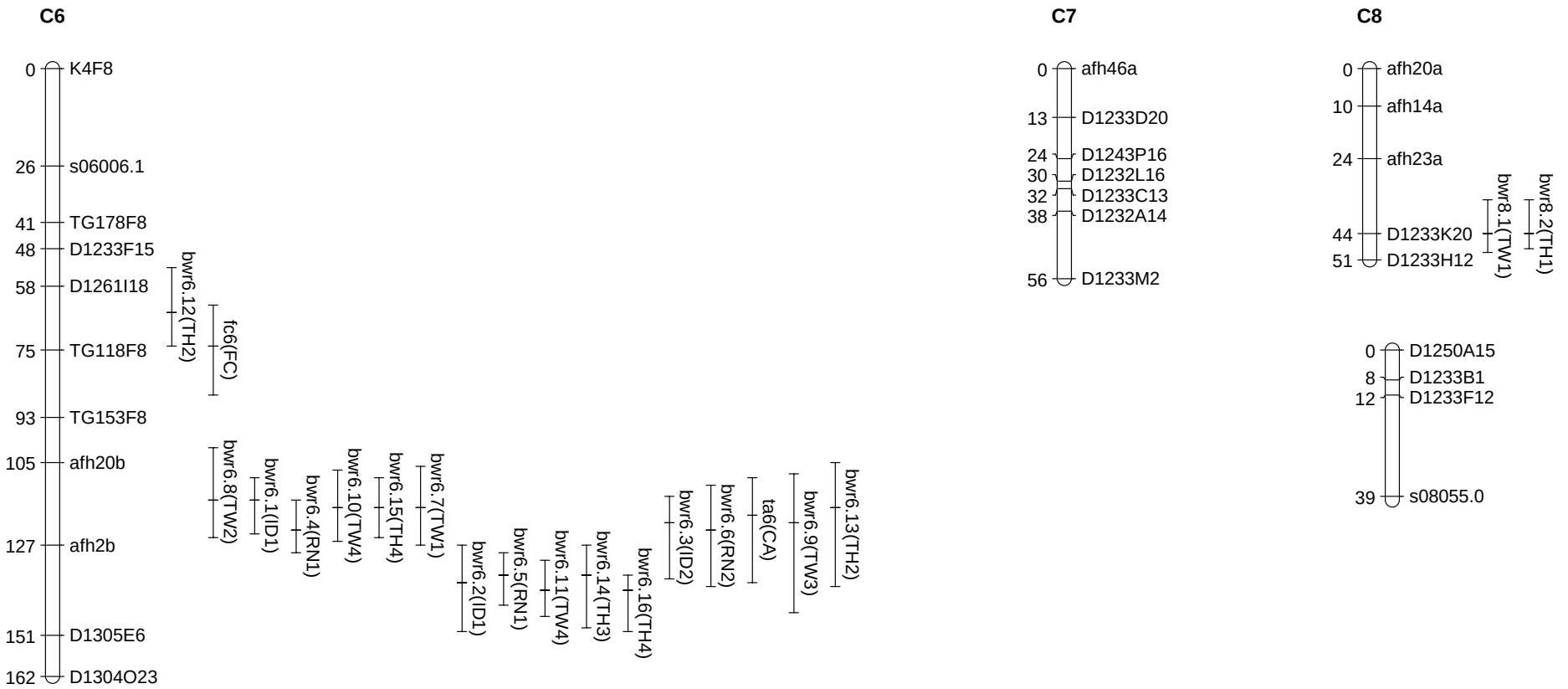


Figure 2.12 continued

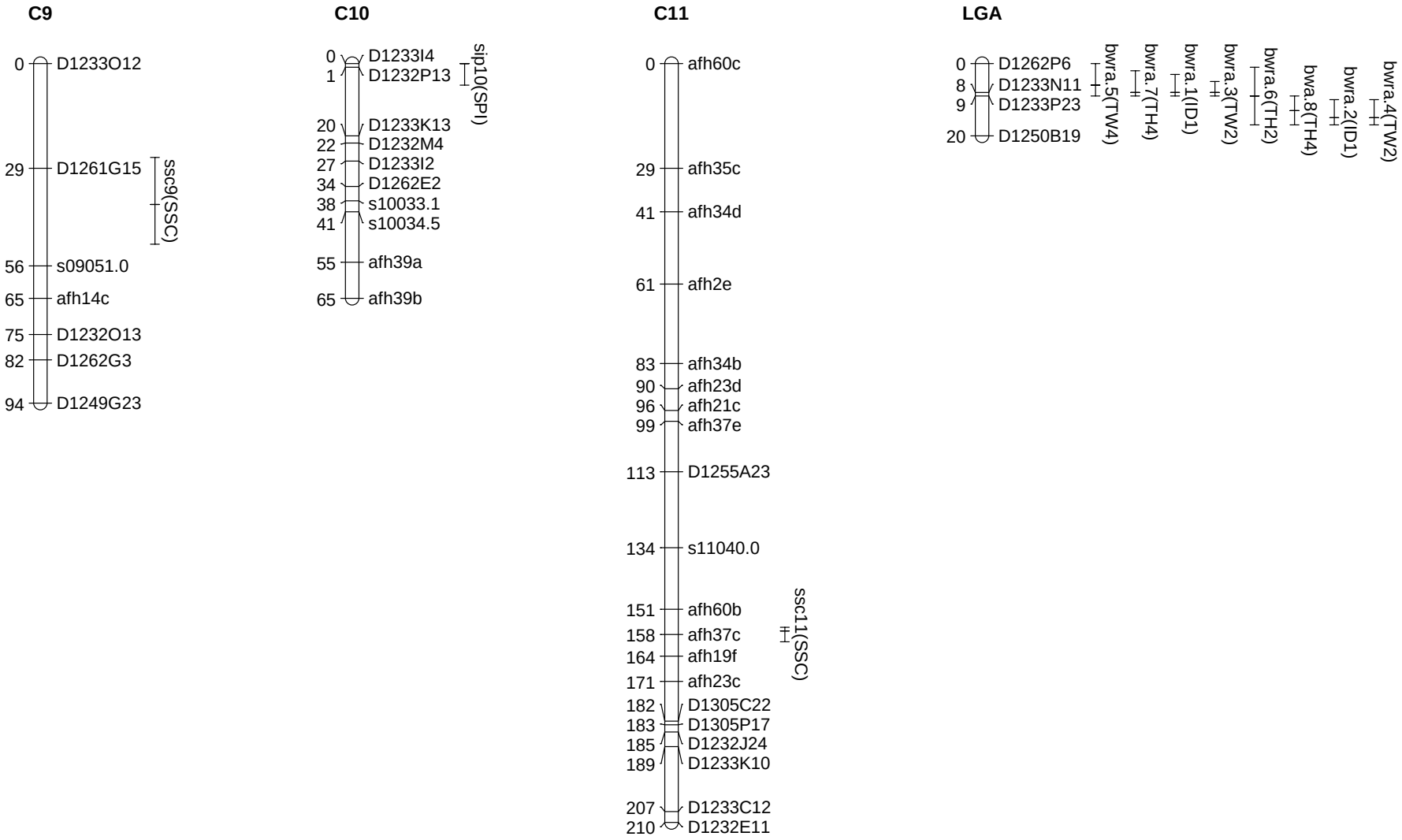


Figure 2.12 continued

A QTL on chromosome 2 *bwr2.1* was detected for ID1, where it explained 5.3% of phenotypic variation, but not detected by ID2. ID1 and ID2 are results of two field trials conducted in different years but at the same farm in Indonesia. Therefore, this QTL could be related to environment effect on the resistance.

Clusters of QTLs were detected on chromosome 6 and LGA presented in this study. There were five QTLs *bwr6.1*, *bwr6.4*, *bwr6.7*, *bwr6.10* and *bwr6.15* in the region between markers afh20b and afh2b were detected for ID1, RN1, TW1, TW4 and TH4, respectively, that explained 10.3-23.9% of phenotypic variation. Another five QTLs *bwr6.2*, *bwr6.5*, *bwr6.11*, *bwr6.14*, and *bwr6.16* were located in the interval between markers afh2b and D1305E6, which explained between 8.2% and 34.7% of the phenotypic variation. However, four out of five QTLs were detected by the same traits, excluded TW1. The large range of phenotypic variation explained at different locations have different disease pressure, which could be related to the strain effect and environmental conditions. In addition, in the overlap region of these two regions, from marker afh20b to D1305E6, four QTLs *bwr6.3*, *bwr6.6*, *bwr6.9*, and *bwr6.13* were detected for the traits ID2, RN2, TW3 and TH2 that explained 23.0%, 12.8%, 9.0% and 8.8% of the phenotypic variation, respectively. Thus, these QTLs in these regions could be related to the stable resistance of H7996. Similarly, numerous QTLs were detected on LGA in the region between markers D1262P6 and D1233P23, and between markers D1233P23 and D1233P23. These QTLs explained phenotypic variations from 6.5% to 20.1%. These QTLs were associated with resistance against Indonesian, Taiwanese, and Thailand strains, but not for Reunion and Indian strains. Thus they could be related to location specificity of the resistance.

2.3.5.2 QTLs affecting morphological traits

For sympodial index, only one QTL *spi10* located on chromosome 10 between markers D1233I4 and D1233K13 was detected explaining 19.4% of the phenotypic variation. The WVa700 allele contributed to this trait. Two QTLs were detected for CA: *ca1* (5.0%) on chromosome 1 and *ca6* (11.2%) on chromosome 6. Both QTLs had equal covariances because the expected value of additive effect is zero. Two QTLs located on different chromosomes affected SSC. While QTL *ssc9* located between a DArT marker D1233O12 and a SSR marker coded s09051.0, resistant parent allele reduced the trait, QTL *ssc11* located between markers afh60b and afh19f, and susceptible parent allele increased the trait. These two QTLs were found to explain 18.7% and 9.7% of the phenotypic variation,

respectively. Only one genomic region on chromosome 6 was found to influence fruit color (a/b), where it explained 16.4 of phenotypic variation.

2.3.5.3 Single marker analysis

We are interested in whether the 13 markers, which belonged to certain chromosomes but not fulfill the criteria to be included in the framework map for composite interval mapping, are linked to QTLs. A single-marker analysis approach using simple linear regression, which is still commonly adopted to identify potential QTL-harboring linked markers, was used and the results are presented in Table 2.8. All markers showed significant with certain traits, excluded marker LEOH36. RFLP marker TG564F8 was highly significant with almost all the disease reaction datasets excepted traits IN2, PH1, and RN2. This QTL seems having large effect as indicated by its significantce level. While the two markers on LGB D1232L19 and D1262M8 had shown significant association with SSC and resistance against Indian, Taiwanese and Thai strains, markers D1232K7 and D1233I1 had shown significant association with CA, FC and resistance against Taiwanese strain. These four markers were strongly associated with FW and SPI as well.

Table 2.8 QTL-linked markers identified by single marker analysis. See table 2.5 for trait abbreviation

Trait	Chromosome/marker[1]												
	1				7	8	9	LGA		LGB			
	D1262C14	LEOH36	D1244H17	s01138.0	s07002.0	s08001.0	s09058.0	D1233J4	TG564F8	D1232L19	D1262M8	D1232K7	D1233I1
IN1									****	**	**		
IN2													
ID1					**				****	*	*	*	
ID2								*	****				
PH1													
PH2					*				****				
RN1									****				
RN2												*	
TW1									****				
TW2					*				****	**	**		
TW3									****				
TW4									****	**	**	*	*
TH1						**			***				
TH2	*				*				****	*	*		
TH3	*								****				
TH4									****	**	**	*	
SPI			*							*	*	*	*
FW							*			****	****	****	****
CA												**	**
SSC										*	*		
pH													*
FC												**	**

[1]Markers were selected from linkage map (Chapter 1).
Significance at the 5%, 1%, 0.1% and 0.01% levels are indicated by *, **, *** and ****, respectively.

2.3.6 Fine mapping

2.3.6.1 Bulk segregant analysis

Twenty-one AFLP primer combinations were screened to identify polymorphisms between the susceptible (SB) resistant (RB) bulks derived from the H7996 x WVa700 RIL population. These primers generated 20 polymorphic bands potentially linked to bacterial wilt resistance. Based on their reproducibility, only nine markers were selected and converted into PCR-based marker form (Table 2.9).

Table 2.9 Polymorphic AFLP fragments between resistant and susceptible pools

No.	AFLP primer pair	Marker name	Band presented in				Fragment size (bp)
			H7996	WVa700	RB	SB	
1	E1-AAC/M2-CAC	afh2a	+	-	+	-	136
2	E1-AAC/M2-CAC	afh2b	-	+	-	+	137
3	E1-AAC/M2-CAC	afh2c	+	-	+	-	221
4	E3-ACA/M4-CAT	afh20b	-	+	-	+	135
5	E3-ACA/M7-CTG	afh23c	-	+	-	+	237
6	E3-ACA/M7-CTG	afh23e	-	+	-	+	378
7	E4-ACC/M5-CTA	afh37b	-	+	-	+	140
8	E4-ACC/M5-CTA	afh37c	+	-	+	-	159
9	E4-ACC/M5-CTA	afh37h	-	+	-	+	322

RB: resistant bulk; SB: susceptible bulk; "+" presence; "-"absence.

2.3.6.2 Conversion of AFLP, DArT and RFLP markers into PCR-based marker form

In order to locate quantitative trait loci effecting bacterial wilt resistance, a preliminary map was constructed by Dr. Elaine Graham (former head of Molecular Lab, AVRDC, personal communication) intergrating AFLP data from F_6 generation (Dr. Bareto, personal communication), RFLP data from F_2 generation (Dr. Grimsley, personal communication) and DArT and SSR data generated from the RILs (present study). Based on the genetic map, we determined a number of loci linked to bacterial wilt resistance on chromosome 2, 3, 4, and 6. The markers located in these regions were converted into sequence specific PCR-based markers. To saturate the regions, another eighteen markers located on chromosome 2, 3, 4, and 6 were selected and tested (Table 2.10).

Table 2.10 Selected markers from QTL regions converted into sequence specific PCR-base markers

No.	Marker code	Marker name	Size (bp)	Actual size (bp)[1]	SNP site
1	2.1[2]	TG31	~2400		
2	2.2	D1261P15 (IB5)		109	
3	2.3	D1232K4 (IC3)		435	
4	2.4	D1305B24 (IIF5)		609	
5	2.5	TG1	~700		
6	2.6	D1233C21 (IVC3)	124		
7	2.7	cLEC7P21	350	350	23
8	2.8	T1616	1500		-
9	3.1	TG324	1300	1276	25, 400, 1192
10	3.2	cLPT-5-E7	1800		
11	3.3	TG130	1500	1390	96
12	3.4	TG585	491	491	85
13	3.5	T1388	1000	100	416, 463, 572, 759
14	3.6	cLPT2E21	1250	1310	106
15	3.7	TG74	2300		
16	4.1	D1233O18 (IIIE11)		107	
17	4.2	D1233J4 (IID9)		301	
18	4.3	D1249B11 (IIA3)		369	
19	4.4	T707	458	458	426
20	4.5	T1405	535		
21	4.6	cLEC7B23	600	600	no
22	6.1	TG178	~1630		
23	6.2	D1261I18 (IB2)		78	
24	6.3	TG118	~2500		
25	6.4	D1304O23 (IIIF12)		128	
26	6.5	T892	1200	1326	131, 1211
27	6.6	T507	800		-

[1]Size after sequenced.
[2]First number is chromosome number and second number is number of marker of each chromosome selected.
" –"sequencing failed; no: no SNP found.

None of the new eighteen RFLP markers showed polymorphism between H7996 and WVa700 (Figure 2.13); and nine of these were successfully cloned and sequenced. A total of fourteen single nucleotide polymorphisms (SNPs), which included three from TG324, one from TG585, one from cLPT2E21, one from cLEC7P21, one from T707, four from T1388, one from TG130, and two from T892, were identified (Table 2.10). Four SNP sites were selected and allele-specific primers were designed (see Materials and Methods). Out of eight primer combinations, only one primer combination 4.4-426b/4.4R (T707-426b/T707R) showed polymorphism when annealing temperature reached 68.4°C (Figure 2.14). However, this result was confirmed with an annealing temperature range of 67-68.5^{o}C and control of 60^{o}C (Figure 2.15). In order to increase the specificity of the allele specific primers, another four allele-specific primers were designed with one mismatch nucleotide before one or two SNP sites using the Web-available program SNAPER (http://ausubellab.mgh.harvard.edu/) (see Materials and Methods). One out of four primer combinations was polymorphic at annealing temperature of 61.8°C; however, this method showed inconstant results.

Out of nine DArT sequences, only three sequence-specific primer pairs were designed from DArT markers located on chromosome 4. Only one sequence-specific primer pair designed from the sequence of the DArT marker, D1233J4, showed polymorphism with a product of 300bp from resistant parental line H7996 using a touch-down PCR program (Figure 2.16).

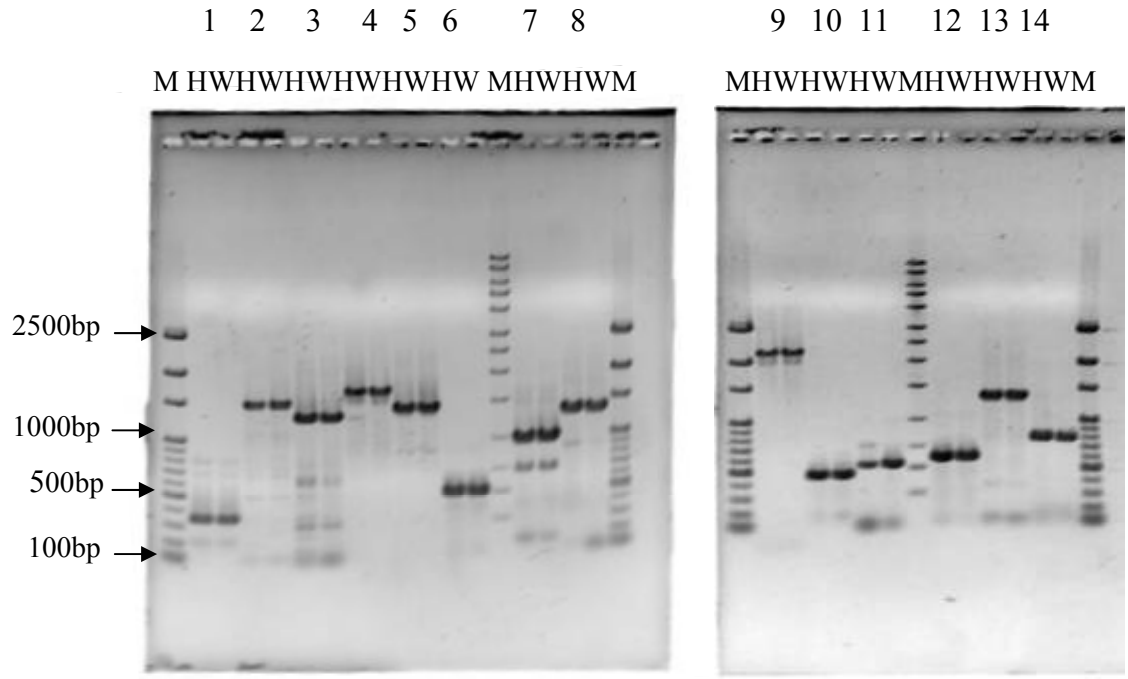


Figure 2.13 Screening polymorphism between H7996 and WVa700 with RFLP markers on 1% agarose gel; marker code 1: 2.7; 2: 2.8; 3: 3.1; 4: 3.2; 5: 3.3; 6: 3.4; 7: 3.5; 8: 3.6; 9: 3.7; 10: 4.4; 11: 4.5; 12: 4.6; 13: 6.5; 14: 6.6 (see Table 2.9). [H = H7996; W = WVa700; M= 100bp ladder (left and right of the gel) and 1kb ladder (middle) (Promega)].

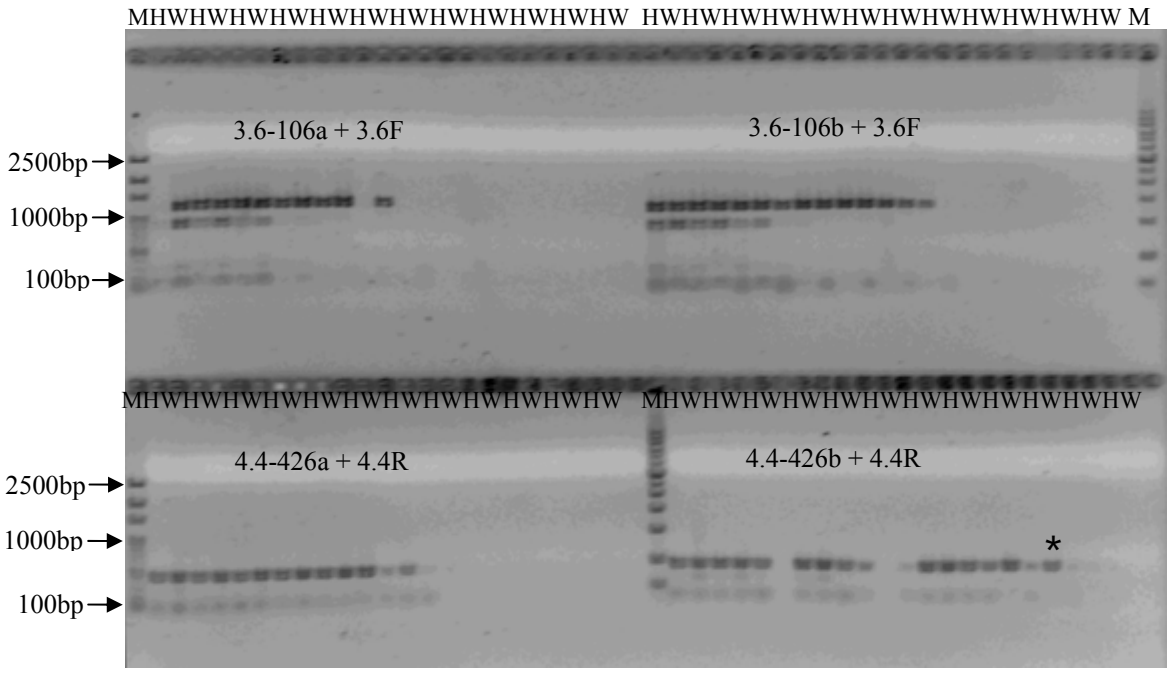


Figure 2.14 Screening polymorphism between H7996 and WVa700 with different primer combinations and annealing temperatures (using gradient of 45-70^{o}C) on 1% agarose gel; *primer showed polymorphism at annealing temperature of 68.4^{o}C. [H = H7996; W = WVa700; M= 100bp ladder (left of the gel) and 1kb ladder (right of the gel) (Promega)].

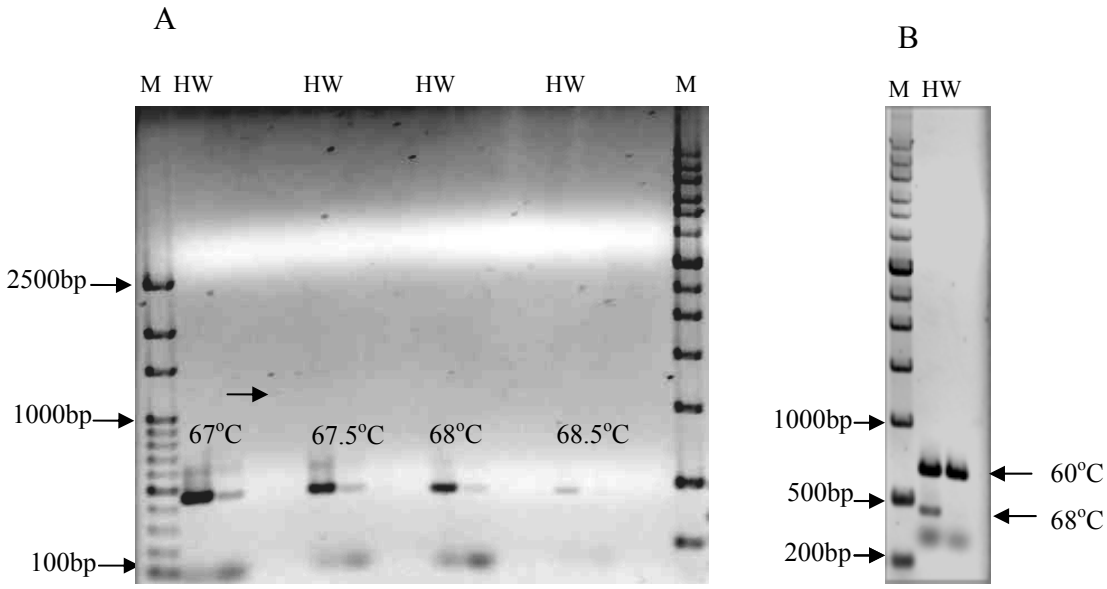


Figure 2.15 Confirmation of primer combination 4.4-426bF/4.4R (T707-426bF/T707R) at different annealing temperatures (A) and annealing temperature at 60°C and 68°C (B) on 1% agarose gel. [H = H7996; W = WVa700; M= 100bp ladder and 1kb ladder (Promega)].

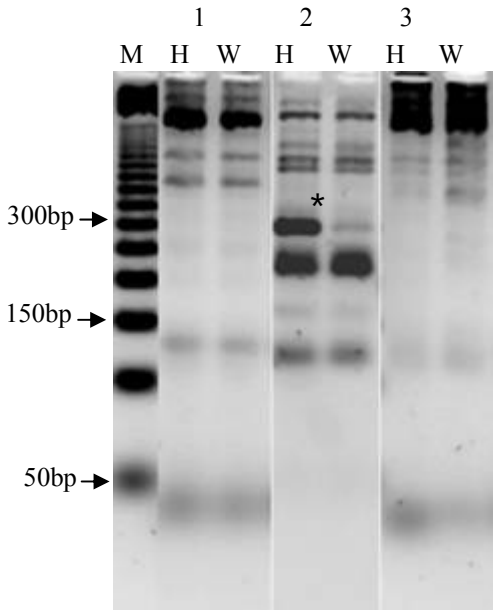


Figure 2.16 Screening polymorphism between H7996 and WVa700 with DArT markers on 6% NuSieve 3:1 agarose gel; *primer combination showed polymorphism; marker code 1: 4.1; 2: 4.2; 3: 4.3. [H = H7996; W = WVa700; M= 50bp ladder (Promega)].

In addition, nine AFLP markers between 135bp and 378bp, which showed in BSA to be linked to bacterial wilt resistance, were chosen for conversion to sequence specific PCR-based markers. Seven out of nine fragments were successfully cloned, sequenced and sequence-specific primers developed. However, using these primer pairs, no polymorphism was detected in H7996 and WVa700. In an attempt to develop SNP markers, the fragments from H7996 and WVa700 were excised from the gel and then amplified for sequencing. Only two markers afh37h and afh23c generated good sequences and only marker afh23c contained SNP, a deletion in H7996.

Due to low polymorphism between H7996 and WVa700, flanking DNA was isolated to obtain more sequence information using inverse PCR method. Genomic DNA of H9776 and WVa700 were digested with several 4-base cuters AluI, HaeIII, and TagI (each was treated as separate template) and 6 base-cuters EcoRV (for marker afh2b) and XbaI (for marker afh23c) and then were amplified with new primers designed from AFLP fragments afh2b and afh23c. The reason we choose these two markers for inverse PCR was the presence of a restriction site for 6-base cuter enzymes. However, no polymorphism was detected between the two parents H7996 and WVa700. To overcome the low polymorphism rate, sixteen randomly amplified microsatellite polymorphism primers were designed in order to combine with either seven forward or seven reverse primers designed from the AFLP fragment sequences. Out of 112 primer combinations, only one primer combinations (TAFLP001.afh20bF + $GC(GA)_4$) showed polymorphism (Figure 2.17) but polymorphic bands were not stable over PCR amplifications. Therefore, those primer combinations were not used to screen the RIL population.

The two primer combinations, one was designed from DArT marker D1233J4 (see Table 2.1) and other was designed from RFLP marker T707 (4.4-426bF/4.4R) (see Table 2.2) were screened on the mapping population. Since both were dominant markers, primer combination 4.4-426bF/4.4R was amplified at annealing temperature of 68°C to get polymorphic product and 60°C as control. The segregation of polymorphism gained from RFLP marker is shown in Figure 2.18. For the converted DArT marker, we were not able to score the RIL population because multiple bands were generated in the individual lines as illustrated in Figure 2.19.

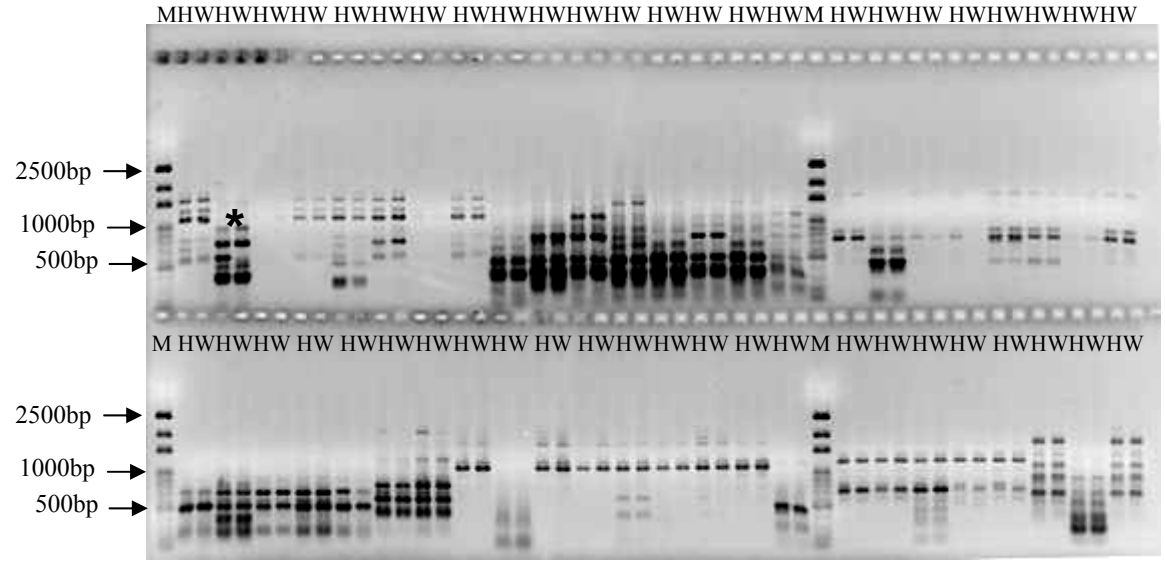


Figure 2.17 Screening polymorphism between H7996 and WVa700 using various primers combinations on 1% agarose gel; *primer showed polymorphism. [H = H7996; W = WVa700; M= 100bp ladder (Promega)].

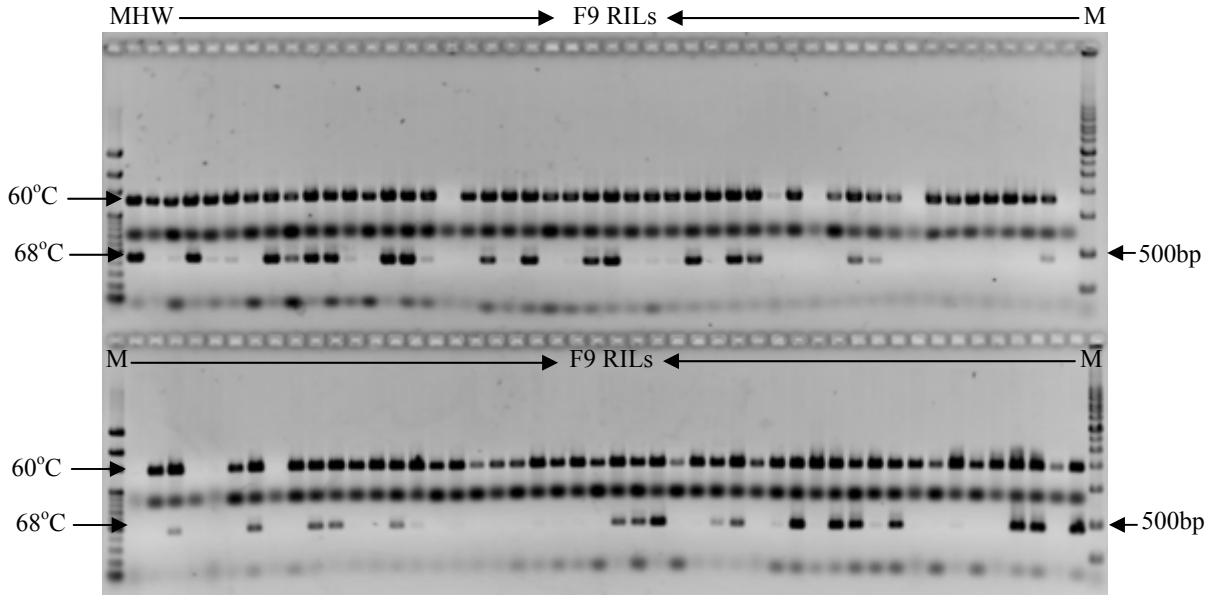


Figure 2.18 Segregation of a converted RFLP marker into PCR-base marker form. Products at annealing temperature at 68°C were run ahead 15 minutes at annealing temperature at 60°C. [H = H7996; W = WVa700; M= 100bp ladder (left of the gel) and 1kb ladder (right of the gel) (Promega)].

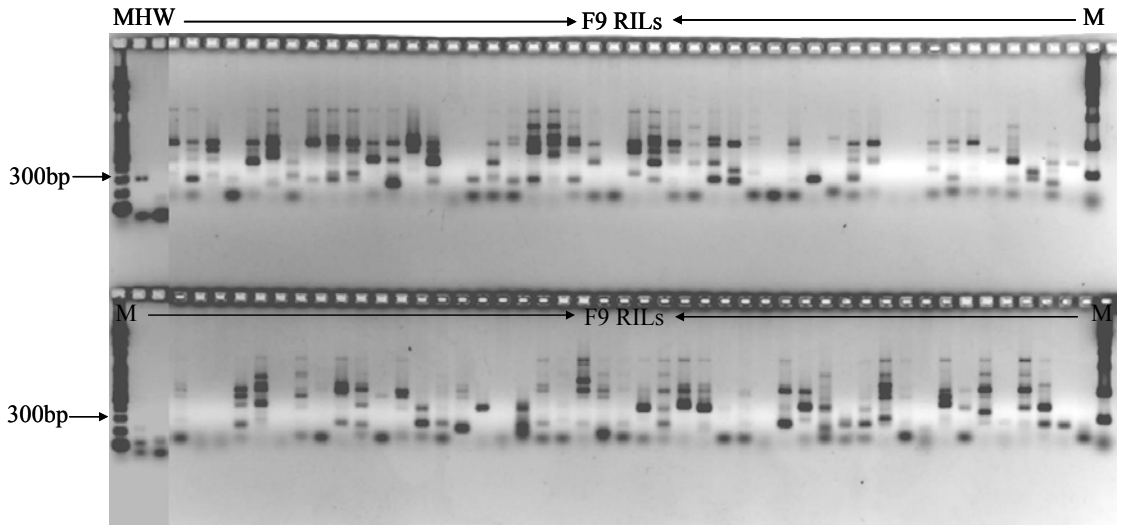


Figure 2.19 Segregation of a converted DArT marker (D1233J4) into PCR-base marker form. [H = H7996; W = WVa700; M= 100bp ladder (left of the gel) and 1kb ladder (right of the gel) (Promega)].

2.4 DISCUSSION

2.4.1 Resistance to bacterial wilt in H7996 and its associated QTLs

Expression of resistance to bacterial wilt in tomato depends on genetic variability of the pathogen as well as environmental and climate conditions (Prior *et al.* 1990). In the present study, performance of the RIL population derived from the cross of H7996 and WVa700 was highly correlated among evaluations inoculated with different strains or conducted in various locations, with the exception of PH1 trial. This indicates that there are common QTLs associated with resistance expression under various environments. The term "environment" here includes those factors that possibly affect the resistance expression, such as pathogen strains, temperature, soil moisture, relative humidity, soil type, and presence of root knot nematode etc. The fact that no QTL was detected for the PH1 dataset indicated the strain TM22, used in PH1 trial, could have a very different interaction with H7996 compared with other strains. More studies need to be conducted to verify this assumption. No QTLs were detected from trial IN2 despite the data were significantly correlated with most of the other trials. This could be due to the low variation of the data points caused by low disease pressure in trial IN2.

2.4.1.1 Common QTLs important for resistance against race 1 strains

Diseae reactions of the RIL population were evaluated in 15 trials against race 1 strains in six countries both in the field or at seedling stage. QTLs commonly detected for the more trials would indicate the more importance of those QTLs contributing to stable resistance. Based on the QTL analysis results, QTLs located in three chromosomal regions fit under this criteria. The most important region would be the segment of 101 to 150 cM of chromosome 6. One to two QTLs in this region were detected for 10 out of the 15 trials. QTLs in this region were associated with resistance expressed under the environment of Taiwan (TW1, TW2, and TW4), Indonesia (ID1and ID2), Thailand (TH2, TH3, and TH4), and Reunion (RN1). The importance of QTLs in this region was also supported by the large percentage contributing to the overall disease variation ranging from 8.2 to 34.7%. The R^2 values of the QTLs in this region were the largest among QTLs detected from trial TW1 (15.5%), TW4 (25.8%), ID1 (23.9%), ID2 (23.0%), TH2 (8.8%), TH3 (10.1), TH4 (22.7%), and RN1 (34.7%). QTLs in this regions have been identified by previous studies;

e.g. a major QTL for resistance to Pss4 (the same strain used in the TW2 trial), JT519 (the same strain used in RN1 trial) and GMI8217 was detected by Wang *et al*. (2000), Carmeille *et al*. (2006) and Thoquet *et al*. (1996a; b), respectively.

There was another QTL detected on chromosome 6 located between 53 to 74 cM that was associated with resistance observed in trial TH2, close to RFLP marker TG118. The same QTL was detected against GMI8217 in both F_2 and F_3 population that derived from the same cross by Thoquet *et al*. (1996a; b), however, it was not detected in studies of Carmeille *et al*. (2006) and Wang *et al*. (2000).

Another chromosomal region that hosted important QTLs was on LGA. First, a QTL located between 0 cM and 17 cM of LGA was associated with resistance expressed under the environment of Taiwan (TW2 and TW4), Indonesia (ID1), and Thailand (TH2 and TH4). The R^2 value of the QTL ranged from 6.5 to 20.1% and was the largest among QTLs detected from trial TW2 (20.1%). Another QTL on LGA is linked with TG564. The linkage with TG564 on this linkage group was detected and strongly associated to resistance observed in all race 1 trials in this study, excluded IN2 and PH1. In the high-density linkage map of tomato, marker TG564 was mapped on chromosome 3 (Tanksly *et al.* 1992), whereas Wang *et al*. (2000) assigned this marker to chromosome 12. In the present study, this marker was nearest to LGA with a recombinant rate of 0.3 and merged into LGA. This linkage group was hypothesized to be belonged to either chromosome 5 or 12. However, in previous studies using the same cross, a QTL linked with TG564 was detected for resistance to strains Pss4 in the F_3 population (Wang *et al.* 2000) and JT519 in the F_8 RIL population (Carmeille *et al.* 2006) on chromosome 12. We thus strongly hypothesized LGA belonging to chromosome 12.

A QTL located on LGB might contribute to stable resistance as well. Results of single marker analysis indicated significant linkage with D1232L19 and D1262M8 on LGB. Since these two markers were only 1.8 cM apart, it is likely that only one QTL is located in the region. This QTL is associated with resistance expressed under the environment of India (IN1), Indonesia (ID1), Taiwan (TW2 and TW4) and Thailand (TH2 and TH4). This linkage group was hypothesized to belong to chromosome 5, since LGA was strongly hypothesized to belong to chromosome 12.

2.4.1.2 Colonization by Pss4 and resistance to bacterial wilt in H7996

Latent infection of *R. solanacearum* has been reported in tomato (Grimault *et al.* 1994; Saile *et al.* 1997) and other crops such as potato (Ciampi and Sequeira, 1980), and geranium (Swanson *et al.* 2005). In our study, we demonstrated that H7996 is not immune to bacterial wilt pathogen, where 12.5 % of the symptomless plants of H7996 were colonized by *R. solanacearum* at the middle of stem. This agreed with the results of Grimault *et al*. (1994) that a race 1 strain 8217 detected in 74% of H7996 plants, while Carmeille *et al*. (2006) observed colonization by a race 3 strain JT516 in H7996 hypocotyls.

Colonization by strain Pss4 at middle stem in F_9 RILs was correlated with disease reactions observed in most trials, except RN2. This could be due to trial RN2 was conducted with a race 3 strain JT516 isolated from potato. The largest correlation coefficient with the Pss4 colonization reaction was the visual symptom variation of Pss4 (trial TW2). QTLs on chromosome 6 (in the interval of 108 to 145 cM) and LGA (linked with TG564) were determined to be associated with variation of Pss4 colonization. And these QTLs were also associated with stable resistance. Our results agreed with Prior *et al*. (1996) and Grimault *et al*. (1994) suggesting resistance to bacterial wilt in tomato is largely depending on suppression of bacterial multiplication inside the plant. Hence, colonization percentage could be used as a selection criterion beside visual symptom in selection for stable resistance, and the screening could be conducted at 6 days after inoculation following our protocol compared to 28 days after inoculation of the visual symptom screening.

2.4.1.3 Plausible strain-specific QTLs to race 1 strains

Strains Pss4 and Pss186 that differ in their aggressiveness but are close in genetic relationship (Jaunet and Wang 1999), were used to evaluate the RIL population using the same method and under similar environments. The objective was to determine plausible strain-specific QTLs. Comparing the QTLs detected to be associated with resistance to Pss4 and Pss186, three QTLs were detected with either one strain but not both. Those detected only with Pss4 (trial TW2) were *bwr2.2* on chromosome 2 and the QTL linked with D1232L19 and D1262M8 on LGB. And that detected only with Pss186 (trial TW1) was *bwr8.1* on chromosome 8. Among these, the allele of *bwr2.2* from Wva700, the susceptible parent, was associated with resistance, which confirmed the results of Wang *et*

al. (2000). Whether these QTLs were really associated with strain-specific resistance requires further studies. One approach could be developing near-isogenic lines carrying different combinations of QTLs and challenging the isogenic lines against different pathogen strains.

2.4.1.4 Plausible environment-specific QTLs to race 1 strains

Several environmental factors could affect disease development caused by *R. solanacearum*. Other than the strain effect, it was difficult to conclude from our results the presence of QTLs that were specific to other environmental factors. Trial ID1 and ID2 were supposedly the best combination to look for environment-specific QTLs. These two trials were conducted in the same plots following the same protocol but at different seasons. Thus, pair-wise comparisons of the QTLs detected from these trials could identify QTLs important for resistance expression under a particular season. QTLs on chromosome 2, 6, LGA and LGB were detected to be associated with resistance reaction of trial ID1. Very few QTLs were detected from trial ID2. Those detected were located on chromosome 6 and LGA, which were detected from trial ID1 as well. The failure of detecting other QTLs as in trial ID1 could be due to the low variation of trial ID2's data. Trial ID2 was conducted in the rainy season, when very high disease pressure of bacterial wilt and other diseases like pith necrosis occurred. Therefore, it is difficult to conclude that QTLs detected only with trial ID1 but not ID2 were environment-specific. The best approach to elucidate plausible QTLs specific to certain environment factors would be to examine disease reactions under controlled environments.

2.4.1.5 Comparison of QTLs associated with resistance to race 1 and 3 strains

Trial RN2 was the only trial that was evaluted against a race 3 strain JT516. The only QTL detected to be associated with resistance to JT516 was *bwr6.6* on chromosome 6. This QTL was close to the RFLP marker TG153, where a major QTL for resistance to this strain was detected in the both $F_{2:3}$ and F_6 RIL population by Carmeille *et al.* (2006). More diverse race 3 strains should be used to evaluate the mapping population to determine whether there are other QTLs associated with race 3 resistance. This QTL was also associated with stable resistance to race 1 strains. This further indicates the importance of this QTL against a broad spectrum of the pathogen strains.

2.4.2 Morphological traits and their associated QTLs

Tomato fruit composition exhibits a quantitative variation, controlled by several genes, more or less influenced by environmental conditions. Molecular markers allow the dissection of such quantitative traits into discrete QTL, which can be located on a genetic map (Causse *et al.* 2004; Fulton *et al.* 2002; Saliba-Colombani *et al.* 2001). The existence of a QTL in a chromosomal region reveals at least one polymorphic locus is segregating in this region, and is responsible for part of the trait variation. Numerous QTLs have been identified for many agronomic and horticultural traits in *Solanum lycopersicum* and related species (Bernacchi *et al.* 1998; Causse *et al.* 2004; Fulton *et al.* 1997; Fulton *et al.* 2002; Fulton *et al.* 2000; Grandillo *et al.* 1999; Grandillo and Tanksley, 1996a; Saliba-Colombani *et al.* 2001; Tanksley *et al.* 1996). Thus far researches were mainly focused on economically important characteristics such as yield, fruit weight and processing performance (soluble solid, color etc.) and the relationship among traits have been analysed with regard to QTL mapping.

2.4.2.1 Sympodial index

In tomato, sympodial index (SPI) is a distinctive feature among species. The wild-type growth habit is classified as 'indeterminate' (SP-) in reference to the continuous production of an unrestricted number of sympodial units; whereas 'determinate' *(sp/sp)* means a limited number of sympodial shoots arise before further extension of the main apex ceases (Pnueli *et al.* 1998).

All of the colored-fruited species plus *S. habrochaites* have a mean SPI of 3, whilst the remaining spp., all green-fruited, average 2 (Rick, 1986). In the present study, *S. lycopersicum* H7996 had a SPI of about 2.5, whereas SPI of 2.9 was obtained in *S. pimpinellifolium* WVa700. The distribution of SPI in the F_9 RILs (Figure 2.9) with a peak at 3 suggested dominant inheritance of the SPI from the parent WVa700, which could carried the SP gene family (Carmel-Goren *et al.* 2003).

In tomato, at least six SELP-PRUNING (SP) genes located on chromosome 2, 3, 5, 6 and 9, control the regulation of the vegetative reproduction (Carmel-Goren *et al*. 2003). However, in the present study, a major QTL for SPI, *ssi10* (R^2 = 19.4%) was detected on chromosome 10. This QTL with small negative effect was located 6 cM from the top of the chromosome. Furthermore, SPI was associated with marker D1244H17 on chromosome 1

and all the 4 markers on LGB (Table 2.8). The observation on LGB indicates that this genomic region may contain multiple, linked QTLs for SPI. These observations suggest that SP gene family could be presented on chromosome 1, 10 and LGB (possibly chromosome 5).

2.4.2.2 Fruit weight

Tomato fruit weight (FW) is considered as a classical example of a quantitative trait displaying continuous variation (MacArthur and Butler, 1938). In the genus *Solanum*, all wild species have a small fruit size compared with the domesticated tomato. Loci *fw*1.1, *fw*2.2, *fw*3.1, and *fw*4.1 were first identified as QTLs controlling fruit weight in a cross between a small-fruited wild tomato and a large-fruited cultivated counterpart; however, locus *fw*2.2 is the only one that has been cloned and studied at the molecular level (Alpert *et al.* 1995). In the present study, small fruit was observed in *S. pimpinellifolium* WVa700 (2.32 g), while larger fruit was observed in *S. lycopersicum* H7996 (33.82 g). Fruit weights of the F_9 RILs were intermediate between the two parents and skewed toward the small-fruited parent suggesting small fruit is dominant and consistent with previous studies (Chen *et al.* 1999; Grandillo and Tanksley, 1996a; Paterson *et al.* 1991).

In this study, FW was also strongly associated with the four markers on LGB suggesting presence of multiple, closely linked QTLs for FW in this region. LGB is supposed to belong to either chromosome 5 or 12. If it is hypothesized to belong to chromosome 12, this could agree with previous investigations that numerous QTLs was detected on chromosome 12 (Causse *et al.* 2002; Causse *et al.* 2004; Chen *et al.* 1999; Fulton *et al.* 1997; Fulton *et al.* 2000; Saliba-Colombani *et al.* 2001). However, it could be hypothesized to belong to chromosome 5 that would agree with investigations of Doganlar *et al.* (2000), and van der Knaap and Tanksley (2003). In addition, an anchor marker on chromosome 9, s09058.0, was significantly linked with FW. This indicates that this region may contain a QTL linked to FW. This agreed with previous studies that several QTLs on chromosome 9 were identified to have significant effects on FW (Causse *et al.* 2004; Chen *et al.* 1999; Fulton *et al.* 1997).

2.4.2.3 Skin color

The skin color of tomato fruit has been classified as yellow or clear (colorless). The dominant gene Y governs the yellow skin, whereas the homozygous recessive y causes

colorless skin (Lindstrom, 1925). Thus, in this study, H7996 carried the dominant gene Y with yellow skin; covertly WVa700 carried the recessive gene y with clear skin. Segregation of skin color in the F_9 RILs followed the expected 1:1 ratio; however, about 21% of the F_9 families showed both skin colors of the two parents suggesting those RILs still had residual heterozygocity.

2.4.2.4 Fruit quality

Since numerous QTLs effecting fruit quality have been found distributed over all 12 chromosomes of tomato (Chen *et al.* 1999; Fulton *et al.* 2002; Fulton *et al.* 2000), we analyzed fruit quality traits of the two parents and found some degree of differences in citric acid, pH value, soluble solid content (Brix^o), and color (a/b) from the two parents (Figure 2.13A, B, C, D). Hence, we were interested to know the relationship between fruit quality traits and bacterial wilt resistance.

Citric acid

Inheritance of citric acid (CA) is largely quantitative, but in some crosses, there was evidence of a single major gene conditioning high acidity; and the component of genetic variance affecting acidity was additive (Lower and Thompson, 1967). The CA of the two parents H7996 and WVa700 was 0.55 and 0.45, respectively, whereas variation of CA was observed in RILs (Figure 2.13A) suggesting that CA is a typical quantitative trait, which agreed with Fulton *et al.* (2002).

CA was mainly controlled by two linked regions on LGB, QTLs on chromosome 1 (*ca1*) and 6 (*ca6*). The QTL *ca1* shared more or less the same position on chromosome 1 in our study as the QTL detected in cross with *S. pimpinellifolium* (Fulton *et al.* 2002). QTL *ca6* is the first QTL affecting CA identified on chromosome 6 so far. Two additional QTLs linked to markers D1232K7 and D1233I1 on LGB were asscociated with CA as well.

Fruit pH value

Fruit pH is important for the flavor of fresh market tomato as variation in sour taste. Low fruit pH of tomato allows the reduction of processing time. In tomato, numerous QTLs have been identified to effect pH value (Bernacchi *et al.* 1998; Causse *et al.* 2002; Chen *et al.* 1999; Fulton *et al.* 2002; Fulton *et al.* 2000; Fulton *et al.* 1997b; Paterson *et al.* 1988; Saliba-Colombani *et al.* 2001) indicating inheritance is largely quantitative. In the present

study, we found the fruit of H7996 had relatively lower pH than WVa700; and the F_9 RILs exhibited a continuous distribution (Figure 2.12B) with the mean in the direction of high pH. However, only one genomic region on LGB was associated with pH value (Table 2.8).

Soluble solid content

Soluble solid contents (SSC) are closely related to organoleptic quality of fresh market tomatoes and determine the price of tomato for processing. The major components of soluble solids are soluble sugar such as glucose and fructose (65%) and organic acids such as citric and malic acid (13%) and their concentrations are responsible for taste and flavor of ripe fruits. Most researches on tomato quality have been focused on these components (Balibrea *et al.* 2006; Baxter *et al.* 2005; Causse *et al.* 2002; Fridman *et al.* 2004). High soluble solid concent has been reported to exhibit dominance and over-dominance (Paterson *et al.* 1988; Rick, 1974; Tanksley and Hewitt, 1988). The differences observed between the two parents and distribution of the RIL population are indicating the high SSC of the population was inherited from the susceptible parent WVa700.

The positions of soluble solid content QTLs on chromosome 9 detected in this study was close to QTLs detected in crosses with *S. pimpinellifolium* (Chen *et al*. 1999; Fulton *et al.* 2002), *S. cheesmanii* (Paterson *et al*. 1991), *S. peruvianum* (Fulton *et al*. 1997). In addition, a QTL controlling SSC located on chromosome 11. This is the first QTLs linked to SSC detected on chromosome 11. Another two genomic regions D1232L19 and D1262M8 on LGB were associated with SSC. Thus, if LGB belongs to chromosome 5, the result was in accordance with results of Bernacchi *et al*. (1998) and Fulton *et al*. (2000). However, if LGB belongs to chromosome 12, the observation agreed with result of Chen *et al*. (1999) and Fulton *et al*. (2002).

Color value (a/b)

The color of tomato is a complex trait associated with tomato fruit quality. The complexity of tomato color is due to the presence of a diverse carotenoid pigment system with appearance conditioned by pigment types and concentrations, and subject to both genetic and environmental regulation (Sacks and Francis, 2001). Many genes influencing tomato color have been identified such as apricot (*at*), beta-carotene (*B*), dark green (*dg*), green flesh (*gf*), green ripe (*Gr*), high pigment-1 (*pm-1*), high pigment-2 (*pm-2*), intense pigment (*Ip*), red color in yellow fruit (*ry*), yellow flesh (*r*) (Jenkins and Mackinney, 1956; Kerr, 1955; Kerr,

1956a; Kerr, 1956b; Lesley and Lesley, 1960; Young, 1956). We found here in the present study a higher a/b value in H7996 (1.58) and a lower value in WVa700 (1.19). This result could be explained by H7996 having red-fruited color and yellow skin, whereas WVa700 has a pink-fruited color with colorless skin. Thus, H7996 fruits could have higher lycopene content than WVa700 fruits. This was in accordance with results observed by Brandt *et al*. (2006) the higher the ratio of *a/b*, the higher the lycopene content; and suggesting typical quantitative trait presented in studied population. The QTL *fc6* on chromosome 6 was located closely to a region QTL as detected by Saliba-Colombani *et al*. (2001), where fruit color was measured by spectrophotometer color measurement. Two additional markers D1232K7 and D1233I1 on LGB were also asscociated with fruit color.

2.4.3 Possible linkage between resistance to bacterial wilt and morphological traits

Linkage of fruit size and resistance to bacterial wilt in tomato has been studied. Monma *et al*. (1997) reported there was no correlation between the resistance index and fruit weight in the F_2 generations of the two crosses (r = -0.074, r = -0.019) involving *S. lycopersicon* indicating it is possible to select plants with both high resistance and large fruits in segregating populations; whereas Scott *et al*. (2003) reported numerous selections over the years with large fruit turned to have good level of resistance, when Hawaii 7997, a sister line of H7996, was used as a source of resistance. This could be due to differences in aggresiveness of strains used. Our results showed significant positive correlations between FW (fruit weight) and disease reaction in trial IN1, RN2, TH2, and TH4, indicating the RIL having larger fruit size tend to have higher severity of bacterial wilt. The QTL analysis results were in agreement with this observation. The three markers on LGB were significantly linked with fruit weight, as well as with disease reactions in trial IN1, ID1, TW2, TW4, TH2 and TH4 (Table 2.8). More molecular markers and anchor markers need to be added on LGB to further examine the importance of the QTLs on fruit weight as well as disease resistance.

Citric acid content (CA) was found negatively correlated with disease reactions in trial ID2 and RN2. This means the higher CA, the lower disease severity. QTL *ca6* on chromosome 6 was detected to be associated with citric acid content and had the same position as QTLs for bacterial wilt resistance. Similarly, a marker on LGB was associated with CA and BW reaction of several trials (Table 2.8). This indicates there are possible associations between

bacterial wilt resistance and CA. This is the first report about association between bacterial wilt resistance and citric acid in tomato.

Fruit color was positively correlated with disease reactions in trial RN2, TW1, TW2, TH2, and TH3. The result indicates higher a/b ratio, i.e., redder-fruited plants would be less resistant to bacterial wilt. This was contrary to the fact that H7996 had a higher a/b ratio and is a resistant line, whereas WVa700 had lower a/b ratio and is a susceptible line. Fruit color is a complex trait and subject to both genetic and environmental regulations (Koskitalo and Ormrod, 1972). QTL *fc6* on chromosome 6 and marker D1232K7 on LGB were found to be associated to FC. Marker D1232K7 was also linked to bacterial wilt resistance traits like ID1, TW4, and TH4. These observations suggest possible linkage between bacterial wilt resistance and fruit color, althought not conclusive.

2.4.4 Fine mapping

The very low rate of polymorphism between H7996 and WVa700 made mapping and fine mapping of QTLs associated with BW resistance difficult. Several techniques were evaluated in this study that showed ability to detect polymorphism. However, these techniques are slow and the success rate is low. Thus, an effective and stable SNP detection technology is needed for fine mapping of QTLs in this RIL population. Nevertheless, several SNPs have been found that would be useful in fine mapping of QTLs to develop closely linked markers for marker-assisted selection and gene cloning. The QTL detected on chromosome 6 showed highly significant resistance to different strains and locations and would be a primary target region for fine mapping. RFLP marker TG153, AFLP markers afh20b and afh2b, and DArT marker D1305E6 have been mapped to this region. Furthermore, the RFLP marker TG564 and the four DArT markers on another significant QTL regions on LGA could be utilized for conversion into sequence specific PCR-based markers to close the gap between linkage groups. Although some AFLP, RFLP, and DArT markers have been converted into sequence-specific PCR-based form, results achieved were not consistent. This could be due to genetic background of the materials used in the present study as discussed in Chapter 1. To overcome the inconsistency problem, real-time PCR also called quantitative real time polymerase chain reaction (QRT-PCR) could be an effective technique to determine whether a specific sequence is present in the samples. Many new technologies are available in detecting SNP polymorphism that are more effective and should improve the fine mapping process.

Lastly, the availability of markers is critical to any mapping experiment. The lack of user-friendly markers in tomato is a challenge and this resource has to be developed to support genetic mapping and marker-assisted selection.

2.5 SUMMARY

Resistance in tomato against race 1 strains of *Ralstonia solanacearum* was evaluated in a F_9 population of recombinant inbred lines (RILs) derived from a cross between *S. lycopersicum* H7996 (resistant) and *S. pimpinellifolium* WVa700 (susceptible). The F_9 population showed an intermediate level of resistance to strain Pss186 (53% of plants were healthy) and a lower level of resistance to strain Pss4 (30% of plants were healthy). To determine the colinization of the pathogen in the F_9 population, a protocol was developed using three resistant RILs and three susceptible RILs. The sampling time was determined to be 6 days after inoculation with strain Pss4, since the difference between the two groups was the largest and the standard error was the lowest at 6 days after inoculation. *R. solanacearum* was detected in 12.5% of symptomless H7996 plants, whereas it was detected in 56.3% of the symptomless WVa700 plants. The percentage of colonized plants of the RILs ranged between 0% and 100%. Beside the nine traits collected in this study, results from 13 additional resistance tests at different locations were used. Visual symptoms of most datasets were significantly correlated in a positive manner with each other, excluded PH1. Colonization by Pss4 (TW3) observed in the mid-stem was significantly correlated with all visual symptom datasets, excluded RN2. Significant correlation between the disease reactions and morphological traits was not consistant among datasets and a few correlations were significant between fruit weight and fruit quality. A total of 106 loci (32 AFLPs, 59 DArTs, 6 RFLPs, 11 SSRs) distributed over 15 linkage groups covering 1089.1 Kosambi cM was used for quantitative trait loci (QTLs) mapping using composite interval mapping. In addition, 13 markers belonged to certain chromosomes were determined association with resistance separately by single marker analysis.

A total of 37 QTLs were identified. Out of 37 QTLs detected, 31 QTLs was identified for bacterial wilt resistance, one for sympodial index, two for citric acid, two for soluble solid content and one for fruit color (a/b). They explained from 5.0% to 34.7% of the phenotypic variation, depending on the traits. The LOD ranged from 2.7 to 10.6. A cluster of QTLs (15 QTLs) was detected in an interval of 50 cM on chromosome 6, which explained between 8.2% and 34.7% of phenotypic variation. The large range of phenotypic variation explained at different locations have different disease pressure, which could be related to the strain effect and environmental conditions. Thus, these

QTLs in these regions could be related to the stable resistance of H7996. Similarly, numerous QTLs were detected on LGA in the region between markers D1262P6 and D1233P23, and between markers D1233P23 and D1233P23 and with TG564. These QTLs explained phenotypic variations from 6.5% to 20.1%. These QTLs were associated with resistance against Indonesian, Taiwanese, and Thailand strains, but not for Reunion and Indian strains. Thus they could be related to location specificity of the resistance. Most of markers showed significant with certain traits, excluded marker LEOH36. Numerous SNPs have been found in this study would be promising for forthcoming fine-mapping work, particularly the chromosome 6 regions, where QTLs were detected to be associated with resistance to different strains and locations.

Chapter 3

Resistance to race 1 of *Ralstonia solanacearum* in wild tomato germplasm*

3.1 INTRODUCTION

Bacterial wilt caused by race 1 strains of *Ralstonia solanacearum* is one of the most important diseases that limit tomato production in the tropics and subtropics. As a soil-borne pathogen, the bacteria enter tomato plants through the roots, colonize vascular tissue and cause wilting symptom (Denny, 2006). Race 1 strains are difficult to control because of their soil-borne nature and their extreme wide host range including many weed hosts favoring its widespread distribution and persistence in the environment (Hayward, 2000). Chemical control for soil-borne diseases is usually unsuccessful and uneconomic and furthermore no commercial pesticides are available for controlling *R. solanacearum* except the application of chemical fumigants. Thus, host plant resistance has been a major strategy for managing bacterial wilt in tomato.

Resistance sources to bacterial wilt in tomato have been identified and cultivars with different levels of resistance have been developed (Scott *et al.* 2005). However, breeding for durable resistance is challenging due to the fact that resistance in tomato to bacterial wilt can be location-specific (Hanson *et al.* 1996). Location specificity can be due to the presence of strains that vary in aggressiveness. For example, all reported strains of *R. solanacearum* are race 1 and biovar 3 or 4 in Taiwan, but they are highly variable in aggressiveness (Jaunet and Wang, 1999). Performance of a resistant line is largely depending on local strain profiles (Lin and Wang, unpublished data). Tomato accessions have been evaluated with different strains and biovars of race 1 of *R. solanacearum* and with different screening methods, and only few accessions were found to be resistant

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(Gonzalez and Summers, 1996; Jaworski *et al.* 1987). There are only few *Solanum pimpinellifolium* or *S. lycopersicum* accessions being used as sources of resistance in breeding programs globally (Scott *et al.* 2005). Thus, there is a need to identify more diverse resistance sources to possibly overcome the highly variable pathogen strains.

Resistance to plant pathogens has been identified from several wild tomato germplasm (Egashira *et al.* 2000; Pico *et al.* 2000; Pim *et al.* 1993; Rosello *et al.* 1999). In addition, several potential sources for resistance to tomato bacterial disease that can be easily crossed with *S. lycopersicum* cultivars, have been used successfully for genetic studies (Astua-Monge *et al.* 2000; Francis *et al.* 2000). However, intensive evaluations of wild tomato germplasm for resistance to bacterial wilt have not been conducted. Jaworski *et al*. (1987) evaluated 2,064 tomato accessions in the field with natural and artificial inoculation of indigenous strains of race 1 biovar 1. The evaluated wild tomato accessions include 72 *S. pimpinellifolium*, 60 *S. peruvianum*, 4 *S. habrochaites*, and 6 *S. habrochaites* f. *glabratum* (previous known as *L. hirsutum* f. *glabratum*). And GA 1405-1-2 BWT, a selection from PI 251323 (*S. pimpinellifolium*) was the only wild accession among the four selected resistant materials.

Therefore, the aim of this study is to evaluate wild tomato germplasm for resistance to race 1 strains of *R. solanacearum*. First, a strain with lower aggressiveness was used. Then selected resistant accessions were evaluated with two other strains with higher aggressiveness. Because the selected resistant accessions were mostly belonged to *S. pennellii*, a population of introgression lines of LA716 (*S. pennellii*) were evaluated in order to identify possible chromosomal location of resistant QTLs.

3.2 MATERIALS AND METHODS

3.2.1 Plant materials

Two core collections of wild tomato were evaluated in this study in order to cover majority of tomato gene pools. They are 109 accessions from AVRDC wild tomato core collection and 143 accessions from core collection of Tomato Genetic Resource Center (TGRC) located at University of California, Davis. All together, there are 252 accessions evaluated including 14 accessions of *S. cheesmaniae,* 17 of *S. chilense,* 10 of *S. chmielewskii,* 52 of *S. lycopersicum,* 37 of *S. habrochaites,* 10 of *S. neorickii,* 19 of *S. pennellii,* 42 of *S. peruvianum,* and 51 of *S. pimpinellifolium*. All seeds of tested accessions were provided by Genetic Resource and Seed Unit (GRSU), AVRDC. TGRC core collection includes chosen accessions representing the genetic diversity of wild tomato, which was established by the late Dr. Charles M. Rick. The collection was acquired and multiplied by GRSU at AVRDC. A population of fifty introgression lines that derived from a cross between *S. lycopersicum* cv M82 and *S. pennelli* LA716 (Eshed and Zamir, 1994), and the two parents were evaluated to explore possible chromosomal locations of QTLs associated with resistance to *R. solanacearum*. The materials were acquired from TGRC and multiplied at AVRDC. However, line LA3487 (IL3-2), which contains a chromosome 3 segment of *S. pennellii* failed to set and was not tested due to lack of seed.

In each evaluation, H7996 and L390 were used as resistant and susceptible controls. Before sowing, seeds were treated by soaking at 4X diluted Chlorox (6% sodium hypochlorite) for 5 minutes and then rinsed under running water for 15 minutes and sown immediately in 2-inch pots. Seeds of H7996 and L390 were sown 2 days later than the wild accessions and introgression lines. The potting mixture used consisted of sand, soil, rice husk and compost in the ratio of 1:3:1:1 and has been steam-sterilized. Three-week old seedlings with four fully expanded true leaves were used for evaluation.

3.2.2 Bacterial strains and plant inoculation

Strains of *R. solanacearum* Pss4 (race 1, biovar 3), Pss186 (race 1, biovar 4), and Pss190 (race 1, biovar 4) (Jaunet and Wang, 1999) isolated from tomato were provided by the Bacteriology Unit, AVRDC. These strains were used because of their difference in aggressiveness. Overall, Pss190 is the most aggressive followed by Pss4 and then Pss186

(Jaunet and Wang, 1999). When screening of wild tomatoes, strain Pss186 was used in the preliminary evaluation. Pss4 and Pss190 were used later to evaluate the durability of the resistance of the selected wild tomatoes. When evaluating the introgression lines of LA716, only strain Pss186 was used.

Stored cultures of Pss4, Pss186 and Pss190 at -80^{o}C were streaked on TTC medium (Kelman, 1954) and incubated at 30°C for 2 days. Several typical fluidal single colonies from TTC were transferred to 523 medium (Kado and Heskett, 1970) for multiplication at 30^{o}C for 24 hours. Then a dense bacterial suspension of each strain was prepared. And a total of 0.1 ml of the suspension was spread on one fresh 523 plate and kept at 30^{o}C for 24 hours. Bacterial masses were harvested and suspended with distilled water until an O.D. value reached 0.3 at the wavelength of 600nm (about 10^8 cfu/ml). Seedlings with four fully expanded true leaves (about three-week old) were inoculated by pouring 20ml of the above suspension on the soil surface of each pot. When evaluating the introgression lines in the field, the suspension was further diluted 5 times with distilled water (approximately $2x10^7$ cfu/ml).

For seedling evaluations, inoculated plants were rated at 1, 2, 3 and 4 weeks after inoculation using a 0-5 scale, where 0 means no symptom; 1 means one leaf partially wilted; 2 means 2-3 leaves wilted; 3 means 4 or more leaves wilted; 4 means all leaves wilted; 5 means death of the plant (Winstead and Kelman, 1952). Percentages of wilted plants (PWP) at 4 weeks after inoculation were calculated following the formula of PWP = (N_W/N_T) x 100, where N_T is number of total tested plants and N_W is number of wilted plants. Plants in the field trial were rated once a week after transplanting using 0-5 scale, where 0 means seedling has no symptom; 1 means less than 20% leaves wilted; 2 means 20% to less than 60% leaves wilted; 3 means 60% to less than 100% leaves wilted; 4 means all leaves wilted; 5 means plant collapsed or dead. PWP at 6 weeks after transplanting was calculated.

Relative area under the disease progress curve (RAUDPC) (Fry, 1978) was calculated following: First PWP was used to calculate area under the disease progress curve (AUDPC), which expresses the dynamics of disease development according to Shaner and Finney (1977), following the formula of AUDPC = $\sum_{i=1 \text{ to } n-1} [(Y_{i+1} + Y_i)/2] \times [X_{i+1} - X_i]$, where Y_i is percentage of wilted plants at the i*th* observation (i = 1 being the first observation point), and X_i is time at the i*th* observation, and n is total number of

observation. Second AUDPC was then divided by the number of days from inoculation to the end of observation period.

To determine the presence of the pathogen in symptomless plants at the time of final rating, a printing method was used. Symptomless plants were harvested, and roots and all leaves were removed. The remaining stem was washed with tap water, rinsed in distilled water, sprayed with 70% alcohol, and blotted dry on paper towels. Each plant was sectioned at the stem midpoint and 2cm from stem tip with a sterilized razor blade. The cut surface of the top section and lower surface of middle section were pressed tightly on the plate surface for 5 seconds per print and five prints were made continuously for each cut surface. The medium used is a semi-selective medium of *R. solanacarum* called SM1 (Tsai *et al* 1985). The SM1 plates were incubated at 30^{o}C for 3 days. When fluidal bacterial mass was observed on at least one print, the plant was scored as positive for pathogen colonization; then percentage of colonized plants (PCP) was calculated for each section following the formula of PCP = $((N_C + N_W)/N_T) \times 100$, where N_T is number of total plants, N_W is number of wilted plants, and N_C is number of plants shown positive colonization. If there were doubts about the identity of cultured bacteria, bacterial mass was streaked on SM1 for observing typical colonies of the pathogen.

3.2.3 Experimental design and data analysis

For evaluating wild tomatoes, the accessions were evaluated over 7 batches with one week apart among batches due to limited space in the greenhouse. Each evaluation was laid out as a randomized complete block design (RCBD). Accessions with final percentage of wilted plants of equal or smaller than 60% were selected for confirmation. Four confirmation trials were conducted overtime at different seasons and greenhouse locations and against different pathogen strains (Table 3.2). When more than one strain was used in one experiment, split-plot design was followed with “strain” as the main-plots and “plant materials” as the subplots. Screening of introgression lines of LA716 at seedling stage was conducted in the same way. All experiments consisted of 2 replications and 10 plants per replication. When evaluating LA716 introgression lines in the field, RCBD was followed with 3 replications and 12 plants per replication at spacing of 60cm between lines and 40cm between rows in a plot size of 2.4m x 1m. Basal fertilizer (15% N, 15 % P2O5, 15% K2O, and 4% MgO) was applied broadcasting over the field before building beds with 120kg per hectare.

All percentage data were transformed by arcsine square-root for the analyses of variance. In the combined analysis of variance across trials, and in trials involving more than one strain where the split plot design was used, the data was analyzed using the PROC MIXED procedure of SAS (SAS Institute, Inc., Cary, NC). The entry mean comparison was performed under each trial or strain when the entry x trial or entry x strain was significant. Significant differences were determined at $P<0.05$ by LSD.

3.3 RESULTS

3.3.1 Resistance to bacterial wilt in wild tomatoes

Evaluation of wild tomato germplasm was conducted over seven batches against *R. solanacarum* strain Pss186 from February 8, 2006 to July 21, 2006. Over the seven screenings, maximum temperature ranged from 31°C to 32.1°C and minimum temperature from 25.7°C to 27.3°C. These conditions were favorable to the development of bacterial wilt, which was indicated by the complete wilting of L390 plants in each test (Table 3.1). The similar disease pressure over batches resulted in a similar severity on the resistant control H7996, which had PWP at four weeks after inoculation ranged from 0% to 5%. Final percentage of wilted plants ranged from 10% to 100% among 253 genotypes screened. Most accessions were highly susceptible excluded a few of S. *pennelli* and *S. chmielewskii* (Table 3.1). Accessions having PWP equal to or less than 60% were selected for confirmation. These included one accession of *S. chmielewskii*, LA1317, and seven accessions of *S. pennellii*, LA1926, LA1943, LA716, LA1272, LA1656, LA1732, and TL01845.

Table 3.1 Summary of preliminary screening of wild tomatoes over seven batches[1] for resistance to a *R. solanacearum* strain Pss186 (race 1, biovar 4).

Species[2]	Percentage of wilted plants	
	Range[3]	Mean[3]
S.cheesmanii (14)	90.0-100.0	98.7
S. chilense (17)	100.0	100.0
S. chmielewskii (10)	50.0-100.0	87.8
S. lycopersicum (52)	85.0-100.0	98.5
S. habrochaites (37)	88.9-100.0	99.4
S. neorickii (10)	90.0-100.0	98.5
S. pennellii (19)	10.0-100.0	69.8
S. peruvianum (42)	90.0-100.0	99.2
S. pimpinellifolium (51)	80.0-100.0	97.5
H7996 (Resistant control)	0.0-5.0	3.0
L390 (Susceptible control)	100.0	100.0

[1]Temperature ranges over batches were 31-32.1°C (Max.)/25.7-27.3°C (Min.); relative humidity ranged from 75.5-86.2% (Max.) to 57.5-74.5% (Min) over batches.

[2]The number of accessions screened of each species was indicated in the parentheses.
[3]Values were ranges and means of percentage of wilted plants of accessions of each species and the controls over batches.

3.3.2 Durability of selected resistant accessions

In order to evaluate the durability of resistance in selected wild accessions, the materials were evaluated under different environments and against different *R. solacearum* strains. Information about each trial was summarized in Table 3.2.

Table 3.2 Information of confirmation trials

Trial	Inoculation date (day/month/year)	Strain used	Max. T[1]	Min. T[1]	Max. RH[1]	Min. RH[1]
1	23/06/2006	Pss186	31.9 ± 1.6	27.3 ± 0.9	85.1 ± 2.1	74.4 ± 4.9
2	09/08/2006	Pss4, Pss186	28.9 ± 1.6	26.4 ± 0.8	90.9 ± 3.8	55.8 ± 5.0
3	13/10/2006	Pss4, Pss186, Pss190	30.5 ± 1.8	25.0 ± 2.5	87.4 ± 6.3	68.4 ± 4.0
4	26/12/2006	Pss190	29.0 ± 2.2	25.7 ± 1.4	62.2 ± 8.2	55.9 ± 6.5

[1]Values were means of each record from inoculation day until the last recording day; Max T: maximum temperature; Min. T: minimum temperature; Max. RH: maximum relative humidity; Min. RH: minimum relative humidity.

Trials 1 and 2 were conducted in the summer. Trial 1 was conducted in a screen-house without temperature control facility, and Trial 2 in a glasshouse with temperature control facility; this made the temperature range narrower and the relative humidity range larger in Trial 2. Trial 3 was conducted in the autumn, while Trial 4 was in the early winter. Both trials were conducted in a glasshouse with heating facility. Reactions of selected accessions to Pss186 were confirmed three times (Table 3.3). Combined analysis showed a significant interaction between trial and entry. The controls, H7996 and L390, were not significantly different over the trials. In general, all tested accessions had similar reactions over trials, excepted LA716, LA1317, and LA1656. These accessions displayed a higher disease incidence in Trial 1. This could be due to the higher temperature and relative humidity in this trial. Resistance in LA1317 could be more sensitive to Trial 1 conditions, which had a significant higher disease incidence. LA1943 and LA1732 were more durable than the others against Pss186, as they had similar reactions as H7996 in two of three confirmation trials.

Table 3.3 Percentage of wilted plants of selected accessions at 28 days after inoculation with Pss186 in 3 confirmation trials

Entry	Trial 1			Trial 2			Trial 3		
S. chmielewskii									
LA1317	80.0	b[1]	A[2]	25.0	cd	B	nt		
S. pennelli									
LA716	40.0	cd	A	35.0	cd	A	0.0	d	B
LA1272	64.3	c	A	55.0	bc	A	nt		
LA1656	40.0	cd	A	0.0	e	B	10.0	cd	AB
LA1732	20.0	de	A	55.0	bc	A	40.0	b	A
LA1926	55.0	cd	A	75.0	ab	A	nt		
LA1943	25.0	cde	A	35.0	cd	A	10.0	cd	A
TL01845	nt[3]			20.0	de	A	25.0	bc	A
H7996	5.0	e	A	0.0	e	A	15.0	bcd	A
L390	100.0	a	A	90.0	a	A	100.0	a	A

[1]Means followed by the same small letters were not significantly different within the same column based on $LSD_{0.05}$ (within trial comparison).
[2]Means with followed by the same capital letters were not significantly different within the same row based on $LSD_{0.05}$ (between trial comparisons).
"nt" means the accession was not tested in that particular trial.

The five *S. pennellii* accessions, which showed a lower incidence to Pss186, were selected and inoculated with two other strains. The aggressiveness displayed by Pss4, Pss186, and Pss190 was as expected (Table 3.4). Pss4 and Pss186 caused similar percentage of wilted plants on H7996 plants. And Pss190 caused a complete wilting on H7996. The entry x strain interaction was significant, indicating individual entries had different reactions against different strains. All entries were highly susceptible to Pss4 and showed similar reactions to Pss186 and Pss190 based on visual wilting symptoms. LA716 was the most resistant, which had 0% of wilted plants against Pss186 and Pss190. The survived plants were assayed to determine whether they were colonized by the pathogen. The percentages of colonized plants were always lower in the top-section than in the middle-section of stems on plants of all entries inoculated with both strains. And all the resistant accessions were not immune to *R. solanacearum*.

Table 3.4 Percentage of wilted plants (PWP) and percentage of colonized plants at mid-stem (PCP-m) and top-stem (PCP-t) of selected accessions at 28 days after inoculation with Pss186, Pss190 and Pss4 in Trial 3

	PWP								PCP-m						PCP-t					
Entry	Pss4			Pss186			Pss190			Pss186			Pss190			Pss186			Pss190		
LA716	100.0	a[1]	A[2]	0.0	c	B	0.0	c	B	25.0	b	A	0.0	c	A	5.0	bc	A	0.0	c	A
LA1656	100.0	a	A	10.0	bc	B	15.0	bc	B	10.0	b	A	35.0	bc	A	5.0	bc	A	10.0	c	A
LA1732	100.0	a	A	40.0	b	B	50.0	b	B	45.0	b	A	55.0	b	A	40.0	b	A	40.0	b	A
LA1943	100.0	a	A	10.0	bc	B	20.0	bc	B	10.0	b	A	35.0	bc	A	0.0	c	A	10.0	c	A
TL01845	100.0	a	A	25.0	b	B	10.0	bc	B	45.0	b	A	15.0	bc	A	30.0	b	A	5.0	c	B
H7996	5.0	b	B	15.0	bc	B	100.0	a	A	15.0	b	B	100.0	a	A	10.0	bc	B	100.0	a	A
L390	100.0	a	A	100.0	a	A	100.0	a	A	100.0	a	A	100.0	a	A	100.0	a	A	100.0	a	A

[1]Means in the same column followed by the same small letters were not significantly different based on $LSD_{0.05}$ (within strain comparison).
[2] Means in the same row followed by the same capital letters were not significantly different based on $LSD_{0.05}$ (between strain comparisons).

The five tested *S. pennellii* accessions had similar tolerance to Pss186 as H7996 resulted in similar percentage of colonized plants. The H7996 was highly susceptible to Pss190, while the *S. pennellii* accessions displayed good tolerance to Pss190, which have 0% to 10% of colonized plants, excepted LA1732.

Reactions of selected accessions to Pss190 were confirmed in another experiment, and combined analysis did not show significant interaction between trial and entry (Table 3.5). The controls, H7996 and L390, were fully susceptible to Pss190 in the two trials. LA1317 and LA1926 showed a susceptible reaction similar to the controls H7996 and L390. Among the entries, LA716 and TL01845 were the most resistant over two trials. Resistance to Pss190 in these wild tomatoes should be confirmed in summer under higher mean temperature and relative humidity conditions to ensure their durability.

Table 3.5 Disease incidence of selected accessions at 28 days after inoculation when inoculated with Pss190 in 2 confirmation trials

Entry	Trial 3		Trial 4	
S. chmielewskii				
LA 1317	nt		100.0	a
S. pennelli				
LA716	0.0	c[1]	15.0	bc
LA1272	nt		55.0	b
LA1656	15.0	bc	25.0	b
LA1732	50.0	b	60.0	b
LA1926	nt		75.0	ab
LA1943	20.0	bc	40.0	b
TL01845	10.0	bc	0.0	c
H7996	100.0	a	95.0	a
L390	100.0	a	100.0	a

[1]Means followed by the same small letters were not significantly different in column-wide comparison based on $LSD_{0.05}$ (within trial comparison).
"nt" means not-tested.

3.3.3 Reactions of LA716 introgression lines to Pss186

Introgression lines (ILs) of LA716 were evaluated in greenhouse and field to find possible locations of QTL associated with the resistance in LA716 to Pss186. Under the greenhouse conditions (mean max. temperature: 31.8^{o}C, mean min. temperature: 29.4^{o}C), ILs of LA716 were highly susceptible when inoculated with inoculum density of 10^{8} cfu/ml. Percentage of wilted plants of ILs ranged from 80% to 100%. All tested ILs had similar severity and disease progress compared to M82 and the susceptible check L390 (data not showed). Presence of high temperature range during this trial should contribute to this high severity, which was observed among the three screening trials of PSs186 (Table 3.3). Seedlings of the ILs were inoculated with lower inoculum dose and transplanted to the field. Here, the mean temperature ranged from 17.9^{o}C to 26.6 ^{o}C and the total rainfall was 47mm during the trial. Judging from the control lines, disease pressure in the field trial was lower than that in the greenhouse trial due to the lower temperature and lower initial inoculum dose. Plants of *S. pennellii* LA716 was not adopted to high soil moisture conditions in the field. During the field trial, the field was irrigated right after transplanting and it rained right after transplanting (9mm in the first 4 days), which prolonged the high soil moisture condition. Only four plants per replication survived one week after transplanting; however, all the ILs grew well in the trial. The percentage of wilted plants of introgression lines ranged from 27.8 % to 100%; meanwhile that of LA716 and M82 were 25% and 86.1%, respectively. When evaluating with final percentage of wilted plants, only LA3501 had significantly lower incidence than M82 and was not significantly different from LA716. Analyzing data of relative area under the disease progress curve (RAUPDC), which expresses the dynamics of the disease development, all the ILs displayed significant higher RAUPDC than LA716 except four ILs LA3476, LA3501, LA3510, and LA3517 showed significantly slower disease progress than M82 (Table 3.6). The resuslt indicated possible QTLs located in the introgressed segments present in these four lines.

Table 3.6 Percentage of wilted plants (PWP) and relative area under disease progress curve (RAUPDC) of selected introgression lines after inoculation with Pss186 in the field in comparisons to LA716 and M82

Entry	PWP[1]	LA716	M82	RAUPDC[2]	LA716	M82
LA3476 (IL1-1)	72.2	**	ns	54.6	**	*
LA3501 (IL6-2)	27.8	ns	**	28.5	*	**
LA3510 (IL8-1)	63.9	**	ns	47.6	**	**
LA3517 (IL10-3)	66.7	**	ns	53.2	**	*
H7996	0.0	*	**	0.0	**	ns
L390	91.7	**	ns	73.4	**	ns
LA716	25.0			18.8		
M82	86.1			67.7		

[1] Means of final percentage of wilted plants (6 weeks after transplanting)
[2] Means of RAUPDC: relative area under the disease progress curve
[3] Mean comparisons between LA 716 and M82 with introgression lines by LSD (** means significant at P<0.01; * means significant at P<0.05; ns: not significant).

3.4 DISCUSSION

Planting resistant materials has been the main strategy to control tomato bacterial wilt caused by race 1 strains of *R. solanacearum*. However, limited resistance sources have been used in breeding programs (Scott *et al*. 2005) and most of them are not durable across locations and strains. This study evaluated resistance in 252 accessions of tomato germplasm belonging to 9 species with the idea to identify new diverse resistance sources to overcome the diverse strains of the pathogen.

In this study, resistance to race 1 strains of *R. solanacarum* was identified from wild tomato accessions, particularly *S. pennellii*. Using a *R. solanacearum* strain, Pss186, a total of 8 wild accessions were found to have significant tolerance to bacterial wilt. The frequency of finding resistance sources from tomato germplasm has been low as shown by previous studies. Jaworski *et al*. (1987) evaluated 2,064 tomato accessions against race 1 biovar 1 strains and only identified four selections to be highly tolerant. Among them, three selections were from *S. lycopersicum* and one from *S. pimpinellifolium*. Similarly, Gonzalez and Summers (1996) found five accessions to have some degree of resistance against 2 strains of race 1 biovar 1 and eight accessions were resistance to 2 strains of race 1 biovar 3 among 233 accessions screened. More recently, partial resistance to a strain of race 3 was detected in one accession belonging to *S. peruvianum*, and one *S. lycopersicon* var. *cerasiforme* accession among the 82 tomato accessions screened (Carmeille *et al.* 2006). Summarizing results from previous studies, it may be worthwhile to evaluate more intensively the accessions of *S. lycopersicon* var. *cerasiforme*, *S. pimpinellifolium*, and *S. pennellii* for resistance to the pathogen.

Durability of selected resistance sources is a concern, as both temperature and strain can affect the final severity of bacterial wilt on tomato. It is known that several tomato varieties displayed higher severity of bacterial wilt under higher temperature under controlled environment (Krausz and Thurston, 1975) and in the field (Prior *et al.* 1996). The resistance to Pss186 in *S. pennelli* LA1943, LA1926, LA1272, LA1732 and TL01845 was consistent under different seasons and environmental conditions. Another two *S. pennelli* LA716 and LA1656 as well as *S. chmielewskii* LA1317 could be sensitive to high

temperature, as they expressed higher disease incidence, but not complete breakdown, in the first trial, when temperature was higher.

Strain specific nature of resistance was found in *S. pennellii* accessions. These accessions were resistant to Pss186 and Pss190, but not Pss4. In tomato, strain specific nature of resistance to *R. solanacearum* has been reported (Krausz and Thurston, 1975). And strain-specific QTLs have already been identified in H7996, a resistant tomato variety (Danesh and Young, 1994; Wang *et al.* 2000). Therefore using well-characterized strains in screening is important for future resistance deployment. H7996 was fully susceptible to Pss190 but resistant to Pss186 and Pss4. In the previous studies, H7996 was found to be the most stable resistant source against to different strains of race 1 and race 3 as well (Carmeille *et al.* 2006; Wang *et al.* 1997). Therefore, integrating resistance in H7996 and *S. pennelli* accessions in breeding lines would increase durability of resistance against the pathogen.

In tomato, resistance to diseases including *Fusarium* wilt (*Fusarium oxysporum* f. sp. *lycopersici*), stem canker (*Alternaria alternata* f. sp. *lycopersic*), tobacco etch virus (Bournival *et al.* 1990; Erik *et al.* 1995; Legnani *et al.* 1996; Reis *et al.* 2004) has been identified in *S. pennellii* accessions. However, this is the first report of *S. pennellii* accessions being resistant to bacterial wilt. Since *S. pennellii* LA716 was found to be resistant to race 1 strain Pss186 of *R. solanacearum* in this study, the introgression lines of LA716, which completely covered the genome of LA716 (Eshed and Zamir, 1994), were evaluated against Pss186. In the field evaluation, which had lower disease pressure, only LA3501 (IL6-2) showed a similar level of resistance as LA716. The IL6-2 carried an introgression segment on chromosome 6, where resistance gene *Bwr-6* located (Carmeille *et al.* 2006). Anchor RFLP markers in the segments, like TG25 (Danesh and Young, 1994), TG153 (Carmeille *et al.* 2006; Danesh and Young, 1994; Mangin *et al.* 1999; Thoquet *et al.* 1996; Wang *et al.* 2000), TG162 and TG240 (Mangin *et al.* 1999; Wang *et al.* 2000) have been detected to be associated with resistance to bacterial wilt in H7996 when challenging with different strains of race 1 and race 3. Therefore, the resistance gene in this region could be essential to durable resistance to bacterial wilt in tomato. Due to the missing of LA3487 (IL3-2) in our study, we could not rule out the possible association of this chromosomal region with the resistance in LA716, although no QTL has been detected in this region.

Our strategy of using a less aggressive strain for preliminary screening and then confirming with more diverse strains has proved to be efficient. In this study, we identified new resistance sources from wild tomato accessions. Among them, the *S. pennellii* accessions LA1656, LA1943 and TL01845 showed durable resistance to Pss186 and Pss190, but not to Pss4. Resistance in these wild accessions needs to be transferred into *S. lycopersicum* background before it can be used in breeding program for improving resistance.

3.5 SUMMARY

A total of 252 wild *Solanum* accessions and one population of forty-nine introgression lines (ILs) were screened for resistance to a race 1 biovar 4 strain Pss186 of *Ralstonia solanacearum*. Most wild tomato accessions were highly susceptible. However, five wild tomato accessions of *S. pennellii*, i.e. LA1943, LA716, LA1656, LA1732 and TL01845 were resistant to strain Pss186. These accessions were challenged against strains Pss4 and Pss190, which were more aggressive. All the five *S. pennellii* accessions were susceptible to Pss4, but displayed high to moderate resistance to Pss190, a virulent strain that made H7996 susceptible, with percentage of wilted plant ranged from 0 to 60%. Thus, the results found in this study evidencing that the presence of strain-specific resistance. Only IL6-2, which has an introgression segment on chromosome 6, was found to be resistant to Pss186 among screened ILs. This confirms the importance of resistance loci on chromosome 6 that were identified by other studies. These new resistant sources will provide breeders more resources to breed for durable resistance to bacterial wilt of tomato.

GENERAL CONCLUSIONS

Resistance to *R. solanacearum* in a tomato line Hawaii 7996 is stable and controlled by several genes. Genetic polymorphism between Hawaii 7996 and WVa700, the two parents of the mapping population used in this study, is limited. Thus, the linkage map constructed in this study still has major gaps among chromosomes. QTLs on chromosome 6, LGA (possibly chromosome 12) and LGB were associated with resistance to several race 1 strains under different environments, suggesting they play a major role in resistance to race 1 strains of *R. solanacearum*. Resistance mechanism in Hawaii 7996 appears to be related to the suppression of the pathogen colonization, as similar QTLs were found from visual symptom data as well as colonization data. In addition, a QTL on chromosome 2 with resistance contributed from the susceptible parent Wv700 was identified. QTL analysis results also suggested plausible strain-specific and enviroment-specific QTLs that could modify the expression of the resistance traits. This is the first report on association between bacterial wilt resistance and fruit weight, citric acid, and fruit color. Strain specific nature of resistance was found in *S. pennellii* accessions. *S. pennellii* accessions LA1656, LA1943 and TL01845 showed durable resistance to Pss186 and Pss190, but not to Pss4 and resistance in LA1317, LA716 and LA1656 could be sensitive to high temperature. Results gained from screening a set of introgression lines of LA716 indicated genetic control of the resistance in *S. pennellii* is not simple and a possible QTL is presumably located on chromosome 6. These new resistant genes in wild accessions need to be transferred into *S. lycopersicum* background before they can be used in breeding programs for improving resistance.

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APPENDIX TABLES

Appendix table 1.1 Summary of polymorphism of AFLP selective primer pairs used in screening F_9 RILs derived from cross H7996 x WVa700

No. of primer combination	Primer combination	No. of bands	No. of polymorphic bands	Percent polymorphism
2	E1&M2	46	5	10.9
4	E1&M4	55	2	3.6
10	E2&M2	50	2	4.0
11	E2&M3	43	3	7.0
14	E2&M6	61	5	8.2
16	E2&M8	58	1	1.7
34	E3&M2	38	5	13.2
19	E3&M3	46	7	15.2
20	E3&M4	53	4	7.5
21	E3&M5	51	4	7.8
23	E3&M7	53	5	9.4
26	E4&M2	32	1	3.1
35	E4&M3	36	4	11.1
36	E4&M4	58	3	5.2
37	E4&M5	43	9	20.9
38	E4&M6	41	4	9.8
39	E4&M7	31	2	6.5
41	E5&M1	32	2	6.3
44	E5&M4	24	3	12.5
46	E5&M6	28	4	14.3
60	E8&M4	34	3	8.8

Appendix table 1.2 Molecular weight (MW), band presented, and χ^2 test for goodness of fit for 1:1 Mendelian segregation ratio of AFLP markers

Marker number	Marker code	MW (bp)	Band presented in		Frequency		χ^2 value	
			H7996	WVa700	H	W		
1	afh	260	+	-	99	86	0.91	
2	afh2a	136	+	-	103	82	2.38	
3	afh2b	137	-	+	101	84	1.56	
4	afh2c	221	+	-	104	81	2.86	
5	afh2d	225	+	-	104	81	2.86	
6	afh2e	434	+	-	140	45	48.78	**
7	afh4a	130	-	+	80	99	2.02	
8	afh4b	243	-	+	55	122	25.36	**
9	afh10a	173	+	-	98	89	0.43	
10	afh10b	270	+	-	100	87	0.90	
11	afh11a	124	-	+	83	99	1.41	
12	afh11b	367	+	-	65	117	14.86	**
13	afh14a	87	-	+	108	80	4.17	**
14	afh14b	121	-	+	62	126	21.79	**
15	afh14c	270	+	-	83	105	2.57	
16	afh14d	289	+	-	140	48	45.02	**
17	afh14e	342	+	-	77	111	6.15	**
18	afh16a	245	-	+	104	79	3.42	
19	afh19a	134	+	-	96	89	0.26	
20	afh19b	169	+	-	77	108	5.19	**
21	afh19c	172	+	-	77	108	5.19	**
22	afh19d	303	-	+	84	101	1.56	
23	afh19e	349	+	-	75	110	6.62	**
24	afh19f	395	-	+	138	47	44.76	**
25	afh19g	450	-	+	141	43	52.20	**
26	afh20a	98	-	+	108	78	4.84	**
27	afh20b	135	-	+	90	96	0.19	
28	afh20c	186	-	+	65	121	16.86	**
29	afh21a	103	-	+	67	120	15.02	**
30	afh21b	194	+	-	116	70	11.38	**
31	afh21c	232	-	+	176	10	148.15	**
32	afh21d	310	+	-	81	105	3.10	
33	afh23a	142	+	-	108	77	5.19	**
34	afh23b	210	-	+	68	117	12.98	**
35	afh23c	237	-	+	126	59	24.26	**
36	afh23d	240	-	+	174	11	143.62	**
37	afh23e	378	-	+	91	88	0.05	
38	afh34a	81	+	-	136	49	40.91	**
39	afh34b	354	-	+	167	18	120.01	**
40	afh34c	386	+	-	100	80	2.22	

Appendix Table 1.2 continued

Marker number	Marker code	MW (bp)	Band presented		Frequency		χ^2 value	
			H7996	WVa700	H	W		
41	afh34d	421	+	-	113	72	9.09	**
42	afh34e	450	+	-	88	97	0.44	
43	afh35a	191	-	+	69	117	12.39	**
44	afh35b	193	-	+	69	117	12.39	**
45	afh35c	271	+	-	112	74	7.76	**
46	afh35d	487	-	+	102	84	1.74	
47	afh36a	122	+	-	145	40	59.59	**
48	afh36b	165	-	+	75	110	6.62	**
49	afh36c	369	+	-	90	95	0.14	
50	afh37a	118	-	+	89	96	0.26	
51	afh37b	140	+	-	105	80	3.38	
52	afh37c	159	+	-	137	48	42.82	**
53	afh37d	162	+	-	137	48	42.82	**
54	afh37e	196	-	+	181	5	166.54	**
55	afh37f	209	-	+	78	107	4.55	**
56	afh37g	297	+	-	53	133	34.41	**
57	afh37h	322	-	+	97	88	0.44	
58	afh37i	445	-	+	115	70	10.95	**
59	afh38a	127	+	-	74	112	7.76	**
60	afh38b	151	+	-	80	106	3.63	
61	afh38c	163	+	-	108	78	4.84	**
62	afh38d	165	+	-	107	79	4.22	**
63	afh39a	109	+	-	82	104	2.60	
64	afh39b	276	-	+	75	111	6.97	**
65	afh41a	164	+	-	77	110	5.82	**
66	afh41b	166	+	-	77	110	5.82	**
67	afh44a	95	+	-	136	49	40.91	**
68	afh44b	104	+	-	136	49	40.91	**
69	afh44c	115	-	+	96	89	0.26	
70	afh46a	158	+	-	104	81	2.86	
71	afh46b	161	-	+	107	77	4.89	**
72	afh46c	370	+	-	72	115	9.89	**
73	afh46d	411	-	+	133	53	34.41	**
74	afh60a	89	-	+	67	116	13.12	**
75	afh60b	138	+	-	138	44	48.55	**
76	afh60c	360	-	+	75	110	6.62	**

Critical χ^2 values for 1 degree of freedom: 3.841 (P=0.05) and 6.635 (P=0.01).

Appendix table 1.3 Summary of polymorphism of SNP primers used in screening the two parents H7996 and WVa700

No.	Primer name	Chr.	Restriction enzyme used	MW (bp)	
				H7996	WVa700
1	LEOH8.1	9		180	180
2	LEOH10	4	BsaJ I	200	200
3	LEOH16.1	5		180	180
4	LEOH16.2	5	BsaW I	200	200
5	LEOH17.1	multiple		400	400
6	LEOH19	12	BsaB I	300	300
7	LEOH23.1	2	Msp I	200	200
8	LEOH31.3	9	Msp I/Mse I	400/300	400/300
9	LEOH36	1	Bcl I	1000	1200
10	LEOH37	4	NmuC I	100	100
11	LEOH40.1	7	NmuC I	450	450

Appendix table 1.4 Molecular weight (MW) and χ^2 test for goodness of fit for 1:1 Mendelian segregation ration of SSR markers

No.	Primer code	Marker code	MW (bp)		Frequency		χ^2 value	
			H7996	WVa700	H	W		
1	01-138.0	s01138.0	211	213	81	102	2.41	
2	02-022.0	s02022.0	144	140	100	76	3.27	
3	02-036.6	s02036.6	180	181	93	79	1.14	
4	03-074.1	s03074.1	184	181	87	86	0.01	
5	03-099.0	s03099.0	281	285	98	82	1.42	
6	04-015.0	s04015.0	391	389	95	79	1.47	
7	04-037.0	s04037.0	249	253	79	90	0.72	
8	04-054.5	s04054.5	374	358	69	111	9.80	**
9	04-056.0	s04056.0	364	348	67	107	9.20	**
10	04-058.0	s04058.0	144	147	68	111	10.33	**
11	04-058.1	s04058.1	266	223	69	110	9.39	**
12	06-006.1	s06006.1	188	191	101	79	2.69	
13	06-099.8	s06099.8	402	399	103	71	5.89	**
14	07-002.0	s07002.0	349	355	101	76	3.53	
15	08-001.0	s08001.0	257	292	100	77	2.99	
16	08-055.0	s08055.0	269	273	96	88	0.35	
17	08-055.1	s08055.1	322	325	96	85	0.67	
18	09-051.0	s09051.0	204	227	75	101	3.84	*
19	09-058.0	s09058.0	268	268	75	102	4.12	**
20	10-033.1	s10033.1	236	232	82	106	3.06	
21	10-033.2	s10033.2	310	306	81	95	1.11	
22	10-034.5	s10034.5	313	306	83	105	2.57	
23	10-075.0	s10075.0	211	208	97	78	2.06	
24	11-040.0	s11040.0	331	342	133	46	42.28	**
25	SSR3	SSR3	111	95	72	89	1.80	

Critical χ^2 values for 1 degree of freedom: 3.841 (P=0.05) and 6.635 (P=0.01).

Appendix table 1.5 Chi-square test (χ^2) for goodness of fit for 1:1 Mendelian segregation ration of RFLP markers

No.	Marker name	Frequency		χ^2 value	
		H	W		
1	K4F8	66	39	6.94	**
2	TG118F8	63	34	8.67	**
3	TG153F8	54	44	1.02	
4	TG178F8	34	34	0.00	
5	TG515F8	72	31	16.32	**
6	TG564F8	37	49	1.67	

Critical χ^2 values for 1 degree of freedom: 3.841 (P=0.05) and 6.635 (P=0.01).

Appendix table 1.6 Chi-square test (χ^2) for goodness of fit for 1:1 Mendelian segregation ration of DArT markers

No.	Marker name	Frequency		χ^2 value	
		H	W		
1	D1232A14	67	86	2.36	
2	D1232A24	51	105	18.69	**
3	D1232B11	118	50	27.52	**
4	D1232B15	104	65	9.00	**
5	D1232B17	96	79	1.65	
6	D1232B18	88	68	2.56	
7	D1232B24	103	49	19.18	**
8	D1232B7	90	70	2.50	
9	D1232C22	85	91	0.20	
10	D1232C8	97	74	3.09	
11	D1232D2	65	92	4.64	**
12	D1232D23	77	88	0.73	
13	D1232D4	57	100	11.78	**
14	D1232E10	96	77	2.09	
15	D1232E11	115	43	32.81	**
16	D1232E12	82	86	0.10	
17	D1232E16	98	52	14.11	**
18	D1232E4	71	94	3.21	
19	D1232E6	38	125	46.44	**
20	D1232F20	89	74	1.38	
21	D1232F3	89	77	0.87	
22	D1232G13	55	109	17.78	**
23	D1232G16	88	75	1.04	
24	D1232G19	83	75	0.41	
25	D1232G3	61	105	11.66	**
26	D1232H15	105	66	8.89	**
27	D1232I10	59	105	12.90	**
28	D1232I17	90	73	1.77	
29	D1232I3	72	100	4.56	**
30	D1232I4	119	49	29.17	**
31	D1232J2	107	42	28.36	**
32	D1232J20	72	100	4.56	**
33	D1232J24	119	51	27.20	**
34	D1232J3	67	92	3.93	**
35	D1232K1	93	81	0.83	
36	D1232K17	61	101	9.88	**
37	D1232K22	77	81	0.10	
38	D1232K4	100	68	6.10	**
39	D1232K7	95	78	1.67	
40	D1232L10	74	75	0.01	
41	D1232L14	87	76	0.74	
42	D1232L16	95	75	2.35	
43	D1232L19	72	95	3.17	
44	D1232L20	39	130	49.00	**
45	D1232L22	75	96	2.58	

Appendix Table 1.6 continued

No.	Marker name	Frequency		χ^2 value	
		H	W		
46	D1232M2	78	85	0.30	
47	D1232M4	48	97	16.56	**
48	D1232M9	79	73	0.24	
49	D1232N10	89	76	1.02	
50	D1232N11	39	126	45.87	**
51	D1232N20	84	88	0.09	
52	D1232N22	96	73	3.13	
53	D1232N23	99	71	4.61	**
54	D1232O10	87	75	0.89	
55	D1232O13	72	101	4.86	**
56	D1232O23	71	104	6.22	**
57	D1232P10	96	78	1.86	
58	D1232P13	54	97	12.25	**
59	D1232P8	68	101	6.44	**
60	D1233A12	86	76	0.62	
61	D1233A16	92	79	0.99	
62	D1233A21	53	107	18.23	**
63	D1233B1	94	82	0.82	
64	D1233B13	101	48	18.85	**
65	D1233B18	50	113	24.35	**
66	D1233B20	108	43	27.98	**
67	D1233B23	100	72	4.56	**
68	D1233B4	92	75	1.73	
69	D1233B9	89	76	1.02	
70	D1233C12	108	52	19.60	**
71	D1233C13	95	79	1.47	
72	D1233C15	91	73	1.98	
73	D1233C17	66	89	3.41	
74	D1233C21	102	67	7.25	**
75	D1233C23	49	112	24.65	**
76	D1233C3	103	64	9.11	**
77	D1233C6	103	64	9.11	**
78	D1233D13	94	73	2.64	
79	D1233D18	64	107	10.81	**
80	D1233D20	96	73	3.13	
81	D1233D21	94	66	4.90	**
82	D1233E10	63	108	11.84	**
83	D1233E13	86	71	1.43	
84	D1233E15	100	75	3.57	
85	D1233E22	54	112	20.27	**
86	D1233E8	100	72	4.56	**
87	D1233E9	97	73	3.39	
88	D1233F12	92	75	1.73	
89	D1233F15	96	80	1.45	
90	D1233F16	95	76	2.11	
91	D1233F3	72	87	1.42	
92	D1233F4	60	109	14.21	**

Appendix Table 1.6 continued

No.	Marker name	Frequency		χ^2 value	
		H	W		
93	D1233G16	115	42	33.94	**
94	D1233G20	96	80	1.45	
95	D1233G23	109	45	26.60	**
96	D1233G6	80	96	1.45	
97	D1233H11	77	95	1.88	
98	D1233H12	70	76	0.25	
99	D1233H14	70	78	0.43	
100	D1233H18	93	78	1.32	
101	D1233H22	96	70	4.07	**
102	D1233H24	85	89	0.09	
103	D1233H3	77	97	2.30	
104	D1233H6	117	56	21.51	**
105	D1233I1	97	75	2.81	
106	D1233I2	76	88	0.88	
107	D1233I24	99	67	6.17	**
108	D1233I4	52	101	15.69	**
109	D1233I6	92	76	1.52	
110	D1233J15	47	108	24.01	**
111	D1233J19	92	72	2.44	
112	D1233J2	93	74	2.16	
113	D1233J20	100	74	3.89	**
114	D1233J21	83	88	0.15	
115	D1233J23	94	75	2.14	
116	D1233J4	27	136	72.89	**
117	D1233J7	92	70	2.99	
118	D1233J8	94	73	2.64	
119	D1233J9	58	112	17.15	**
120	D1233K10	118	55	22.94	**
121	D1233K13	39	111	34.56	**
122	D1233K15	140	25	80.15	**
123	D1233K19	91	74	1.75	
124	D1233K2	48	111	24.96	**
125	D1233K20	90	71	2.24	
126	D1233K23	91	78	1.00	
127	D1233K24	70	98	4.67	**
128	D1233K3	119	50	28.17	**
129	D1233K6	96	71	3.74	
130	D1233K8	83	78	0.16	
131	D1233L11	83	87	0.09	
132	D1233L15	96	74	2.85	
133	D1233L22	73	86	1.06	
134	D1233L5	94	76	1.91	
135	D1233L6	86	75	0.75	
136	D1233L7	95	75	2.35	
137	D1233L9	59	111	15.91	**
138	D1233M1	75	74	0.01	
139	D1233M10	92	75	1.73	

Appendix Table 1.6 continued

No.	Marker name	Frequency		χ^2 value	
		H	W		
140	D1233M11	37	131	52.60	**
141	D1233M12	61	110	14.04	**
142	D1233M15	111	40	33.38	**
143	D1233M2	61	99	9.03	**
144	D1233M23	85	82	0.05	
145	D1233M5	48	110	24.33	**
146	D1233M7	84	71	1.09	
147	D1233N11	50	108	21.29	**
148	D1233N13	71	88	1.82	
149	D1233N17	100	73	4.21	**
150	D1233N4	42	108	29.04	**
151	D1233N8	62	83	3.04	
152	D1233O12	46	115	29.57	**
153	D1233O14	77	80	0.06	
154	D1233O18	38	111	35.77	**
155	D1233O4	57	113	18.45	**
156	D1233O9	77	96	2.09	
157	D1233P11	80	79	0.01	
158	D1233P17	89	73	1.58	
159	D1233P2	82	89	0.29	
160	D1233P22	72	100	4.56	**
161	D1233P23	60	115	17.29	**
162	D1242D24	83	82	0.01	
163	D1242F11	117	49	27.86	**
164	D1242G22	79	93	1.14	
165	D1242G23	110	66	11.00	**
166	D1242L15	103	73	5.11	**
167	D1242M23	78	87	0.49	
168	D1242N22	79	82	0.06	
169	D1243A10	100	70	5.29	**
170	D1243B6	75	83	0.41	
171	D1243E24	86	66	2.63	
172	D1243E8	103	73	5.11	**
173	D1243I17	97	71	4.02	**
174	D1243P12	101	75	3.84	*
175	D1243P16	105	70	7.00	**
176	D1244D2	111	65	12.02	**
177	D1244D6	67	92	3.93	**
178	D1244G10	80	94	1.13	
179	D1244G13	123	48	32.89	**
180	D1244G16	103	73	5.11	**
181	D1244G17	112	64	13.09	**
182	D1244H17	109	42	29.73	**
183	D1244L17	74	86	0.90	
184	D1244M23	100	62	8.91	**
185	D1249B11	71	86	1.43	
186	D1249D9	55	102	14.07	**
187	D1249E23	74	93	2.16	

Appendix Table 1.6 continued

No.	Marker name	Frequency		χ^2 value	
		H	W		
188	D1249F11	119	48	30.19	**
189	D1249G23	59	113	16.95	**
190	D1249I6	52	106	18.46	**
191	D1249J22	91	78	1.00	
192	D1249K24	52	104	17.33	**
193	D1249P18	42	96	21.13	**
194	D1249P19	45	107	25.29	**
195	D1250A15	52	89	9.71	**
196	D1250B11	104	68	7.53	**
197	D1250B19	68	98	5.42	**
198	D1250E2	59	105	12.90	**
199	D1250H23	65	102	8.20	**
200	D1250K17	91	48	13.30	**
201	D1250L12	71	103	5.89	**
202	D1250M22	57	114	19.00	**
203	D1250O17	63	91	5.09	**
204	D1250P9	54	101	14.25	**
205	D1255A23	151	21	98.26	**
206	D1255F18	110	60	14.71	**
209	D1261G15	74	99	3.61	
210	D1261H3	99	74	3.61	
211	D1261I18	102	68	6.80	**
212	D1261N4	101	60	10.44	**
213	D1261O23	70	91	2.74	
214	D1261P15	95	63	6.48	**
215	D1262C14	105	71	6.57	**
216	D1262C8	59	116	18.57	**
217	D1262E2	68	94	4.17	**
218	D1262G3	66	93	4.58	**
219	D1262M18	79	76	0.06	
220	D1262M8	67	95	4.84	**
221	D1262P6	46	118	31.61	**
222	D1304A1	98	45	19.64	**
223	D1304A13	103	71	5.89	**
224	D1304A23	102	73	4.81	**
225	D1304A6	111	65	12.02	**
226	D1304B24	74	96	2.85	
227	D1304B5	102	70	5.95	**
228	D1304C20	77	78	0.01	
229	D1304C3	103	73	5.11	**
230	D1304D18	101	74	4.17	**
231	D1304E11	103	73	5.11	**
232	D1304E22	103	73	5.11	**
233	D1304F18	140	23	83.98	**
234	D1304F19	110	65	11.57	**
235	D1304F6	100	67	6.52	**
236	D1304F7	99	74	3.61	

Appendix Table 1.6 continued

No.	Marker name	Frequency		χ^2 value	
		H	W		
237	D1304G14	125	48	34.27	**
238	D1304G15	107	67	9.20	**
239	D1304G21	103	73	5.11	**
240	D1304H10	99	75	3.31	
241	D1304H14	100	72	4.56	**
242	D1304H2	98	75	3.06	
243	D1304I4	76	90	1.18	
244	D1304J1	103	73	5.11	**
245	D1304J19	100	70	5.29	**
246	D1304J20	75	78	0.06	
247	D1304J24	145	23	88.60	**
248	D1304J6	103	73	5.11	**
249	D1304K8	102	74	4.45	**
250	D1304L17	93	82	0.69	
251	D1304L19	76	90	1.18	
252	D1304M18	102	73	4.81	**
253	D1304M19	97	61	8.20	**
254	D1304M6	103	54	15.29	**
255	D1304N13	105	38	31.39	**
256	D1304N16	103	73	5.11	**
257	D1304N24	136	40	52.36	**
258	D1304O11	101	73	4.51	**
259	D1304O19	102	73	4.81	**
260	D1304O2	103	73	5.11	**
261	D1304O20	103	68	7.16	**
262	D1304O23	108	67	9.61	**
263	D1304P7	100	74	3.89	**
264	D1305A19	104	65	9.00	**
265	D1305A2	110	43	29.34	**
266	D1305A4	124	45	36.93	**
267	D1305B24	88	65	3.46	
268	D1305B4	76	84	0.40	
269	D1305C17	76	95	2.11	
270	D1305C19	100	72	4.56	**
271	D1305C22	126	49	33.88	**
272	D1305E6	103	62	10.19	**
273	D1305F12	103	69	6.72	**
274	D1305F18	103	73	5.11	**
275	D1305F19	105	54	16.36	**
276	D1305F2	99	73	3.93	**
277	D1305F21	101	73	4.51	**
278	D1305F8	103	73	5.11	**
279	D1305G16	77	95	1.88	
280	D1305G19	102	73	4.81	**
281	D1305G2	100	70	5.29	**
282	D1305G22	126	49	33.88	**
283	D1305G6	100	62	8.91	**

Appendix Table 1.6 continued

No.	Marker name	Frequency		χ^2 value	
		H	W		
284	D1305G8	103	73	5.11	**
285	D1305H1	103	73	5.11	**
286	D1305H11	103	73	5.11	**
287	D1305H17	102	74	4.45	**
288	D1305H24	103	73	5.11	**
289	D1305I10	103	73	5.11	**
290	D1305I11	103	73	5.11	**
291	D1305I16	66	95	5.22	**
292	D1305I21	107	67	9.20	**
293	D1305I3	101	72	4.86	**
294	D1305J11	99	68	5.75	**
295	D1305J13	103	73	5.11	**
296	D1305J14	92	71	2.71	
297	D1305J20	121	48	31.53	**
298	D1305J6	126	41	43.26	**
299	D1305L1	103	73	5.11	**
300	D1305L14	77	93	1.51	
301	D1305L6	101	74	4.17	**
302	D1305L9	103	73	5.11	**
303	D1305M18	121	47	32.60	**
304	D1305N19	74	92	1.95	
305	D1305N21	99	69	5.36	**
306	D1305N4	47	121	32.60	**
307	D1305O1	103	73	5.11	**
308	D1305O10	74	94	2.38	
309	D1305O9	103	73	5.11	**
310	D1305P1	98	75	3.06	
311	D1305P12	103	73	5.11	**
312	D1305P15	103	73	5.11	**
313	D1305P17	122	51	29.14	**

Critical χ^2 values for 1 degree of freedom: 3.841 (P=0.05) and 6.635 (P=0.01).

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3. Truong, T. H. H., Esch, E., Wang, J. F., Graham, E., and Hanson, P. M. Construction of a genetic linkage map for mapping bacterial wilt resistance in the tomato cultivar Hawaii7996 (in preparation).
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3. Truong, T. H. H., Esch, E., Wang, J. F., Graham, E., and Hanson, P. M. Construction of a genetic linkage map for mapping bacterial wilt resistance in the tomato cultivar Hawaii7996 (in Vorbereitung).
4. Wang, J.-F., Truong, T. H. H., Esch, E., Graham, E. Hanson, P. M. and de la Peña, R.C. Mapping quantitative resistance loci to bacterial wilt in tomato line Hawaii 7996 (in Vorbereitung).

Tagungsbeiträge:

Truong, T. H. H., Esch, E., Wang, J. F. (2007). Screening of wild tomato germplasm for resistance to race 1 strains of *Ralstonia solanacearum*. Second International Symposium on Tomato Diseases at Kusadasi, Turkey, 8-12. Oktober 2007.