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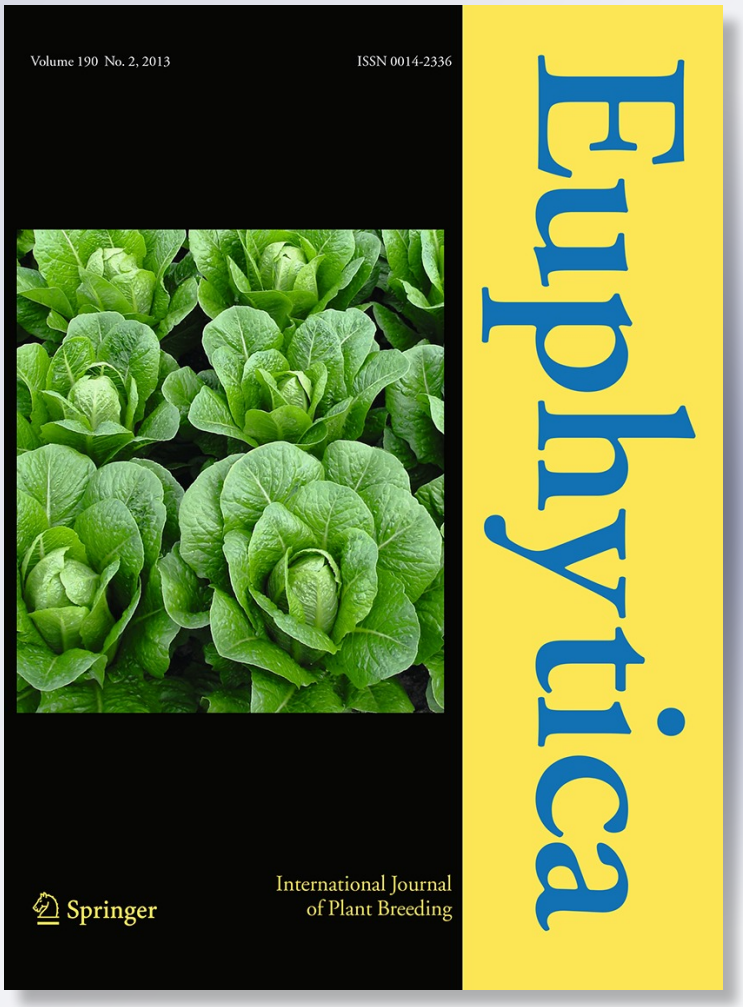

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Identification of major QTLs associated with stable resistance of tomato cultivar 'Hawaii 7996' to *Ralstonia solanacearum*

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Abstract Bacterial wilt caused by *Ralstonia solanacearum* is one of the most devastating diseases of tomato. Tomato cultivar 'Hawaii 7996' has been shown to have stable resistance against different strains under different environments. This study aimed to locate quantitative trail loci (QTLs) associated with stable resistance using 188 recombinant inbred lines (RILs) derived from 'Hawaii 7996' and 'West Virginia 700.' A new linkage map with good genome coverage was developed, mainly using simple sequence repeat markers developed from anchored bacterial artificial chromosome or scaffold sequences of tomato. The population was evaluated against phylotype I and phylotype II strains at seedling stage or in the field in Indonesia, Philippines, Taiwan, Thailand, and Reunion. Two major QTLs were identified to be associated with stable resistance. *Bwr-12*, located in a 2.8-cM interval of chromosome 12, controlled 17.9–56.1 % of total resistance variation. The main function of *Bwr-12* was related to suppression of internal multiplication of the pathogen in the stem. This QTL was not associated with resistance against race 3-phylotype II strain. *Bwr-6* on chromosome 6 explained 11.5–22.2 % of the phenotypic variation. Its location differed with phenotype datasets and was distributed along a 15.5-cM region. The RILs with the resistance allele from both *Bwr-12* and *Bwr-6* had the lowest disease incidence, which was significantly lower than the groups with only *Bwr-12* or *Bwr-6*. Our studies confirmed the polygenic nature of resistance to bacterial wilt in tomato, and that stable resistance in 'Hawaii 7996' is mainly associated with *Bwr-6* and *Bwr-12*.

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Introduction

Bacterial wilt caused by *Ralstonia solanacearum* is one of the most devastating diseases of tomato, particularly in tropical and subtropical humid countries (Elphinstone 2005). The pathogen is soil-borne, enters tomato roots via the secondary root emergence site or at root tips, colonizes xylem vessels, and spreads rapidly throughout the vascular system of susceptible tomato genotypes (Vasse et al. 1995). *R. solanacearum* has an extremely wide host range covering more than 200 monocotyledonous and dicotyledonous species and can survive for long periods under a wide range of environmental conditions (Denny 2006). The pathogen's ability to colonize the weed rhizosphere and not cause symptoms allows it to survive over seasons in tomato production areas (Lin et al. 2009).

Traditionally *R. solanacearum* has been classified into five races and six biovars according to host range and biochemical properties, respectively (Denny 2006). Recent genetic and phylogeny studies conclude the presence of four different phylotypes related to the geographical origin of the strains, namely Asiaticum (phylotype I), Americanum (phylotype II), Africanum (phylotype III), and Indonesian (phylotype IV) (Fegan and Prior 2005). Variation in virulence of *R. solanacearum* strains within phylotypes has been observed, and tomato has high susceptibility to diverse *R. solanacearum* strains (Lebeau et al. 2011). Examining the interactions between a set of core resistant accessions of tomato, pepper and eggplant and a core collection of the pathogen strains representing the phylogenetic diversity found bacterial wilt resistance was generally high in eggplant and pepper, but not in tomato.

The use of genetic resistance is the cheapest, most efficient and most environmentally friendly method available to control tomato bacterial wilt. Resistance to race 1 strains (phylotype I and II) has been identified from several sources, but there are few commercial cultivars that possess stable resistance (Scott et al. 2005). Resistance to bacterial wilt in tomato can be location-specific (Hanson et al. 1996) and strain-specific (Lopes et al. 1994; Prior et al. 1990). 'Hawaii 7996' (*Solanum lycopersicum*) is one of the most stable sources of resistance against *R. solanacearum* in tomato; the cultivar exhibited a high survival rate (97 %) over 12 field trials conducted in 11 countries in Asia, America, Australia and Indian Ocean region (Wang et al. 1998). Several quantitative trait locus (QTL) analyses have been performed with the tomato cross 'Hawaii 7996' (resistant cultivar) × 'West Virginia 700' (WVa700; *S. pimpinellifolium*, susceptible cultivar). Inoculation of the F_3 population with strain GMI8217 (race 1, phylotype II) highlighted, among seven different loci detected, an important resistance QTL on chromosome 6 of 'Hawaii 7996' (Thoquet et al. 1996a, b). The same population was challenged with race 1-phylotype I Pss4 strain and the same QTL on chromosome 6 was found together with a strain-specific major QTL on chromosome 12 and a few other minor QTLs (Wang et al. 2000). Carmeille et al. (2006b) examined the resistance in 'Hawaii 7996' against a race 3-phylotype II strain and proposed a phylotype-specific rather than strain-specific trait. A study on L285, another tomato genotype resistant against the race 1-phylotype I UW364 strain, also described polygenic resistance (Danesh et al. 1994), showing that resistance to bacterial wilt in tomato is a complex strain-specific trait.

Symptom expression of tomato bacterial wilt could be affected by pathogen strain, inoculation method, and environmental factors, such as temperature (Hayward 1991). The genotype × environment interaction could be expected to have great influence on the phenotypic variation of the polygenic resistance trait, which could reduce the mapping precision. Use of recombinant inbred lines (RILs) for mapping QTLs could alleviate such a problem (Burr and Burr 1991). RILs are produced by selfing individual F_2 plants for several generations; the increased recombination events compared with a simple F_2 population will enhance the precision of mapping. Moreover, RILs can be used as a permanent mapping population for replicated trials under different environments. Thus, a RIL population derived from 'Hawaii 7996' was used in this study.

Two linkage maps have been reported for the cross between 'Hawaii 7996' and 'WVa700' (Thoquet et al. 1996a, b; Wang et al. 2000; Truong et al. 2010). However, there were either major gaps or unidentified chromosomes in these maps. Geethanjali et al. (2010, 2011) reported the use of bacterial artificial chromosome (BAC) sequences of tomato to design simple sequence repeat (SSR) markers and demonstrated their usefulness in genetic diversity and mapping studies.

SSR marker assays are highly reproducible and the set-up cost of gel-based SSR assays is much lower than assays based on single nucleotide polymorphisms, making gel-based SSRs more accessible for breeders to use for marker-assisted selection. The tomato genome sequence is available at Solanaceae Genomics Network (SGN) (http://sgn.cornell.edu/), provided by the Tomato Genome Consortium (2012). Sequences of scaffolds covering the entire genome are available for developing SSR markers of targeted regions.

In this study, we report on the use of SSR markers in a RIL population derived from a cross between 'Hawaii 7996' and 'WVa700' to identify QTLs conferring stable resistance against several race 1-phylotype I strains. Important and major QTLs associated with resistance to phylotype I strains were identified. The same mapping population was challenged with a race 3-phylotype II strain in Reunion (Carmeille et al. 2006b). Mapping results of phylotypes I and II were compared and discussed.

Materials and methods

Plant materials and DNA extraction

A set of 188 F_9 RILs derived from a cross between *S. lycopersicum* cultivar 'Hawaii 7996' (resistant) and *S. pimpinellifolium* cultivar 'West Virginia 700' (WVa700; susceptible) was used for genotyping and phenotyping. The F_2 and F_3 generations were used in earlier studies (Thoquet et al. 1996a, b; Wang et al. 2000). The population was later advanced to F_5 at the Institute of Plant Breeding, University of the Philippines Los Baños (UPLB) and then to F_9 at AVRDC—The World Vegetable Center. Genomic DNA was isolated from fresh leaves using GenElute™ plant genomic DNA miniprep kit (Sigma, USA) following the instruction manual.

Bacterial strains

Ralstonia solanacearum strains originating from Taiwan and Philippines were used for seedling screening (Table 1). Pss4 and Pss186 were isolated from tomato. Both strains belong to the predominant virulence group in Taiwan, and Pss4 has higher virulence than Pss186 (Jaunet and Wang 1999). Tm-151 is a highly virulent strain isolated from tomato in the Philippines. Cultures of *R. solanacearum* were routinely grown (2 days, 30 °C) on 2,3,5-triphenyl tetrazolium chloride medium (Kelman 1954). For inoculum preparation, the bacteria were multiplied on 523 medium plate (Kado and Heskett 1970) at 30 °C for 24 h, and a bacterial suspension was prepared in sterilized distilled water and adjusted to approximately 10^8 CFU per ml ($OD_{600nm} = 0.3$). Experiments of JT516 and JT519 were conducted by Carmeille et al. (2006b). JT516 isolated from potato was the only phylotype II strain used in this study.

Resistance assays at seedling stage

The assays for Pss4 and Pss186 were conducted at AVRDC. In each trial, the 188 F_9 RIL, two parents and one susceptible control (L390) were used. The experiments were conducted following a randomized complete block design (RCBD) with two replications and eight plants per line. Evaluation with Pss4 was conducted twice. Trial TW-Pss4a was conducted in a temperature-controlled greenhouse and the temperature ranged from 28.4 to 31.2 °C during the trial. Trials TW-Pss4b and TW-Pss186 were conducted in screenhouses with temperatures from 21.2 to 30.6 °C. The assay for Tm-151 was conducted at UPLB using the F_6 RILs, and only one replication was conducted with 16 plants per line (Balatero 2000, Balatero et al. 2005). Night temperatures ranged from 24 to 28 °C; day temperatures from 31 to 35 °C. Plants were inoculated by drenching the bacterial suspension (10^8 CFU/ml) on the soil surface near the base of plant at the 5 fully expanded leaves stage (Wang et al. 2000). The ratio between the applied inoculum and the potting mixture was about 1–10 (v/v). Percentages of wilted plants were recorded once a week for 4 weeks.

Data of trial RE-JT516 and RE-JT519 were provided by Dr. P. Besse, Université de La Réunion and have been used by Carmeille et al. (2006b). The inoculation was conducted by dipping uprooted seedlings in inoculum (10^8 CFU/ml) for 30 min then immediately transplanting the seedlings into individual pots. RE-JT516 was conducted under lower temperatures (15–25 °C), while RE-JT519 was conducted under higher temperatures (20–30 °C), which are conducive environments for the respective strains.

Table 1 Means of maximum percentage of wilted plants and percentage of colonized plants (for TW-Pss4c only) on two parents, susceptible control and RIL population derived from the cross between 'Hawaii 7996' (H7996) and 'West Virginia 700' (WVa700)

Trial[a]	Location[b]	Pathogen information[c]	Parents and control			RIL
			H7996	WVa700	L390	
TW-Pss4a	AVRDC, Taiwan	Tomato, race 1, bv 3, ph I	24.0	99.0	100.0	80.4
TW-Pss4b	AVRDC, Taiwan	Tomato, race 1, bv 3, ph I	19.8	96.9	100.0	70.4
TW-Pss186	AVRDC, Taiwan	Tomato, race 1, bv 4, ph I	4.2	86.5	100.0	56.7
TW-TC	Taichung, Taiwan	Race 1, bv 3/4, ph I	4.2	86.5	100.0	56.7
TW-Pss4c	AVRDC, Taiwan	Tomato, race 1, bv 3, ph I	12.5	56.3	81.3	52.0
PH-Tm151	UPLB, Philippines	Tomato, race 1, bv 3	18.8	100.0	100.0	83.3
TH-CM	Chiangmai, Thailand	Race 1, bv 3	0.0	100.0	100.0	41.3
ID-PW	Purwakarta, Indonesia	Race 1, bv 3	0.0	100.0	100.0	66.7
RE-JT519	CIRAD, Reunion	Geranium, race 1, bv 3, ph I	0.0	79.7	n.d.[b]	20.6
RE-JT516	CIRAD, Reunion	Potato, race 3, bv 2, ph II	19.9	89.2	n.d.	76.4

[a] Trial code consists of two parts, first the country abbreviations followed by strain code or location abbreviation

[b] *AVRDC* AVRDC—The World Vegetable Center, *UPLB* Institute of Plant Breeding, University of Philippines Los Baños, *CIRAD* Centre de cooperation international en recherché agronomique pour le développement, *n.d.* not determined

[c] Host origin, race, biovar (bv) and phylotype (ph) of respective *R. solanacearum* strain used in each trial or present in the infested field

Resistance assays assessing the degree of colonization

Trial TW-Pss4c was conducted to determine the variation among RILs in their ability to limit bacterial colonization following the method described by Hai et al. (2008). The experiment was conducted in the same manner as that for TW-Pss4a. Symptomless plants were harvested 6 days after inoculation. Each plant was cut at the stem midpoint and printed on semi-selective medium SM1 (Tsai et al. 1985). Percentage of colonized plants (PCP) was calculated following the formulas 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 showing positive colonization.

Resistance assays conducted in natural infested fields

Three field trials were conducted in Taiwan, Thailand and Indonesia (Table 1). The fields have a known history of tomato bacterial wilt and have been used regularly for evaluating plant materials for resistance to bacterial wilt. The trials were conducted following RCBD with two replications and 8 plants per replication with spacing of 70 cm between lines and 30 cm between rows in a plot size of 1.5 m × 1.2 m. Seedlings about one-month-old were transplanted on 28 June, 28 July and 1 November 2004 for trials ID-PW, TW-TC, and TH-CM, respectively. Normal crop management practices were used. Seedlings for trial ID-PW were inoculated with *R. solanacearum* strain Ps18 (isolated from eggplant, race 1, biovar 3) by the soil drenching method 1 day before transplanting. Additional plants of L390 were transplanted in front of each plot (ID-PW), between lines (TW-TC), or in the same transplanting holes (TH-CM) to promote even distribution of the pathogen. Strains of *R. solanacearum* were isolated from wilted tomato plants collected from these infested fields, and they were identified to be biovar 3 or 4. Disease development was determined weekly until the disease reaction reached maximum severity. Percentage of wilted plants was calculated for each entry and used for further analyses.

Marker development and linkage map construction

Search of SSR motifs, SSR primer design, polymerase chain reaction amplification and visualization of fragments of SSR markers were conducted as

described by Geethanjali et al. (2010). SSR markers with even distribution on each chromosome were developed from BAC clones. Fourteen and 12 SSR markers on chromosomes 6 and 12, respectively, reported by Geethanjali et al. (2010, 2011) were used in this study. Special effort was made to saturate the reported QTL region on chromosomes 6 and 12. Four BAC clones and three scaffolds spanning SLM6-10 and SLM6-53 from SGN were searched for SSR motifs. Similarly, six scaffolds for the SLM12-19 to SLM12-9 and SLM12-5 and SLM12-31 regions were mined for marker development.

Eighteen additional anchored SSR markers from SGN and selected amplified fragment length polymorphism (AFLP) and diversity array technology (DArT) markers from Truong et al. (2010) were used for linkage map construction to fill gaps. In total, 16 AFLP, 47 DArT and 145 SSR polymorphic markers between two parents were used for genotyping the 188 RIL. Marker segregation types were identified in a Chi-square test (χ^2) for deviation from the segregation ratios of 1:1 ratio. Linkage map was constructed using the JoinMap® program version 4 (van Ooijen 2006). The genotyped markers were classified into 12 linkage groups, using the grouping module of JoinMap® at a minimum logarithm of odds (LOD) value of 4.0. Marker order and genetic distance were calculated using a regression mapping algorithm with the following parameters: Kosambi map function, recombination frequency <0.4, and LOD score >3.0.

QTL detection

For QTL analysis, the percentage data of resistance assays were transformed using arcsine square root to obtain a normal distribution of the trait values. QTL detection was conducted using simple interval mapping (SIM) (Lander and Botstein 1989) and composite interval mapping (CIM) (Zeng 1994) in QGene Version 4.3.6 (Joehanes and Nelson 2008). A window size of 2 cM was used. Markers outside the interval are considered as co-factors and they were selected by the program using stepwise regression in CIM. Thresholds of significance for the test statistics were estimated by 1,000 permutations at a significance level of $p < 0.01$ for SIM and CIM, except RE-JT519 at a level of $p < 0.05$ for CIM. LOD thresholds for SIM ranged from 3.5 to 3.8 and ranged from 4.3 to 5.3 for CIM. The proportion of phenotypic variation explained by each QTL was calculated as the R^2 value. Additive gene effects of the QTL also were estimated.

Effects and interactions of QTLs

Two of the mapped QTLs in our studies co-located with *Bwr-6* and *Bwr-12* were reported by Carmeille et al. (2006b). Both *Bwr-6* and *Bwr-12* were associated with resistance variation observed in six trials. The datasets of TW-Pss4b, TW-Pss186, TW-TC, TH-CM, and ID-PW had replications and were used to evaluate the effect and interaction of QTLs. RILs were divided into groups according to the presence of 'Hawaii 7996' or 'WVa700' alleles on *Bwr-6* and/or *Bwr-12*. The genotype of SLM12-2 or SLM12-10 was used to represent *Bwr-12*, and similarly SLM6-118 for *Bwr-6a*, SLM6-136 for *Bwr-6b*, and SLM6-94 for *Bwr-6c* (Fig. 1). The analysis of variance with contrast partitioning was performed using the PROC GLM procedure of SAS (SAS v 9.2, SAS Institute, Cary, NC, USA).

Results

Phenotypes of parental lines and RIL population

To identify QTLs associated with the stable resistance of 'Hawaii 7996', the RILs were evaluated for their resistance variation against different strains at the seedling stage in the greenhouse as well as in the field in five countries. Disease incidence data were collected from nine trials (Table 1). L390 was highly susceptible and all plants wilted; this indicates sufficient disease pressure was present in the experiments. The resistant parent 'Hawaii 7996' was found to be resistant against race 1-phylotype I and race 3-phylotype II strains with the mean percentage of wilted plant ranged from 0 to 24 %. The mean percentage of wilted plants for susceptible parent 'WVa700' ranged from 80 to 100 %.

A continuous frequency distribution of RILs between parents in all trials was observed with the mean maximum percentage of wilted plants ranging from 20.6 to 83.3 % (Table 1). The parents, susceptible 'WVa700' and resistant 'Hawaii 7996,' were at or near the frequency distribution extremes for all trials. A skewed distribution toward the susceptible parent was observed in TW-Pss4a, TW-Pss4b, PH-

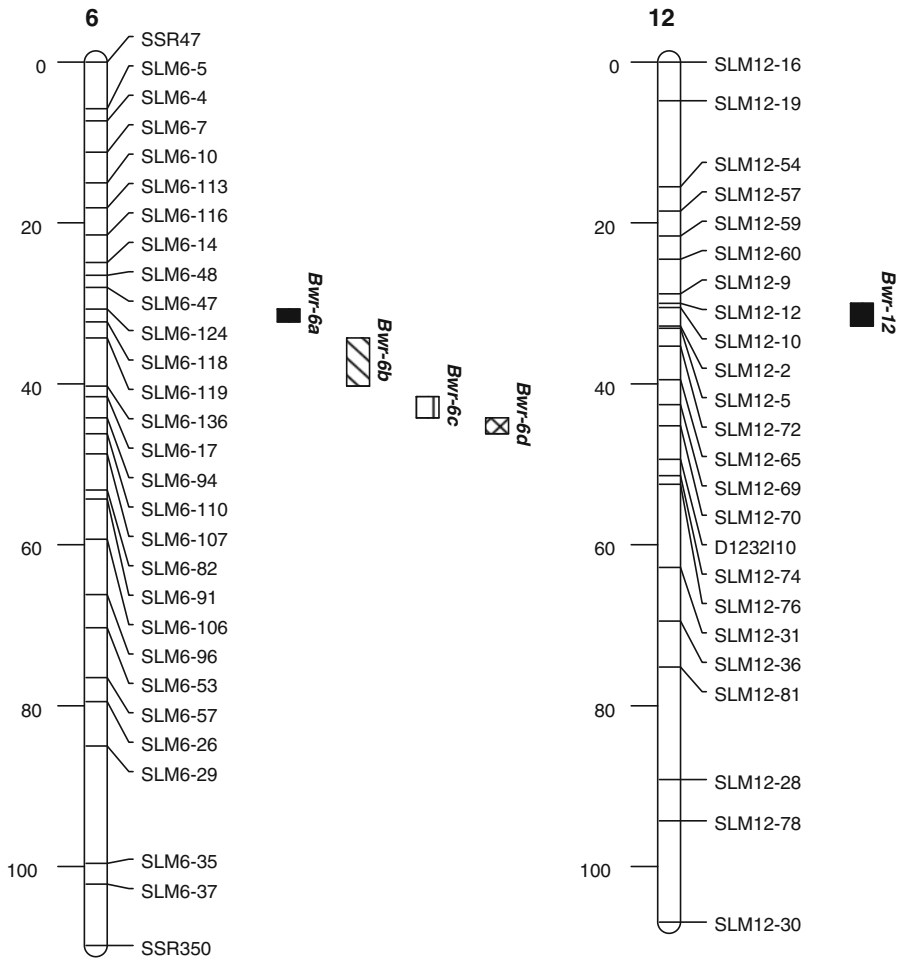

Fig. 1 Linkage map of molecular markers on chromosomes 6 and 12 used for bacterial wilt resistant QTL analysis. The marker order and relative distances are based on the RIL population derived from the cross between 'Hawaii 7996' and 'WVa700.' Putative QTL are indicated in boxes. The QTL names and interval markers are show in Table 2

Tm151, and ID-PW, while skewed distribution toward the resistant parent was observed in TW-TC and RE-JT519 (data not shown).

Variation in the degree of colonization by strain Pss4 was observed between parents and among RILs. A total of 12.5 % of 'Hawaii 7996' plants were colonized by the bacterium 6 days after inoculation; although no symptom was observed (Table 1). At the same time, 56.3 % of the susceptible 'WV 700' plants were colonized. The RIL followed a near-normal distribution with a mean of 52 % of colonized plants.

Construction of the genetic linkage map

In total, 16 AFLP, 47 DArT and 145 SSR polymorphic markers over the 12 chromosomes were used to construct a 'Hawaii 7996' × 'WVa700' linkage map. Thirty-three percent of the mapped markers deviated significantly ($p < 0.01$) from the expected 1:1 segregation ratio. The degree of segregation distortion varied from chromosome to chromosome; all chromosomes showed distortion for less than 50 % of the mapped loci, except on chromosome 11, where all markers showed significant distortions. The map has a total length of 1061.8 cM, which is comparable with another linkage map of *S. lycopersicum* × *S. pimpinellifolium* (Ashrafi et al. 2009). The average distance between markers is 5.1 cM and no gaps exceeding 25 cM are present in the linkage map.

To saturate the known QTL region on chromosomes 6 and 12, a total of 59 primer pairs were designed and tested. Thirteen polymorphic SSR markers were added between SLM6-10 and SLM6-

53 (Fig. 1). Four and six markers were added in the interval of SLM12-19/SLM12-9 and SLM12-5/SLM12-31, respectively (Fig. 1).

Comparison of QTLs for BW resistance in Hawaii '7996'

Using the newly constructed linkage map and the 10 phenotype datasets, QTLs associated with resistance in 'Hawaii 7996' were mapped on chromosomes 3, 6, and 12 (Table 2). They matched the QTLs detected in previous studies using $F_{2:3}$ populations for resistance to both phylotype I and II strains, which also found QTL on chromosomes 2, 4, 5, 8 and 11 (Thoquet et al. 1996a, b; Wang et al. 2000; Carmeille et al. 2006b). Considering that the QTL map position shifts with environmental factors and phenotype evaluation, we followed the same assumption as Carmeille et al. (2006b) and considered QTLs reported in the different studies identical when the estimated positions were within the range of 20 cM. Although QTLs on chromosome 6 were mapped on several intervals, they are all located in the TG153-TG240 interval of *Bwr-6* (about 20 cM of total length) reported by Carmeille et al. (2006b). QTLs on chromosome 12 co-located with *Bwr-12* were reported by Wang et al. (2000) and Carmeille et al. (2006b). *Bwr-6* and *Bwr-12* were associated with nearly all phenotype datasets. In contrast, QTLs on chromosome 3 were detected only in association with PH-Tm151 when using the SIM method; the effect was smaller than *Bwr-6* and *Bwr-12*. This QTL co-located with *Bwr-3*, as reported by Carmeille et al. (2006b).

A major QTL (*Bwr-12*) on chromosome 12

Using the linkage map constructed in this study, a major QTL conferring resistance to the race 1-phylotype I strain of *R. solanacearum* on chromosome 12 was identified (Table 2). The QTL, *Bwr-12*, was located in a 2.8-cM interval between SLM12-12 and SLM12-2 (Fig. 1). The resistance was contributed by the 'Hawaii 7996' allele. It was detected using phenotype data of all the trials except RE-JT516 (Table 2). Phenotypic variation explained by *Bwr-12* ranged from 17.9 to 56.1 %, depending on the trial. These results suggest that *Bwr-12* has a large effect and is a major contributor to the stable resistance of 'Hawaii 7996.'

Bwr-12 also was associated with variation in the colonization of the RILs caused by strain Pss4, and it explained 28.2 % of this trait (Table 2). This suggests *Bwr-12* suppresses internal multiplication of the pathogen.

QTL (*Bwr-6*) on chromosome 6

A QTL of resistance to race 1-phylotype I and race 3-phylotype II strain of *R. solanacearum* on chromosome 6 were identified based on CIM analysis results along the 15.5-cM segment of SLM6-124 and SLM6-110 (Table 2). QTLs located in this region on chromosome 6 have been reported by Thoquet et al. (1996a, b), Wang et al. (2000) and Carmeille et al. (2006a, b) and have been named as *Bwr-6*. Here we named them as *Bwr-6a*, *Bwr-6b*, *Bwr-6c* and *Bwr-6d* according to their position order from the distal end. This allows easy orientation for future studies to dissect this region. These QTLs explained 11.5–22.2 % of the phenotypic variation. For all QTLs on chromosome 6, only alleles from the resistant parent 'Hawaii 7996' contributed to the resistance phenotype. *Bwr-6* was not detected with phenotype data of TW-Pss4a, TW-Pss4c and PH-Tm151, and it was the only QTL detected against JT516, the race 3-phylotype II strain.

Interactions of *Bwr-12* and *Bwr-6*

Both *Bwr-12* and *Bwr-6* were associated with resistance phenotypes observed in 6 of the 10 trials. *Bwr-12* had a larger or similar effect than *Bwr-6*, except in the case of RE-JT519 (Table 2). The RILs with the *Bwr-12* resistance allele showed a significantly lower percentage of wilted plants with a 33.2–53.0 % reduction in disease incidence than the group with the susceptible allele (Table 3). The RILs with the *Bwr-6* resistance allele also showed a significantly lower percentage of wilted plants than the group with the susceptible allele, but the reduction in disease incidence was smaller than that of *Bwr-12* ranged from 16.1 to 27.9 %. *Bwr-12* and *Bwr-6* displayed an additive interaction. The group with both resistance alleles from *Bwr-12* and *Bwr-6* was the least susceptible and had the lowest disease incidence, which was significantly lower than the groups with only a single resistance allele from *Bwr-12* or *Bwr-6*. With the presence of both *Bwr-12* and *Bwr-6*, the resistant groups showed a 45.5–70.4 % reduction in disease incidence compared with the susceptible group, which

Table 2 QTLs detected for resistance to diverse *R. solanacearum* strains in 10 trials based on CIM and SIM analysis in a RIL population derived from the cross between 'Hawaii 7996' and 'WVa700.'

Trial	QTL[a]	Marker interval	CIM			SIM		
			LOD[b]	R^2 (%)	Add effect[c]	LOD	R^2 (%)	Add effect
TW-Pss4a	*Bwr-12*	SLM12-10/SLM12-2	31.1	53.9	−14.9	27.9	50.1	−14.7
TW-Pss4b	*Bwr-12*	SLM12-10/SLM12-2	26.8	48.1	−15.678	22.4	42.2	−15.5
	Bwr-6a	SLM6-124/SLM6-118	–	–	–	3.7	8.7	−6.8
TW-Pss186	*Bwr-12*	SLM12-12/SLM12-10	19.6	38.2	−11.3	–	–	–
	Bwr-6a	SLM6-124/SLM6-118	5.0	11.5	−4.9	3.9	9.1	−5.6
	Bwr-12	SLM12-10/SLM12-2	–	–	–	17.8	35.4	−11.5
TW-TC	*Bwr-12*	SLM12-10/SLM12-2	12.4	27.7	−13.4	11.6	26.2	−13.7
	Bwr-6b	SLM6-119/SLM6-136	9.6	22.2	−10.4	11.1	25.2	−12.8
TW-Pss4c	*Bwr-12*	SLM12-12/SLM12-10	10.2	28.2	−11.5	10.6	29.4	−12.5
PH-Tm151	*Bwr-12*	SLM12-10/SLM12-2	17.0	34.0	−11.3	16.3	32.9	−11.8
	Bwr-3	D1304J20/afh34a	–	–	–	4.5	10.4	−7.2
TH-CM	*Bwr-12*	SLM12-10/SLM12-2	20.0	38.7	−17.2	18.9	37.0	−18.7
	Bwr-6c	SLM6-17/SLM6-94	8.2	18.2	−9.6	–	–	–
	*Bwr-6**	SLM6-82/SLM6-91	–	–	–	7.0	15.7	−11.6
ID-PW	*Bwr-12*	SLM12-10/SLM12-2	33.6	56.1	−20.9	28.8	50.7	−21.5
	Bwr-6b	SLM6-119/SLM6-136	–	–	–	5.7	13.1	−10.4
RE-JT519	*Bwr-12*	SLM12-10/SLM12-2	4.6	17.9	−8.1	7.0	26.1	−11.1
	Bwr-6d	SLM6-94/SLM6-110	5.0	19.5	−7.9	6.2	23.4	−9.8
RE-JT516	*Bwr-6b*	SLM6-119/SLM6-136	5.3	20.3	−7.2	5.5	21.0	−7.9

[a] QTL, was following the name given by Carmeille et al. (2006b). The name *Bwr* stands for bacterial wilt resistance and the number after *Bwr*—indicates the chromosome location. All the QTLs were reported in previous studies (Wang et al. 2000; Carmeille et al. 2006b). Names of *Bwr-6a*, *Bwr-6b*, *Bwr-6c* and *Bwr-6d* were used to indicate the intervals mapped with different phenotype dataset. *Bwr*-6* was not considered, as it was detected only with one dataset by SIM analysis

[b] LOD, significant at 1 % level, except RE-JT519 for CIM. Maximum value of the log-likelihood in the marker interval (values are superior to the LOD-thresholds 4.6–5.3 for CIM and 3.5–3.8 for SIM)

[c] The negative values of additive effect indicated that the resistance alleles are from 'Hawaii 7996'

indicated these are the most important QTLs associated with the resistance trait.

Discussion

'Hawaii 7996' was identified as the tomato cultivar with the most stable resistance to *R. solanacearum* based on field evaluations conducted worldwide (Wang et al. 1998). However, poor genome coverage of the linkage map and the use of only a few phenotype datasets in previous studies hindered progress in understanding the genetic control of this resistance. In this study, we evaluated a set of RILs derived from 'Hawaii 7996' against different strains under different environments. Each phenotype trial represents a different disease pressure situation with the combination of pathogen strain, inoculation method, and environment. A new linkage map with good genome coverage was developed to allow more precise locating of QTLs. As a result, we were able to identify major QTLs associated with stable resistance in 'Hawaii 7996.'

New linkage map and quantitative nature of BW resistance

The linage map used in previous studies has only about 75 % genome coverage (Thoquet et al. 1996a, b; Wang et al. 2000; Carmeille et al. 2006b). Truong et al. (2010) added AFLP, DArT, and anchored SSR markers. Although the genome coverage was enhanced, chromosomes 5 and 12 could not be formed due to the lack of anchored markers. In this study we focused on SSR markers for two main reasons. First, the utilization of

Table 3 Group comparison among RIL groups based on *Bwr*-12 or *Bwr*-6 alleles (2 groups) and both *Bwr*-12 and *Bwr*-6 alleles (4 groups)

Trial	*Bw-12*		*Bwr-6*[a]		*Bwr-12*/*Bwr-6*[a]			
	HH[b]	WW[b]	HH	WW	HH/HH	HH/WW	WW/HH	WW/WW
TW-Pss4b								
Sample size	66	116	100	82	41	25	56	55
PWP[c]	32.2 (33.0) a[d]	77.3 (65.5) b	51.8 (46.1) a	70.3 (61.1) b	27.3 (28.2) a	46.0 (40.9) b	70.6 (59.7) c	83.2 (71.6) d
TW-Pss186								
Sample size	70	108	98	79	40	30	55	50
PWP	38.2 (39.5) a	71.4 (58.6) b	49.4 (44.8) a	65.5 (55.7) b	33.5 (33.8) a	44.6 (40.7) b	60.7 (52.6) c	79 (65.3) d
TW-TC								
Sample size	56	68	71	57	35	17	33	35
PWP	20.4 (21.8) a	61.7 (56.6) b	29.6 (31.0) a	54.2 (47.5) b	13.0 (16.1) a	36.7 (36.1) b	41 (37.9) b	74.4 (64.8) c
TH-CM								
Sample size	64	110	101	77	35	21	55	49
PWP	14.6 (16.4) a	57.8 (51.6) b	30.2 (29.2) a	58.1 (51.6) b	9.9 (12.0) a	23.9 (24.8) b	44.4 (41.0) c	74 (64.2) d
ID-EW								
Sample size	66	116	100	81	39	24	57	51
PWP	16.9 (17.9) a	69.9 (60.5) b	39.0 (35.4) a	62.1 (54.6) b	9.5 (11.7) a	29.4 (28.6) b	60.3 (52.6) c	79.9 (68.8) d

[a] Genotype data of respective *Bwr-6* QTL associated with each resistance assay was used (see Table 2)

[b] *HH* homozygous for the 'Hawaii 7996' allele, *WW* homozygous for the 'WVa700' allele

[c] *PWP* means of percentage of wilted plants; the transformed values with arcsine square root are shown in the parentheses

[d] Mean comparisons were conducted using the transformed data in the same trial, separately for *Bwr-12*, *Bwr-6* and *Bwr-12*/*Bwr-6* genotypes. Means with the same letter are not significantly different based on least significant difference at $p < 0.05$

anchored BAC clone sequences to develop SSR markers has been demonstrated to be an efficient approach in tomato (Geethanjali et al. 2010, 2011). Secondly, SSR marker assays are highly reproducible and simple to use. This allows wider application of the results in marker-assisted selection. We were able to develop a new linkage map consisting of 12 chromosomes with an average of 5.1 cM distance between markers. Using both BAC clone and scaffold sequences from SGN allowed the addition of more SSR markers in the known QTL regions on chromosomes 6 and 12. This permitted the detection of possible QTLs associated with the complex stable resistance in 'Hawaii 7996.'

Phylotype I-specific QTL *Bwr-12* with major effect

Bwr-12 was detected in all resistance assays except RE-JT516. The results agreed with Carmeille et al. (2006b) that *Bwr-12* is phylotype I-specific. *Bwr-12* by far is the most important QTL associated with the stable resistance in 'Hawaii 7996.' Not only was it detected under various disease pressures, but it also had the largest effect among all QTLs detected, controlling 17.9–56.1 % of the phenotype variation. Under high disease pressure environments, such as that in trial TW-Pss4a, it was the only detected QTL. The interval (SLM12-12 to SLM12-2) spans only 2.8 cM. Effectiveness of the linked SSR markers in marker-assisted selection should be explored.

Resistance to race 1 strains in tomato was shown to be associated with limiting bacterial colonization rather than bacterial penetration (Grimault and Prior 1993; Grimault et al. 1994). *Bwr-12* was found to be associated with this mechanism. Major QTLs associated with resistance to *R. solanacearum* GMI1000 (race 1-phylotype I) strain have been identified in *Arabidopsis thaliana* and *Medicago truncatula* (Deslandes et al. 1998; Vailleau et al. 2007). A resistance gene, *RRS1-R*, has been cloned from the resistant Nd-1 accession of *A. thaliana* and a corresponding Avr protein, PopP2 from GMI1000, was identified (Deslandes et al. et al. 2003). We are in the process of cloning *Bwr-12* to determine if it is a classical resistance gene.

Broad-spectrum QTL *Bwr-6* on chromosome 6

Bwr-6 was associated with resistance to race1-phylotype I and race 3-phylotype II strains. The presence of *Bwr-6* QTLs enhanced the resistance level together with *Bwr-12* against race 1-phylotype I strains. Therefore, it is desirable to select both *Bwr-6* and *Bwr-12* in resistance breeding programs. The location of *Bwr-6* differed with phenotype datasets and was distributed along a 15.5-cM region on the short arm of chromosome 6. Mangin et al. (1999) has suggested the presence of two QTLs on chromosome 6, which were detected at different time points in the development of the disease. However, QTL map position could shift with environmental factors present in each phenotype evaluation. Further studies using near-isogenic lines to dissect the *Bwr-6* region would be needed to fine-map the QTLs in this region.

The short arm of chromosome 6 harbors many genes and QTLs conferring resistance to diverse pathogens such as root knot nematode (*Mi-1*), *Cladosporium fulvum* (*Cf-2*, *Cf-5*) (Dickinson et al. 1993), *Tomato yellow leaf curl virus* (*Ty-1* and *Ty-3*) (Verlaan et al. 2011), and *Oidium neolycopersici* (*Ol-4* and *Ol-6*) (Seifi et al. 2011). Deberdt et al. (1999) has shown that *Mi* and *Bwr-6* could be allelic or linked in repulsion. Identification of molecular markers closed linked with *Bwr*-6 would facilitate pyramiding of resistance genes in the region.

Resistance to JT516, a race 3-phylotype II strain, under cool temperatures was confirmed to be associated with *Bwr-6*, as reported by Carmeille et al. (2006b). Tomato is highly susceptible to race 3-phylotype II strains, the typical potato brown rot strains (Carmeille et al. 2006a; Lebeau et al. 2011). These strains are well-adapted to cooler temperatures and could compromise tomato production in temperate regions. JT516 is placed under phylotype IIB sequevar 1 based on sequence variation of partial endoglucanase (*egl*) gene, where typical potato brown rot strains belonged. Although they are genetically similar, variation in virulence on tomato among IIB-1 strains was observed (Cellier and Prior 2010). More phylotype IIB sequevar 1 strains should be evaluated to conclude the genetic control of resistance against this group of strains.

A minor QTL *Bwr-3* on chromosome 3

Bwr-3 with minor effect was detected only in association with PH-Tm151. Carmeille et al. (2006b) suggested that *Bwr-3* might be a phylotype II-specific

QTL, because it was found to be active against GMI8217 and JT516 phylotype II strains and not against Pss4 and JT519 phylotype I strains. Tm151 is a biovar 3 strain, which should belong to phylotype I. It is plausible that *Bwr-3* has a broad-spectrum effect like *Bwr-6*, but its effect is too small to be detected with all phenotype assays.

In conclusion, this study confirmed the quantitative nature of resistance to *R. solanacearum* in tomato 'Hawaii 7996.' By examining the RIL under various disease pressure conditions, we concluded that *Bwr-6* and *Bwr-12* were the most important QTLs contributing to the stable resistance of 'Hawaii 7996.' Future research should focus on these two QTLs using marker-assisted selection and functional studies of plant-pathogen interactions.

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