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ORIGINAL ARTICLE

Identification of a molecular marker tightly linked to bacterial wilt resistance in tomato by genome-wide SNP analysis

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Abstract

Key message **Genotyping of disease resistance to bacterial wilt in tomato by a genome-wide SNP analysis**

Abstract Bacterial wilt caused by *Ralstonia pseudosolanacearum* is one of the destructive diseases in tomato. The previous studies have identified *Bwr-6* (chromosome 6) and *Bwr-12* (chromosome 12) loci as the major quantitative trait loci (QTLs) contributing to resistance against bacterial wilt in tomato cultivar 'Hawaii7996'. However, the genetic identities of two QTLs have not been uncovered yet. In this study, using whole-genome resequencing, we analyzed genome-wide single-nucleotide polymorphisms (SNPs) that can distinguish a resistant group, including seven tomato varieties resistant to bacterial wilt, from a susceptible group, including two susceptible to the same disease. In total, 5259 non-synonymous SNPs were found between the two groups. Among them, only 265 SNPs were located in the coding DNA sequences, and the majority of these SNPs were located on chromosomes 6 and 12. The genes that both carry SNP(s) and are near *Bwr-6* and *Bwr-12* were selected. In particular, four genes in chromosome 12 encode putative leucine-rich repeat (LRR) receptor-like proteins. SNPs within these four genes were used to develop SNP markers, and each SNP marker was validated by a high-resolution melting method. Consequently, one SNP marker, including a functional SNP in a gene, *Solyc12g009690.1*, could efficiently distinguish tomato varieties resistant to bacterial wilt from susceptible varieties. These results indicate that *Solyc12g009690.1*, the gene encoding a putative LRR receptor-like protein, might be tightly linked to *Bwr-12*, and the SNP marker developed in this study will be useful for selection of tomato cultivars resistant to bacterial wilt.

Introduction

Bacterial wilt, caused by the soil-borne bacterium *Ralstonia pseudosolanacearum*, is one of the most destructive diseases to tomatoes (Caldwell et al. 2017; Hayward 1991). The pathogen invades tomatoes through wounds or natural openings of roots occurring when lateral roots grow horizontally from the taproots. The pathogen continues to infect xylem tissues resulting in blockage of water flow from vascular system of susceptible plants (Peeters et al. 2013; Vasse et al. 1995). Diverse strategies, such as chemical and biological control, have been used to control this devastating disease, but these methods proved ineffective (Denny 2007; Huet 2014). Thus, the best strategy for controlling the disease is to breed cultivars with stable resistance to *R. pseudosolanacearum* (Salgon et al. 2017).

Tomato cultivar 'Hawaii7996' has stable resistance to *R. pseudosolanacearum* (Wang et al. 1998). The polygenic basis of resistance to bacterial wilt was established with a set of recombinant inbred lines (RILs) derived from 'Hawaii7996' (resistant cultivar) and 'West Virginia 700' (susceptible cultivar, *S. pimpinellifolium*) (Thoquet et al. 1996a, b; Truong et al. 2010; Wang et al. 2000, 2013). Using SSR markers in the mapping population, two major quantitative trait loci (QTLs), named *Bwr-6* and *Bwr-12* and located on chromosomes 6 and 12, respectively, were closely associated with resistance to bacterial wilt (Carmeille et al. 2006a, b; Geethanjali et al. 2010, 2011;

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Thoquet et al. 1996a, b; Wang et al. 2000, 2013). Although there have been QTL mapping studies for bacterial wilt resistance in tomato, a few molecular markers have actually been applied in tomato breeding for resistant hybrids due to limitation of the accessibility of molecular markers. Thus, for practical breeding, new molecular markers closely linked to disease resistance are needed.

The advent of next-generation sequencing (NGS) technologies has accelerated discovery of genome-wide genetic variations via resequencing of whole genomes (Bentley 2006). Resequencing the genome of individuals that can be compared with a reference genome permits comparison of the sequence variation between susceptible and resistant phenotypes (Stratton 2008). These variations are considered the essential factors determining the phenotypic variations in disease resistance (Subbaiyan et al. 2012). The genome sequence of tomato cultivar 'Heinz1706' was reported through NGS technologies and used as the reference genome (Tomato Genome Consortium 2012). Comparison of DNA sequences with the reference genome sequence through whole-genome sequencing can discover a large number of genome-wide DNA polymorphisms, such as simple sequence repeats (SSRs), insertion/deletions (InDels), and single-nucleotide polymorphisms (SNPs), which are important materials of development of molecular markers for marker-assisted selection (MAS) in tomato (Foolad and Panthee 2012; Ganal et al. 2009; Jones et al. 2009; Ribaut and Hoisington 1998; Subbaiyan et al. 2012; Zalapa et al. 2012).

Molecular markers were tightly linked to genes controlling agronomically important traits and used as efficient tools when plant breeders separated the tomato individuals into different genotypic groups based on the presence or absence of locus of interest (Collard et al. 2005). Among diverse molecular markers, researchers have shown preference for developing SNP markers over others due to inherent advantages of SNPs: enormous numbers, high-throughput ability, and cost-effectiveness among individuals of the same species (Mammadov et al. 2012; Subbaiyan et al. 2012). In addition, SNP markers are co-dominant, enable discrimination between heterozygous and homozygous alleles, and use SNP detecting post-PCR analysis such as high-resolution melting analysis (HRM), which facilitates detection and can be easily automated unlike SSR and InDel markers (Lehmensiek et al. 2008; Thomson 2014).

In this study, we hypothesized that tomato varieties have several SNPs that could distinguish bacterial wilt resistance genotypes and that are located near both *Bwr-6* and *Bwr-12*. To explore genome-wide SNPs, whole-genome resequencing of tomato varieties was conducted. As a result, we found a functional SNP within a putative leucine-rich repeat (LRR) receptor-like protein gene and developed an efficient SNP marker that could efficiently distinguish resistant genotypes to bacterial wilt in tomato.

Materials and methods

Plant materials and growth conditions

Forty-two tomato genotypes (cultivars and inbred lines) were used (Table 1). For genome-wide SNP analysis and development of SNP markers, seven resistant genotypes ('Hawaii7996', 'Hawaii7998', 'BWR-1', 'BWR-22', 'BWR-23', '10-BA-3-33', and '10-BA-4-24') (Dannon and Wydra 2004; Hwang et al. 2012; Wang et al. 1998; Fig. 1) and two susceptible genotypes ('BWS-3' and 'Heinz1706') (Tomato Genetics Resource Center, http://tgrc.ucdavis.edu/index.cfm; Fig. 1) were first used. For validation of the SNP marker, the F_1 hybrid from crossing between a resistant parent, '10-BA-4-24' and a susceptible parent, 'IL12-2', which is one of introgression lines between *S. lycopersicum* 'M82' and *S. pennellii* 'LA716' (Eshed and Zamir 1995), was generated and used. In addition, F_2 population from selfing of the F_1 hybrid was generated, and a total of 79 F_2 seeds were used to determine marker accuracy (Table 3). Moreover, 31 tomato cultivars, including commercial F_1 hybrids, were genotyped. Seeds of each genotype were sown in a tray with 32 wells filled with Baroker soil (Seoul Bio, Korea). The trays were kept in a growth chamber at 28 °C under a 14 h/10 h light/dark cycle with 60% humidity and 63.03 µmol/m s light intensity for 4–5 weeks. Genomic DNA was extracted from 3 to 4 young leaves of each plant.

Genomic DNA extraction

Genomic DNA samples were isolated from the young leaves of tomato seedlings using the modified cetyl-tri-methyl-ammonium bromide (CTAB) method (Murray and Thompson 1980). The young leaves were rapidly frozen with liquid nitrogen and ground before they melted. Samples were mixed with CTAB buffer and incubated at 65 °C for 30 min. Chloroform was added and the samples were centrifuged at 13,000 rpm for 5 min. The supernatant was transferred to a new microfuge tube and cold 100% isopropanol added. The DNA pellets were washed with 70% ethanol after centrifugation, dried, and dissolved in sterilized water. The final concentration of isolated genomic DNA was adjusted to 10 ng/ml with a NanoDrop 2000/UV–Vis spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).

Disease assay for bacterial wilt with the bacterial pathogen, *R. pseudosolanacearum*

For disease assay, *R. pseudosolanacearum* strain SL882 (race 1, biovar 4; phylotype 1) isolated from tomato in Korea

Table 1 Forty-two tomato cultivars and breeding lines, and their source, bacterial wilt resistance phenotype, and SNP genotype with the KHU-1 primer set

Tomato cultivar or line	Supplier	Bacterial wilt phenotype[a]	SNP marker genotype[b]
For resequencing and marker development			
Hawaii7996	PNU	R	R
Hawaii7998	PNU	R	R
BWR-1	PNU	R	R
BWR-22	PNU	R	R
BWR-23	PNU	R	R
10-BA-3-33	PNU	R	R
10-BA-4-24	PNU	R	R
BWS-3	PNU	S	S
Heinz1706[c]	–	S	–
For marker validation			
IL12-2	KNU	S	S
F_1 (10-BA-4-24 × IL12-2)	KNU	R	H
For genotyping			
Highpower	KNU	R	R
Super high power	KNU	R	R
Doctor Q	Nongwoo Bio Co., Ltd	R	R
Ailsa craig	RDA-Gene Bank	R	R
Divisoria	RDA-Gene Bank	R	S
Moneymaker	–	S	S
M82	–	S	S
Miniheckssu	Asia Seed Co., Ltd	S	S
Mulya	RDA-Gene Bank	S	S
Sigyo 1 ho	RDA-Gene Bank	S	S
Broadley	RDA-Gene Bank	S	S
Yulwon	RDA-Gene Bank	S	S
Tiara	Nongwoo Bio Co., Ltd	S	S
TYsenseQ	Nongwoo Bio Co., Ltd	ND(S)[d]	S
Minimaru	Nongwoo Bio Co., Ltd	ND(S)	S
Redpang	Nongwoo Bio Co., Ltd	ND(S)	S
Titichal	Nongwoo Bio Co., Ltd	ND(S)	S
Goldminichal	Nongwoo Bio Co., Ltd	ND(S)	S
TYaltorang	Nongwoo Bio Co., Ltd	ND(S)	S
Pinktop	Nongwoo Bio Co., Ltd	ND(S)	S
Betatiny	Nongwoo Bio Co., Ltd	ND(S)	S
TY tiny	Nongwoo Bio Co., Ltd	ND(S)	S
Prime alexander	Nongwoo Bio Co., Ltd	ND(S)	S
Cupirang	Nongwoo Bio Co., Ltd	ND(S)	S
Minichal	Nongwoo Bio Co., Ltd	ND(S)	S
Rapsody	Syngenta Korea	ND(S)	S
Medison	Syngenta Korea	ND(S)	S
Ricophin-9	Syngenta Korea	ND(S)	S
Duine	Syngenta Korea	ND(S)	S
Kaedilrak	Asia Seed Co., Ltd	ND(S)	S
Dotaerang TY winner	Takii Korea Co., Ltd	ND(S)	S

PNU Pusan National University, *KNU* Kyungpook National University, *RDA* Rural Development Administration

[a]Disease response of resistant (R) or susceptible (S) cultivars after inoculation with *R. pseudosolanacearum* strain SL882

[b]SNP marker genotype, using the KHU-1 primer set, developed in the present study (R, resistant; H, heterozygous; S, susceptible)

[c]'Heinz1706' was used as the reference tomato genome

[d]ND(S), not determined in this study, but susceptible, based on supplier information

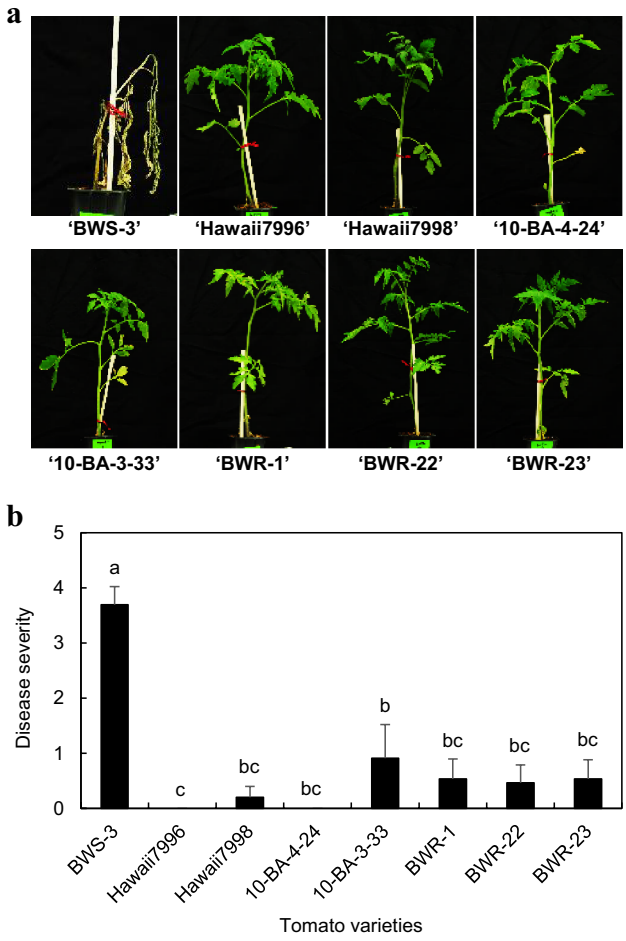


Fig. 1 Evaluation of disease resistance to bacterial wilt in eight tomato varieties. **a** Photographs taken 14 days after inoculation with *R. pseudosolanacearum*. **b** Numerical value of disease severity of bacterial wilt. Disease severity was measured 14 days after inoculation by scoring from 0 to 5. The degree of symptoms to bacterial wilt disease is shown on the *y*-axis: 0, no symptoms; 1, one leaf partially wilted; 2, one or two leaves completely wilted; 3, most leaves wilted; 4, all leaves wilted; 5, plant died. Mean values with different letters on the bars are significantly different ($p < 0.05$) according to Duncan's multiple range test

was used (Lee et al. 2011; Safni et al. 2014). Bacterial cells were streaked and grown on casamino acid-Peptone-Glucose (CPG) medium (casamino acid 1 g/l; bactopeptone 10 g/l; glucose 5 g/l; agar 15 g/L; pH 7.2) in the culture chamber at 30 °C for 2 days (Wicker et al. 2007). A bacterial culture suspension was diluted with distilled water to adjust the final concentration to 10^8 CFU per ml ($OD_{600nm} = 0.1$) with a Nanodrop 2000/UV–Vis spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Then, 4- to 5-week-old seedlings were inoculated by pouring 10 ml of bacterial suspension per pot on top of soil near tomato roots, and then, disease symptoms were observed under controlled conditions of 28 °C with 60% humidity for 2 weeks. In totally, 10–15 plants per tomato variety were inoculated. The individual plants were observed daily for wilting. Depending on the extent of wilting disease symptoms in tomato plants, the severity of bacterial wilt caused by *R. pseudosolanacearum* strain SL882 was estimated on a scale from 0 to 5 (0; no wilting, 1; one leaf partially wilted, 2; one or two leaves completely wilted, 3; most of the tomato leaves wilted, 4; all leaves wilted, 5; plant died) (Kelman 1954).

Discovery of genome-wide SNPs using whole-genome sequencing (WGS)

Genomic DNAs extracted from eight tomato varieties were used for whole-genome resequencing and genotyping. Then, genome resequencing was conducted using the HiSeq-X platform in Macrogen Inc. (Seoul, Korea). Genome coverage of the whole genome was acquired based on the tomato reference 'Heinz1706' genome (SL2.5) (Tomato Genome Consortium 2012). Consecutively, polymorphic SNPs that could distinguish between a group susceptible to bacterial wilt (S2: 'Heinz1706' and 'BWS-3') and a group resistant to bacterial wilt (R7: 'Hawaii7996', 'Hawaii7998', '10-BA-3-33', '10-BA-4-24', 'BWR-1', 'BWR-22', and 'BWR-23') was found by Seeders Inc. (Daejeon, Korea). DynamicTrim and LengthSort programs of solexaQA (v.1.13) package were used for sequence pre-processing of short reads (Cox et al. 2010). The cleaned short reads were aligned to the reference genome for raw SNP detection of samples using the programs BWA (0.6.1-r104) and SAMtools (0.1.16) (Li and Durbin 2009; Li et al. 2009). Then, SEEDERS in-house script was used to generate SNP matrix among samples and SNP filtering (Kim et al. 2014). Sequence alignment of the two groups was conducted by the Multiple Sequence Alignment (MSA) and the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology for Information (NCBI) to discover non-synonymous SNPs within several genes among the genome-wide SNPs.

PCR amplification and high-resolution melting (HRM) analysis

To confirm amplification of one-band in the PCR reaction before HRM analysis, the conventional PCR was first used. Total reaction volumes of 20 µl constitute 50 ng genomic DNA, 2× Taq^{Basic} PCR Master mix 2 (BIOFACT, Daejeon, Korea), and 10 pmol of the primer sets (Table S1). The PCR reactions were performed using T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA), and the first step was 2 min predenaturation at 95 °C followed by 30 cycles of 95 °C for 2 min, 55–58 °C for 40 s, and 72 °C for 15 s; and, finally, 72 °C for 5 min. For characterization of tomato genotypes, standard PCRs with 11 primer sets, which produced single bands, were performed in a 20 µl mixture, including 50 ng

genomic DNA, 2× Real-Time PCR Master Mix, including EvaGreen™ fluorescent dye in the mixture (BIOFACT, Daejeon, Korea), and 10 pmol of each primer set. The short amplicons were produced with the following conditions: 95 °C for 15 min; 30 cycles of 95 °C for 20 s, 55–58 °C for 40 s, and 72 °C for 15 s; and 72 °C for 5 min. For HRM analysis, the amplicons were melted in a range of 65–95 °C with a 0.2 °C temperature increase every 10 s on a CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA) to genotype tomato varieties with an SNP marker via Precision Melt Analysis Software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

The differences between the mean values of disease scores of the tomato plants were evaluated using Duncan's multiple range tests, and $p < 0.05$ was considered a significant difference. For statistical analysis, R version 3.4.1 program (https://cran.cnr.berkeley.edu/bin/windows/base/) was used.

Results

Evaluation of disease resistance against bacterial wilt caused by *R. pseudosolanacearum* in tomato varieties

The disease assay was conducted to evaluate disease resistance or susceptibility of tomato varieties against bacterial wilt and to analyze SNPs between susceptible (S) and resistant (R) groups to bacterial wilt. Eight tomato varieties, 'Hawaii7996', 'Hawaii7998', '10-BA-4-24', '10-BA-3-33', 'BWR-1', 'BWR-22', 'BWR-23', and 'BWS-3', were used in this assay. All tomato plants, except those receiving mock treatment, were inoculated with *R. pseudosolanacearum* strain SL882 under the controlled experimental conditions. During observation for 14 days after inoculation, disease symptoms of inoculated tomato plants were measured by scoring from 0 to 5. As shown in Fig. 1a, seven tomato varieties, all except 'BWS-3', survived for 14 days after inoculation. In addition, the mean values of disease severity of the seven tomato varieties were less than 1 (Fig. 1b). Thus, 'Hawaii7996', 'Hawaii7998', '10-BA-4-24', '10-BA-3-33', 'BWR-1', 'BWR-22', and 'BWR-23' showed strong resistance and were grouped together as 'R7', while only 'BWS-3' was susceptible to bacterial wilt and was grouped as 'S2' with 'Heinz1706', of which the whole-genome sequence is available (Tomato Genome Consortium 2012). Our phenotype results were consistent with the previous reports, and we used these tomato varieties for the whole-genome resequencing analysis to discover polymorphic SNPs among tomato varieties, compared with the genome sequence of 'Heinz1706'.

Whole-genome resequencing of tomato varieties for detection of genome-wide SNPs

Whole-genome resequencing of eight tomato varieties was carried out to detect genome-wide SNPs. After sequencing, sequence pre-processing of short reads resulted in the final generation of about 1.6 billion 25-bp paired-end reads using the software solexaQA (v.1.13) package including DynamicTrim and LengthSort (Cox et al. 2010) (Table S2). Based on alignment with the tomato reference genome, 'Heinz 1706', mapping of short reads of eight tomato varieties was conducted (Tomato Genome Consortium 2012). The short reads provided 23–31× coverage of the entire genome and covered approximately a minimum of 85.15% in '10-BA-4-24' to a maximum of 89.15% in 'BWS-3' by comparing eight tomato varieties with the reference genome sequence (Table S2). For discovery of raw SNPs, the two programs BWA (0.6.1-r104) and SAMtools (0.1.16) were used and SEEDERS in-house script (Kim et al. 2014; Li and Durbin 2009; Li et al. 2009) found a total of 4,857,161 SNPs (Table 2). Next, the SNPs were classified into three types depending on SNP index: SNP index ≥ 0.9 was considered homozygous, $0.4 \leq$ SNP index ≤ 0.6 was heterozygous, and the rest of SNPs not included in both index ranges ($0.6 <$ SNP index < 0.9 and SNP index < 0.4) were grouped as others (Table 2). A total of 3,926,501 homozygous SNPs (about 80% of the total SNPs) were found. Their positions were subclassified into coding DNA sequence (CDS), intron, untranslated region (UTR), and intergenic region based on genomic location of the reference genome and were used to identify common SNPs in tomato varieties belonging to "R7" or "S2" groups and to discover polymorphic SNPs between "R7" and "S2" (including cultivar 'Heinz1706') groups. Within "R7" and "S2" groups, 346,985 and 1,589,380 SNPs were found to be common, respectively, while 5259 SNPs (about 0.13% of the total homozygous SNPs) were found to be polymorphic between the two groups. Among these polymorphic SNPs, 265 (about 5% of polymorphic SNPs), 619, 25, and 4350 were located in CDS, intron, UTR, and intergenic regions of the reference genome, respectively (Fig. 2a). Overall, whole-genome sequencing and genome sequence analysis of groups resistant and susceptible to bacterial wilt successfully identified polymorphic SNPs between groups.

Identification and distribution of polymorphic SNPs in genes located near *Bwr-6* and *Bwr-12*

Polymorphic SNPs located in CDS were further analyzed to determine their chromosomal positions. The largest number of coding SNPs (168) was located on chromosome 12, and the second (53) was located on chromosome 6 (Fig. 2a). The density of coding SNPs was also highest in chromosome 12, followed by chromosome 6 and accounted for 63 and 20%

Table 2 Summary of SNPs detected from each tomato variety

Tomato variety	No. of total SNPs	No. of homozygous SNPs (SNP index ≥ 0.9)	No. of heterozygous SNPs (0.4 ≤ SNP index ≤ 0.6)	No. of others (0.6 < SNP index < 0.9 and SNP index < 0.4)
BWS-3	423,723	310,770	36,606	76,347
Hawaii7996	871,026	752,192	39,925	78,909
Hawaii7998	1,281,871	1,156,489	41,234	84,148
10-BA-4-24	521,769	396,630	40,672	84,467
10-BA-3-33	552,425	420,643	39,666	92,116
BWR-1	401,529	296,092	34,575	70,862
BWR-22	401,498	296,811	34,470	70,217
BWR-23	403,320	296,874	34,259	72,187
Total	4,857,161	3,926,501	301,407	629,253

SNPs were found based on the genome sequence of 'Heinz1706'

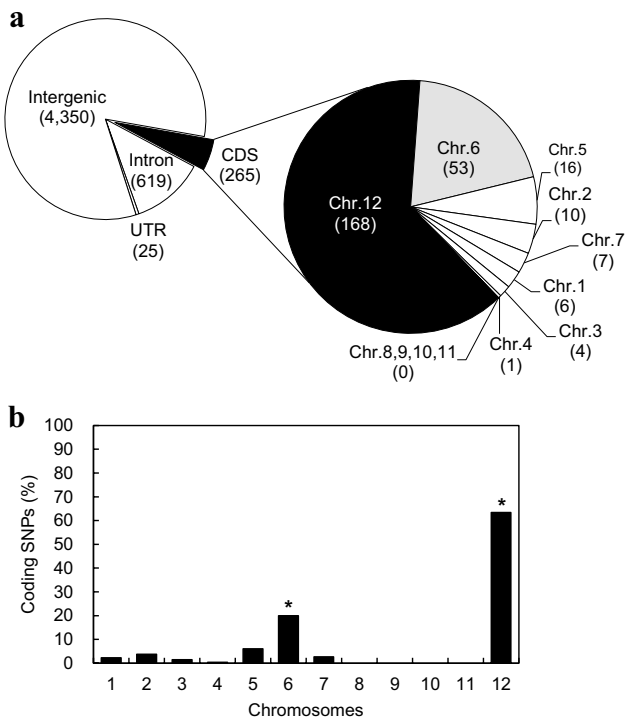


Fig. 2 Genomic distribution of polymorphic SNPs. **a** Total number of SNPs classified by genomic locations and coding SNPs identified in each chromosome. Polymorphic SNPs were categorized into intergenic or genic region and then subdivided into coding DNA sequence (CDS), introns, and untranslated regions (UTR). The number of SNPs in each genomic region is shown in parentheses. **b** Percentage of coding SNPs in each chromosome. The SNP density was calculated by dividing the number of coding SNPs in each chromosome by the total number of coding SNPs. The asterisks indicate chromosomes harboring two major QTLs for disease resistance to bacterial wilt

of the total SNPs, respectively (Fig. 2b). *Bwr-6* and *Bwr-12*, which have been identified as major QTLs related to resistance to bacterial wilt, are located on chromosome 6 and 12, respectively (Wang et al. 2013). Thus, we hypothesized that the detected polymorphic coding SNPs are closely related to phenotypic differences in bacterial wilt resistance.

Based on this hypothesis, we focused on polymorphic coding SNPs located near *Bwr-6* and *Bwr-12*. In recent studies, several SSR markers have been identified near *Bwr-6* (SLM6-118 and SLM6-17) and *Bwr-12* (SLM12-12 and SLM12-2) (Geethanjali et al. 2010, 2011; Wang et al. 2013). Thus, polymorphic coding SNPs near these SSR markers were analyzed. From this analysis, 18 non-synonymous SNPs in 15 genes near two SSR markers on chromosome 6 and 59 non-synonymous SNPs in 26 genes near two SSR markers on chromosome 12 were discovered, and the identities of the annotated proteins encoded by a total of 41 genes were analyzed (Fig. 3; Tables S3, S4). Based on gene annotation, none of genes near SLM6-118 and SLM6-17 SSR markers on chromosome 6 seemed to be candidates responsible for disease resistance to bacterial wilt. However, four genes, *Solyc12g009690.1*, *Solyc12g009740.1*, *Solyc12g009770.1*, and *Solyc12g009780.1,* located near SLM12-12 and SLM12-2 SSR markers on chromosome 12, seemed to be candidates, because they encode homologs of the leucine-rich repeat (LRR) receptor-like serine/threonine-protein kinase (RLP), which are well-known protein families to regulate disease resistance in plants (Fig. 4). Thus, SNPs only in those four genes were further analyzed.

Seven non-synonymous SNPs (A/G, G/A, C/T, C/A, C/T, A/C, and A/G) were located at 699, 813, 866, 888, 981, 1106, and 1196 bp sites of *Solyc12g009690.1* and produced different amino acids (histidine/arginine, serine/asparagine, proline/serine, alanine/aspartate, serine/leucine, asparagine/histidine, and arginine/glycine, respectively) (Fig. 4; Fig. S1). Two functional SNPs were found at 3676 and 4062 bp sites of *Solyc12g009740.1*. The first SNP changes the amino acid from asparagine to threonine, and the second changes from asparagine to aspartate (Fig. 4; Fig. S2). Next, six polymorphic SNPs

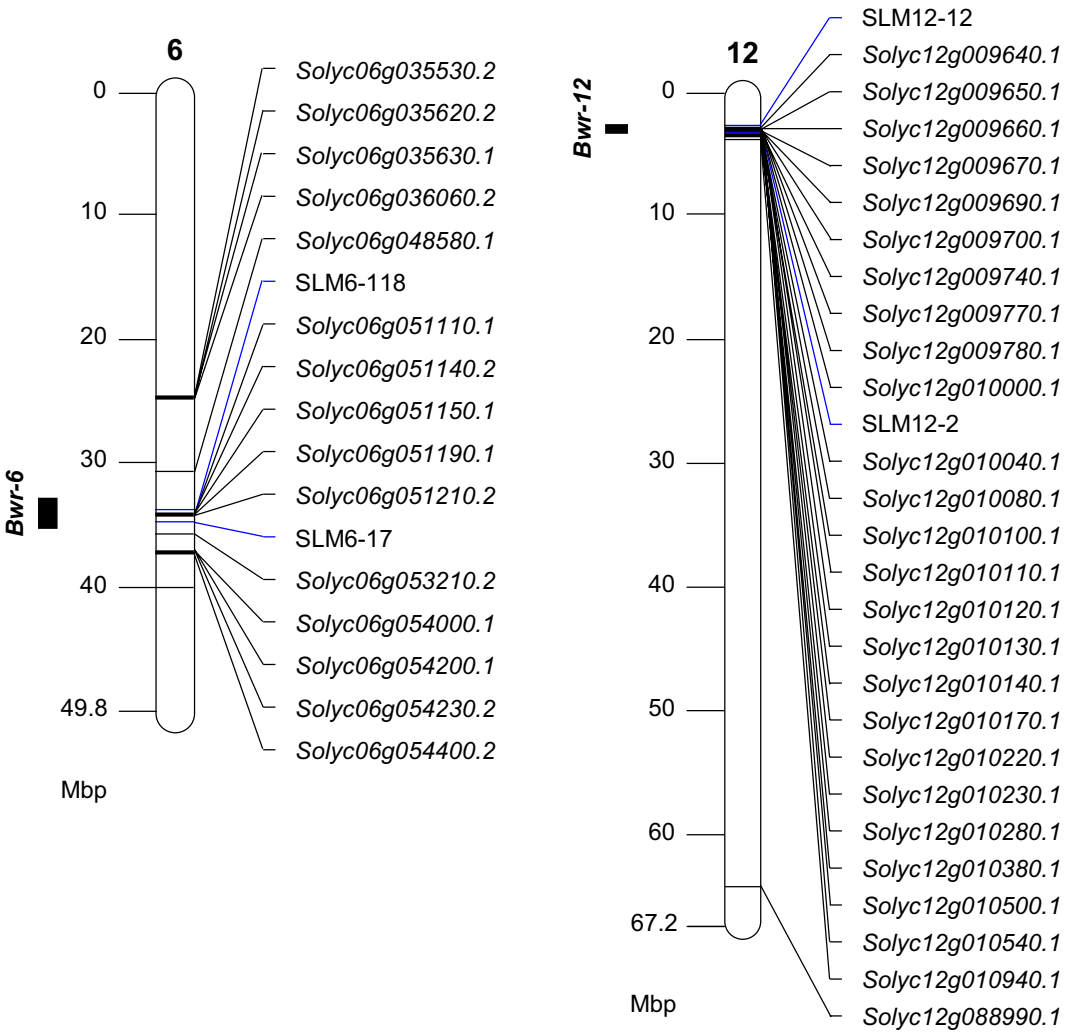
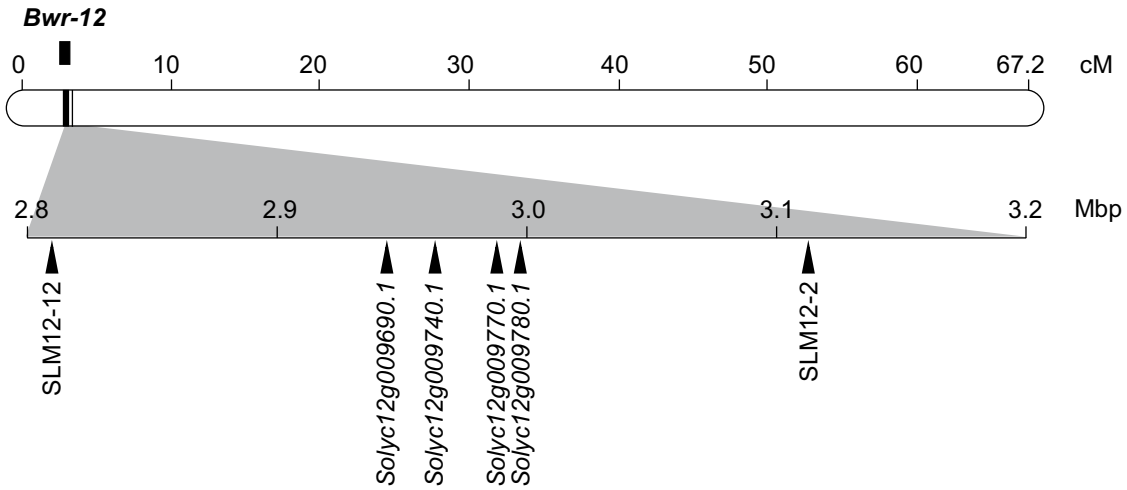


Fig. 3 Schematic locations of putative genes containing polymorphic SNPs on chromosome 6 or 12. The SNPs were found in 15 and 26 genes on chromosomes 6 and 12, respectively. *Bwr-6* and *Bwr-12* were reported as major QTLs controlling bacterial wilt disease (Wang et al. 2013). Black boxes, predicted locations of the two major QTLs; blue lines, positions of SSR markers developed in the previous studies (Geethanjali et al. 2010, 2011; Wang et al. 2013) (color figure online)

(A/G, T/C, A/C, A/C, T/C, and A/G) in *Solyc12g009770.1* positioned at 1201, 1549, 1681, 1720, 1742, and 1954 bp sites, changed the amino acids (asparagine/aspartate, tyrosine/histidine, isoleucine/leucine, isoleucine/leucine, isoleucine/threonine, and isoleucine/valine, respectively) (Fig. 4; Fig. S3). In the *Solyc12g009780.1*, one SNP (G/A) were found at the 3847 bp site and altered the amino acid from alanine to threonine (Fig. 4; Fig. S4). These results indicate that four genes encoding homologs of RLP proteins might be candidate genes of the *Bwr-12* responsible for disease resistance to bacterial wilt in tomato.

Development and validation of SNP markers to genotype tomato varieties for disease resistance to bacterial wilt

To examine if any of the four genes is responsible for *Bwr-12*, 13 different SNP markers were developed. The 13 primer sets (Fig. S1, S2, S3, S4, and Table S1) were designed to produce 100–200 bp amplicons from four candidate genes, and some amplicons contain more than one SNP, because they are located in close proximity to one another. After verifying that these primer sets amplify only one PCR band



Gene ID	Position (bp)	SNPs		Amino acids		Gene annotation
		S2	R7	S2	R7	
Solyc12g009690.1	2,941,301	A	G	H	R	LRR receptor-like serine/threonine-protein kinase, RLP
	2,941,415	G	A	S	N	
	2,941,468	C	T	P	S	
	2,941,490	C	A	A	D	
	2,941,583	C	T	S	L	
	2,941,708	A	C	N	H	
	2,941,798	A	G	R	G	
Solyc12g009740.1	2,967,484	A	C	N	T	
	2,967,870	A	G	N	D	
Solyc12g009770.1	2,987,224	A	G	N	D	
	2,987,572	T	C	Y	H	
	2,987,704	A	C	I	L	
	2,987,743	A	C	I	L	
	2,987,765	T	C	I	T	
	2,987,977	A	G	I	V	
Solyc12g009780.1	2,993,505	G	A	A	T	

Fig. 4 Schematic locations and list of putative *RLP* genes having functional SNPs on chromosome 12, based on the genome sequence of 'Heinz1706'. SLM12-12 and SLM12-2 are SSR markers linked with *Bwr-12* reported by Geethanjali et al. (2011). Arrows indicate physical locations of the putative resistance *RLP* genes and SSR markers on chromosome 12. *cM* centimorgan

from each tomato variety, HRM analysis was conducted to determine if any of these SNP markers could clearly distinguish tomato varieties belonging to either a susceptible or resistant group to bacterial wilt. Consequently, only one SNP marker using a KHU-1 primer set designed from the *Solyc12g009690.1* gene (Fig. 5) could very efficiently determine the genotype of tomato varieties used in this study, as follows. HRM analysis with this SNP marker perfectly grouped eight varieties for marker development as resistant or susceptible varieties consistent with phenotyping results (Table 1). For further validation of this SNP, we generated F_1 from crossing a resistant parent, '10-BA-4-24' and a susceptible parent, 'IL12-2'. HRM analysis with this SNP perfectly distinguished the three genotypes (Fig. 6). The melting peaks for '10-BA-4-24', F_1 hybrid, and 'IL12-2' were 78.6, 78.4, and 78.2 °C, respectively.

Furthermore, F_2 segregation population generated from crossing between '10-BA-4-24' and 'IL12-2' was used to determine accuracy of the SNP marker on chromosome 12 (Table 3). A total of 79 F_2 plants were genotyped with the SNP marker and also phenotyped by inoculation of bacterial wilt pathogen. Thirty-five plants showed both resistant

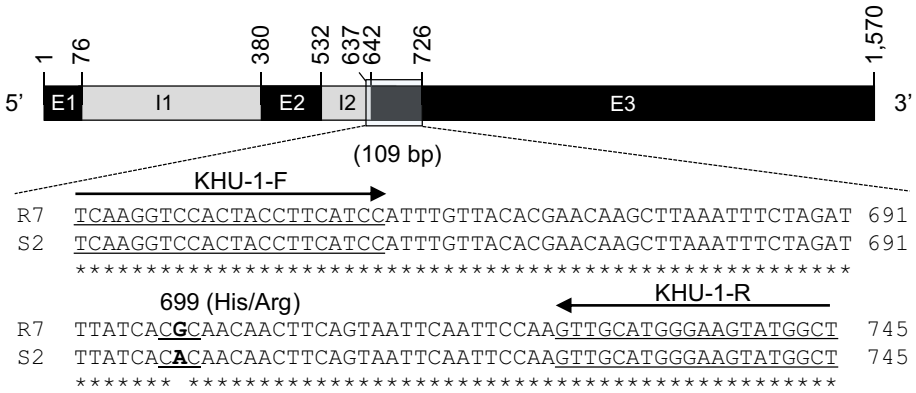


Fig. 5 Identification of an SNP from alignment of sequence between S2 and R7. Sequence comparison was conducted using CLUSTALW in the putative resistance gene *Solyc12g009690.1*. The sequence of the S2 group was derived from the 'Heinz1706' reference genome (SL2.5). The location of the KHU-1 primer set and a functional SNP were underscored in the gene structure. S2 and R7 are different groups showing susceptibility and resistance to bacterial wilt, respectively. The numbers indicate the positions of exons and introns within the gene. The arrows indicate the locations of the two primers. Asterisk indicates the consensus sequence. *E* exon, *I* intron

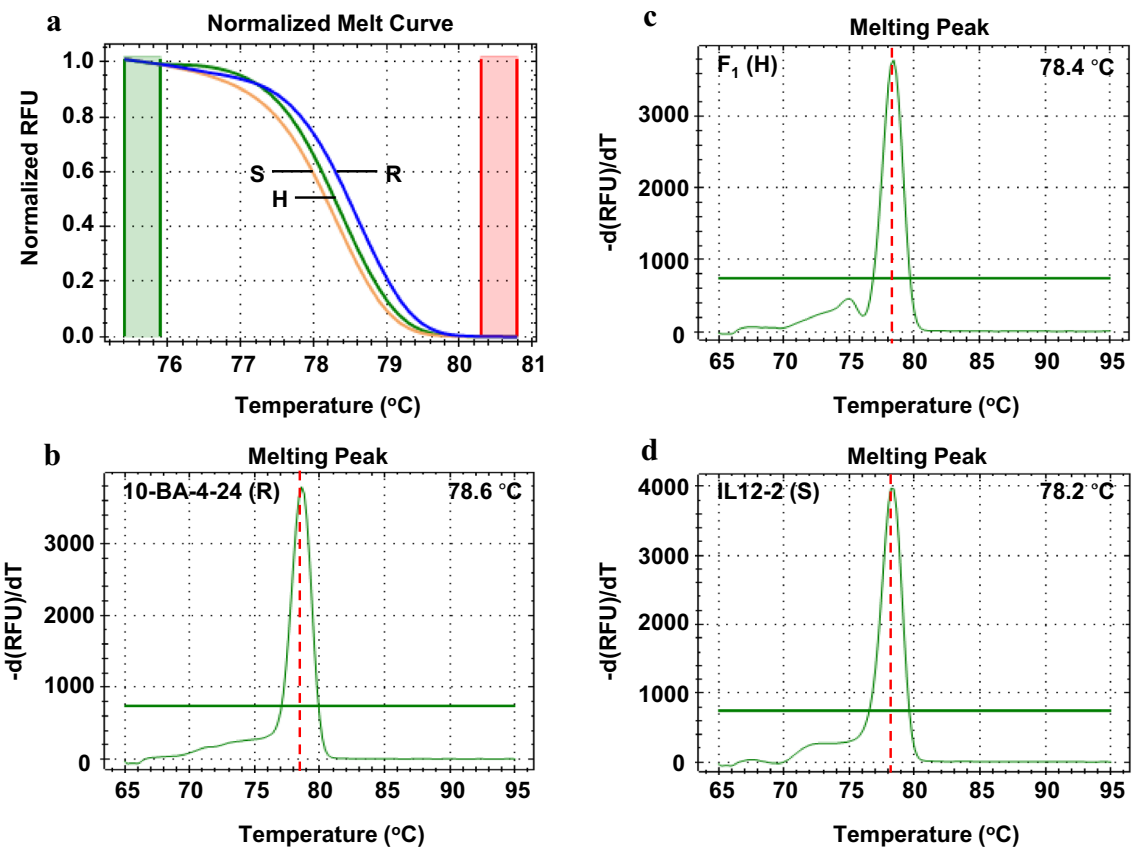


Fig. 6 Validation of an SNP marker by HRM analysis with the KHU-1 primer set. **a** Three normalized melting curves are shown from a resistant parent, '10-BA-4-24' (R, blue curve), a heterozygous F_1 (H, green curve), and a susceptible parent, IL12-2 (S, orange curve). The first negative derivative of the change in fluorescence (d*F*) with respect to the change in temperature (d*T*) is shown as $-$ d*F*/d*T* on the *y*-axis. In **b**, **c**, and **d**, melting peaks are obtained from a resistant parent, a heterozygous F_1 and a susceptible parent, respectively. RFU, relative fluorescence units (color figure online)

Table 3 Analysis of 79 F_2 population generated from crossing between '10-BA-4-24' × 'IL12-2'

Phenotype[b]	SNP marker genotype[a]		
	Resistant (homozygous)	Resistant (heterozygous)	Susceptible
Resistant	14	21	36[c]
Susceptible	0	0	8
Accuracy of genotyping (%)	100	100	18

[a]SNP marker genotype, using the KHU-1 primer set, developed in the present study

[b]Disease response of F_2 population 14 days after inoculation with *R. pseudosolanacearum* strain SL882

[c]Resistant phenotype probably due to *Bwr-6* on the chromosome 6

genotypes and phenotypes and, among them, 14 and 21 genotypes were classified into homozygous or heterozygous alleles, respectively. The others were grouped as a susceptible genotype, but only 8 plants consistently showed susceptible phenotypes, while 36 plants showed resistant phenotype. Thus, all F_2 plants showing resistant genotypes with SNP marker, regardless of homozygous or heterozygous genotypes, were 100% consistent with those phenotypes, indicating that this SNP marker is useful to trace down *Bwr-12* on the chromosome 12. On the other hand, only 18% accuracy of the SNP marker for susceptible genotypes was shown, indicating that 36 plants showing resistance only by phenotyping might be due to *Bwr-6* on the chromosome 6 (Danesh et al. 1994; Miao et al. 2009; Wang et al. 2013).

To further assess the range of usage of this marker, 31 tomato varieties, including commercial F_1 hybrids, were added to HRM analysis (Table 1). Among a total of 41 tomato cultivars other than 'Heinz1706', cultivars '10-BA-4-24', 'IL12-2', and F_1 hybrid were used as standard genotypes showing resistant, heterozygous, and susceptible genotypes, respectively. The genotypes were divided into six groups based on normalized melting curves in HRM analysis using the KHU-1 primer set (Fig. 7a). In this analysis, 11 tomato cultivars, including '10-BA-4-24' and 'Hawaii7996' carrying the resistance allele to bacterial wilt, produced two melting curves (blue and orange), while 29 cultivars, including 'IL12-2' and 'BWS-3', generated three melting curves (red, green, and pink). In HRM analysis, information on melting peaks from resistant and susceptible groups was also obtained. A total of 11 tomato cultivars categorized into the resistant group and displaying two melting curves had melting temperatures in the range of 78.4–78.6 °C, while 29 tomato cultivars classified into the susceptible group displaying three melting curves had slightly low melting temperatures in the range of 78.0–78.2 °C (Fig. 7b, c). These genotyping results were well correlated with phenotyping results (Table 1). However, there was one exception, the variety 'Divisoria'. This variety was categorized into the susceptible group, although it showed resistance to bacterial wilt (Table 1). Overall, these results indicate that *Solyc12g009690.1*, the gene encoding a putative RLP protein, might be tightly linked to *Bwr-12* and the SNP marker developed in the present study will be useful for selection of tomato cultivars resistant to bacterial wilt.

Discussion

In the present study, we analyzed genome-wide polymorphic SNPs between two tomato groups, one resistant and one susceptible to bacterial wilt, by whole-genome sequencing. In addition, using information on polymorphic SNPs in CDS, we developed a single SNP marker in the *Solyc12g009690.1* gene on the chromosome 12 that could efficiently distinguish the resistance genotypes of tomatoes by HRM analysis. Based on these results, the *Solyc12g009690.1* gene is tightly linked to *Bwr-12*, which has been detected in the variety 'Hawaii7996'. This gene was present in other tomato varieties resistant to bacterial wilt such as 'BWRs', '10-BA-3-33', and '10-BA-4-24', and contained the same SNP (G/A) at the 699 bp position.

Among five resistant varieties confirmed as resistant phenotypes in this study, all varieties except 'Divisoria' were grouped as resistant genotypes. This indicates that these resistant varieties might carry resistant alleles of the *Solyc12g009690.1* gene with G/A SNP at the same position and all of these resistant varieties except 'Divisoria' might have been originated from the same genetic origin. Moreover, the presence of resistant varieties like 'Divisoria' indicates that there are multiple origins of genetic alleles for disease resistance to bacterial wilt. As expected, the SNP at the 699 bp position of *Solyc12g009690.1* gene of 'Divisoria' was A, a susceptible SNP. Previously, genetic analyses with *S. lycopersicum* 'T51A' and wild tomato relative *S. lycopersicum* var. *cerasiforme* 'L285' (Danesh et al. 1994; Miao et al. 2009), which are resistant to bacterial wilt, were performed, and these varieties might carry major QTLs for disease resistance to bacterial wilt on different chromosomes from 'Hawaii7996'.

Based on analysis of F_1 and F_2 populations from crossing between '10-BA-4-24' × 'IL12-2' with the SNP marker, all F_1 and resistant F_2 plants showed resistant genotypes, confirming that resistant alleles are dominant and this SNP marker could be useful for tracing down resistant allele on the chromosome 12. However, 82% of susceptible F_2 plants based on genotyping with the SNP marker showed resistant phenotypes (Table 3). This discrepancy can be easily explained by the presence of *Bwr-6* on the chromosome 6. As mentioned above, resistant alleles in both 'Hawaii 7996' and '10-BA-4-24' varieties might be originated from the

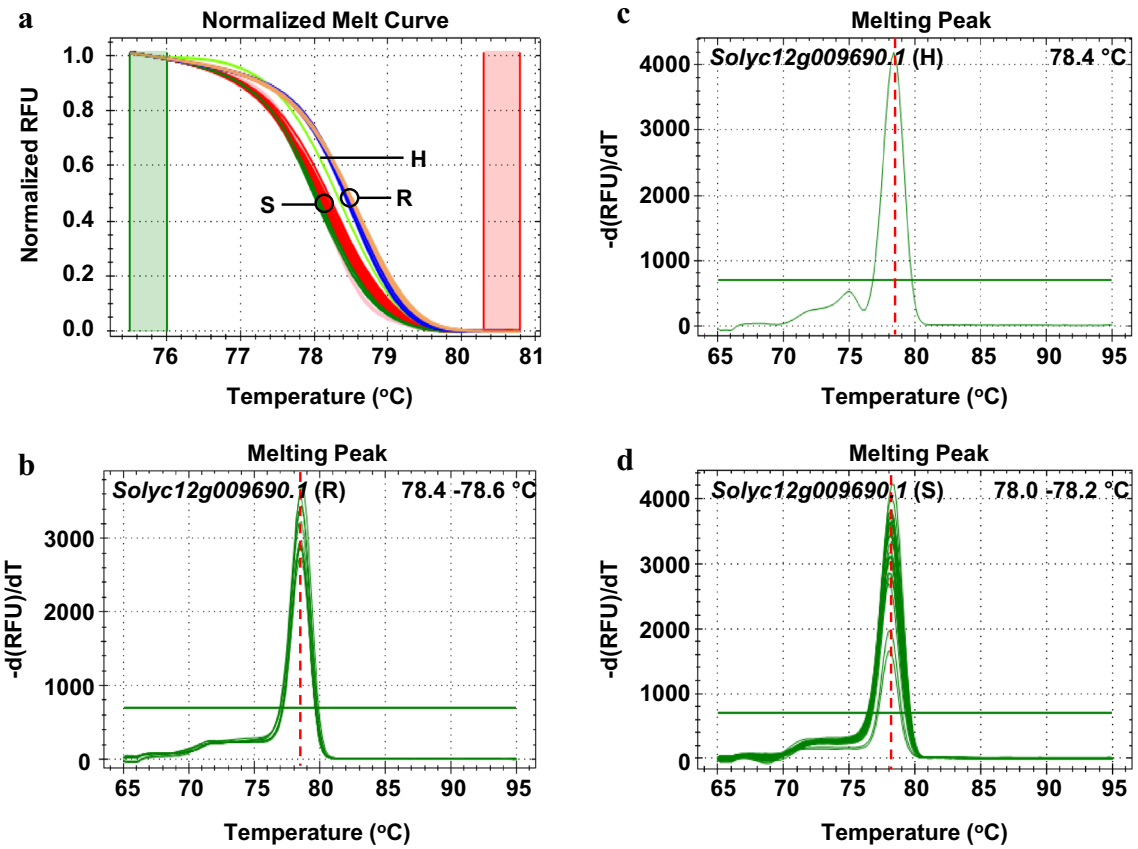


Fig. 7 Genotyping of 41 tomato varieties by HRM analysis with the KHU-1 primer set. The results of normalized melting curves (**a**) are simultaneously obtained with melting curves of tomato varieties carrying resistant (**b**), heterozygous (**c**), and susceptible alleles (**d**). **a** Six normalized melting curves are shown, and "R", "H", and "S" indicate the groups of resistant (blue and orange curves), heterozygous (light green curve), and susceptible (red, green, and pink curves) varieties, respectively. The first negative derivative of the change in fluorescence (d*F*) with respect to the change in temperature (d*T*) is shown as $-\mathrm{d}F/\mathrm{d}T$ on the *y*-axis. In **b**, **c,** and **d**, melting peaks are obtained from resistant and susceptible tomato varieties, including commercial F_1 hybrids. *RFU* relative fluorescence units (color figure online)

same genetic origin, and 'Hawaii 7996' carries two QTLs in the chromosome 6 and 12, which is very likely the same case in '10-BA-4-24'. In addition, contribution of two QTLs to bacterial wilt resistance seems to be very similar in terms of the number of F_2 plants showing resistant phenotypes, i.e., 35 and 36 F_2 plants grouped as resistant and susceptible genotypes, respectively, with the SNP marker showed resistant phenotypes.

The *Solyc12g009690.1* gene encodes a homolog of RLP (LRR receptor-like serine/threonine-protein kinase) proteins with LRR domains as extracellular domains and a transmembrane (TM) domain. The G/A SNP at the 699 bp position of this gene changes arginine in the resistant allele to histidine in the susceptible allele. This amino acid is located in the LRR domain of the protein (Fig. S5), and the change in amino acids from arginine to histidine might influence the protein structure and ultimately protein function. The overall protein structure of a predicted *Solyc12g009690.1* gene product is very similar to that of Cf proteins, which are disease resistance (R) proteins to leaf mold fungus, *Cladosporium fulvum* (Wulff et al. 2009). The previous reports have shown that some amino acid changes in the LRR domain of Cf9 protein directly affects the resistance function of this protein, probably by disrupting recognition of target pathogen proteins (Kim et al. 2017; Parniske et al. 1997; Wulff et al. 2009). Moreover, RLPs have been reported to be associated with functions in plant–pathogen interaction, in particular, the initiation and transduction of signals in the major plant defense pathways (Yang et al. 2012). Why the amino acid changes from arginine to histidine in LRR domains of the *Solyc12g009690.1* gene product affects the protein function remains to be determined.

Prior to development of an SNP marker in this study, several molecular markers such as sequence-characterized amplified region (SCAR) and cleaved amplified polymorphism sequences (CAPS), as well as SSR markers linked to disease resistance to bacterial wilt have been developed (Danesh et al. 1994; Miao et al. 2009; Wang et al.

2000). In particular, RFLP and SSR markers linked to two major QTLs of 'Hawaii7996' were developed (Geethanjali et al. 2010, 2011; Thoquet et al. 1996a; Wang et al. 2013). Because their genetic identities have not been determined, it was not clear how far these markers are located from QTLs. Instead, the new SNP marker developed in this study is tightly linked to the *Solyc12g009690.1* gene, which might be responsible for the *Bwr-12*. Therefore, our SNP marker could be more useful and efficient than other markers to identify resistant alleles during marker-assisted breeding.

Whole-genome sequencing to discover polymorphic SNPs between resistant and susceptible tomato groups combined with genotyping by SNP markers is a powerful means to track candidate resistance genes that may be responsible for bacterial wilt resistance. SNPs allow the unification of the candidate gene approach and association-based fine mapping to identify genes of interest. Recent studies have shown similar analyses to find resistance genes in plants. For example, Silva et al. (2012) used whole-genome sequencing of 13 inbred rice lines to identify non-synonymous SNPs and candidate genes responsible for resistance to sheath blight caused by the fungal pathogen *Rhizoctonia solani*. In pepper, Liu et al. (2014) developed SNP markers locating putative genes underlying a major QTL for *Phytophthora capsici* resistance using a combination of bulked segregant analysis (BSA) and microarrays. They found two *NBS-LRR* genes and *SARS.2A* as candidates linked to the resistance locus on chromosome 5 and used them for SNP marker development. Both of these studies and the present study suggest that the usage of genome-wide polymorphic SNPs can be an efficient strategy to track candidate genes associated with disease resistance in plants. However, further studies using overexpression of transgenes and/or gene knock-out are needed to demonstrate whether the candidate genes actually play a key role in disease resistance.

Author contribution statement BYK, ISH, and CSO performed all experiments, analyzed all data, and wrote the paper. HJL contributed to the bioassay. JML, ES, and DC contributed to data analysis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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