Development of diagnostic molecular markers for marker-assisted breeding against bacterial wilt in tomato

Alebel Mekuriaw Abebe†1), Jinwoo Choi†1), Youngjun Kim1), Chang-Sik Oh2), Inhwa Yeam3), Ill-Sup Nou4) and Je Min Lee*1)

1) Department of Horticultural Science, Kyungpook National University, Daegu 41566, South Korea
2) Department of Horticultural Biotechnology, College of Life Science, Kyung Hee University, Yongin, Gyeonggi-do 17104, South Korea
3) Department of Horticulture and Breeding, Andong National University, Andong, Gyeongbuk, 36729, South Korea
4) Department of Horticulture, Sunchon National University, Suncheon, Jeonnam 57922, South Korea

Bacterial wilt, caused by the *Ralstonia pseudosolanacearum* species complex, is an important vascular disease that limits tomato production in tropical and subtropical regions. Two major quantitative trait loci (QTL) of bacterial wilt resistance on chromosome 6 (*Bwr-6*) and 12 (*Bwr-12*) were previously identified in *Solanum lycopersicum* ‘Hawaii 7996’; however, marker-assisted breeding for bacterial wilt resistance is not well established. To dissect the QTL, six cleaved amplified polymorphic sites (CAPS) and derived CAPS (dCAPS) markers within the *Bwr-6* region and one dCAPS marker near *Bwr-12* were developed, and resistance levels in 117 tomato cultivars were evaluated. Two markers, RsR6-5 on chromosome 6 and RsR12-1 on chromosome 12, were selected based on the genotypic and phenotypic analysis. The combination of RsR6-5 and RsR12-1 effectively distinguishes resistant and susceptible cultivars. Furthermore, the efficiency of the two markers was validated in the F3 generation derived from the F2 population between E6203 (susceptible) and Hawaii 7998 (resistant). Resistant alleles at both loci led to the resistance to bacterial wilt. These markers will facilitate marker-assisted breeding of tomato resistant to bacterial wilt.

**Key Words:** tomato, bacterial wilt, polygenic resistance, molecular marker, single nucleotide polymorphism, marker-assisted breeding.

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**Introduction**

Bacterial wilt, caused by *Ralstonia solanacearum*, is one of the most destructive diseases that affects many plant species. The *R. solanacearum* species complex (RSSC) has been classified into races, biovars, and phylotypes based on host range, carbon source usage, 16S-23S rRNA gene sequence of the strains, respectively (Cho et al. 2018, Hayward 1991, Jeong et al. 2007). Genomic analysis and proteomic profiling of various strains of the pathogen collected from different countries classified RSSC into three species: *R. solanacearum* (Phylotype II), *R. pseudosolanacearum* (Phylotypes I and III), and *R. syzygii* (Phylotype IV). Further genomic analysis of the species classified *R. syzygii* into three subspecies named syzygii, indonesiensis, and celebesensis (Prior et al. 2016).

The pathogen has a wide host range. Tomato and other *Solanaceae* plants are major hosts. The disease threatens the cultivation of these crops in tropical and subtropical regions and heated greenhouses in temperate regions because high temperatures are better suited for the pathogen and disease development. As a result, the pathogen causes substantial economic losses (Hayward 1991, Lopes and Rossato 2018). The pathogen moves into plant roots via natural openings, such as hydathodes, or damaged areas and proliferates in the xylem tissues. It then damages the xylem tissues and blocks the water flow, leading to the total collapse and death of susceptible plants (Bae et al. 2015, Lowe-Power et al. 2018). Xylem colonization and spread are necessary for bacterial wilt disease progress because mutations in xylem colonization rendered pathogen strains incapable of causing wilting in tomato plants (Schell 2000). Evaluation of core collections of the three fruit vegetables of *Solanaceae* crops (tomato, eggplant, and pepper) against different strains of the pathogen showed that resistance of tomato collections is low compared with eggplant and pepper (Lebeau et al. 2011).

Different control strategies, such as chemical, biological,
and cultural practices, can reduce bacterial wilt severity, but none of them are effective (Yuliar et al. 2015). Development and use of resistant cultivars is the most effective approach to control bacterial wilt (Abebe et al. 2016, Huet 2014, Scott et al. 2005, Wang et al. 2018). Breeding for bacterial wilt-resistant cultivars has been challenging due to the polygenic nature of resistance, broad host range, variability of the pathogen strains, and effect of environmental factors that directly influence the phenotypic expression of the disease (Danesh et al. 1994, Fegan and Prior 2005, Hayward 1991, Lee et al. 2011, Thoquet et al. 1996b, Tran and Kim 2010). Moreover, the resistance locus is linked to undesirable horticultural traits (Scott et al. 2005). Solanum lycopersicum ‘Hawaii 7996’ (hereafter Hawaii 7996) is a stable resistant resource against bacterial wilt across various geographic locations and different bacterial strains with the highest average survival rate of 97% (Wang et al. 1998). Analysis of bacterial wilt resistance using F2, F3, and recombinant inbred line (RIL) populations derived from a cross between Hawaii 7996 (resistant) and West Virginia 700 (susceptible) identified QTL on chromosomes 4, 6, and 11 (Thoquet et al. 1996a); 3, 4, 6, 8, and 10 (Thoquet et al. 1996b); 6 (Mangin et al. 1999); 6, 8, and 12 (Wang et al. 2000); 3, 4, and 6 (Carmeille et al. 2006); and 3, 6, and 12 (Wang et al. 2013). Among them, a QTL on chromosome 6 (Bwr-6) was stable when measured with different phenotyping criteria (area under disease progress curve and bacterial colonization), in different bacterial strains (race 3-phytophthora II and race 1-phytophthora I), and as measured in two different seasons (hot and cold) (Carmeille et al. 2006). Bwr-12 was an active QTL specifically against Pss4 (race 1, biovar 4) (Wang et al. 2000). QTL on chromosome 6 (Bwr-6) and 12 (Bwr-12) are thought to be responsible for the stable resistance of Hawaii 7996. Bwr-12 covers 2.8 cM between the SSR markers SLM12-12 and SLM12-2, controlling more than 50% of the phenotypic variation in some trials. Bwr-6 was localized between SLM6-124 and SLM6-110 covering 15.5 cM and controlling up to 22.2% of the phenotypic variation (Wang et al. 2013). Although two QTL, Bwr-6 and Bwr-12, were repeatedly confirmed as major contributors to bacterial wilt resistance in Hawaii 7996, the genetic nature of these critical QTL remained unidentified. We have previously conducted whole-genome resequencing of two susceptible cultivars (Heinz 1706 and BWS-3) and seven resistant cultivars (Hawaii 7996, Hawaii 7998, 10-BA-3-33, 10-BA-4-24, BWR-1, BWR-22, and BWR-23) of tomato and identified genome-wide single nucleotide polymorphisms (SNPs) in resistant and susceptible groups of cultivars (Kim et al. 2018). The highest number of polymorphic SNPs in coding regions were found on chromosome 12 (168 SNPs) followed by chromosome 6 (53 SNPs). These SNPs might be associated with resistance to bacterial wilt. Based on the SNP information generated by re-sequencing, an HRM marker (KHU-1) that is tightly linked to Bwr-12 was developed; however, no tightly linked SNP-based molecular marker was developed to trace Bwr-6 resistance due to the large interval (~12.7 Mbp) (Kim et al. 2018).

In this study, we further analyzed the Bwr-6 region to dissect and develop a diagnostic molecular marker for this important QTL. Cleaved amplified polymorphic site (CAPS) and derived CAPS (dCAPS) markers were developed within Bwr-6 and screened for their diagnostic potential using 117 tomato genotypes. Phenotypic and genotypic analysis using a wide range of germplasms enabled us to select RsR6-5 as a diagnostic marker for Bwr-6 among the newly developed markers in the region. Consequently, this marker, in combination with RsR12-1, effectively distinguished bacterial wilt-resistant and wilt-susceptible tomato cultivars. The newly developed marker RsR6-5 together with RsR12-1 will promote marker-assisted breeding of tomato by targeting two major resistance QTL against bacterial wilt.

### Materials and Methods

#### Plant materials

In total, 117 tomato cultivars were collected (either seed or genomic DNA sample) from different sources, including the Tomato Genetics Resource Center (TGRC), UC Davis; Kyung Hee University, Korea; National Agrobiodiversity Center (RDA-Genebank), Korea; and various commercial seed companies in Korea. The phenotype of 27 cultivars was confirmed by inoculation test in this study, 12 cultivars were inferred from previous reports and the phenotype of 78 cultivars was received from the respective company/supplier along with the genomic DNA sample (Table 1). Seeds were first sown in Petri dishes for germination, and germinated seeds were transferred to 128 cell seedling trays “(28 × 28 × 40, bottom 15 mm)” filled with bio mix (JM bio, Korea). The seedlings were grown in the Agricultural Experiment Station of Kyungpook National University in a glasshouse at an average temperature of 25–28°C and 16–8 h light-dark cycles. The seedlings were moved from the glasshouse to the growth chamber 3–4 days before inoculation for acclimatization to growth chamber conditions where they were kept post-inoculation. Four-week-old seedlings were used for inoculation.

| Phenotyping                  | Number of cultivars | Bacterial wilt phenotype |
|-----------------------------|---------------------|--------------------------|
|                             |                     | Resistant | Susceptible |
| This study                  | 27                  | 11        | 16          |
| Previous report             | 12                  | 4         | 8           |
| Company/supplier            | 78                  | 2         | 76          |
| Total                       | 117                 | 17        | 100         |

Table 1. Summary of the phenotypic composition of tomato cultivars used in this study
Disease evaluation of tomato cultivars for bacterial wilt resistance

*R. pseudosolanacearum* strain SL882, classified as race 1, biovar 4, and phytophotype I (Lee et al. 2011), was cultured on casamino acid-peptone-glucose (CPG) medium (casamino acid, 1 g; peptone, 10 g; glucose, 5 g; and agar, 15 g per liter of distilled water) and incubated at 28°C for 48 h (Kelman 1954). The bacterial culture from the Petri dish (90 × 15 mm) was rinsed with distilled water and washed using a cotton swab to make the inoculum suspension and its concentration was adjusted to approximately 10^8 CFU/ml (OD_600~0.1) using a NanoDrop 2000/UV–Vis spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).

Seedlings at one month after sowing were inoculated by dipping the roots in the bacterial suspension (Caldwell et al. 2017). Each seedling was grown in a 128-cell seedling tray (28 × 28 × 40, bottom 15 mm) was pulled out, and its roots were dipped in the bacterial suspension. The inoculated seedlings were transplanted into 50-cell seedling trays (45 × 45 × 50, bottom 32 mm) and kept in a growth chamber (temperature = 28°C, relative humidity = 70%, and 16–8 h light-dark cycles). The disease severity was evaluated based on a disease scale of 0 to 4, where 0 = no visible symptoms; 1 = 25% of leaves wilting; 2 = 50% of leaves wilting; 3 = 75% leaves wilting; 4 = all foliage is wilted, and the plant dies (Morel et al. 2018). The disease scale was determined based on visual observation of the degree of wilting. The average value of disease severity for ten plants was calculated per each line. Cultivars with mean disease severity scores of <2 were classified as resistant, while those with scores >2 were classified as susceptible to bacterial wilt.

Genomic DNA extraction

Genomic DNA of 27 cultivars which were phenotyped in this study was extracted from young leaf tissues with a modified cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). The concentration and quality of genomic DNA were measured using NanoDrop 2000/UV–Vis spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).

Sequence analysis of *Bwr-6* region and marker development

Non-synonymous SNPs between resistant and susceptible groups of tomato cultivars near *Bwr-6* within 15 candidate genes (18 SNPs) were previously identified by whole-genome resequencing (Kim et al. 2018). Each candidate gene contained one non-synonymous SNP except two genes, *Solyc06g051110.1* and *Solyc06g051140.2*, which have two and three SNPs, respectively. The nucleotide changes between bacterial wilt-resistant and wilt-susceptible groups of tomato varieties for these SNPs, along with the respective amino acid changes, are presented in Table 2. To dissect *Bwr-6* and develop diagnostic markers, selected SNPs were converted to CAPS/dCAPS markers. Based on six non-synonymous SNPs located at 24669159, 34398374, 34399541, 35950028, 37049726, and 37186202, respectively) in *Bwr-6* (Kim et al. 2018), five CAPS (RsR6-1~RsR6-5) and one dCAPS (RsR6-6) markers were developed. An HRM molecular marker (KHU-1),

### Table 2. List of candidate genes containing non-synonymous SNPs near *Bwr-6* and *Bwr-12*, adapted from Kim et al. (2018). SNPs indicated in bold were used to develop CAPS/dCAPS markers in this study

| Candidate gene | SNP position (bp) | Nucleotide change | Amino acid change | Gene annotation (ITAG2.4) |
|----------------|------------------|-------------------|------------------|--------------------------|
| *Solyc06g035530.2* | 24,482,686 | T | C | F | L | Gibberellin 20-oxidase-2 |
| *Solyc06g035620.2* | 24,667,815 | A | G | Y | C | Scarecrow-like 1 transcription factor |
| *Solyc06g035630.1* | 24,669,159 | T | C | L | P | GRAS family transcription factor |
| *Solyc06g036060.2* | 25,438,944 | A | G | I | V | Zinc finger family protein |
| *Solyc06g048580.1* | 31,287,788 | T | C | L | S | Unknown protein |
| *Solyc06g051110.1* | 31,287,788 | T | C | L | S | Unknown protein |
| *Solyc06g051140.2* | 31,287,788 | T | C | L | S | Unknown protein |
| *Solyc06g051140.2* | 31,287,788 | T | C | L | S | Unknown protein |
| *Solyc06g051140.2* | 31,287,788 | T | C | L | S | Unknown protein |
| *Solyc06g051140.2* | 31,287,788 | T | C | L | S | Unknown protein |
| *Solyc06g051140.2* | 31,287,788 | T | C | L | S | Unknown protein |

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Table 3. DNA marker information used in this study

| Marker name | SNP position (bp) | Marker type | Primer sequence (5’→3’) | T_m (°C) | Restriction enzyme | Expected size (bp) |
|-------------|------------------|-------------|--------------------------|----------|--------------------|-------------------|
| RsR6-1      | 24,669,159       | CAPS        | F: GGAATATGGTTAACAATCCAGTG | 57.5     | MnlI               | 227, 173, 54      |
|             |                  |             | R: GAAATACAAAATCCACACGCCGTG | 59.3     |                    |                   |
| RsR6-2      | 34,389,374       | CAPS        | F: CTTCTTGATAGGACGCTTGATAT | 59.3     | Rsal               | 87, 116, 203      |
|             |                  |             | R: CAATCAAGCCATGCACCCCCATTTTC | 59.3     |                    |                   |
| RsR6-3      | 34,399,541       | CAPS        | F: CTTCTTGGCCAGATCTTGAATAG | 57.5     | MnlI               | 214, 116, 98      |
|             |                  |             | R: CCAAGGTCAGCTCAAAATTTCCA | 57.5     |                    |                   |
| RsR6-4      | 35,950,028       | CAPS        | F: GTTTTCCCTGGAAATCATTGGGC | 57.5     | Mosel              | 116, 97, 213      |
|             |                  |             | R: GTATAGTGTGATGTCACAATTCGC | 57.5     |                    |                   |
| RsR6-5      | 37,049,726       | CAPS        | F: CTCAGAAAAGCTGGATAAATCTGAAG | 59.3     | HinfI              | 204, 129, 75      |
|             |                  |             | R: GGAGAAAGGCACGCCGACTTTTT | 60.6     |                    |                   |
| RsR6-6      | 37,186,202       | dCAPS       | F: CGGTGATGACGGAGTGTAGTAAA | 59.3     | HpyCH4III          | 234, 200, 34      |
|             |                  |             | R: AGTCTTGCCTTGGACGGTAGCACAGAAC | 60.6     |                    |                   |
| RsR12-1     | 2,941,301        | dCAPS       | F: GTTACGAGCAAAGCTTTAATCTGATTTATCCC | 58.8     | AciI               | 203, 168, 35      |
|             |                  |             | R: GTAATCAATTCGAGGACCTGTC | 64.9     |                    |                   |

**PCR amplification and gel electrophoresis**

PCR reactions were carried out according to the manufacturer’s instructions (SolGent Co., Ltd., Daejeon, Korea) in a total volume of 25 μl containing 1 μl genomic DNA, 2.5 μl 10X e-Tag reaction buffer, 0.5 μl of 10 mM dNTP mix, 1 μl of each forward and reverse primers, 0.125 μl Solg e-Tag DNA polymerase, and 18.875 μl of ddH2O. PCR amplification was carried out using a Bio-Rad T100 thermocycler (Bio-Rad Laboratories, Inc.) with the following conditions: denaturing for 3 min at 95°C, followed by 34 cycles of 30 s at 95°C denaturation, 30 s at annealing temperature (which varied for different primer sets (Table 3)), 1 min at 72°C extension, and a final elongation step at 72°C for 5 min. PCR products were digested with the respective restriction enzymes. The reaction mixture consisted of 5 μl template PCR product, 1 μl reaction buffer, 0.1 μl of restriction enzyme, and 3.9 μl ddH2O. The mixture was incubated at 37°C for 16 hrs and was carried out using Bio-Rad T100 thermal cycler (Bio-Rad Laboratories, Inc.). The digested product was mixed with 2 μl 6X DNA loading buffer and subjected to gel electrophoresis on a 3% agarose gel to visualize the polymorphic DNA bands. The details of CAPS/dCAPS marker information, including primer sequences, restriction enzymes, and the expected band sizes for resistant and susceptible tomato groups, are presented in Table 3.

**Selection of F3 and marker validation**

To confirm the efficiency of the two QTLs, F3 generation developed from a cross between E6203 (susceptible) and Hawaii 7998 (resistant) were screened with two markers RsR6-5 (Bwr-6) and RsR12-1 (Bwr-12). Ten F2 plants homoyzous for both markers (five resistant and five susceptible), four F3 plants harboring only RsR6-5, and four F2 plants harboring only RsR12-1 were selected for harvesting the F3 seeds. The F3 generation was inoculated and evaluated for disease resistance. Ten plants of each F3 progeny were inoculated. The resistant and susceptible parents were included as controls during disease evaluation. The mean disease severity of ten plants was used to designate the resistance level of each progeny. Furthermore, the markers were evaluated for their diagnostic value using tomato cultivars (Yang et al. 2015).

**Results**

**Phenotyping of tomato cultivars for bacterial wilt resistance**

The resistance level of 27 cultivars against bacterial wilt disease was inoculated and confirmed in this study. Hawaii 7996, B-Blocking, Shincheonggang, BWR-20, Spider, High Power, 10-BA-3-33, 10-BA-4-24, IT 201664, Hawaii 7998, and Fighting were resistant with low mean disease severity scores (<2) while UC-134, LA1589, Purple Calabash, Florida8516, Heinz 1706, A-1, E6203, Moneymaker, Super Dotaerang, Anahu, Red Strong, Bluck Plum, Gold Nugget, VF-36, M82, and Dotaerang Red were susceptible with a mean disease severity of ten plants was used to designate the resistance level of each progeny. Furthermore, the markers were evaluated for their diagnostic value using tomato cultivars (Yang et al. 2015).
The primer sets were tested for polymorphism using six susceptible and five resistant cultivars. All primer sets resulted in a clear polymorphic band between susceptible and resistant tomato cultivars after enzyme digestion (Fig. 2). For selecting an accurate and reliable marker for Bwr-6, six markers (RsR6-1~6-6) were screened using 117 tomato cultivars (Table 4). The Bwr-12 genotype of these cultivars was determined using RsR12-1. Hawaii 7996, Hawaii 7998, 10-BA-3-33, 10-BA-4-24, BWR-20, BWR-1, BWR-22, and BWR-23 had homozygous resistant genotypes with all six markers near Bwr-6. IT201664, High Power, Spider, SVTX6258, Super High Power, B-Blocking, Shincheonggang, Fighting, and Geumgang are either susceptible or heterozygous to RsR6-1, RsR6-2, and RsR6-3 while they are resistant to RsR6-4, RsR6-5, and RsR6-6 except for High Power, which is heterozygous to RsR6-4 and RsR6-5. Comparing the six markers based on the genotype of resistant cultivars, RsR6-1, RsR6-2, and RsR6-3 did not seem better candidate markers for Bwr-6 because IT201664, High Power, Spider, and Super High Power are susceptible to these markers.

Therefore, we considered RsR6-4, RsR6-5, and RsR6-6 for further analysis using the susceptible set of cultivars. Almost all bacterial wilt-susceptible cultivars had susceptible or heterozygous genotypes to RsR6-5 except Gold Sugar, Sinheukjinju, SV7160TC, and LA1589. Red Strong shows the resistant genotype to RsR6-4 and the susceptible genotype to RsR6-5. Cultivars resistant to RsR6-6 and heterozygous with RsR12-1, such as SV02444 TG, SV4224 TH, and SV0339TG, are expected to be resistant to bacterial wilt, but all were susceptible. In addition, Red Strong and SkyBall have homozygous resistant genotypes with RsR6-6 and RsR12-1, although both exhibit susceptible phenotype (Table 4).

The presence of resistant alleles in both Bwr-6 and Bwr-12 resulted in resistant phenotype while the absence of either of the two resulted in susceptible phenotype. This marker analysis indicated that cultivars homozygous resistant to RsR6-5 and either a homozygous resistant...
### Table 4. SNP marker genotype of resistant and susceptible tomato cultivars or lines used in this study

| No. | Tomato cultivar/line | Type | Company/Supplier | SNP marker genotype | Reference |
|-----|----------------------|------|------------------|---------------------|-----------|
|     |                      |      |                  | RsR6-1 | RsR6-2 | RsR6-3 | RsR6-4 | RsR6-5 | RsR6-6 | RsR6-12 |       |
| 1   | Hawaii 7996          | Inbred line | KHU | R | R | R | R | R | R | This study |
| 2   | Hawaii 7996          | Inbred line | KHU | R | R | R | R | R | R | This study |
| 3   | 10-BA-3-33           | Inbred line | KHU | R | R | R | R | R | R | This study |
| 4   | 10-BA-4-24           | Inbred line | KHU | R | R | R | R | R | R | This study |
| 5   | BWR-20               | Inbred line | KHU | R | R | R | R | R | R | This study |
| 6   | BWR-1                | Inbred line | KHU | R | R | R | R | R | R | Kim et al. (2018) |
| 7   | BWR-22               | Inbred line | KHU | R | R | R | R | R | R | Kim et al. (2018) |
| 8   | BWR-23               | Inbred line | KHU | R | R | R | R | R | R | This study |
| 9   | IT 201664            | Inbred line | RDA | H | S | S | R | R | R | This study |
| 10  | High Power           | F<sub>1</sub> hybrid | Dae Yeon seed co. | H | S | S | H | H | R | This study |
| 11  | Super High Power     | F<sub>1</sub> hybrid | Dae Yeon seed co. | S | S | S | R | R | R | Kim et al. (2018) |
| 12  | Spider               | F<sub>1</sub> hybrid | Takii Korea | H | H | H | R | R | R | This study |
| 13  | B-Blocking           | F<sub>1</sub> hybrid | Takii Korea | H | H | H | R | R | H | This study |
| 14  | Shinchonrugang       | F<sub>1</sub> hybrid | Farm Hannong | H | H | H | R | R | H | This study |
| 15  | Fighting             | F<sub>1</sub> hybrid | Takii Korea | H | H | H | R | R | R | This study |
| 16  | SYTX6258             | F<sub>1</sub> hybrid | Monsanto Korea | H | H | H | R | R | R | Supplier |
| 17  | Geunggang            | F<sub>1</sub> hybrid | Monsanto Korea | H | H | H | R | R | R | Supplier |

**Bacterial wilt susceptible**

| 18  | MB2                  | Inbred line | TGRC | S | S | S | S | S | S | S | This study |
| 19  | E6203                | Inbred line | TGRC | S | S | S | S | S | S | S | This study |
| 20  | VF36                 | Inbred line | TGRC | R | S | S | S | S | S | S | This study |
| 21  | Monemakeso           | Inbred line | TGRC | R | S | S | S | S | S | S | This study |
| 22  | A-1                  | Inbred line | TGRC | S | S | S | S | S | S | S | This study |
| 23  | Jonahu               | Inbred line | TGRC | S | S | S | S | S | S | R | S | This study |
| 24  | Black Plum           | Inbred line | TGRC | S | S | S | S | S | S | S | S | This study |
| 25  | Florida 8516         | Inbred line | TGRC | S | S | S | S | S | S | S | S | This study |
| 26  | Gold Nugget          | Inbred line | TGRC | S | R | S | S | S | S | R | S | This study |
| 27  | Purple Calabash      | Inbred line | TGRC | H | H | S | H | S | R | S | This study |
| 28  | UC-134               | Inbred line | TGRC | S | S | S | S | S | S | S | S | This study |
| 29  | Heinz 1206           | Inbred line | TGRC | S | S | S | S | S | S | S | S | This study |
| 30  | New Yorker           | Inbred line | TGRC | S | S | S | S | S | S | R | S | Jung et al. (2014) |
| 31  | Shinheksu            | F<sub>1</sub> hybrid | Asia Seed Co., Ltd. | R | S | S | S | S | S | R | S | Kim et al. (2018) |
| 32  | TY Unique            | F<sub>1</sub> hybrid | Asia Seed Co., Ltd. | R | S | S | S | H | R | H | Supplier |
| 33  | Shinho Yellow        | F<sub>1</sub> hybrid | Asia Seed Co., Ltd. | H | H | H | H | R | H | H | Supplier |
| 34  | Red Strong           | F<sub>1</sub> hybrid | Bunong Seed | R | S | S | S | R | S | R | This study |
| 35  | Sun Star             | F<sub>1</sub> hybrid | Bunong Seed | H | S | S | S | H | S | H | Supplier |
| 36  | Black Eagle          | F<sub>1</sub> hybrid | Bunong Seed | H | S | H | H | S | H | S | Supplier |
| 37  | Tamla                | F<sub>1</sub> hybrid | Bunong Seed | H | S | H | S | H | H | Supplier |
| 38  | Bntoska              | F<sub>1</sub> hybrid | Bunong Seed | H | S | H | S | H | S | H | Supplier |
| 39  | TY Izzang            | F<sub>1</sub> hybrid | Bunong Seed | H | S | H | H | S | R | S | Supplier |
| 40  | Candy Plus           | F<sub>1</sub> hybrid | Bunong Seed | H | S | H | S | H | S | H | Supplier |
| 41  | Super Star           | F<sub>1</sub> hybrid | Bunong Seed | H | S | S | S | S | H | H | Supplier |
| 42  | Red Zenith           | F<sub>1</sub> hybrid | Bunong Seed | H | S | H | H | S | R | H | Supplier |
| 43  | TY Hunter            | F<sub>1</sub> hybrid | Bunong Seed | R | S | H | H | S | H | H | Supplier |
| 44  | TY One Top           | F<sub>1</sub> hybrid | Bunong Seed | S | S | S | H | S | H | H | Supplier |
| 45  | Yureka               | F<sub>1</sub> hybrid | Bunong Seed | S | S | H | H | S | H | H | Supplier |
| 46  | Black Ace            | F<sub>1</sub> hybrid | Bunong Seed | R | S | S | S | S | S | S | Supplier |
| 47  | Oasis                | F<sub>1</sub> hybrid | Bunong Seed | S | S | H | S | S | H | S | Supplier |
| 48  | TY Megaton           | F<sub>1</sub> hybrid | Bunong Seed | S | S | S | S | R | S | R | Supplier |
| 49  | Gold Sugar           | F<sub>1</sub> hybrid | Bunong Seed | R | S | S | S | H | R | R | S | Supplier |
| 50  | Daezaeang Red        | F<sub>1</sub> hybrid | Dongseon seed | H | H | S | S | S | R | S | This study |
| 51  | Black Ball           | F<sub>1</sub> hybrid | Dongseon Seed | R | S | H | H | S | H | H | Supplier |
| 52  | Kolmi                | F<sub>1</sub> hybrid | Dongseon Seed | S | S | S | H | H | S | H | Supplier |
| 53  | Sky Ball             | F<sub>1</sub> hybrid | Dongseon Seed | R | S | H | H | S | S | R | R | Supplier |
| 54  | Starbuck             | F<sub>1</sub> hybrid | Farm Hannong | R | S | S | S | S | S | S | Supplier |
| 55  | Olleh TY             | F<sub>1</sub> hybrid | Farm Hannong | S | S | H | H | S | R | S | Supplier |
| 56  | Raffito              | F<sub>1</sub> hybrid | Farm Hannong | H | S | S | S | S | H | R | Supplier |
| 57  | Big Wonderful        | F<sub>1</sub> hybrid | Genong Seed | R | S | H | H | S | H | S | Supplier |
| 58  | TY Carnival          | F<sub>1</sub> hybrid | Gyeongwon | R | S | H | H | S | H | H | Supplier |
| 59  | Legend Summer        | F<sub>1</sub> hybrid | Haesung Seed plus | S | S | S | S | S | S | S | Supplier |
| 60  | Daewang              | F<sub>1</sub> hybrid | Jeil Seed Bio | H | S | S | S | S | S | S | Supplier |
| No. | Tomato cultivar/line | Type | Company/Supplier* | SNP marker genotype* | Reference* |
|-----|----------------------|------|-------------------|----------------------|------------|
| 61  | Hongboreuk            | F₁ hybrid | Jeil Seed Bio | S S S S S S S | Supplier |
| 62  | Jeilheukju            | F₁ hybrid | Jeil Seed Bio | S S S R R | Supplier |
| 63  | Dotaerang Myeonggum   | F₁ hybrid | Jeil Seed Bio | S S S S S | Supplier |
| 64  | Heukryong             | F₁ hybrid | Jeil Seed Bio | S S S S S S | Supplier |
| 65  | Minjaok               | F₁ hybrid | Jeil Seed Bio | S S S R R | Supplier |
| 66  | Sinheukjuin           | F₁ hybrid | Jeil Seed Bio | H R S R R | Supplier |
| 67  | Super Dotaerang       | F₁ hybrid | Koreogon seed   | H H S S S | This study |
| 68  | Lezaforta F₁          | F₁ hybrid | Mifko seed     | S S S S H | Supplier |
| 69  | Unicorn               | F₁ hybrid | Monsanto Korea | H S S S S | Supplier |
| 70  | SV 7160 TC            | F₁ hybrid | Monsanto Korea | R S H R R | Supplier |
| 71  | SV02444 TG            | F₁ hybrid | Monsanto Korea | S S H S S | Supplier |
| 72  | Bacchus               | F₁ hybrid | Monsanto Korea | R S H H S | Supplier |
| 73  | SV4224 TH             | F₁ hybrid | Monsanto Korea | S S H S R | Supplier |
| 74  | SV0339TG              | F₁ hybrid | Monsanto Korea | S S H S S | Supplier |
| 75  | Tiara                 | F₁ hybrid | Nongwoo Bio Co., Ltd. | H S S S R | Kim et al. (2018) |
| 76  | TY SenseQ             | F₁ hybrid | Nongwoo Bio Co., Ltd. | S S H H S | Supplier |
| 77  | Tadang                | F₁ hybrid | Nongwoo Bio Co., Ltd. | S S S R | Supplier |
| 78  | Titchal               | F₁ hybrid | Nongwoo Bio Co., Ltd. | S S H S R | Supplier |
| 79  | TY Altorang           | F₁ hybrid | Nongwoo Bio Co., Ltd. | S S H H S | Supplier |
| 80  | Beta Tiny             | F₁ hybrid | Nongwoo Bio Co., Ltd. | S S H H S | Supplier |
| 81  | TY Tiny               | F₁ hybrid | Nongwoo Bio Co., Ltd. | S S H H S | Supplier |
| 82  | Cupirang              | F₁ hybrid | Nongwoo Bio Co., Ltd. | S S S S S | Supplier |
| 83  | Minichal              | F₁ hybrid | Nongwoo Bio Co., Ltd. | S S H H S | Supplier |
| 84  | TY Sispen             | F₁ hybrid | Nongwoo Bio Co., Ltd. | S S H H S | Supplier |
| 85  | Black Change          | F₁ hybrid | Nongwoo Bio Co., Ltd. | H S H H S | Supplier |
| 86  | Malya                 | F₁ hybrid | RDA              | S S S S R | Kim et al. (2018) |
| 87  | Sibgyo 1 ho           | F₁ hybrid | RDA              | S S S S S | Kim et al. (2018) |
| 88  | Broadley              | F₁ hybrid | RDA              | S S S S S | Kim et al. (2018) |
| 89  | Yulwon                | F₁ hybrid | RDA              | S H H R | Kim et al. (2018) |
| 90  | Hoyong                | F₁ hybrid | Sakata Korea     | H S S S S | Kim et al. (2018) |
| 91  | Tosama                | F₁ hybrid | Sakata Korea     | S S S H S | Supplier |
| 92  | Super Sun Road        | F₁ hybrid | Sakata Korea     | H S S H S | Supplier |
| 93  | Super Top             | F₁ hybrid | Sakata Korea     | S S S H H | Supplier |
| 94  | Lokosuan Maru         | F₁ hybrid | Sakata Korea     | S S S S S | Supplier |
| 95  | Taiyau                | F₁ hybrid | Sakata Korea     | S S S S S | Supplier |
| 96  | Taihu                 | F₁ hybrid | Sakata Korea     | R S H S S | Supplier |
| 97  | Super Top             | F₁ hybrid | Sakata Korea     | S S S S S | Supplier |
| 98  | Tiger                 | F₁ hybrid | Samsung Seeds   | H S S S S | Supplier |
| 99  | Chalstone TY          | F₁ hybrid | Sky seed        | S S H H S | Supplier |
| 100 | TY Marathon            | F₁ hybrid | Sky seed        | S S S S R | Supplier |
| 101 | Rapsody               | F₁ hybrid | Syngenta Korea  | H S S S H | Supplier |
| 102 | Madison               | F₁ hybrid | Syngenta Korea  | H S S S S | Supplier |
| 103 | Ricophin-9            | F₁ hybrid | Syngenta Korea  | H S S H H | Supplier |
| 104 | Duine                 | F₁ hybrid | Syngenta Korea  | H S S S S | Supplier |
| 105 | Dafnis                | F₁ hybrid | Syngenta Korea  | S S H H S | Supplier |
| 106 | Komodo                | F₁ hybrid | Syngenta Korea  | S S H H S | Supplier |
| 107 | Terry                 | F₁ hybrid | Syngenta Korea  | S S H H S | Supplier |
| 108 | Mamirio               | F₁ hybrid | Syngenta Korea  | H S H S H | Supplier |
| 109 | European Rapsodie     | F₁ hybrid | Syngenta Korea  | S S H S H | Supplier |
| 110 | Tria Plus             | F₁ hybrid | Taeyang seed    | R S H H H | Supplier |
| 111 | Kang Jeok             | F₁ hybrid | Taeyang seed    | R S H H S | Supplier |
| 112 | Dotaerang TY Winner   | F₁ hybrid | Takii Korea     | S S H S S | Supplier |
| 113 | Dotaerang Plus        | F₁ hybrid | Takii Korea     | S S S S S | Supplier |
| 114 | Dotaerang Solar       | F₁ hybrid | Takii Korea     | S S S S S | Supplier |
| 115 | Cuty                  | F₁ hybrid | Takii Korea     | S S S S S | Supplier |
| 116 | Dotaerang Diamond     | F₁ hybrid | Takii Korea     | S S S S S | Supplier |
| 117 | LA1589                | Wild species | TGRGC | S R R R S | This study |

* Company/supplier of seed or DNA sample: KHU = Kyung Hee University, RDA = National Agrobiodiversity Center (RDA-Genebank), TGRGC = Tomato Genetics Resource Center. All tomato genotypes belong to *Solanum lycopersicum* species except LA1589 (*Solanum pimpinellifolium*).

* SNP marker genotype: R = resistant, S = susceptible, H = heterozygous.

* Reference for the phenotypic information of the tomato cultivars used in the study. Supplier’s phenotypic information was obtained via personal communication from the company.
or heterozygous genotype to RsR12-1 exhibited a resistant phenotype. However, homozygous susceptible or heterozygous genotypes with RsR6-5 exhibited a susceptible phenotype regardless of the RsR12-1 genotype. One exception to this was High Power, which exhibits a resistant phenotype although it has a heterozygous genotype with RsR6-5. The genotype of RsR6-5 is highly correlated with the bacterial wilt phenotype of tomato cultivars and selected for tracing Bwr-6. The diagnostic accuracy of the markers was evaluated and RsR6-5 and RsR12-1 combination was resulted in 94.1% true positive rate and 100% true negative rate (Supplemental Table 1). Taken together, RsR6-5 and RsR12-1 should be used for effective marker-assisted selection of bacterial wilt resistance in tomatoes.

**Validation of the RsR6-5 and RsR12-1 markers using F3 populations**

To validate the efficiency of the two markers for selecting resistant lines in the segregating population, 10 F3 generations homozygous resistant and susceptible (each five lines), and eight F3 generation carrying only Bwr-6 or Bwr-12 (each four lines) were developed from E6203 (susceptible) and Hawaii 7998 (resistant) were selected based on RsR6-5 and RsR12-1 genotype. Hawaii 7998 is one of the entries in international set of bacterial wilt resistant lines evaluated in twelve fields and showed an average of 90% survival rate (Wang et al. 1998). The resistance of Hawaii 7998 and Hawaii 7996 was derived from the same origin, PI 127805A (S. pimpinellifolium) (Daunay et al. 2010, Scott et al. 2005). Hence, genetic resistance to bacterial wilt in these two lines might be governed by same gene.

Ten plants of each F3 generation, along with the two parental lines, were inoculated with *R. pseudosolanacearum* strain SL882 for disease evaluation. The two parental lines exhibited distinct differences in bacterial wilt resistance as expected, with mean disease scores of 4.0 ± 0.0 and 1.2 ± 0.61, respectively. The mean disease severity between homozygous resistant and susceptible genotypes in the F3 generation was significantly different (Fig. 3A). Lines with homozygous resistant genotypes exhibited highly resistant phenotypes with mean disease severity scores ranging from 0.4 ± 0.40 to 1.6 ± 0.65, which were not significantly different from that of the resistant parent. On the other hand, homozygous susceptible F3 exhibited highly susceptible phenotypes with mean disease severity scores of 4.0 ± 0.0, which were similar to the susceptible parent (Fig. 3A, 3B).

**Discussion**

Genetic resistance is the most effective control strategy for bacterial wilt of tomato, and multiple breeding programs have been engaged in developing resistant lines by incorporating resistance from different resistant sources (Daunay et al. 2010, Wang et al. 1998). Genomic regions linked to bacterial wilt resistance in the well-known resistant cultivar, Hawaii 7996, were identified on different chromosomes, and some regions were detected against specific strains (Thoquet et al. 1996a, 1996b, Wang et al. 2000). Bwr-3 and Bwr-4 were associated with resistance against phytophate II strains, while Bwr-12 was specific to
Phytoype-1 (Carmeille \textit{et al.} 2006). In contrast, \textit{Bwr-6} was associated with various strains from different phylootypes (I and II) (Thoquet \textit{et al.} 1996a) and consistently detected under various conditions (Carmeille \textit{et al.} 2006, Geethanjali \textit{et al.} 2010, Mangin \textit{et al.} 1999, Wang \textit{et al.} 2013). Genetic analysis of bacterial wilt resistance in \textit{S. lycopersicum var. cerasiforme} ‘L285’ identified three QTL on chromosomes 6, 7, and 10 (Danesh \textit{et al.} 1994). The genomic regions associated with bacterial wilt resistance on chromosome 6 in both \textit{Hawaii 7996} and L285 were co-localized. Furthermore, the most stable QTL for bacterial wilt resistance in eggplant was also identified in chromosome 6 and syntenic with \textit{Bwr-6} of tomato (Salgon \textit{et al.} 2018). All these suggest the significance of \textit{Bwr-6} in bacterial wilt resistance in tomato and likely in other Solanaceae crops. The development of functional markers for such broad-spectrum QTL is essential to facilitate marker-assisted breeding.

Genetic analysis using only segregating populations is time-consuming, cost-inefficient, and the developed markers may be specific to certain resistant lines (Pascual \textit{et al.} 2016). In this regard, we used a complementary approach to dissect the \textit{Bwr-6} region, using germplasm collections and F$_2$ generation to validate our results. SNP-based CAPS/dCAPS markers near \textit{Bwr-6} were developed and validated. A total of 117 tomato germplasms were screened with newly developed markers for \textit{Bwr-6} genotypes, and the corresponding phenotypic information was used to explore the efficiency of each marker.

Among 17 resistant cultivars used to screen the markers in the \textit{Bwr-6} region, four cultivars (IT 201664, High Power, Super High Power, and Spider) had susceptible genotypes with RsR6-1, RsR6-2, and RsR6-3. On the other hand, all resistant cultivars had homozygous resistant genotypes with RsR6-4, RsR6-5, and RsR6-6 except High Power, which has a heterozygous genotype with RsR6-4 and RsR6-5. Similarly, all cultivars exhibiting resistant phenotypes have homozygous resistant genotypes with RsR12-1, except B-Blocking, Shincheonggang, Fighting, and Geumgang which are heterozygous to this marker. The true positive rate of RsR6-1, RsR6-2, and RsR6-3 combined with RsR12-1 was 47.1\% whereas that of RsR6-4 and RsR6-5 was 94.1\% and that of RsR6-6 was 100\%. Based on these observations, we hypothesized that the three markers (RsR6-4, RsR6-5, and RsR6-6) are better predictors of resistance conferred by \textit{Bwr-6} than RsR6-1, RsR6-2, and RsR6-3.

The markers were further compared using the susceptible panel of germplasms. Some cultivars exhibiting susceptible phenotypes, such as Red Strong, are resistant to RsR6-4, RsR6-6, and RsR12-1. However, all cultivars having homozygous resistant genotypes with RsR6-5 and RsR12-1 exhibited resistant phenotypes. In summary, 99\% and 87\% true negative rate was obtained for RsR6-4 and RsR6-6 while RsR6-5 resulted in 100\% true negative rate genotypes in combination with RsR12-1 (Supplemental Table 1). These results suggest that RsR6-5 is the best diagnostic marker to trace \textit{Bwr-6} associated with bacterial wilt resistance. Diagnostic markers developed based only on a segregating population may not fully correlate with the trait when tested in diverse germplasms, hindering their utilization for marker-assisted selection in a broad set of breeding germplasms (Niewohner \textit{et al.} 1995). Therefore, utilization of a wide range of germplasms, including inbred lines, commercial F$_1$ hybrids, and wild species, for validating developed markers is essential before deployment to end-users, including breeders and farmers.

The diagnostic potential of RsR6-5 coupled with RsR12-1 for bacterial wilt resistance in tomato was tested using a broader set of germplasms (Bartkiewicz \textit{et al.} 2018, Yang \textit{et al.} 2015) and can be used for marker-assisted selection in commercial breeding programs. Without determining the genotyping results for another major resistance QTL, \textit{Bwr-12}, determination of \textit{Bwr-6} genotype with the RsR6-5 marker alone is able to predict resistant and susceptible phenotypes in the tomato cultivars used in this study with 94.1\% and 96\% accuracy, respectively. High Power, SV7160TC, Gold Sugar, Sinheukjinju, and LA1589 showed non-matching genotypes. On the other hand, \textit{Bwr-12} genotyping determined by RsR12-1 alone was able to predict resistant and susceptible phenotypes with 100\% and 66\% accuracy, respectively. Resistance conferred by the \textit{Bwr-12} genotype shows a dominant inheritance pattern (Kim \textit{et al.} 2018). RsR6-5 and RsR12-1 combination was resulted in 94.1\% of true positive rate and 100\% true negative rate showing the highest diagnostic accuracy compared to other marker combinations.

Heterozygous to RsR6-5 and either heterozygous or homozygous resistant to RsR12-1 yielded susceptible phenotypes except for High Power, which suggests that the \textit{Bwr-6} resistance allele might be recessive; however, this should be further validated using a segregating population. Recessive gene resistance to bacterial disease has been reported in Arabidopsis and rice. \textit{R. solanacearum} resistance in \textit{Arabidopsis thaliana} is governed by a recessively inherited gene (\textit{RRSI-R}) (Deslandes \textit{et al.} 2002). Similarly, among more than 43 resistance genes identified so far for bacterial blight in rice caused by \textit{Xanthomonas oryzae pv. oryzae}, 16 are inherited as recessive traits (Kim 2018, Vikal and Bhatia 2017).

Extensive QTL mapping studies have been conducted to identify genomic regions associated with bacterial wilt resistance (Carmeille \textit{et al.} 2006, Geethanjali \textit{et al.} 2010, Mangin \textit{et al.} 1999, Thoquet \textit{et al.} 1996a, 1996b, Wang \textit{et al.} 2000, 2003). The release of the tomato reference genome (Tomato Genome Consortium 2012) and availability of whole-genome resequencing data for various tomato cultivars (Lin \textit{et al.} 2014, The 100 Tomato Genome Sequencing Consortium 2014) facilitated comparisons among tomato genotypes using genome-wide SNP markers for different traits. The adequate number of SNPs among tomato genotypes can be used to saturate markers nearby previously identified QTL regions. Accordingly, whole-genome
Marker-assisted selection for bacterial wilt resistance in tomato

Resequencing of bacterial wilt resistant and susceptible tomato cultivars revealed genome-wide SNPs that were candidates for distinguishing the two groups of tomato, with the highest number of non-synonymous SNPs identified on chromosomes 12 and 6 (Kim et al. 2018). Analysis of SNPs near Bwr-12 in the same study discovered molecular marker (KHU-1) tightly linked to bacterial wilt resistance; this marker was used to discriminate resistant and susceptible tomato cultivars. The analysis of SNPs near Bwr-6 and the development of diagnostic markers in this study will pave the way toward identifying candidate genes and facilitating resistance gene pyramiding.

RsR6-5 is located in the coding region of Solyc06g054230.2, which encodes a putative calmodulin protein kinase, suggesting that this gene could be a possible candidate gene for bacterial wilt resistance in tomato. The nucleotide substitution of guanine to thymine at 128 bp changes the amino acid aspartate to glutamate. The majority of plant disease resistance genes encode nucleotide-binding site-leucine-rich repeat (NBS-LRR) proteins (McHale et al. 2006). However, the role of calmodulin proteins in the response of plants to both biotic and abiotic stresses has also been reported (Cheval et al. 2013, Zeng et al. 2015). Calmodulin is a calcium-binding protein and regulates downstream calcium signal-related responses. The expression of tomato calmodulin genes is significantly altered upon pathogen infection. Functional analysis revealed that the silencing of SICaM2 (Solyc10g081170.1.1) and SICaM6 (Solyc03g098050.2.1) in tomato reduced its resistance to tomato rattle virus and Pityrum aphanidermatum and decreased the expression of downstream signaling and defense-related genes (Zhao et al. 2013). Transcriptome analysis of bacterial wilt-resistant (LS-89) and susceptible (Ponderosa) cultivars indicated an approximately 30-fold increase of a putative calmodulin-binding family protein in response to R. solanacearum infection in resistant cultivars, while the analogous response in susceptible cultivars was very limited (Ishihara et al. 2012). These findings suggest that Solyc06g054230.2 may play an important role in bacterial wilt resistance in tomato; although, further analysis is required to elucidate the gene function.

In conclusion, SNPs near Bwr-6 were analyzed to search for markers tightly linked to this QTL. A total of 117 tomato germplasms were used to validate newly developed markers near this QTL. Among the analyzed markers, RsR6-5 is tightly linked to bacterial wilt resistance derived from Bwr-6. Consequently, this marker, in combination with RsR12-1, effectively predicted bacterial wilt-resistant and susceptible cultivars. The significance of these markers was further validated using an F3 generation developed from the crossing between resistant and susceptible parents. F3 lines that had resistant genotypes with RsR6-5 and RsR12-1 exhibited resistant phenotypes, while susceptible to the same markers exhibited susceptible phenotypes. The SNP-based diagnostic marker to Bwr-6 was not identified in previous studies, and the newly developed marker in this study (RsR6-5) will help to trace this locus in marker-assisted breeding of tomato cultivars that are resistant to the devastating effects of bacterial wilt.

Author Contribution Statement

JML conceived and designed the experiments. AMA, JC and YK performed the experiments. CSO, IY, and ISN provided experimental materials. AMA and JML wrote and revised the manuscript, and all co-authors contributed and approved the final draft of the manuscript.

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