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# Gene-based molecular marker system for multiple disease resistances in tomato against *Tomato yellow leaf curl virus*, late blight, and verticillium wilt

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**Abstract** Marker assisted selection (MAS) for disease resistance is widely applied in practical tomato breeding programs in the public and private sectors. Due to its commercial value and importance as a model crop, tomato has taken the lead in MAS among the horticultural crops. *Tomato yellow leaf curl virus*, which is transmitted by the whitefly (*Bemisia tabaci*), is a major threat to tomato production worldwide. The *Ty1* and *Ty3* resistance loci originated from *Solanum chilense* LA1969 and LA1932/LA2779, respectively. Recently, the gene responsible for *Ty1* resistance was identified as a DFDGD-class RNA-dependent RNA polymerase and was demonstrated to be allelic with *Ty3* resistance. The *Ph3* resistance locus from *S. pimpinellifolium* (L3708), which confers incomplete resistance against a widerange of *Phytophthora infestans* isolates, is considered the most effective source of resistance against tomato late blight. A coiled-coil nucleotide-binding leucine-rich repeat gene on chromosome 9 was determined to be responsible for *Ph3* resistance. Resistance against verticillium wilt diseases in tomato is conferred by the *Ve* locus that contains two closely linked, inversely oriented genes: *Ve1* and *Ve2*. The *Ve* locus provides resistance against *Verticillium alboatrum* race 1 and encodes an extracellular leucine-rich repeat receptor-like protein class of disease resistance proteins. We developed reliable and comprehensive molecular markers based on either

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the single nucleotide polymorphisms or insertions/deletions directly responsible for the resistance provided by the *Ty1*, *Ph3*, and *Ve1* loci. These gene-based functional molecular markers are expected to enhance the effectiveness and accuracy of MAS for disease resistance in tomato breeding programs.

**Keywords** *Solanum lycopersicum* · Functional markers · Allele-specific markers · SNP · HRM

## Introduction

In tomato (*Solanum lycopersicum* L.) breeding programs, marker assisted selection (MAS) has been widely and successfully deployed for disease resistance by applying genetic markers to select single resistance genes and to combine (assemble) multiple resistance genes (Foolad and Sharma 2005). Single nucleotide polymorphisms (SNPs) are one of the most common types of genetic variation and have been widely utilized inplant genomics for genome mapping, association studies, diversity analysis, and the tagging of economically important genes (Jehan and Lakhanpaul 2006). Various technological innovations have accelerated the discovery of novel SNPs and the detection of known SNPs, allowing SNPs to be the most expeditious and cost effective genetic markers available for MAS (Giancola et al. 2006; Caicedo et al. 2007; Choi et al. 2007; Jones et al. 2009). A number of SNPs in tomato have been discovered and verified (Labate and Baldo 2005; Yang et al. 2004) and have been successfully used to generate plants with resistance to multiple diseases (Yang et al. 2005).

Gene-based markers are derived from DNA polymorphisms that are physically located within genic regions that are directly associated with phenotypic outcomes, whereas randomly generated molecular markers are mostly generated by fine mapping approaches (Bagge et al. 2007; Salgotra et al. 2014). Gene-based markers, rather than randomly generated markers based on DNA polymorphisms located adjacent to the gene of interest, are clearly advantageous for increasing the accuracy of MAS (Salgotra et al. 2014). A "functional" marker is a gene-based marker based on SNPs or insertions/deletions (InDels) that causes critical phenotypic change(s), such as a conformational shift of the protein's tertiary structure or a premature stop codon (Bagge et al. 2007). Generally, gene-based markers are developed based on SNPs or InDels detected among different alleles and require sequence information about the functional motifs of the genes responsible for the phenotypes of interest. DNA markers derived from functionally defined sequences are developed and applied in multiple types of breeding programs, including applications for cultivar identification, parental line selection, and the selection of progenies in segregating populations (Lagudah et al. 2009; Yeam et al. 2005; Collard and Mackill 2008). The release of the tomato genome sequence has stimulated novel gene characterization in the tomato, and the corresponding gene-based markers are expected to expedite practical tomato breeding programs.

*Tomato yellow leaf curl virus* (TYLCV), which is transmitted by the whitefly (*Bemisia tabaci*), is one of the major threats to tomato production worldwide. Several sources ofTYLCV resistance have been identified among wild tomato species. The *Ty1* resistance locus on chromosome 6 originated from *S. chilense* LA1969 (Zamir et al. 1994). The *Ty3* resistance locus on chromosome 6 originated from *S. chilense* LA1932 and LA2779 (Ji et al. 2007a, b; Agrama and Scott 2006). Recently, a gene responsible for *Ty1* resistance was identified as a DFDGD-class RNA-dependent RNA polymerase and was demonstrated to be allelic with *Ty3* resistance (Verlaan et al. 2013). The *Ty3*-carrying *S. chilense* LA1932 and LA2779 have an additional resistance locus, *Ty4*, on chromosome 3 (Ji et al. 2009b). *Ty2* resistance originated from *S. habrochaites* B6013 (Hanson et al. 2000; Ji et al. 2009a; Yang et al. 2014) and was recently mapped to a 300 kb interval on chromosome 11 (Yang et al. 2014). Recessive resistance against TYLCV was identified in *S. peruvianum* and designated *ty5* (Friedmann et al. 1998; Anbinder et al. 2009).

Late blight (LB) in tomato is caused by *Phytophthora infestans*, which is notorious for the devastating LB in the potato. LB resistance in the potato has been extensively studied, and over 60 resistance genes were characterized or located on the genetic map (Rodewald and Trognitz 2013). Although fewer studies of LB have been conducted in the tomato, several resistance loci have been identified in wild tomato species and applied in practical breeding programs. *Ph1* and *Ph2*, two race-specific resistance genes from

*S. pimpinellifolium*, were mapped to chromosome 7 and 10, respectively (Peirce 1971; Moreau et al. 1998; Foolad et al. 2008). *Ph3*, another resistance gene from *S. pimpinellifolium* L3708 conferring incomplete resistance against a widerange of *P. infestans* isolates, is considered the most effective source of resistance against tomato LB (Black et al. 1996; Zhang et al. 2013). A coiled-coil nucleotide-binding leucine-rich repeat (NBS–LRR) gene on chromosome 9 was determined to be responsible for *Ph3* resistance (Zhang et al. 2014). *Ph4* was discovered in *S. habrochaites* LA1033 (AVRDC 1998; Kim and Mutschler 2006). *S. pimpinellifolium* PSLP153, a novel resistance source, was determined to carry *Ph5-1* and *Ph5-2* on chromosome 1 and 10, respectively (Merk et al. 2012; Merk and Foolad 2012). In addition, quantitative resistance to LB has been reported in *S. habrochaites* and *S. penellii* (Brouwer and St Clair 2004; Li et al. 2011; Smart et al. 2007; Cai et al. 2013).

Tomato verticillium wilt, which is caused by *Verticillium dahliae* and *V. alboatrum*, is a soil-borne fungal disease. The *Ve* gene was reported to confer resistance to tomato verticillium wilt and was mapped to tomato chromosome 9 (Schaible et al. 1951; Diwan et al. 1999; Kawchuk et al. 2001). In 2001, the *Ve* locus was characterized and found to contain two closely linked, inversely oriented genes, *Ve1* and *Ve2*. *Ve* resistance was shown to be effective against *V. alboatrum* race 1 and was found to encode the extracellular leucine-rich repeat receptor-like protein class of disease resistance proteins (Kawchuk et al. 2001). The resistance spectrums of *Ve1* and *Ve2* have been determined (Fradin et al. 2009), and cleaved amplified polymorphic sequence (CAPS) markers for *Ve1* and *Ve2* have been reported (Acciarri et al. 2007; Kuklev et al. 2009).

In this study, we developed a series of allele-specific molecular markers for the *Ty1*, *Ph3*, and *Ve1* resistance loci based on the genic sequences responsible for each type of resistance. We converted the PCR-based CAPS, derived cleaved amplified polymorphic sequences (dCAPS), and sequence characterized amplified region (SCAR) markers into high-resolution melt (HRM) markers to facilitate high-throughput SNP detection. The markers generated in this study, which are based on the SNPs or InDels directly responsible for the resistance phenotype, can be considered as functional markers. These functional markers are expected to contribute to expediting the efficiency and accuracy of MAS for disease resistance in tomato breeding programs.

## Materials and methods

### Plant materials

A total of 27 tomato genotypes including released or commercial cultivars and accessions were used in this research (Table 1). Nine tomato accessions (LA series) were provided by the C. M. Rick Tomato Genetics Resource Center, Department of Plant Sciences, University of California, Davis, CA 95616 (http://tgrc.ucdavis.edu); 4 lines (IT series) were obtained from the gene bank of the Rural Development Administration (RDA), Republic of Korea; and 14 cultivars were purchased from commercial sources. The following *S. chilense* accessions harbouring TYLCV resistance were included: LA1969 for *Ty-1* resistance and LA1932 and LA2779 for *Ty-3* resistance (Zamir et al. 1994; Agrama and Scott 2006). *S. pimpinellifolium* LA1269 (also known as L3708) and *S.lycopersicum* LA4285 and LA4286 were used for *Ph-3* resistance (Black et al. 1996). The 14 commercial cultivars included: one accession each from Sakata Seed Co. (Suzhou, Japan), Takii Seed Inc. (Kyoto, Japan), and Punong Seed (Suwon, Korea); two accessions from Asia Seed Co. (Seoul, Korea); and three accessions each from Syngenta-Korea Inc. (Seoul, Korea), Monsanto-Korea (Seoul, Korea), and Nongwoo Bio (Suwon, Korea).

### DNA extraction

DNA was extracted from young leaves using the cetyltrimethylammonium bromide (CTAB) method with minor modifications (Murray and Thompson 1998). Fresh, young leaves (0.2 g) were ground in a 2.2 ml microfuge tube with 800 µl extraction buffer [(2 % w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl(pH 8.0), 1 % PVP, 0.1 % sodium bisulfite, and 0.2 % (v/v) 2-mercaptoethanol] and incubated in a water bath at 60 °C for 30 min with occasional swirling. The contents of the tube were then mixed with an equal volume of chloroform:isoamyl alcohol (24:1, v/v) and centrifuged at 13,000 rpm for 15 min. The aqueous phase was separated and mixed

**Table 1** Tomato genotypes used in this study

| | Name | Species | References |
|---|---|---|---|
| 1 | LA1932 | *S. chilense* | Ji et al. (2007a) |
| 2 | LA1938 | *S. chilense* | Ji et al. (2007b) |
| 3 | LA1969 | *S. chilense* | Zamir et al. (1994) |
| 4 | LA2779 | *S. chilense* | Agrama and Scott (2006) |
| 5 | LA3473 | *S. lycopersicum* | TGRC |
| 6 | LA3474 | *S. lycopersicum* | TGRC |
| 7 | LA4285 (CLN2264F) | *S. lycopersicum* | TGRC |
| 8 | LA4286 (CLN2264G) | *S. lycopersicum* | TGRC |
| 9 | LA1269 (L3708) | *S. pimpinellifolium* | Black et al. (1996) |
| 10 | IT229371 | *S. lycopersicum* | RDA |
| 11 | IT229370 | *S. lycopersicum* | RDA |
| 12 | IT236514 | *S. lycopersicum* | RDA |
| 13 | IT236513 | *S. lycopersicum* | RDA |
| 14 | SKT-1 | *S. lycopersicum* | |
| 15 | TKI-1 | *S. lycopersicum* | |
| 16 | SGT-1 | *S. lycopersicum* | |
| 17 | SGT-2 | *S. lycopersicum* | |
| 18 | STG-3 | *S. lycopersicum* | |
| 19 | MST-1 | *S. lycopersicum* | |
| 20 | MST-2 | *S. lycopersicum* | |
| 21 | MST-3 | *S. lycopersicum* | |
| 22 | AS-1 | *S. lycopersicum* | |
| 23 | AS-2 | *S. lycopersicum* | |
| 24 | PN-1 | *S. lycopersicum* | |
| 25 | NW-1 | *S. lycopersicum* | |
| 26 | NW-2 | *S. lycopersicum* | |
| 27 | NW-3 | *S. lycopersicum* | |

*SKT* Sakata, *MST* Monsanto, *SGT* Syngenta, *NW* Nongwoo Bio, *TKI* Takii, *AS* Asiaseed, *PN* Punong, *TGRC* tomato genetics resource center, *RDA* Rural Development Administration

(3:2, v/v) with iced isopropanol. The DNA precipitate was centrifuged at 13,000 rpm for 15 min, and then the pellet was washed with 70 % ethanol, dried overnight at room temperature, and resuspended in 100 µl TE buffer [10 mM Tris–HCl (pH 8.0), 0.1 mM EDTA]. Finally, the contents were centrifuged at 13,000 rpm for 5 min, after which the dissolved DNA in the aqueous phase was quantified using a spectrophotometer (Gene-Quant, Pharmacia Biotech) and diluted to 10 ng/µl for PCR amplification.

## Sequencing analysis

The *Ty1*, *Ph3*, and *Ve1* reference sequences were obtained from the GenBank database (HG975445 for *Ty1*, KJ563933 for *Ph3*, and AF272367 for *Ve1*). The primers used to sequence *Ty1*, *Ph3*, and *Ve1* were designed using Primer 3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The PCR products were cloned using the TOPO cloning kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Sequencing was conducted by the dye termination method using an ABI3730 capillary DNA sequencer (ElimBio Inc., CA, USA). Sequence alignments and SNP detection were performed using SeqMan and MegAlign (DNASTAR, Inc., Madison, WI, USA). Restriction enzyme sites revealing sequence variations among the lines were investigated and confirmed using the sequence analysis software package Laser gene 7.2 (DNASTAR, Inc., Madison, WI, USA).

## Molecular markers based on the *Ty1* polymorphism

PCR amplification for the Ty1-*Ssp*I, Ty1-*Bgl*II, and Ty1-*Taq*I markers was conducted using a single pairof primers (F: 5′TGAAGACAAAAACTGCTTC3′, R: 5′TCAGGGTTTCACTTCTATGAAT3′) derived from the *Ty1* genic sequence. The PCR conditions described by Kim et al. (2011) were used. Each 25 µl reaction mixture contained 20 ng DNA, 0.4 mM each of the forward and reverse primers (Bioneer, Korea), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris–HCl (pH 8.0), 2 mM $MgSO_4$, and 5 units Taq polymerase (TaKaRa, Shiga, Japan). Three restriction enzymes, *Taq*I, *Bgl*II, and *Ssp*I, were used to differentiate each targeted SNP. The PCR amplicons were incubated with each restriction enzyme separately for 3 h. The samples were then separated on a 2.5 % agarose gel containing TBE and visualized under UV light using the Gel Doc 2000 (BIO-RAD, CA, USA) after staining with ethidium bromide.

## Molecular markers based on the *Ph3* polymorphism

A fragment spanning the SNP responsible for *Ph3* resistance was amplified using two primer sets, Ph3-

**Table 2** Primers and reaction conditions for the markers developed

| Marker name | Forward primer (5′–3′) | Reverse primer (5′–3′) | Tm (°C) | Product size (bp) | Type of marker |
|---|---|---|---|---|---|
| Ty1-*Ssp*I | ATGAAGACAAAAACTGCTTC | TCAGGGTTTCACTTCTATGAAT | 55 | 608 | CAPS with *Ssp*I |
| Ty1-*Bgl*II | ATGAAGACAAAAACTGCTTC | TCAGGGTTTCACTTCTATGAAT | | | CAPS with *Bgl*II |
| Ty1-*Taq*I | ATGAAGACAAAAACTGCTTC | TCAGGGTTTCACTTCTATGAAT | | | CAPS with *Taq*I |
| Ph3-*Msp*I | TCG ATC GTA TGT AGA CGA TG | AGG CAA ATC TTG AAG AAG CA | 55 | 400 | CAPS with *Msp*I |
| Ph3-SCAR | CTACTCGTGCAAGAAGGTAC | TCCACATCACCTGCCAGTTG | 55 | | SCAR |
| Ve1-*Xba*I | CGA ACT TGA CTA CAT TGA CC | CAG TCT TGA AAG GTT GCT CA | 55 | 743 | CAPS with *Xba*I |
| Ty1-*Ssp*I-HJ | GGTTGGTCTCCTTGATAGTCATGT | TCCACTTGAAGCTTAATAGTCTTTGA | 55 | 118 | SNP |
| Ph3-*Msp*I-HJ | CAACATCACGGATACAAGTAACAA | CATGATCCAAACCGATGACC | 53 | 107 | SNP |

*Msp*I and Ph3-SCAR (Table 2). Each 25 µl PCR mixture contained 20 ng DNA, 0.4 mM each of the forward and reverse primers (Bioneer, Korea), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris–HCl (pH 8.0), 2 mM $MgSO_4$, and 5 units Taq polymerase (TaKaRa, Shiga, Japan). For the CAPS marker analysis, the PCR product was digested by *Msp*I for 2 h at 37 °C. Electrophoresis was conducted on a 2.5 % agarose gel containing TBE and visualized under UV light after staining with ethidium bromide.

### Molecular markers based on the *Ve1* polymorphism

A primer set for the *Ve1* CAPS marker was designed based on the *Ve1* gene sequence (AF272367; Table 2). PCR amplification was performed following the method described above. The PCR product was digested by *Xba*I for 90 min at 37 °C. Electrophoresis was conducted on a 2.5 % agarose gel containing TBE and visualized under UV light after staining with ethidium bromide.

### High-resolution melting marker conversion and analysis

The Ty1-*Ssp*I-HJ (F: 5′GGTTGGTCTCCTTGATAG TCATGT3′, R: 5′TCCACTTGAAGCTTAATAGTC TTTGA) and Ph3-*Msp*I-HJ (F: 5′CAACATCACGG ATACAAGTAACAA, R: CATGATCCAAACCGA TGACC3′) primer pairs were designed for the development of HRM markers. PCR was carried out in 20 µl reaction mixtures containing 10× h-Taq reaction buffer (25 mM $MgCl_2$ mix), 10 mM each dNTP Mix, 10 pmol each primer, 2.5 U/µl DNA polymerase, 20× EvaGreen™ (Solgent, Daejeon, Korea), and 10 ng genomic DNA using a CFX Connect™ Real-Time PCR Detection system (BIO-RAD, Hercules, USA). The cycling conditions were 95 °C for 15 min, followed by 40 cycles of 95 °C for 20 s, 60 °C for 15 s, and 72 °C for 30 s. The HRM markers were analyzed at 0.2 °C increments between 65 and 95 °C.

## Results

### Gene-based CAPS markers for *Ty1/3* resistance

A primer set generating an ampliconencompassing the complete *Ty1/3* coding sequence was designed based on the genic sequence characterized by Verlaan et al. (2013). Sequence information for the *Ty1/3* locus, including the *Ty1* and *Ty3* resistance genotypes, was retrieved from 20 tomato accessions (data not shown). A comparative analysis of the resistance and susceptible alleles of the diverse tomato genotypes identified two haplotypes. A total of nine polymorphisms were

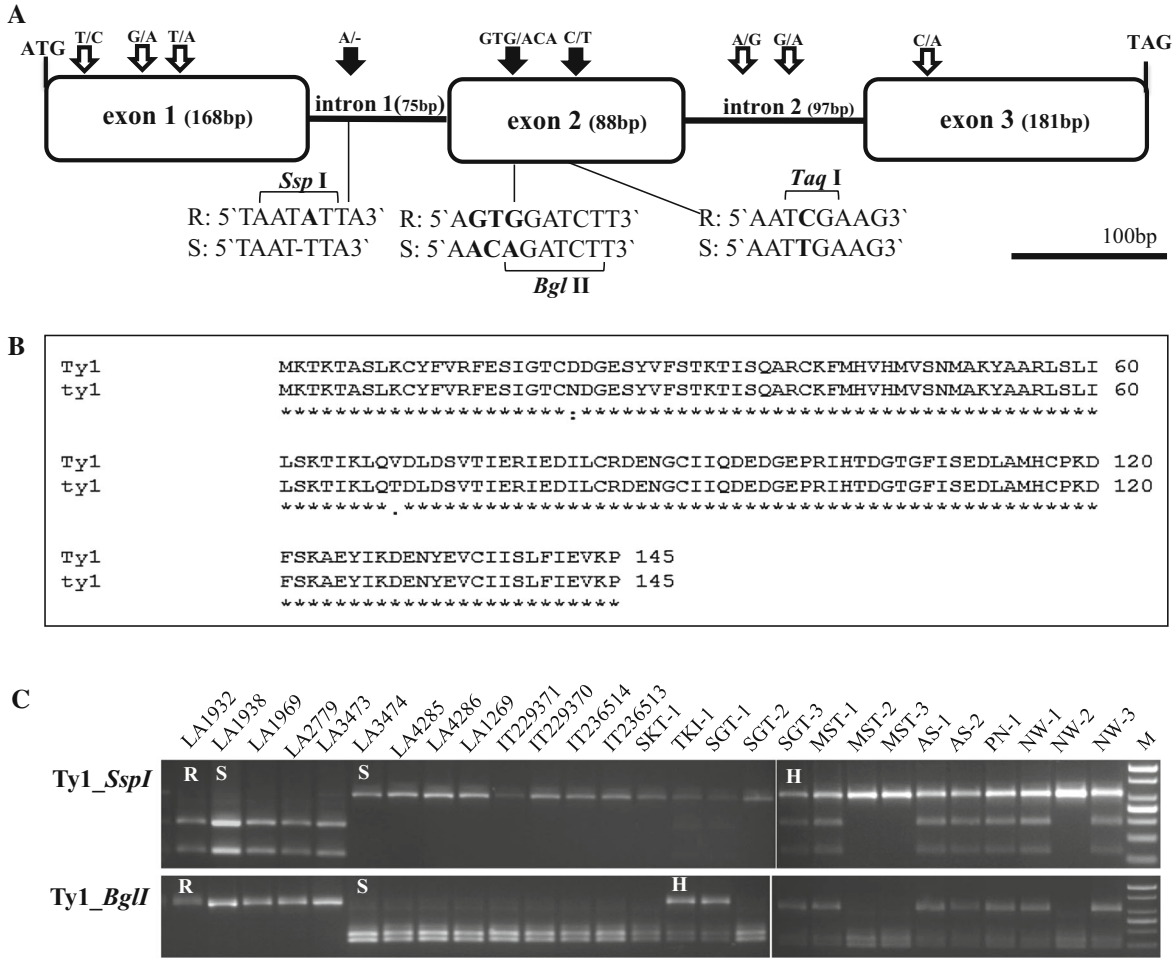


**Fig. 1** Representation of SNP information for *Ty1* gene-based marker development. **a** Schematic representation of the *Ty1* gene structure with exons and introns.The positions of SNPs used for gene-based markers and restriction endonuclease sites (*Ssp*I, *Bgl*II, and *Taq*I) used for CAPS markers are indicated. *Hollow arrows* indicate SNPs that were not used in marker development, and *black arrows* indicate SNPs that were used in marker development. Sequence information for SNPs is shown in *bold*. **b** The aminoacid sequence alignment of Ty1 and ty1 proteins via Clustal W. The SNP in exon 2 (G→A) corresponds to the V–T change at the amino acid level. **c** Genotyping of the Ty1-*Ssp*I and Ty1-*Bgl*I markers in 27 tomato genotypes (1–27). *SKT* Sakata, *MST* Monsanto, *SGT* Syngenta, *NW* Nongwoo, *TKI* Takii, *AS* Asiaseed, *PN* Punong. *Scale bar* indicates 100 bp

detected and three of those were converted into CAPS markers (Fig. 1). The nucleotide polymorphisms utilized for distinguishing the resistant and susceptible haplotypes were an A/− deletion in intron 1, a GTG/ACA three-base polymorphismin exon 2, and a C/T SNP in exon 2. The A/− deletion in intron 1 provided a target to develop a co-dominant marker. The *Ssp*I (5′AATAT3′) endonuclease cleaved the resistance allele but not the susceptible allele (Fig. 1). Therefore, that restriction polymorphism was used to develop the Ty1-*Ssp*I CAPS marker. The *Bgl*II (5′AGATCT3′) endonuclease cleaved the susceptible allele due to the G/A SNP within the three-base polymorphism (GTG/ACA). That reaction produced three bands of contrasting size that were easily resolved on a 2.5 % agarose gel: a 608 bp fragment specific for the resistance allele and two fragments of 327 and 282 bp specific for the susceptible allele. The three-base polymorphism resulted in a single amino acid change of V–T (Fig. 1b). The C/T SNP found in exon 2 provided an additional candidate for converting a co-dominant *Ty1/3* gene-specific marker. The *Taq*I (5′TCGA3′) endonuclease cleaved the resistance allele but not the susceptible allele (Fig. 1c).

**Table 3** Tomato genotypes used to evaluate gene-based markers for resistances to TYLCV, late blight, and verticillium wilt

| | Name | Species | Known resistance | Reference | DNA markers | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Ty1-*Ssp*I | Ty1-*Bgl*II | Ph3-*Msp*I | Ph3-SCAR | Ve1-*Xba*I |
| 1 | LA1932 | *S. chilense* | Ty3 | Ji et al. (2007a) | R | R | Nd | Nd | Nd |
| 2 | LA1938 | *S. chilense* | TYLCV (R) | Ji et al. (2007b) | R | R | Nd | Nd | Nd |
| 3 | LA1969 | *S. chilense* | Ty1 | Zamir et al. (1994) | R | R | Nd | Nd | Nd |
| 4 | LA2779 | *S. chilense* | Ty3 | Agrama and Scott (2006) | R | R | Nd | Nd | Nd |
| 5 | LA3473 | *S. lycopersicum* | Ty1 | TGRC | R | R | Nd | Nd | Nd |
| 6 | LA3474 | *S. lycopersicum* | TYLCV (S) | TGRC | S | S | S | S | Nd |
| 7 | LA4285 (CLN2264F) | *S. lycopersicum* | Ph3 | TGRC | S | S | R | R | Nd |
| 8 | LA4286 (CLN2264G) | *S. lycopersicum* | Ph3 | TGRC | S | S | R | R | Nd |
| 9 | LA1269 (L3708) | *S. pimpinellifolium* | Ph3 | Black et al. (1996) | S | S | R | R | Nd |
| 10 | IT229371 | *S. lycopersicum* | ND | RDA | S | S | R | R | R |
| 11 | IT229370 | *S. lycopersicum* | ND | RDA | S | S | R | R | S |
| 12 | IT236514 | *S. lycopersicum* | ND | RDA | S | S | S | S | S |
| 13 | IT236513 | *S. lycopersicum* | ND | RDA | S | S | S | S | R |
| 14 | SKT-1 | *S. lycopersicum* | TYLCV (R), Ve (R) | | S | S | S | S | R |
| 15 | TKI-1 | *S. lycopersicum* | TYLCV (R), Ve (R) | | H | H | S | S | R |
| 16 | SGT-1 | *S. lycopersicum* | TYLCV (R), Ve (R) | | H | H | S | S | R |
| 17 | SGT-2 | *S. lycopersicum* | LB (R) | | S | S | R | R | S |
| 18 | STG-3 | *S. lycopersicum* | TYLCV (R), Ve (R) | | H | H | S | S | H |
| 19 | MST-1 | *S. lycopersicum* | TYLCV (R), LB (R), Ve (R) | | H | H | R | R | H |
| 20 | MST-2 | *S. lycopersicum* | Ve (R) | | S | S | S | S | R |
| 21 | MST-3 | *S. lycopersicum* | LB (R) | | S | S | R | R | S |
| 22 | AS-1 | *S. lycopersicum* | TYLCV (R), Ve (R) | | H | H | S | S | H |
| 23 | AS-2 | *S. lycopersicum* | TYLCV (R), Ve (R) | | H | H | H | H | R |
| 24 | PN-1 | *S. lycopersicum* | TYLCV (R), Ve (R) | | H | H | S | S | R |
| 25 | NW-1 | *S. lycopersicum* | TYLCV (R) | | H | H | H | S | S |
| 26 | NW-2 | *S. lycopersicum* | Ve (R) | | S | S | S | S | R |
| 27 | NW-3 | *S. lycopersicum* | TYLCV (R), Ve (R) | | H | H | S | S | R |

*TYLCV* Tomato yellow leaf curl virus, *LB* late blight,*Ve* verticillium wilt, *R* resistance, *S* susceptible, *Nd* not determined, *SKT* Sakata, *MST* Monsanto, *SGT* Syngenta, *NW* Nongwoo Bio, *TKI* Takii, *AS* Asiaseed, *PN* Punong

Although the markers generated for *Ty1/3* resistance were gene-based markers, these markers were tested in 27 tomato genotypes in order to verify their applicability (Fig. 1c). In the 27 genotypes tested, LA1932, LA1938, LA1969, LA2779, and LA3473 served as positive controls and LA3474 and LA1269 served as negative controls for *Ty1/3* resistance. Two accessions from TGRC, four accessions from the Rural Development Administration (RDA, Korea) and 14 commercial cultivars were genotyped using the Ty1-*Ssp*I and Ty1-*Bgl*II markers. Both markers clearly discriminated between the*Ty1/3* and *ty1/3* alleles (Table 3). The two markers revealed that 9 of the 14 commercial cultivars tested were heterozygous.

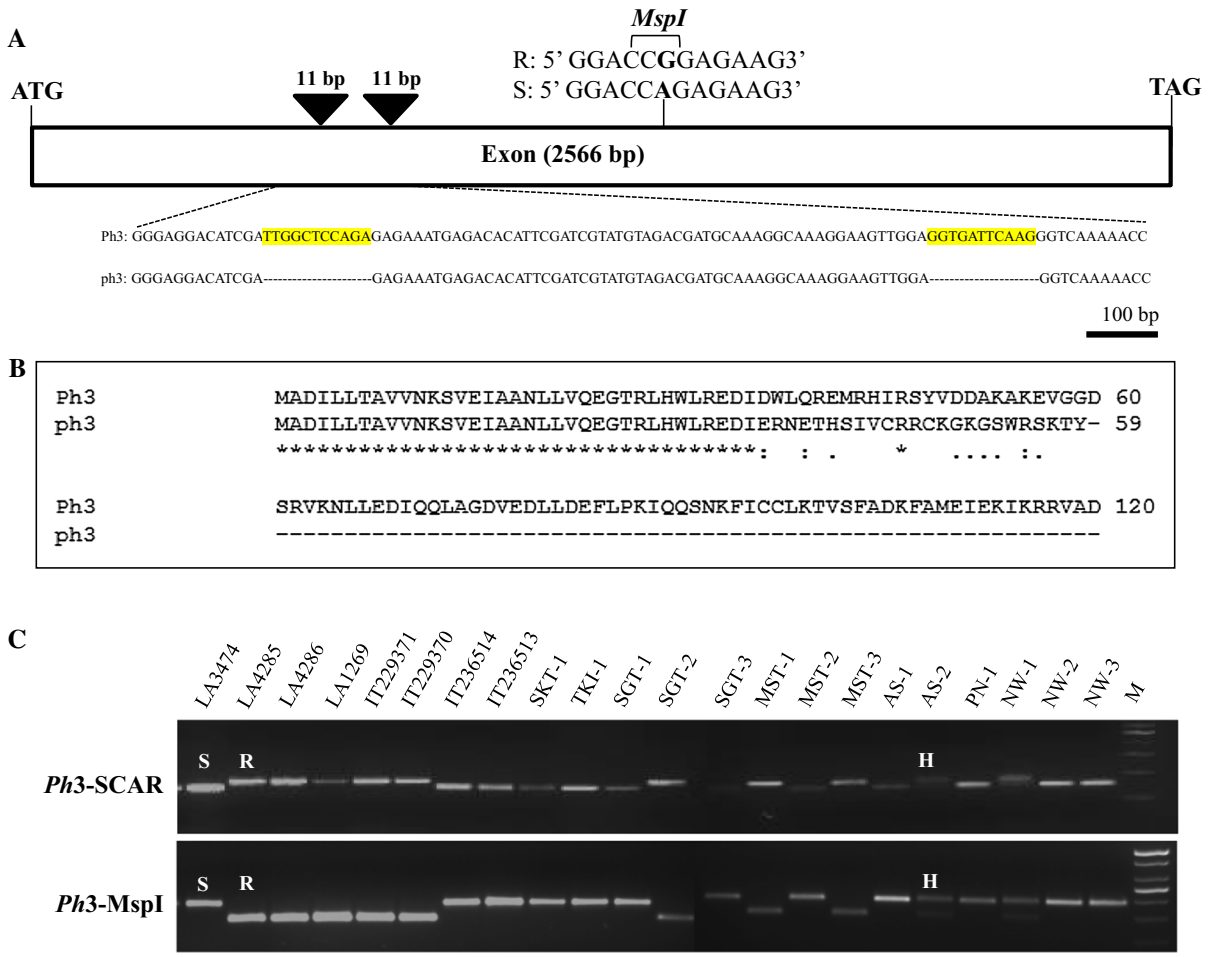


**Fig. 2** Representation of SNP information for *Ph3* gene-based marker development. **a** Schematic representation of the *Ph3* gene structure. The positions of two 11 bp deletions and SNP information (shown in *bold*) are indicated. The *Msp*I endonuclease site used for CAPS marker development is indicated. **b** The aminoacid sequence alignment of the Ph3 and ph3 proteins via Clustal W. **c** Genotyping of the cultivated lines using the gene-based markers. *SKT* Sakata, *MST* Monsanto, *SGT* Syngenta, *NW* Nongwoo, *TKI* Takii, *AS* Asiaseed, *PN* Punong. *Scale bar* indicates 100 bp

Because of the dominant inheritance of *Ty1/3* resistance, $F_1$ cultivars heterozygous at the *Ty1/3* locus are expected to be TYLCV resistant. Our results imply that *Ty1/3* resistance is only maintained in one of the maternal or paternal sources of the commercial F1 cultivars.The genotyping results obtained using the Ty1-*Ssp*I and Ty1-*Bgl*II markers matched perfectly with the phenotypic expectations provided by the seed companies, except SKT1 (Table 3). In the case of SKT1, the phenotype description indicates that it is TYLCV resistant; however, the genotype of the *Ty1/3* locus was susceptible based on the markers developed in this study. Using a *Ty2*-linked marker, T0302 (Ji et al. 2009a; Yang et al. 2014), we were able to confirm that the *Ty1/3* locus contained the *Ty2* gene, another resistance gene against TYLCV located on chromosome 11 (data not shown).

## Gene-based PCR markers for *Ph3* resistance

The *Ph3* gene was recently isolated, and its complete nucleotide sequence was determined by Zhang et al. (accession no. KJ563933). Two allele-specific markers for *Ph3* resistance were developed based on a G/A sequence polymorphism and two 11 bp deletions in exon (Fig. 2a). Figure 2b shows that two 11 bp deletions separated by 56 bp generate a premature stop codon in the protein encoded by the *ph3*

(susceptible) allele, strongly suggesting that the truncated protein generated by the premature stop codon is crucial for the loss of *Ph3* resistance. The Ph3-SCAR marker is designed based on those two 11 bp deletions, which appear to be directly responsible for the loss of resistance.A PCR assay was developed using a single pair of primers (F: 5′CTACTCGTGCAAGAA GGTAC3′, R: 5′TCCACATCACCTGCCAGTTG3′) to amplify two bands of contrasting size that can be resolved in a 2.5 % agarose gel: a 176 bp fragment specific for the *Ph3* allele and a 154 bp fragment specific for the *ph3* allele. That single PCR-based marker provided a co-dominant marker that allowed for the rapid, economical, and reliable tracking of the *Ph3* and *ph3* alleles (Fig. 2c). Ph3-*Msp*I, another PCR-based, allele-specific, co-dominant CAPS marker, was derived from a G/A SNP; *Msp*I (5′CCGG3′) cleaved the resistance allele but not the susceptible allele (Fig. 2). Although the G/A polymorphism does not cause anamino acid change, it appears to co-segregate with the two 11 bp deletions, allowing the CAPS marker to be applied in MAS for *Ph3* resistance.

In order to verify the applicability of the Ph3-SCAR and Ph3-*Msp*I markers, the markers were tested with 22 tomato genotypes, including the *Ph3* resistance sources LA1269, LA4285, and LA4286 (Fig. 2c). Both markers were clearly effective in discriminating the *Ph3* and *ph3* alleles (Table 3). Among the 14 commercial cultivars tested, 3 cultivars were homozygous for the *Ph3* allele, 2 cultivars were heterozygous, and 9 cultivars were homozygous for the *ph3* allele. Considering the dominant inheritance of *Ph3* resistance, heterozygous F1 cultivars are expected to be *Ph3* resistant. The genotyping results obtained using the Ph3-SCAR and Ph3-*Msp*I markers matched with the phenotypic expectations provided by the seed companies, although the phenotype for LB resistance was not indicated for two cultivars, AS-2 and NW1. The markers suggested that these cultivars are LB resistant. We also tested three previously reported, closely linked markers for *Ph3* resistance, TG328, R2M1S, and M67-3(Zhang et al. 2013). The three markers perfectly matched the genotyping results obtained from the Ph3-SCAR and Ph3-*Msp*I markers (data not shown).

## A CAPS marker for *Ve1* resistance

There are several SNPs in the *Ve* locus (Kawchuk et al. 2001; Fradin et al. 2009). In order to design a functional marker based on aSNP that determines the gain/loss of *Ve* resistance, the sequence information of the alleles at the *Ve* locus was explored. A single-bp deletion (TCA/T-A) at nucleotide position 1220 resulting in a premature stop codon was selected and used to generate a CAPS marker for *Ve1* resistance (Fig. 3a, b). A primer set (F: 5′CGAACTTGACTAC ATTGACC3′, R: 5′CAGTCTTGAAAGGTTGCTC A3′; Table 2) was designed for PCR amplification. The *Xba*I (5′TCTAGA3′) endonuclease digested the susceptible allele but not the resistance allele. Based on the sequence alignment, the resistance genotype generated DNA fragments with sizes of 410 and 332 bp, while the susceptible genotype generated DNA fragments with sizes of 410, 310, and 22 bp (Fig. 3c).

The Ve1-*Xba*I marker was applied to17 tomato genotypes.We did not include the original source of *Ve1* resistance, but we included commercial cultivars with resistance to verticillium wilt disease. Because *Ve1* resistance has been deployed in commercial breeding programs for many years, there are many commercial cultivars available. Among the 14 commercial cultivars tested, 8 cultivars were homozygous for the *Ve1* allele, 3 cultivars were heterozygous, and 3cultivars were homozygous for the *ve1* allele. Considering the dominant inheritance of *Ve1* resistance, heterozygous F1 cultivars are expected to be *Ve1* resistant. The genotyping results obtained using the Ve1-*Xba*I marker matched perfectly with the phenotypic expectations provided by the seed companies.

## Conversion of the PCR markers to HRM markers

To convert the SNP-based PCR markers to SNP markers, primer sets for *Ty1*, *Ve1*, and *Ph3* were designed based on the respective DNA polymorphisms and sizes of the amplicons. HRM was deployed for SNP detection. As shown in Fig. 4, two SNPs (A/− in *Ty1* and G/A in *Ph3*) were detectable by HRM analysis, and the genotypes with different SNP alleles were differentiated by distinct melting profiles. The Ty1-*Ssp*I-HJ primer set was designed to include the SNP (A/−) and the expected size of the PCR product was 118 bp. The amplicons generated by theTy1-*Ssp*I-HJ primer set clearly distinguished the two genotypes at temperatures between ∼75 and ∼80 °C. The green and red curves shown in Fig. 4b

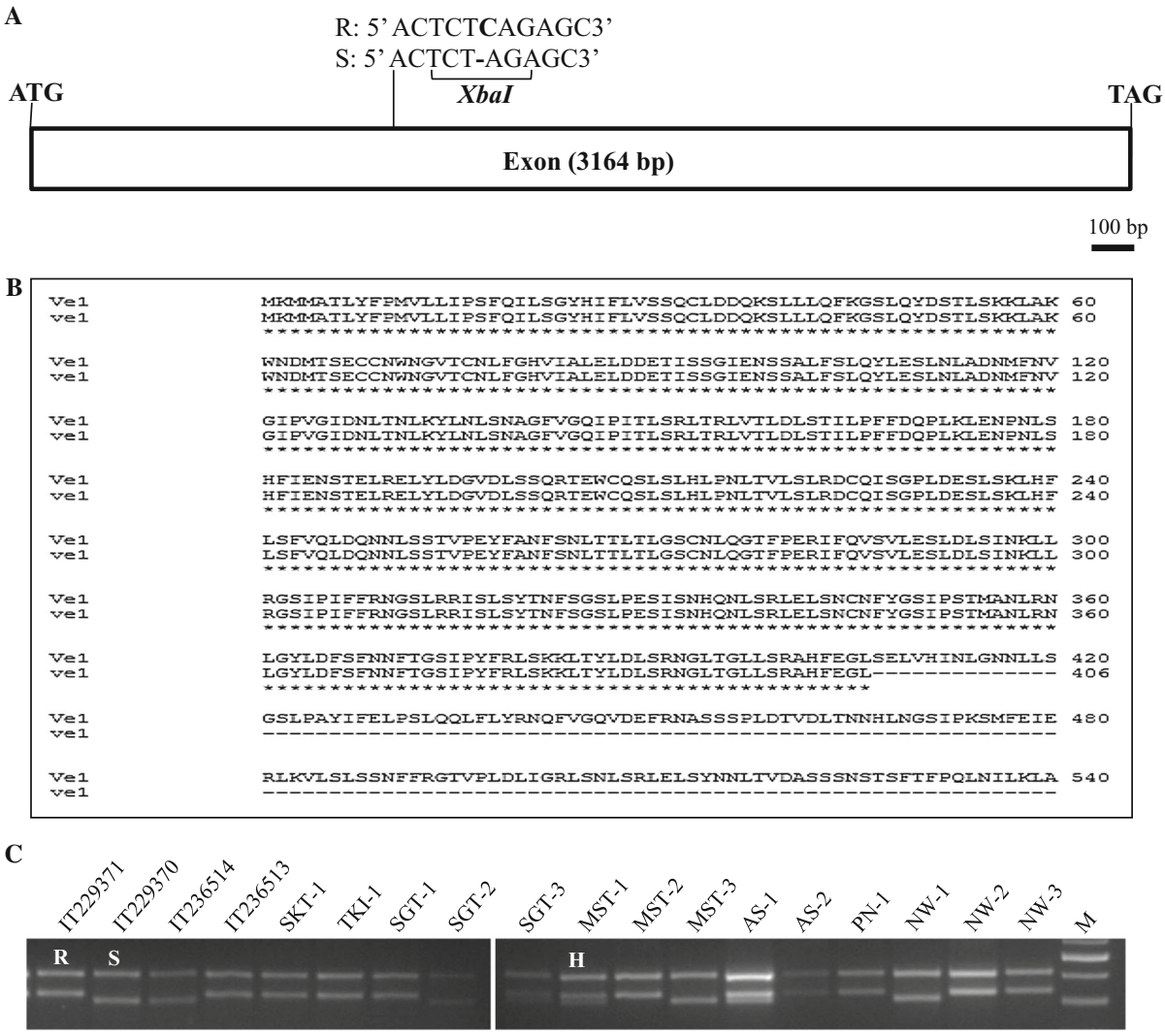


**Fig. 3** Representation of SNP information for *Ve1* gene-based marker development. **a** Schematic representation of the Ve1 gene structure. The position of SNP (shown in *bold*) and restriction endonuclease site *Xba*I used for CAPS marker development are indicated. **b** The amino acid sequence alignment of the Ve1 and ve1 proteins via Clustal W. **c** Genotyping of the cultivated lines using gene-based markers. *SKT* Sakata, *MST* Monsanto, *SGT* Syngenta, *NW* Nongwoo, *TKI* Takii, *AS* Asiaseed, *PN* Punong. *Scale bar* indicates 100 bp

indicate the susceptible and resistant tomato lines, respectively. In the case of the Ph3-*Msp*I-HJ primer set, the G/A variation was clearly distinguished (Fig. 4c), and that SNP was sufficient for significant HRM differentiation between the two genotypes. The 107 bp amplicons distinguished the two genotypes at temperatures between ~78 and ~81 °C. The green and red curves in Fig. 4d indicate the susceptible and resistant tomato lines, respectively. However, when both the Ty1-*Ssp*I-HJ and Ph3-*Msp*I-HJ HRM markers were test with heterozygous genotypes, we found that the *Ty1*/*ty1* genotype was indistinguishable from the*Ty1*/*Ty1* genotype and the *Ph3*/*ph3* genotype was in distinguishable with *Ph3*/*Ph3* genotype (Supplemental Fig. 1). Considering the dominant inheritance of *Ty1* and *Ph3* resistance, the HRM markers developed in this study were sufficient for selecting resistant individuals. However, these markers could not discriminate heterozygous resistance genotypes from homozygous resistance genotypes.

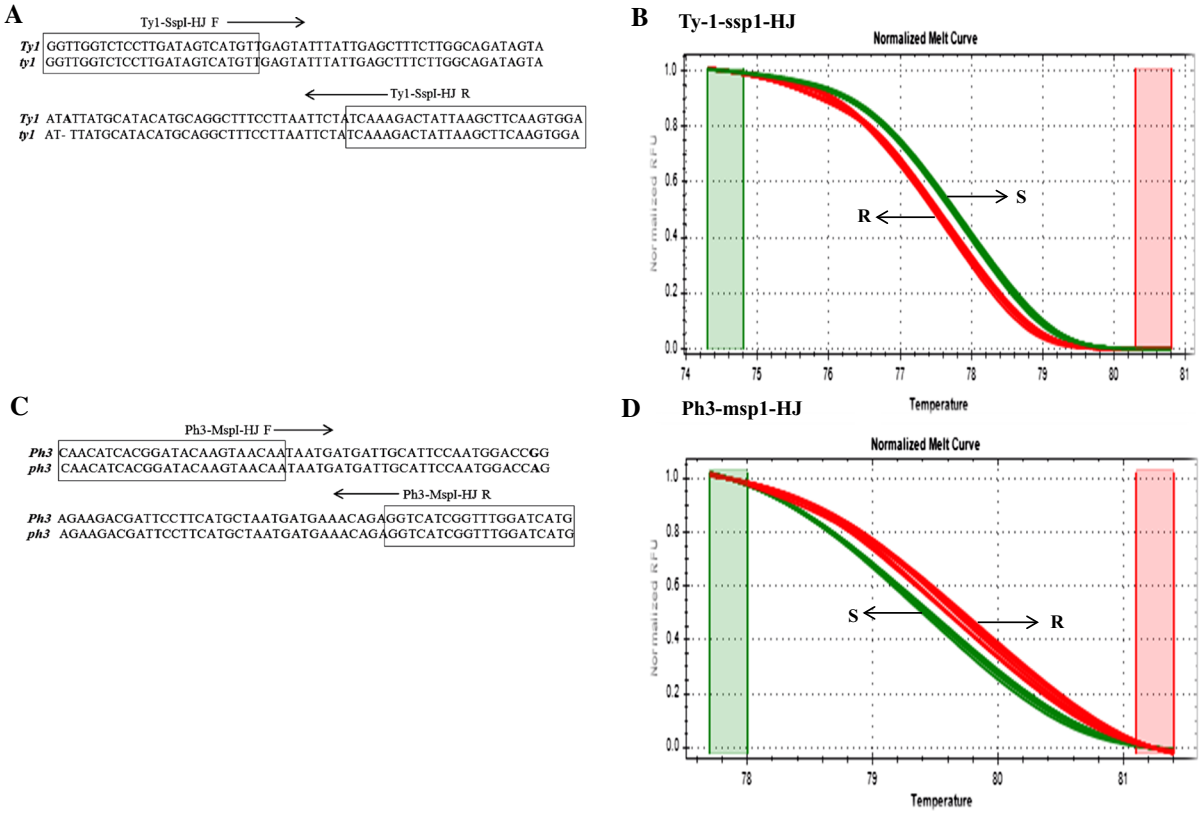


**Fig. 4** HRM analysis of Ty1-*Ssp*I-HJ and Ph3-*Msp*I-HJ. **a** The priming sites and the SNP existing between the *Ty1* and *ty1* genotypes targeted for HRM analysis are indicated. **b** Melting curve analysis of Ty1-*Ssp*I-HJ. Two different melting-curve types were identified (*R* homozygous resistant genotype; *S* homozygous susceptible genotype). **c** The priming sites and the SNP existing between the Ph3 and ph3 genotypes targeted for HRM analysis are indicated. **d** Melting curve analysis of Ph3-*Msp*I-HJ. Two different melting curve types were identified (*R* homozygous resistant genotype; *S* homozygous susceptible genotype)

## Discussion

### Exploring the DNA polymorphisms that are suitable for generating functional molecular markers for *Ty1/3*, *Ph3*, and *Ve1*

Although the genes responsible for *Ty1/3* and *Ph3* resistance were previously characterized, the complete open reading frame sequence information for the resistant and susceptible alleles was not easily accessible (Verlaan et al. 2013). To explore the DNA polymorphisms required to generate the molecular markers, we cloned each allele from the resistant and susceptible genotypes and conducted sequence analysis. First, we excluded DNA polymorphisms located in the intron regions. We then focused only on the nonsynonymous substitutions or nucleotide changes resulting in premature stop codons, which allowed us to generate functional molecular markers.

The DFDGD-class RNA-dependent RNA polymerase is encoded at the *Ty1/3* locus (Verlaan et al. 2013). Recently, the *Ty1* resistance gene was reported to affect the DNA replication and methylation of TYLCV (Butterbach et al. 2014). We focused on a GTG/ACA changein exon 2 for the development of the Ty1-*Bgl*II marker. The three-nucleotide polymorphism (GTG in *Ty1* and ACA in *ty1*) changes a threonine in the *Ty1* allele to a valine in the *ty1* allele, which may be crucial for the gain/loss of resistance against TYLCV. It is plausible that a conformational change caused by the substitution between threonine and valine is responsible for resistance and susceptibility, because threonine contains an aliphatic hydroxyl group and is therefore hydrophilic and more

reactive than valine, which is hydrophobic.To test this hypothesis, it would be necessary to evaluate the direct effect of the GTG/ACA change in exon 2 on the gain/loss of resistance.

*Ph3*, a tomato resistance gene against *P. infestans*, encodes a coiled-coil NBS–LRR protein (Zhang et al. 2014). A large proportion (more than 60) of the *P. infestans* resistance genes in potato are also NBS–LRR proteins (Rodewald and Trognitz 2013). The Ph3-SCAR marker was developed based on two 11 bp deletions separated by 56 bp that generate a premature stop codon in the *ph3* susceptible genotype. *Ve1* resistance is conferred by the extracellular leucine-rich repeat receptor-like protein class of disease resistance proteins (Kawchuk et al. 2001), which appear to be critical for switching on effector-triggered immunity in thehost plant. The Ve1-*Xba*I marker was generated based on a single-bp deletion, TCA/T-A, resulting in a premature stop codon. In the case of the*Ph3* and *Ve1* resistance loci, DNA polymorphisms resulting in truncated proteins in the susceptible genotypes are predicted to be the critical changes responsible for the loss of resistance. Therefore, the Ph3-SCAR and Ve1-*Xba*I molecular markers can be considered functional markers for the *Ph3* and *Ve1* resistance loci, respectively.

## Application of co-dominant and PCR-based markers for allelic selection of the *Ty1/3*, *Ph3*, and *Ve1* resistance loci

For MAS in breeding programs, molecular markers should allow for the analysis of a large number of samples with comprehensive techniques while incurring minimal cost (Lande and Thompson 1990). PCR-based and co-dominant DNA markers are the most useful molecular markers in practical breeding programs in terms of reliability and cost-efficiency, maximizing the advantages of applying molecular markers (Thomson 2014). The CAPS and SCAR markers developed in this study satisfy those requirements. In addition, each marker was derived from a DNA polymorphism in the gene responsible for the specific disease resistance, so each marker targets a polymorphism directly associated with the phenotype of interest. These gene-based markers, which are potentially functional markers, display great advantages over neutral markers that are merely closely linked with the gene of interest. The applicability of gene-based markers, in addition to the accuracy of the selection, can also be broadened due to the nature of gene-based markers (Lagudah et al. 2009). The use of linked markers in MAS is often restricted to progenies where the DNA polymorphisms exist in the parental lines, which narrows the applicability of the markers (Thiel et al. 2004; Komori and Nitta 2005). Gene-based markers, on the other hand, can be applied to diverse plant materials in much broader contexts (Thomson 2014). However, sources containing mutations other than those utilized in this study may exist in nature. Hence, the molecular markers developed in this study may not be suitable for characterizing novel resistance sources. To characterize novel resistance sources, the full sequence information of the targeted resistance gene should be re-investigated.

The unique markers developed in this study for the allelic selection of the *Ty1/3*, *Ph3*, and *Ve1* resistance loci will be powerful tools for tomato breeding programs. We consider these markers to be excellent tools for pyramiding resistance genes in tomato. There are other resistance genes against TYLCV, LB, and verticillium wilt disease that have already been incorporated in tomato breeding programs, including *Ty2*, *Ph1*, and *Ph2*. It is often difficult to monitor the presence of individual resistance genes and to pyramid the genes in breeding lines using traditional phenotypic screening because the action of one resistance gene may mask the actions of others (Hittalmani et al. 2000). The markers generated in this study can facilitate the selection of *Ty1/3*, *Ph3*, and *Ve1* based on genetic information at the DNA level. In addition, molecular markers make it possible to substantially reduce the breeding period. Therefore, molecular markers for *Ty1/3*, *Ph3*, and *Ve1* are very useful tools for introducing and maintaining resistance alleles in tomato breeding programs. The markers developed in this study would beequally useful for selecting populations made by crossing plants that are either resistant or susceptible to TYLCV, LB, and verticillium wilt disease. By using our gene-based markers, such laborious crossing and progeny testing to genotype the *Ty1/3*, *Ph3*, and *Ve1* loci could be avoided.

## Conversion of HRM markers allows for high-throughput determination of *Ty1/3* and *Ph3* resistance

HRM analysis provides a high-throughput SNP detection system with cost-efficiency, promptness, and

convenience. HRM can be an alternative toa CAPS marker for large-scale MAS in a practical breeding program. To obtain reproducible results with HRM, however, several criteria should be satisfied. In order to maximize the performance of the HRM assay, PCR conditions must be optimized to amplify only a clean single fragment without primer dimers and the amplicon should not exceed 300 bp in length (White and Potts 2006; Reed and Wittwer 2004; White and Potts 2006; Montgomery et al. 2007). In this study, high-quality template DNA was essential because the partial degradation or uneven concentration of DNA can result in inconclusive outcomes in HRM assays (data not shown). We designed a total of 10 primers for HRM analysis to develop SNP markers for *Ty1*, *Ph3*, and *Ve1*. The HRM primer sets showed clean separation between the resistance and susceptible alleles for the determination of *Ty1* and *Ph3* resistance. In the case of *Ve1*, the HRM result did not separate the resistance and susceptible alleles clearly (data not shown). Among the SNPs detected between the *Ve1* and *ve1* alleles, the SNP used for the CAPS marker development was the only SNP that could be considered a functional SNP. Some tomato lines were not included in the resistance cluster or in the susceptible cluster possibly due to differences in the template concentrations in the PCR.

This study demonstrates the successful application of the HRM technique for the development of markers for the *Ty1* and *Ph3* resistance genes. The markers can be used to screen large populations. All the markers presented in this study are suitable for MAS, and end users can choose which marker system fits well with their MAS protocols.

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