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Molecular Markers for *Tm*-2 Alleles of Tomato Mosaic Virus Resistance in Tomato

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ABSTRACT

Tomato mosaic virus (*ToMV*) *is one of the most infectious virus diseases in tomato* (*Solanum lycopersicum L*). *The practical and effective method of controlling this disease is through genetic control by using major resistance genes. So far, three genes Tm*-1, *Tm*-2 *and Tm*-2^2 *conferring resistance to ToMV have been reported and utilized in tomato cultivar development. Marker assisted selection* (*MAS*) *has become very important and useful tool in selection of ToMV resistant tomato lines or hybrids. The objective of this research was to identify allele-specific PCR-based, cleaved amplified polymorphic sequence* (*CAPS*), *and allele-derived single nucleotide polymorphism* (*SNP*) *markers for Tm*-2 *loci. Four allele-specific PCR-based markers were identified: one for Tm*-2, *one for Tm*-2^2, *and two for the susceptible allele tm*-2. *Three allele-derived CAPS markers were identified, which can identify and distinguish three alleles, tm*-2, *Tm*-2 *and Tm*-2^2 *in tomato germplasm. Three SNP markers were developed specific for Tm*-2 *locus. These markers will provide breeders with a tool in selection of Tm*-2 *and Tm*-2^2 *resistance genes in tomato breeding program.*

***Keywords*:** *Cleaved Amplified Polymorphic Sequence*, *Marker-Assisted Selection*, *Single Nucleotide Polymorphism*, *Solanum Lycopersicum*, *Tomato*, *Tomato Mosaic Virus* (ToMV), *Tm*-2

1. Introduction

Tomato mosaic virus (ToMV) is one of the most infectious virus diseases in tomato (*Solanum lycopersicum* L. formerly, *Lycopersicon esculentum* Mill). The practical and effective method of controlling this disease is through introducing major resistance gene(s). So far, three genes, *Tm*-1, *Tm*-2 and *Tm*-2^2 of ToMV resistance have been reported and used in tomato cultivar development [1-5]. The *Tm*-1 gene was introgressed into *S. lycopersicum* from the wild tomato species *S. habrochaites* and mapped to chromosome 5 and it conferred resistance to ToMV strains 0 and 2 [1,4,6,7]. The *Tm*-1 gene has been cloned and the sequences were stored in GenBank with five accessions DJ344478 to DJ344481, and DJ344505. The other two genes *Tm*-2 and *Tm*-2^2 were introgressed from *S. peruvianum* and were located closely to the centromere of chromosome 9 and are considered to be allelic and *Tm*-2 conferred resistance to ToMV strains 0 and 1, whereas allele *Tm*-2^2 conferred resistance to ToMV strains 0, 1 and 2 [2,3,5,8-10].

Molecular markers have been widely used in genetic mapping and marker-assisted selection (MAS) for disease resistance in tomato [6]. For *Tm*-2 genes of ToMV resistance, Ohmori *et al.* [11] reported 13 random amplified polymorphic DNA (RAPD) markers linked to the *Tm*-2 locus. From the 13 RAPD markers, Motoyoshi *et al.* [11] found two markers, OPE16 (900) and OPN31 (1000), nearest to the *Tm*-2 locus. Six out of the 13 RAPD markers distributed within 0.7 cM on chromosome 9 were cloned and sequenced to be converted into sequence characterized amplified region (SCAR) markers [11]. Sobir *et al.* [12] and Dax *et al.* [13] identified co-dominant SCAR markers tightly linked to *Tm*-2^2. Three alleles, *Tm*-2, *Tm*-2^2, and *tm*-2 at *Tm*-2 locus have been cloned and sequenced (GenBank Accessions: AF536199, AF536200, and AF536201) and three cleaved amplified polymorphic sequences (CAPS) markers were developed to distinguish the three alleles in tomato [14]. Based on the two CAPS markers reported by Lanfemeijer *et al.*

[14], Arens *et al.* [15] developed a co-dominant assay combining tetra primers designed from the SNP regions of the two CAPS markers using a method called ARMS-PCR SNP and this Tetra-primer ARMS-PCR assays can amplify PCR products with different DNA fragment sizes from tomato lines carrying *Tm*-2 or *Tm*-2^2, and susceptible allele *tm*-2.

Single nucleotide polymorphism (SNP) is becoming to be the most useful as molecular marker in genome mapping, association studies, diversity analysis, and tagging genes for economically important traits in crop plants because of their abundance and automated highthroughput genotyping [16-18]. SNPs have been discovered and verified in tomato [19-22] and successfully used in selecting resistance to bacterial speck and bacterial spot in tomato [22,23]. The objective of this research was to identify *Tm*-2 allele-specific PCR-based markers, CAPS markers, and SNP markers for MAS in tomato breeding.

2. Materials and Methods

2.1. Plant Materials

Twenty-three tomato genotypes including released or commercial cultivars and accessions were used in this research (**Table 1**). Seeds of 15 tomato accessions (LA series) were obtained from the C.M. Rick Tomato Genetics Resource Center, Dept. of Plant Sciences, University of California, Davis, CA 95616 (http://tgrc.ucdavis.edu), six cultivars were purchased from commercial sources, and two lines NY07-461 and NY07-464 were obtained from Cornell University; refined selections from a cross between "Brandywine" and "Rose de Berne" (received as NY07-461 (Brandyrose #1) and NY07-464 (Brandyrose #2)). Among the 23 genotypes, three cultivars, Royal Red (LA2088), CLN2264F (LA4285), and LA3433 are homozygous for *Tm*-2 (*Tm*-2/*Tm*-2); Mogeor (LA3471), Sophya, and VFNT Cherry (LA1221) are homozygous for *Tm*-2^2 (*Tm*-2^2/*Tm*-2^2); Swt Cluster, Bush Celebrity, and Golden Girl are heterozygous for *Tm*-2^2 (*Tm*-2^2/*tm*-2); and the other 14 genotypes contained the susceptible allele *tm*-2 (**Table 1**).

2.2. DNA Extraction, PCR Amplification and DNA Sequencing

Genomic DNA was extracted from fresh leaves of greenhouse-grown plants using the CTAB (Cetyltrimethylammonium Bromide) method [24,25]. The gene- specific primers for *Tm*-2, *Tm*-2^2, or tm-2 at *Tm*-2 locus were designed from the sequences of the GenBank accessions AF536199, AF536200, and AF536201 using the primer design tool—Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). AF536199 is the accession in GenBank that contains the sequence of the *tm*-2 susceptible allele; AF536200 is the sequence *Tm*-2 resistance allele; and AF536201 is the sequence of *Tm*-2^2 resistance allele. The size of the AF536199 sequence of the allele *tm*-2 is 2820 bp; AF536200 is 2819 bp; and AF536201 is 9837 bp [3]. Primers Tm2RS-f2, Tm2RS-r2, Tm2RS-f3, and Tm2RS-r3 were designed for the three sequences of AF536199, AF536200, and AF536201; Tm2S-f1, Tm2S-r1, Tm2S-f2, and Tm2S-r2 for AF536199 only; Tm2R-f1c and Tm2R-r4 for both AF536200 and AF536201; Tm2R-r3 for AF536200 only; and Tm2aR-r3 for AF536201 only (**Table 2**).

PCR amplification was performed in an eppendorf thermal cycler (Eppendorf, Westbury, NY) following standard PCR procedures with minor modifications. Briefly, each 50 µl PCR reaction mixture consisted of 29.8 µl sterilized ddH_2O, 10 µl 5x Mango *Taq* reaction buffer (Bioline, London, UK), 3 µl $MgCl_2$ (25 *mM*), 1.5 µl dNTP (2.5 mM each), 1.5 µl each primer (5 µM), 0.2 µl Mango *Taq* DNA polymerase (5 U/µl) (Bioline, London, UK), and 2.5 µl template DNA (30 ng/µl). For the primer pairs, Tm2RS-f2/r2 and Tm2RS-f3/r3, PCR procedure consisted of an initial denaturation step of 94˚C for 4 min followed by 36 to 38 cycles of 15 to 30 second at 94˚C for denaturation, 15 to 30 s at 51 to 56˚C for annealing, and 40 to 75 s at 72˚C for extension depending on primer pairs with a final extension step at 72˚C for 5 min (**Table 3**). The PCR fragments were separated by gel electrophoresis with 1.5% agarose gel in 0.5 X TAE buffer, stained with ethidium bromide, and visualized with UV light.

The PCR fragments were sequenced in the Purdue Genomics Core Facility, Purdue University, West Lafayette, IN 47907. Before sequencing of PCR products, PCR products were purified using QIA quick PCR Purification Kit (Qiagen Inc, Valencia, CA) following the manufacturer's instructions of the protocol. The sequences amplified from the two primer pairs Tm2RS-f2/-r2 and Tm2RS-f3/-r3 in tomato were submitted to GenBank using a DNA sequence submission and update tool—Sequin (http://www.ncbi.nlm.nih.gov/Sequin/).

2.3. SNP Identification and Genotyping

SNP discovery was postulated from available *Tm*-2 sequences of AF536199, AF536200, and AF536201. The multiple sequence alignment showed that 68 SNPs and one InDel were observed among the three sequences. One of the approaches to SNP validation is through re-sequencing of the same gene among various genotypes. Two *Tm*-2 gene-specific primer pairs Tm-2RS-f2/r2 and Tm-2RS-f3/r3 were designed from the sequences of AF536199, AF536200, and AF536201 using Primer-BLAST (**Table 2**) and used to run PCR among tomato

Table 1. Allele-specific PCR-based, allele-derived CAPS, and allele-derived SNP markers for *Tm*-2 loci in 23 tomato genotypes.

Cultivar/ accession	Genotype at *Tm*-2 locus	Allele-specific PCR[a]					Allele-derived CAPS				SNP genotyping by Sequenom[b]		
		Tm2S-f1/ Tn2S-r1	Tm2S-f2/ Tm2S-r2	Tm2R-f1c/ Tm2R-r3	Tm2R-f1c/ Tm2aR-r3	Tm2R-fic/Tm2-r4	Tm2rs-f3/r3	PshAI	HpaI	BsiHKAI	Tm2-snp1	Tm2-snp46	Tm2-snp53
NY07-461	*tm*-2/*tm*-2	+	+	−	−	−	703 bp	538 bp + 165 bp	703 bp	703 bp	T T	C C	G G
NY07-464	*tm*-2/*tm*-2	+	+	−	−	−	703 bp	538 bp + 165 bp	703 bp	703 bp	T T	C C	G G
Riesentraube	*tm*-2/*tm*-2	+	+	−	−	−	703 bp	538 bp + 165 bp	703 bp	703 bp	T T	C C	G G
LA0656	*tm*-2/*tm*-2	+	+	−	−	−	703 bp	538 bp + 165 bp	703 bp	703 bp	T T	C C	G G
LA1792	*tm*-2/*tm*-2	+	+	−	−	−	703 bp	538 bp + 165 bp	703 bp	703 bp	T T	C C	G G
LA1802	*tm*-2/*tm*-2	+	+	−	−	−	703 bp	538 bp + 165 bp	703 bp	703 bp	T T	C C	G G
LA3386	*tm*-2/*tm*-2	+	+	−	−	−	703 bp	538 bp + 165 bp	703 bp	703 bp	T T	C C	G G
Anahu (LA0655)	*tm*-2/*tm*-2	+	+	−	−	−	703 bp	538 bp + 165 bp	703 bp	703 bp	T T	C C	G G
Motelle (LA2823)	*tm*-2/*tm*-2	+	+	−	−	−	703 bp	538 bp + 165 bp	703 bp	703 bp	T T	C C	G G
Mobox (LA2821)	*tm*-2/*tm*-2	+	+	−	−	−	703 bp	538 bp + 165 bp	703 bp	703 bp	T T	C C	G G
Ontario 7710 (LA2396)	*tm*-2/*tm*-2	+	+	−	−	−	703 bp	538 bp + 165 bp	703 bp	703 bp	T T	C C	G G
Peto 95-43 (LA3528)	*tm*-2/*tm*-2	+	+	−	−	−	703 bp	538 bp + 165 bp	703 bp	703 bp	T T	C C	G G
Rehovot 13 (LA3129)	*tm*-2/*tm*-2	+	+	−	−	−	703 bp	538 bp + 165 bp	703 bp	703 bp	T T	C C	G G
UC-204C (LA3130)	*tm*-2/*tm*-2	+	+	−	−	−	703 bp	538 bp + 165 bp	703 bp	703 bp	T T	C C	G G
Royal Red Cherry (LA2088)	*Tm*-2/*Tm*-2	−	−	+	−	+	703 bp	703 bp	458 bp + 245 bp	358 bp + 353 bp	C C	T T	A A
CLN2264F (LA4285)	*Tm*-2/*Tm*-2	−	−	+	−	+	703 bp	703 bp	458 bp + 245 bp	358 bp + 353 bp	C C	T T	A A
LA3433	*Tm*-2/*Tm*-2	−	−	+	−	+	703 bp	703 bp	458 bp + 245 bp	358 bp + 353 bp	C C	T T	A A
Mogeor (LA3471)	$Tm\text{-}2^2$/ $Tm\text{-}2^2$	−	−	−	+	+	703 bp	703 bp	458 bp + 245 bp	703 bp	C C	T T	A G
Sophya	$Tm\text{-}2^2$/ $Tm\text{-}2^2$	−	−	−	+	+	703 bp	703 bp	458 bp + 245 bp	703 bp	C C	T T	A A
VFNT Cherry (LA1221)	$Tm\text{-}2^2$/ $Tm\text{-}2^2$	−	−	−	+	+	703 bp	703 bp	458 bp + 245 bp	703 bp	C C	T T	A A
Swt Cluster	$Tm\text{-}2^2$/*tm*-2	+	+	−	+	+	703 bp	703bp + 538 bp + 165 bp	703 bp + 458 bp + 245 bp	703 bp	C T	C T	A G
Bush Celebrity	$Tm\text{-}2^2$/*tm*-2	+	+	−	+	+	703 bp	703 bp + 538 bp + 165 bp	703 bp + 458 bp + 245 bp	703 bp	C T	C T	A G
Golden Girl	$Tm\text{-}2^2$/*tm*-2	+	+	−	+	+	703 bp	703 bp + 538 bp + 165 bp	703 bp + 458 bp + 245 bp	703 bp	C T	C T	A G

[a] '+' signifies the PCR fragment (band) present and '−' signifies the PCR fragment (band) absent; [b] The SNP type such as [A T] signifies a heterogeneous SNP type and [A A] signifies a homogenous SNP type.

Table 2. Allele-derived PCR primers and their locations in the sequences of *Tm*-2 loci.

Primer [a] name	Sequence (5' -> 3')	Tm (˚C)	AF536199 (tm-2)	AF536200 (*Tm*-2)	AF536201 (*Tm*-2^2)
Tm2RS-f2	TGCCAAACAAATTGGACTGA	60.1	869 - 888 [b]	868 - 887	6929 - 6948
Tm2RS-r2	AAGCACGAATTTCATGGTCC	60	1458 - 1439	1457 - 1438	7518 - 7499
Tm2RS-f3	TGGAGGGGAATATTTGTGGA	60.1	1966 - 1985	1965 - 1984	8026 - 8045
Tm2RS-r3	ACTTCAGACAACCCATTCGG	60	2668 - 2649	2667 - 2648	8728 - 8709
Tm2S-f1	CAGTGATCCGAGTGAGCAAA	60	2357 - 2376	–	–
Tm2S-r1	TTCCGATAAACTGATTCCGC	60	2750 - 2731	–	–
Tm2S-f2	CTTCCTTCTGGTGTTTGGGA	60.1	2058 - 2077	–	–
Tm2S-r2	CAGAACCTTTAGCGCCTTTG	60	2342 - 2323	–	–
Tm2R-f1c	CTCCTTCTGGTGTTTGGGAG	59.7	–	2058 - 2077	8119 - 8138
Tm2R-r3	CGGTCTACCGTAAAGTTGGC	59.6	–	2502 - 2483	–
Tm2aR-r3	CGGTCTACACTAAAGTAGGC	59.6	–	–	8563 - 8544
Tm2R-r4	AGTACTGCCAGTATATAACG	53.9	–	2520 - 2501	8579 - 8562

[a]All primers were designed from the GenBank accession AF536199, AF536201, and AF536200 using the tool—PrimerBLAST and selected depending their allele-specificity The forward primers, Tm2RS-f2, and Tm2RS-f3, and the reverse primers, Tm2RS-r2, and Tm2RS_r3 were specific for the AF536199, AF536201, and AF536200; the forward primers, Tm2S-f1 and Tm2S-f2, and reverse primers, Tm2S-r1 and Tm2S-r2 specific for AF536199; and the forward primer, Tm2R-f1c for AF536199 and AF536200; the reverse primer Tm2R-r3 for AF536200; and the reverse primer Tm2aR-r3 for AF536201; [b]The primer location presenting the corresponding site in the GenBank accession For example, the primer Tm2RS_f2 is located at 869-888 sites of the GenBank accession AF536199; and '–' signifies the primer sequence does not exist in the corresponding sequence.

Table 3. PCR conditions for different primer pairs.

	Denaturation		Annealing		Extension	
Primer pair	Cycle	Temperature	Time	Temperature	Time	Temperature	Time
Tm2RS-f2/Tm2RS-r2,Tm2RS-f3/Tm2RS-r3	38	94˚C	30 s	53˚C	30 s	72˚C	75 s
Tm2S-f1/Tm2S-r1, Tm2S-f2/Tm2S-r2	36	94˚C	15 s	56˚C	25 s	72˚C	40 s
Tm2R-f1c/Tm2R-r3 and Tm2R-f1c/Tm2aR-r3	36	94˚C	20 s	56˚C	25 s	72˚C	55 s
Tm2R-f1c/Tm2R-r4	36	94˚C	20 s	51˚C	25 s	72˚C	55 s

genotypes with and without *Tm*-2. The PCR fragments were sequenced in the Purdue Genomics Core Facility, Purdue University. The sequences amplified from the primer pairs Tm2RS-f2/r2 and Tm2RS-f3/r3 were aligned using the software BioEdit v 7.0.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). SNPs were extracted from the alignment by TASSEL2.1 [26]. SNP genotyping was done at the Sequenom Technology Core, the Division of Human Genetics in the Department of Genetics, Washington University, St. Louis, MO 63110 (http://hg.wustl.edu/info/Sequenom_description.html). Three SNPs were used for SNP genotyping in 23 tomato genotypes using Sequnom. The primers and the multiplex assay are list in supplementary **Table S1** consisting of 3 SNPs iPLEX, which was designed by the tool—Assay Design 3 specific for iPLEX genotyping on MassARRAY system by Sequenom; the software Assay Design can be downloaded from Sequnom web site (https://www.mysequenom.com) after registration.

2.4. CAPS Marker

From the three sequences of AF536199, AF536200, and AF536201 at *Tm*-2 locus, seven enzymes, *Bse*YI, *Bsi*HKAI, *Bsm*I, *Bts*I, *Hpa*I, *Pci*I, and *Psh*AI can cut at different sites and produce different DNA fragments using the tool—NEBcutter V2.0 (http://tools.neb.com/NEBcutter2) (**Table 4**). Three out of the seven enzymes, *Psh*AI, *Hpa*I, and *Bsi*HKAI were used to digest the PCR fragments amplified from the primer pair Tm2RS-f3/r3 among 23 tomato genotypes with and without *Tm*-2 following the manufacturer's instructions (New England Biolabs Inc.) (**Table 4**). For enzyme *Hpa*I, each digestion

Table S1. Primer properties in the SNP assay for Sequenom SNP genotyping.

SNP_ID	SNP type	2nd-PCRP	1st-PCRP	AMP _LEN	UEP _MASS	UEP_SEQ
Tm2-snp1	C/T	ACGTTGGATGCACACGTCTA-GAGACCATAG	ACGTTGGATGTGTTTA-GACTCCCAAACACC	95	6326	cTCGACG-TAGCCTCATTCAAC
Tm2-snp46	C/T	ACGTTGGATGTAGTTGGGCCAA TGCTTCAC	ACGTTGGATGTATGGCTAGCGGT ATACCTC	102	7495	TGGTGAATATTGGTA GAAATATAG
Tm2-snp53	A/G	ACGTTGGAT-GATCTTCTCAATCTCCATAGC	ACGTTGGATGCTCTTAGAT-GACTTCCTTCC	124	6937	gCAATCTCCATAG-CAAACTCATC

Table 4. Enzyme cut sites in the sequences of *Tm*-2 locus.

Enzyme	Specificity	Cut position [a]		
		AF536199[b]	**AF536200**	**AF536201**
		(*tm*-2)	**(*Tm*-2)**	**(*Tm*-2^2)**
BseYI	C CCAG C	1230/1234	−	−
BsiHKAI	G WGCW C	−	2322/2318 2675/2671	3776/3772, 8736/8732 9836/9832
BsmI	GAATG CN	2451/2449	−	3000/2998
BtsI	GCAGTG NN	2521/2519	−	3576/3574
HpaI	GTT AAC	−	2422	2835, 5643, 8483, 9325
PciI	A CATG T	2307/2311	−	3508/3512
PshAI	GACNN NNGTC	2503	−	3509
		PCR fragments amplified from Tm2RS-f3/r3		
Enzyme	**Specificity**	**FJ817600 FJ817607**	**FJ817605 FJ817604**	**FJ817606 FJ817602**
		(tm-2)	***(Tm-2)***	***(Tm-2^2)***
BsiHKAI	G WGCW C	−	358/354	−
HpaI	GTT AAC	−	458	458
PshAI	GACNN NNGTC	538	−	−

[a]Cut position location by the enzyme in the corresponding accession sequence such as the enzyme BseYI cut the AF536199 sequence at 1230 and 1234 base but not cut the sequences of AF536200 and AF536201. [b]AF536199b, AF536200, AF536201, FJ817600, FJ817607, FJ817605, FJ817604, FJ817606, and FJ817602 are GenBank accessions at *Tm*-2 locus.

reaction consisted of 2.0 µl 10X NEBuffer 4, 0.4 µl restriction enzyme (5 U/µl), and 17.6 µl direct PCR product. For enzyme *Bsi*HKAI, each digestion reaction consisted of 2.0 µl 10X NEBuffer 3, 0.2 µl acetylated BSA (10 µg/µl), 0.2 µl restriction enzyme (10 U/µl), and 17.6 µl direct PCR product. For enzyme *Psh*AI, each digestion reaction consisted of 2.0 µl 10X NEBuffer 4, 0.2 µl acetylated BSA (10 µg/µl), 0.2 µl restriction enzyme (10 U/µl), and 17.6 µl direct PCR product. Digestion mixture was incubated at 37˚C in an eppendorf thermal cycler for 3 hr. The digestion products were visualized in 1.5% agarose gels in 0.5X TBE, stained with ethidium bromide.

3. Results

3.1. Allele-Specific PCR Markers

Two primer pairs, Tm2S-f1/Tm2S-r1 and Tm2S-f2/Tm2S-r2, amplified to produce DNA fragment only for the 14 tomato genotypes containing susceptible allele *tm*-2 with homozygous (*tm*-2/*tm*-2) or heterozygous (*Tm*-2^2/*tm*-2) condition as codominant marker (**Table 1**),

indicating the two primers can be used as PCR-based markers to select the susceptible allele *tm*-2 in tomato. The primer pair Tm2R-f1c/Tm2R-r3 only amplified the tomato genotypes containing resistance allele *Tm*-2. However, the primer pair Tm2R-f1c/Tm2aR-r3 amplified the tomato genotypes with *Tm*-2^2 with homozygous or heterozygous condition. The primer pair Tm2R-f1c/ Tm2R-r4 amplified the DNA from tomato genotypes containing either *Tm*-2 or *Tm*-2^2 with homozygous or heterozygous condition, indicating the primer pair can be used to select either *Tm*-2 or *Tm*-2^2 gene (**Table 1**).

3.2. Allele-Derived CAPS Markers

The enzyme PshAI cut the PCR fragment amplified with the primer pair Tm2RS-f3/r3 at the site 537 base into two fragments with the size of 538 bp and 165 bp for the tomatoes containing susceptible allele *tm*-2; The enzyme HapI cut the PCR fragment into 458 bp and 245 bp among those tomatoe lines carrying either *Tm*-2 or *Tm*2^2 and; BsiHKAI cut the fragment into 358 bp and 353 bp in those containing *Tm*-2 (**Table 1**). This indicated that combination of the three markers can be used to identify and distinguish the three alleles at *Tm*-2 locus in tomato lines.

3.3. Gene-Derived SNP Markers

Both SNP Tm2-snp1 and Tm2-snp46 had three types of SNP [C C], [T T], and [C T] showing co-dominant pattern (**Table 1**). The type [T T] in Tm2-snp1 and [C C] in Tm2-snp46 were specific for those tomato genotypes containing susceptible homozygous alleles *tm*-2/*tm*-2. On the other hand, the type [C C] in Tm2-snp1 and [T T] in Tm2-snp46 were specific to those tomato genotypes carrying resistance homozygous alleles *Tm*-2/*Tm*-2 or *Tm*2^2/*Tm*2^2. [C T] in both SNPs was for the three tomato genotypes containing heterozygous *Tm*2^2/*tm*-2. The SNP Tm2-snp53 had three types [A A], [A G], and [G G]. The tomato lines containing homozygous or heterozygous alleles for resistance allele *Tm*-2 or *Tm*2^2 had the SNP Tm2-snp53 type [A A] or [A G], but those carrying homozygous susceptible allele *tm*-2 had [G G] type in Tm2-snp53 (**Table 1**). Results indicated that these SNPs can differentiate resistance and susceptible allele at *Tm*-2 locus.

3.4. Sequence Analysis and Potential SNP Identification

The primer pairs Tm2RS-f2/r2 and Tm2RS-f3/r3 produced DNA fragments with the size of 490 bp, and 703 bp, respectively in all 23 tomato genotypes (**Table 1**). The 13 DNA fragment sequences amplified from the two primer pair were stored at GenBank with the accessions no. from FJ17595 to FJ17607.

From the multiple sequence alignment among the eight PCR fragments (FJ17600 to FJ17607) amplified from the primer pair Tm2RS-f3/r3 with the corresponding DNA segments of the AF536201, AF536200, AY742887, and AF536199, 40 SNPs were found (Supplementary **Table S2**). Among them, 36 SNPs were capable to discriminate the tomato genotypes with the susceptible allele *tm*-2 and those with the resistance alleles either *Tm*-2 or *Tm*-2^2. Four SNPs, SNP9, SNP30, SNP32, and SNP33 were further capable to differentiate the genotypes carrying *Tm*-2 and *Tm*-2^2. Two SNPs, SNP9 and SNP32 had triple alleles. SNP1 [C/T] was selected as one of the multiplex assay for SNP genotyping in 23 tomato genotypes by Sequenom.

From the multiple sequence alignment among the five PCR fragments (FJ17595 to FJ17599) amplified from the primer pair Tm2RS-f2/r2 with the corresponding DNA segments of the AF536201, AF536200, AY742887, and AF536199, eight SNPs were observed (Supplementary **Table S3**). Among them, six SNPs showed the differences between the tomato lines with susceptible allele *tm*-2 and resistance alleles including *Tm*-2 or *Tm*-2^2. Two SNPs, SNP42 and SNP45 showed the differences between those carrying *Tm*-2 and *Tm*-2^2. SNP46 [C/T] was also selected as one of the multiplex assay for SNP genotyping in 23 tomato genotypes by Sequenom.

Besides the 48 SNPs verified from the re-sequencing of the PCR products amplified the two primer pairs, ten SNPs and one InDel were also postulated among the three *Tm*-2 sequences AF536201, AF536200, and AF536199 (Supplementary **Table S4**). Among them, nine SNPs and one InDel showed the difference between the tomato genotypes with susceptible allele *tm*-2 and resistance alleles including *Tm*-2 or *Tm*-2^2. The SNP SNP53 showed the difference between *Tm*-2 and *Tm*-2^2 and was selected as one of the multiplex assay for SNP genotyping in 23 tomato genotypes by Sequenom.

4. Discussion

Three types of molecular marker development for *Tm*-2 loci were developed in this research. From known *Tm*-2 gene cloning sequences AF536199 (*tm*-2), AF536200 (*Tm*-2), and AF536201 (*Tm*-2^2), two gene-specific primer pairs, Tm2RS-f2/r2 and Tm2RS-f3/r3 were designed. The PCR products amplified from tomato genotypes contained either resistance allele *Tm*-2, *Tm*-2^2 or susceptible allele *tm*-2 using the two primer pairs were sequenced. After multiple sequence alignment, 40 SNPs were found and validated from the sequences amplified from Tm2RS-f3/r3 (**Table S2**) and eight SNPs and one InDel from Tm2RS-f2/r2 (**Table S3**). Two SNPs, snp1 and snp46 selected from sequences amplified from the two primer pairs plus the SNP snp53 picked up from other location of the sequence (**Table S4**) were selected

Table S2. Forty potential SNPs and their types and locations in corresponding sequences amplified by the primer pair Tm-2RS-f3/r3.

GenBank accession	Cultivar/accession	Allele at *Tm*-2 locus	SNP type and location in corresponding sequence a																			
			Snp1	Snp2	Snp3	Snp4	Snp5	Snp6	Snp7	Snp8	Snp9	Snp10	Snp11	Snp12	Snp13	Snp14	Snp15	Snp16	Snp17	Snp18	Snp19	Snp20
AF536201	MoneyMaker	*Tm*-2^2	8119C	8222T	8348T	8360G	8368T	8371T	8373A	8383T	8384T	8393G	8394G	8412T	8416C	8423C	8432A	8441G	8450G	8457C	8459T	8464C
AF536200	Craigella GCR236	*Tm*-2	2058C	2161T	2287T	2299G	2307T	2310T	2312A	2322T	2323C	2332G	2333G	2351T	2355C	2362C	2371A	2380G	2389G	2396C	2398T	2 403C
AY742887	Yukang 2	*Tm*-2	1871C	1974T	2100T	2112G	2120T	2123T	2125A	2135T	2136C	2145G	2146G	2164T	2168C	2175C	2184A	2193G	2202G	2209C	2211T	2216C
AF536199	ATV840	*tm*-2	2059T	2162C	2288A	2300A	2308C	2311G	2313C	2323C	2324A	2333A	2334A	2352G	2356T	2363T	2372G	2381C	2390C	2397A	2399A	2404T
FJ817600	Anahu /LA0655	*tm*-2	94T	197C	323A	335A	343C	346G	348C	358C	359A	368A	369A	387G	391T	398T	407G	416C	425C	432A	434A	439T
FJ817601	LA0656	*tm*-2	94T	197C	323A	335A	343C	346G	348C	358C	359A	368A	369A	387G	391T	398T	407G	416C	425C	432A	434A	439T
FJ817607	Peto 95-43/LA3528	*tm*-2	94T	197C	323A	335A	343C	346G	348C	358C	359A	368A	369A	387G	391T	398T	407G	416C	425C	432A	434A	439T
FJ817604	LA3432	*Tm*-2	94C	197T	323T	335G	343T	346T	348A	358T	359C	368G	369G	387T	391C	398C	407A	416G	425G	432C	434T	439C
FJ817605	LA3433	*Tm*-2	94C	197T	323T	335G	343T	346T	348A	358T	359C	368G	369G	387T	391C	398C	407A	416G	425G	432C	434T	439C
FJ817606	Mogeor /LA3471	*Tm*-2^2	94C	197T	323T	335G	343T	346T	348A	358T	359T	368G	369G	387T	391C	398C	407A	416G	425G	432C	434T	439C
FJ817602	VFNT Cherry /LA1221	*Tm*-2^2	94C	197T	323T	335G	343T	346T	348A	358T	359T	368G	369G	387T	391C	398C	407A	416G	425G	432C	434T	439C
FJ817603	Royal Red Cherry/LA2088	*Tm*-2^2	94C	197T	323T	335G	343T	346T	348A	358T	359T	368G	369G	387T	391C	398C	407A	416G	425G	432C	434T	439C

GenBank accession	Cultivar/accession	Allele at *Tm*-2 locus	SNP type and location in corresponding sequence																			
			Snp21	Snp22	Snp23	Snp24	Snp25	Snp26	Snp27	Snp28	Snp29	Snp30	Snp31	Snp32	Snp33	Snp34	Snp35	Snp36	Snp37	Snp38	Snp39	Snp40
AF536201	MoneyMaker	*Tm*-2^2	8484A	8495G	8508C	8511T	8516T	8526C	8528C	8543A	8545C	8547T	8548A	8554G	8555T	8562C	8566A	8568A	8579A	8591T	8616T	8647A
AF536200	Craigella GCR236	*Tm*-2	2423A	2434G	2447C	2450T	2455T	2465C	2467C	2482A	2484C	2486A	2487A	2493C	2494G	2501C	2505A	2507A	2518A	2530T	2555T	2 586A
AY742887	Yukang 2	*Tm*-2	2236A	2247G	2260C	2263T	2268T	2278C	2280C	2295A	2297C	2299A	2300A	2306C	2307G	2314C	2318A	2320A	2331A	2343T	2368T	2399C
AF536199	ATV840	*tm*-2	2424G	2435A	2448G	2451G	2456C	2466A	2468T	2483T	2485T	2487T	2488G	2494T	2495G	2502A	2506G	2508C	2519G	2531A	2556G	2587C
FJ817600	Anahu /LA0655	*tm*-2	459G	470A	483G	486G	491C	501A	503T	518T	520T	522T	523G	529T	530G	537A	541G	543C	554G	566A	591G	622C
FJ817601	LA0656	*tm*-2	459G	470A	483G	486G	491C	501A	503T	518T	520T	522T	523G	529T	530G	537A	541G	543C	554G	566A	591G	622C
FJ817607	Peto 95-43/LA3528	*tm*-2	459G	470A	483G	486G	491C	501A	503T	518T	520T	522T	523G	529T	530G	537A	541G	543C	554G	566A	591G	622C
FJ817604	LA3432	*Tm*-2	459A	470G	483C	486T	491T	501C	503C	518A	520C	522A	523A	529C	530G	537C	541A	543A	554A	566T	591T	622A
FJ817605	LA3433	*Tm*-2	459A	470G	483C	486T	491T	501C	503C	518A	520C	522A	523A	529C	530G	537C	541A	543A	554A	566T	591T	622A
FJ817606	Mogeor /LA3471	*Tm*-2^2	459A	470G	483C	486T	491T	501C	503C	518A	520C	522T	523A	529G	530T	537C	541A	543A	554A	566T	591T	622A
FJ817602	VFNT Cherry /LA1221	*Tm*-2^2	459A	470G	483C	486T	491T	501C	503C	518A	520C	522T	523A	529G	530T	537C	541A	543A	554A	566T	591T	622A
FJ817603	Royal Red Cherry/LA2088	*Tm*-2^2	459A	470G	483C	486T	491T	501C	503C	518A	520C	522T	523A	529G	530T	537C	541A	543A	554A	566T	591T	622A

SNP type and location in corresponding sequence such as Snp1 is base C located at 8119 base of AF536201; C at 2058 base of AF536200; C at 1871 base of AY742887; T at 2059 base of AF536199; T at 94 base of FJ817600, FJ817601, and FJ817607; and C at 94 base of FJ817604, FJ817605, FJ817606, FJ817602, and FJ817603.

Table S3. Eight potential SNPs and their types and locations in corresponding sequences amplified by the primer pair Tm-2RS-f2/r2.

GenBank accession	Cultivar/ accession	Allele at *Tm-2* locus	SNP type and location in corresponding sequence [a]							
			SNP41	SNP42	SNP43	SNP44	SNP45	SNP46	SNP47	SNP48
AF536201	MoneyMaker	*Tm*-2^2	6962G	7017A	7043G	7085T	7106G	7291T	7454T	7485G
AF536200	Craigella GCR236	*Tm*2	901G	956T	982G	1024T	1045T	1230T	1393T	1424G
AF536199	ATV840	*tm*-2	902T	957A	983T	1025C	1046G	1231C	1394G	1425A
AY742887	Yukang 2	*Tm*2	714G	769T	795G	837T	858T	1043T	1206T	1237G
FJ817595	Anahu/LA0655	*tm*2	34T	89A	115T	157C	178G	363C	526G	557A
FJ817596	LA0656	*tm*2	34T	89A	115T	157C	178G	363C	526G	557A
FJ817599	Peto 95-43/LA3528	*tm*2	34T	89A	115T	157C	178G	363C	526G	557A
FJ817597	VFNT Cherry/LA1221	*Tm*-2^2	34G	89A	115G	157T	178G	363T	526T	557G
FJ817598	LA3433	*Tm*2	34G	89T	115G	157T	178T	363T	526T	557G

[a]SNP type and location in corresponding sequence such as Snp41 is base G located at 6962 base of AF536201; G at 901 base of AF536200; T at 902 base of AF536199; G at 714 base of AY742887; T at 34 base of FJ817595, FJ817596, and FJ817599; and G at 34 base of FJ817597 and FJ817598.

Table S4. Ten potential SNPs and one InDel and their types and locations in corresponding sequences among *Tm*-2 alleles except above 48 SNPs in Table S1 and S2.

GenBank accession	Cultivar	Allele at *Tm*-2 locus	SNP type and location in corresponding sequence [a]										
			SNP49	**SNP50**	**SNP51**	**SNP52**	**SNP53**	**SNP54**	**SNP55**	**SNP56**	**SNP57**	**SNP58**	**InDel1**
AF536199	ATV840	*tm*-2	73A	109A	457G	487C	500G	612T	704C	849G	2672A	2733G	89A
AF536200	Craigella GCR236	*Tm*-2	73C	108T	456A	486G	499G	611C	703T	848A	2671G	2732C	88-89d
AF536201	MoneyMaker	*Tm*-2^2	6134C	6169T	6517A	6547G	6560A	6672C	6764T	6909A	8732G	8793C	88-89d

[a]SNP type and location in corresponding sequence such as Snp49 is base A located at 73 base of AF536199; C at 73 base of AF536200; and C at 6134 base of AF536201.

to use SNP genotyping for identification of *Tm*-2 alleles and verified that they were useful to identify difference alleles, *tm*-2, *Tm*-2, and *Tm*-2^2 in 23 tomato genotypes by Sequnom technology (**Table 1**). Meanwhile, allele-specific PCR primers were designed for each allele at *Tm*-2 locus and five allele-specific PCR-based markers were identified: one for *Tm*-2, one for *Tm*-2^2, one for both *Tm*-2 and *Tm*-2^2, and two for the susceptible allele *tm*-2 in 23 tomato genotypes (**Table 1**). The allele-derived CAPS markers were designed from the sequences amplified from primer pair Tm2RS-f3/r3 digested by the three enzymes, BsiHKAI, HpaI, and PshAI, which can identify and distinguish the three alleles, *tm*-2, *Tm*-2, and *Tm*-2^2 among 23 tomato genotypes (**Table 1**).

The five allele-specific PCR markers were dominant markers, which can't distinguish the homozygous and heterozygous genotypes at *Tm*-2 locus individually, but the combination of these markers can do (**Table 1**). The three CAPS markers performed the same way as the allele-specific PCR-based markers did (**Table 1**). The two SNP markers, snp1 and snp46 were co-dominant ones and they can distinguish the homozygous and heterozygous genotypes at *Tm*-2 locus individually. However, we did not identify a SNP marker that can distinguish *Tm*-2^2 from *Tm*-2. The SNP53 should distinguish *Tm*-2^2 from *Tm*-2 because AF53200 (*Tm*-2) has a "G" at 499 base of the sequence but AF536201 (*Tm*-2^2) has an "A" base at the corresponding site of the sequence (**Table S4**). But our SNP genotyping data did not support *Tm*-2-carrying tomato lines carried a "G" base in the corresponding site, such as Royal Red Cherry (LA2088), CLN2264F (LA4285), and LA3433 contained *Tm*-2 allele but they had [A A] type for snp53 (**Table 1**). Besides the snp53, SNP9, SNP30, SNP32, SNP33, SNP42, and SNP45 also showed difference in their SNP types for *Tm*-2^2 from *Tm*-2 (**Tables S2** and **S3**) and maybe they can be used in SNP genotyping for distinguishing *Tm*-2^2 from *Tm*-2. But, they need to be validated in further research. However, the snp9 was validated to be used to distinguish *Tm*-2^2 from *Tm*-2 by the CAPS marker through digestion

the PCR products amplified from Tm-2RS-f3/r3 by enzyme EsiHKAI (**Tables 1** and **4**). Only those tomato lines contained *Tm*-2 allele were digested by EsiHKAI for the DNA segments amplified from T2RS-f3/r3 because *Tm*-2 carrying tomato genotypes had a "C" base at 359 base of the sequences but others had an "A" base at 359. The SNP32 in supplementary **Table S2** and SNP41 in **Table S3** were used as a tetra-primer ARMS-PCR assays by Arens *et al.* [15] to produce different PCR fragment sizes for detection of three alleles *tm*-2, *Tm*-2, and *Tm*-2^2 in tomato genotypes.

Three types of molecular markers, allele-specific PCR, allele-derived CAPS, and allele-derived SNP were identified for the *Tm*-2 locus for ToMV resistance (**Table 1**). Lanfermeijer *et al.* in 2005 [14] reported three CAPS markers which can distinguish the three alleles, *tm*-2, *Tm*-2, and *Tm*-2^2 in tomato genotypes and the enzyme HapI was also reported that cut the same site of the sequences from resistant cultivars, Craigella GCR236 (*Tm*-2), Craigella GCR267 (*Tm*2^2), and ATV847 (*Tm*2^2), but not in the susceptible cultivars, GCR26 (*tm*-2) and ATV840 (*tm*-2). However, in their research, the primer pair, PrRuG151 (GAGTTCTTCCGTTCAAATCCTAAGCTTGAGAAG)/PrRuG086 (CTACTACACTCACGTTGCTGTGATGCAC) was used to amplify DNA fragments among the five cultivars. The primer PrRuG151 was located at 7824 to 7856 base and PrRuG086 at 8908 to 8881 base of the AF536201 sequence and the primer pair produced a 1085 bp DNA fragment. The enzyme HpaI cut the DNA fragments into two with 660 bp and 424 bp fregments for Craigella GCR236 (*Tm*-2), Craigella GCR267 (*Tm*-2^2), and ATV847 (*Tm*-2^2), but not for GCR26 (*tm*-2) and ATV840 (*tm*-2). In our research, the primer pair Tm-2RS-f3/r3 was used and produced a 703 bp DNA fragment of PCR products. The fragment was digested with HpaI into two fragments with 538 bp and 165 bp for tomato genotypes carrying *Tm*-2 and *Tm*-2^2 but not for *tm*-2 (**Table 1**). HpaI can cut the sequence "GTT AAC" in DNA fragments amplified from prmier pair of either PrRuG151/PrRuG086 or Tm-2RS-f3/r3. Because the resistant genotypes contained *Tm*-2 and *Tm*-2^2 had the sequence "GTT AAC", but the susceptible genotypes carrying *tm*-2 had the "GTT GAC" in corresponding site of the sequence. The SNP snp21 in **Table S2** was the same site of HpaI cutting site. These results indicated that the CAPS marker digested by HpaI and the SNP marker SNP21 [A/G] can be used as a markers to identify and distinguish the susceptible allele *tm*-2 from the resistance allele *Tm*-2 or *Tm*-2^2. After all, these allele-specific PCR-based, CAPS, and gene-derived SNP markers for *Tm*-2 locus will provide breeders to select the allele *tm*-2, *Tm*-2, and *Tm*-2^2 of ToMV resistance in advancing the MAS for tomato breeding.

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