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EVALUATION OF SOME MOLECULAR MARKERS AS SELECTION TOOLS FOR TOMATO MOSAIC VIRUS (*TOMV*) RESISTANCE IN *SOLANUM LYCOPERSICUM* L.

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Abstract

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Tomato mosaic virus (*ToMV*) is one of the most economically important viruses on tomato (*Solanum lycopersicum* L.*)* in Bulgaria, easily transmitted mechanically and by seeds. Breeding, based on genetic controlled resistance, is the most effective and successful approach for combating the *ToMV*-tomato problem due to the dominant monogenic genetic control, governed by three single genes for resistance (*Tm-1*, *Tm-2* and *Tm-2²*). The aim of the current study was to investigate the reliability of some SCAR, CAPS and AS molecular markers for identification of *Tm* genes for resistance in tomato breeding lines. Parallel to molecular study, screening tests with three *ToMV* pathotypes (0, 1 and 2) were carried out as control tests. The resistance to the three pathotypes was found in 6 breeding lines. Twenty *ToMV* resistant plants were subjected to molecular analysis. The CAPS marker revealed the presence of *Tm-2²* allele in homozygous condition in 12 ToMV resistant plants, whereas the AS markers (AS1, AS2 and AS3) confirmed this result only in two plants. The remaining 10 reacted as heterozygous with genotype *Tm-2/Tm-2²*. Another eight *ToMV* resistant plants were determined by CAPS marker as heterozygous for *Tm-2²* gene (*tm-2/Tm-2²*), which was confirmed only in two plants by AS markers. The results obtained for the remaining six plants by AS markers were rather contradictory. Successful application of CAPS marker for genotyping the *Tm-2* locus as well as confusing behavior of AS 2 marker during genotyping the Maritsa VCRI tomato breeding lines is discussed.

Key words: tomato, Tomato mosaic virus, resistance genes, SCAR, CAPS, allele-specific markers
List of abbreviations: AS – allele-specific; CAPS – Cleaved Amplified Polymorphic Sequences; dpi – days post inoculation; MAS – Marker Assistant Selection;RFLP – Restriction Fragment Lenght Polymorphism; SCAR – Sequence Characterized Amplified Region; *ToMV* – Tomato mosaic virus

Introduction

Tomato mosaic virus (*ToMV*) is a rod like virus which belongs to tobamovirus group. The genome of the virus is consisted of positive-sense single-stranded RNA (Goelet et al., 1982; Ohno et al., 1984; Canto et al., 2004). The virus is one of the most economically important pathogens in the world and particularly in Bulgaria causing serious losses in yield and quality in tomato (*Solanum lycopersicum* L). The virus can be transmitted easily by mechanical contact with infected plants or contaminated cultivation tools. *ToMV* can penetrate into plants also through injured roots by infected plant debris in the soil. The virus is seed transmitted and is present in the external testa and sometimes in the endosperm of tomato seeds, but was not proved to be within the embryo (Broadbent, 1976).

Three major genes derived from wild accessions of tomato serve as a source of resistance. *Tm-1* is found in *Lycopersicon hirsutum* and *S. habrochaites* while *Tm-2* and *Tm-2²*

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is present in *Lycopersicon peruvianum* (Foolad, 2007; Hall, 1989; Lanfermeijer et al., 2003; Levesque et al., 1990; Pelham, 1966; Pelham, 1972). *Tm-1* confers resistance to pathotypes 0 and 2 (Foolad, 2007; Foolad and Sharma, 2005; Levesque et al., 1990; Ohmori et al., 1996) and may block the function of two virus-coded proteins which are necessary for replication of the virus RNA (Meshi et al., 1988). In contrast, *Tm-2* confers resistance against pathotypes 0 and 1 while the allelic *Tm-2²* gene is effective against all three *ToMV* pathotypes. It has been observed that the *Tm-2* and *Tm-2²* resistance in heterozygous tomato plants is often accompanied by systemic necrosis (hypersensitive response), especially at high temperatures (Hall, 1980; Pelham, 1966; Pelham, 1972). While *Tm-1* and *Tm-2* can be overcome by a large number of naturally occurring ToMV isolates (Rast, 1975), *Tm-2²* was shown to be more effective than the other resistance genes (Hall, 1980; Lanfermeijer et al., 2003; Jiang and Yang, 2003). There are only few reports on ToMV isolates which are able to overcome it (Hall, 1980; Rast, 1975).

Tm-1 gene was mapped and located on chromosome 2 using Restriction Fragment Lenght Polymorphism (RFLP) markers by Tanksley et al. (1992). Ohmori et al. (1995b, 1996) identified six RAPD markers linked to the same gene which have been converted to SCAR markers. A number of RAPD markers associated with *Tm-2* locus have been reported (Dax et al., 1994 and Ohmori et al., 1995a). Young et al. (1988) identified two RFLP markers tightly linked to the *Tm-2²* allele. Later, co-dominant Sequence Characterized Amplified Region (SCAR) markers, linked to the same allele, were developed by Dax et al. (1998) and Sobir et al. (2000). Three allele-specific (AS) (Shi et al., 2011) and several Cleaved Amplified Polymorphic Sequences (CAPS) markers for distinguishing dominant *Tm-2, Tm-2²* and recessive *tm-2* alleles have been suggested (Shi et al., 2011; Panthee et al., 2013).

The aim of the current study is to prove the reliability of some SCAR, CAPS and AS molecular markers for identification of *Tm* genes for *ToMV* resistance in order to be used as selection tools in tomato breeding program. For this purpose several, available in the literature, SCAR, CAPS and AS markers for identification of alleles in *Tm-1* and *Tm-2* resistance loci were selected. Parallel serological and biological tests with three *ToMV* pathotypes were carried out to separate the susceptible genotypes and also to prove the consistency of the results from the molecular analysis.

Materials and Methods

Plant material

Twenty tomato breeding lines of Maritsa VCRI, derived from different crosses, were used for evaluation of their resistance to the three ToMV pathotypes. Seeds were sown in trays with peat perlite mixture (2.5:1). Seedlings were cultivated in a growth chamber at 22-26°C with photoperiod 14 h day and 10 h night. Two lines possessing *Tm-2* and *Tm-2²* genes respectively were used as positive controls, while the sensitive to all pathotypes cultivar Ideal was used as negative control.

The tomato plants, possessing the dominant *Tm-2* gene, are expected to remain symptomless after inoculation with pathotype 0 and 1 below 30°C, but to develop mosaic symptoms after treatment with pathotype 2, while *Tm-2²* genotypes remain symptomless providing resistance to all three pathotypes. Usually, *Tm-2* and *Tm-2²* heterozygous individuals develop local and/or systemic hypersensitive reaction over 30°C.

Virus inocula

The three pathotypes of *ToMV* (0, 1 and 2) were propagated on susceptible tomato plants, possessing different genes combinations for *ToMV* resistance. Usually 15 days following the virus inoculation the susceptible genotypes develop clear mosaic symptoms and leaves with such symptoms were collected for viral inoculum.

Screening tests

Three sets of plants (10 plants per set) of every breeding line were inoculated mechanically with one of the three pathotypes (0, 1 and 2) in cotyledon phase. Inocula were prepared from fresh tomato leaves with symptoms, ground in universal buffer (1g K_2HPO_4, and 0.1g Na_2SO_3 in 100 ml H_2O, pH 9) in 1:10 ratio w/v in a mortar. A small amount of carborundum (600 mesh) was added to the homogenates as abrasive. After inoculation plants were washed with tap water to remove the plant debris. Symptoms were scored for a period about 40 days post inoculation (dpi). System spread of the virus in symptomless tomato plants was checked by back inoculation on hypersensitive host *N. glutinosa*.

Serological tests

DAS-ELISA (Clark and Adams, 1977) was used to check the virus presence in symptomless plants. Polyclonal antibodies (AS-0104, DSMZ) for detection of *ToMV* coat protein were utilized according to the standard procedures of the manufacturer. Color reaction was developed by adding 10 mg/ml p-nitrophenylphosphate (Sigma, Germany) in substrate buffer. Color intensity was measured on Biotek ELx 808 (Biotek Inc. USA) one hour after adding the substrate. Samples were considered as positive, if OD_{405} was at least two times over the negative control.

Molecular analyses

DNA isolation

DNA was extracted from young leaves of visually and serologically virus-free tomato plants, 40 days after inoculation with *ToMV*. About 0.1 g fresh tissue was collected in an Eppendorf tube and processed according to standard CTAB protocol (Edwards et al., 1991). Pellet was resuspended in 100 µl distillated water.

Identification of ToMV genes for resistance by SCAR, CAPS and allele-specific markers

The following markers were used for genotyping *Tm-1* and *Tm-2* loci (Table 1). Amplifications for all markers were performed in 25 µl reaction mixture consisted of 1x reaction buffer, 1.5 mM $MgCl_2$, µl 0.3 mM dNTPs mix, 0.4 µm of each primer, 1 U BIOTAQ™ (Bioline Reagents Ltd., London, UK) and 50 ng genomic DNA as template. Thermal profile consisted of initial denaturation step at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing at 55°C/54°C and 58°C for SCAR, CAPS and AS markers respectively for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min was executed on Biorad T100 (Biorad Laboratories).

Restriction analysis

Restrictions were performed using endonuclease MboI for SCI10 marker (Ohmori et al., 1996) and BoxI, KspAI and Alw21I for the CAPS marker (Shi et al., 2011). For both markers digestion reactions were carried out with 5 µl of the related PCR product in a final volume of 15 µl according to manufacturer's protocol (Fast Digest, Fermentas).

Agarose electrophoresis

The PCR fragments and products of digestions were separated by electrophoresis on 1% agarose gel in 0.5x TAE buffer for 75 min at 5 V/cm. DNA fragments were stained with DNA Stain G (Serva), and visualized with UV light.

Table 2
Screening test with three ToMV pathotypes (0, 1 and 2)

Breeding line	Pathotype 0	Pathotype 1	Pathotype 2	ELISA
81	S[a]	S	S	+[d]
709	R[b]	R	R	-[e]
717	S	S	S	+
720	R	R	R	-
768	S	S	S	+
776	S	S	S	+
782	S	S	S	+
799	S	S	S	+
868	R	R	R	-
899	S	S	S	+
914	S	S	S	+
934	S	S	S	+
941	S	S	S	+
943	S	S	S	+
944	S	S	S	+
949	S	S	S	+
981	HG[c]	R	R	+/-; -
1042	HG	HG	HG	+/-[f]
1048	HG	HG	HG	+/-
1471	S	S	S	+
Susceptible control cv. Ideal	S	S	S	+

In bold are lines with resistance to ToMV
[a] susceptible; [b] resistant; [c] heterogeneous;
[d] positive for virus presence;
[e] negative for virus presence;
[f] line with virus positive and virus negative individuals.

Table 1
Markers for genotyping Tm-1 and Tm-2 loci

Marker	Resistance alleles	Primer	Sequence 5'-3'	Reference
SCI10	tm1, Tm1 (with Mbo1)	OPI10U	ACAACGCGAGGCCAAATCCCATCA	Ohmori et al. 1996
		OPIl10T	ACAACGCGAGTAGGTTTAGGGTG	
SCA15	Tm1	OPA15U	CCGAACCCCTTAAAAATAGTTTC	Ohmori et al. 1996
		OPA15T	CCGAACCCAATCAGGAGGCTCAT	
SCN20	Tm1	OPN20U	GGTGCTCCGTCGATGCAAAGTGCA	Ohmori et al. 1996
		OPN20T	GGTGCTCCGTAGACATAAAATCTA	
CAPS	tm2, Tm2, Tm22	Tm2RS-f3	TGGAGGGGAATATTTGTGGA	Shi et al. 2011
	(with BoxI, KspAI and Alw21I)	Tm2RS-r3	ACTTCAGACAACCCATTCGG	
AS 1	tm2	Tm2S-f1	CAGTGATCCGAGTGAGCAAA	Shi et al. 2011
		Tm2S-r1	TTCCGATAAACTGATTCCGC	
AS 2	Tm2	Tm2R-f1c	CTCCTTCTGGTGTTTGGGAG	Shi et al. 2011
		Tm2R-r3	CGGTCTACCGTAAAGTTGGC	
AS 3	Tm22	Tm2R-f1c	CTCCTTCTGGTGTTTGGGAG	Shi et al. 2011
		Tm2aR-r3	CGGTCTACACTAAAGTAGGC	

Results

Serological and biological testing of breeding lines with ToMV, pathotypes 0, 1, 2

Following the inoculation of 20 lines with *ToMV* pathotypes 0, 1 and 2 visual evaluation identified 14 susceptible and three resistant. Two lines segregated to susceptible and resistance plants with all three pathotypes, while one was heterogenic only with pathotype 0 and resistant to the others. Resistant genotypes remained symptomless during the whole testing period and ELISA negative for virus presence; the susceptible ones developed clear mosaic symptoms (Table 2).

Subjects for molecular testing were the plants from three breeding lines (709, 720, 868), regarded as resistant to the three pathotypes as well as the resistant plants from three heterogeneous lines (981, 1042 and 1048).

Molecular analysis

The three SCAR markers for identification of *Tm-1* locus (according to Ohmori et al., 1996), produced the following results: Co-dominant marker SCI10 amplified a fragment of about 1100 bp for all tested individuals. Digestion of this product with MboI did not reveal any polymorphism between the investigated samples and the susceptible control. Primers for SCA15 and SCN20 did not produce any fragments in investigated germplasm.

The CAPS marker for identification of alleles in *Tm-2* locus, used according to Shi et al. (2011), produced the following results: A fragment of 703 bp was amplified for each of the tested plants and the product was digested using three endonucleases. All individual plants of lines 709, 720, 868, 1042 and one of line 1048 (№4) generated restriction products only with enzyme KspAI (458 and 245 bp), while BoxI and Alw21I did not produce any fragments (Fig. 1a, Table 3). Such restriction profile corresponded to *Tm-2²/Tm-2²* homozygous genotype (Fig. 1b). The digestion of PCR products in all plants of line 981 and the remaining five of line 1048 generated two fragments (538 and 165 bp) with BoxI and two other fragments (458 and 245 bp) using KspAI. No restriction products were obtained using Alw21I (Figure 1). These profiles are typical for heterozygous genotype *Tm-2²/tm-2* (Table 3). *Tm-2* allele was not identified in any of the tested genotypes.

The application of the three AS markers for genotyping *Tm-2* locus (Shi et al., 2011) produced the following results: All investigated plants of breeding lines 709 and 720, three plants of 1042 (№1, 3 and 4), two plants of 868 (№1 and 3) and one plant of 1048 (№ 4) produced fragments with markers AS2 and AS3, which corresponded to heterozygous genotype *Tm-2/ Tm-*

Table 3
Proposed genotypes of ToMV resistant individuals according to the results of molecular assay

Breeding line	№ of individual plant	CAPS marker				AS markers			
		Proposed genotype	Identification of alleles			Proposed genotype	Identification of alleles		
			tm2	*Tm2*	*Tm2²*		*tm2* by AS1	*Tm2* by AS2	*Tm2²* by AS3
709	1	*Tm-2²/Tm-2²*	-[a]	-	+[b]	*Tm-2/Tm-2²*	-	+	+
	2	*Tm-2²/Tm-2²*	-	-	+	*Tm-2/Tm-2²*	-	+	+
720	1	*Tm-2²/Tm-2²*	-	-	+	*Tm-2/Tm-2²*	-	+	+
	2	*Tm-2²/Tm-2²*	-	-	+	*Tm-2/Tm-2²*	-	+	+
868	1	*Tm-2²/Tm-2²*	-	-	+	*Tm-2/Tm-2²*	-	+	+
	2	*Tm-2²/Tm-2²*	-	-	+	*Tm-2²/Tm-2²*	-	-	+
	3	*Tm-2²/Tm-2²*	-	-	+	*Tm-2/Tm-2²*	-	+	+
981	1	*tm-2/Tm-2²*	+	-	+	*tm-2/Tm-2/Tm-2²*	+	+	+
	2	*tm-2/Tm-2²*	+	-	+	*tm-2/Tm-2²*	+	-	+
	3	*tm-2/Tm-2²*	+	-	+	*tm-2/Tm-2/Tm-2²*	+	+	+
1042	1	*Tm-2²/Tm-2²*	-	-	+	*Tm-2/Tm-2²*	-	+	+
	2	*Tm-2²/Tm-2²*	-	-	+	*Tm-2²/Tm-2²*	-	-	+
	3	*Tm-2²/Tm-2²*	-	-	+	*Tm-2/Tm-2²*	-	+	+
	4	*Tm-2²/Tm-2²*	-	-	+	*Tm-2/Tm-2²*	+	+	+
1048	1	*tm-2/Tm-2²*	+	-	+	*tm-2/Tm-2/Tm-2²*	+	+	+
	2	*tm-2/Tm-2²*	+	-	+	*tm-2/Tm-2/Tm-2²*	+	+	+
	3	*tm-2/Tm-2²*	+	-	+	*tm-2/Tm-2/Tm-2²*	+	+	+
	4	*Tm-2²/Tm-2²*	-	-	+	*Tm-2/Tm-2²*	-	+	+
	5	*tm-2/Tm-2²*	-	-	+	*tm-2/Tm-2/Tm-2²*	+	+	+
	6	*tm-2/Tm-2²*	-	-	+	*tm-2/Tm-2/Tm-2²*	+	+	+

Identical genotypes proposed of CAPS and AS markers are in bold
[a] absence of allele; [b] presence of allele

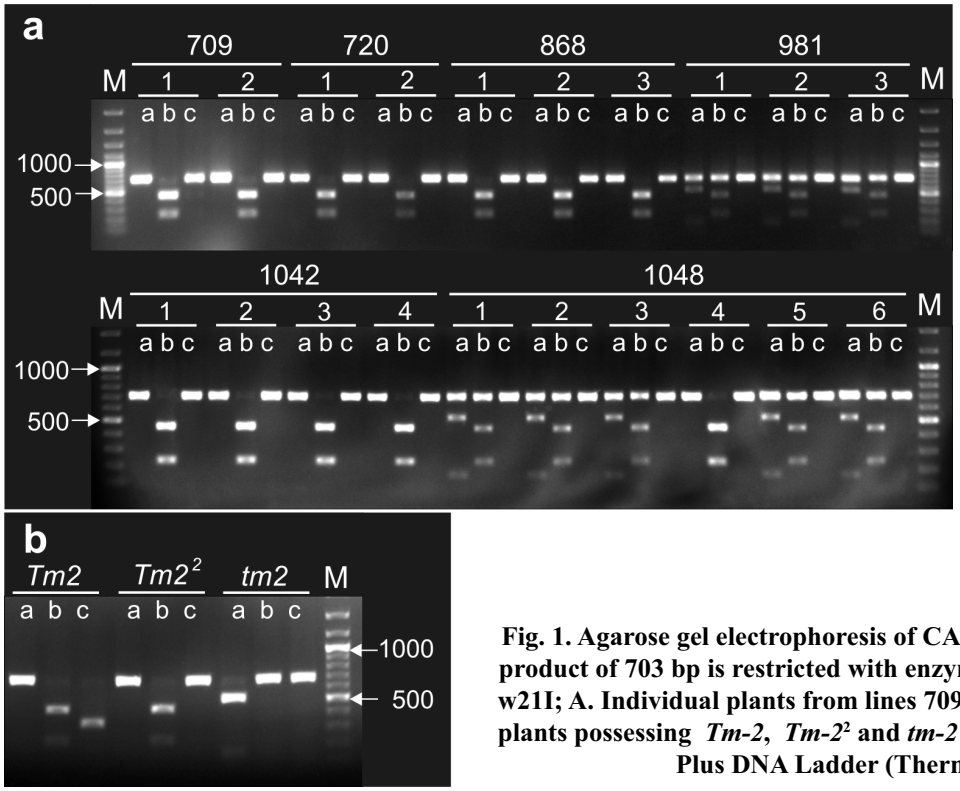

Fig. 1. Agarose gel electrophoresis of CAPS marker for *Tm-2* locus. PCR product of 703 bp is restricted with enzymes: a – BoxI; b – KspAI; c – Alw21I; A. Individual plants from lines 709 to 1048; B. Homozygous control plants possessing *Tm-2*, *Tm-2*² and *tm-2* alleles. M – GeneRuler™ 100 bp Plus DNA Ladder (Thermo Scientific Inc.)

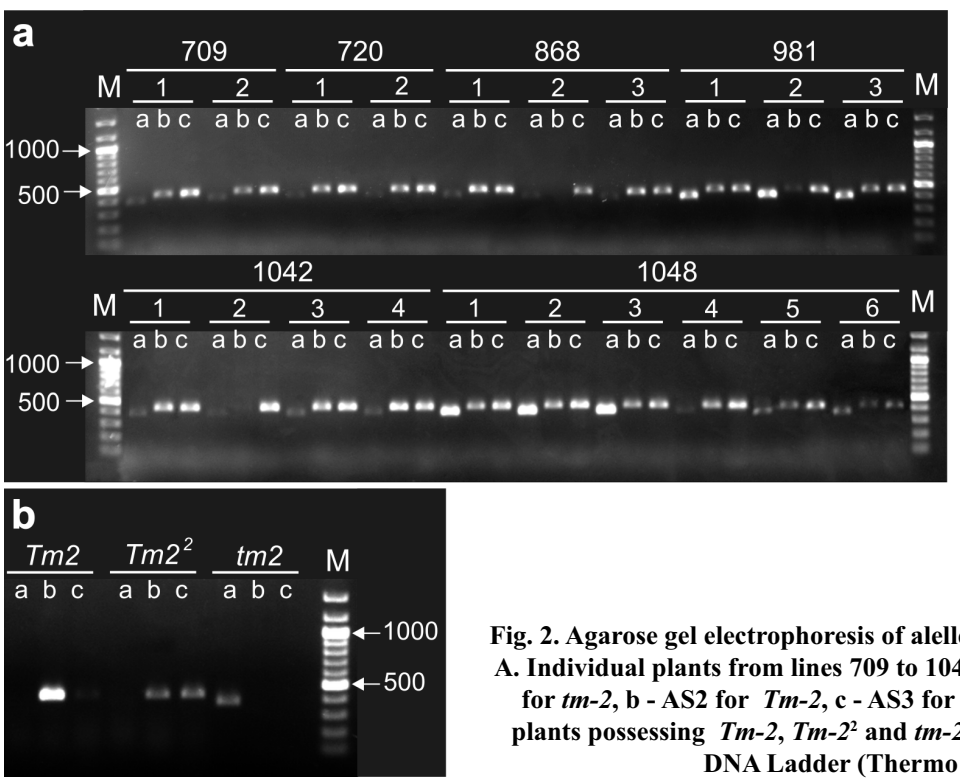

Fig. 2. Agarose gel electrophoresis of alelle-specific markers for *Tm-2* locus. A. Individual plants from lines 709 to 1048; 1-6: number of plants; a - AS1 for *tm-2*, b - AS2 for *Tm-2*, c - AS3 for *Tm-2*²; B. Homozygous control plants possessing *Tm-2*, *Tm-2*² and *tm-2*. M – GeneRuler™ 100 bp Plus DNA Ladder (Thermo Scientific Inc.)

2² (Figure 2; Table 3). Signals for homozygous genotypes (*Tm-2²/Tm-2²*) were produced for plant № 2 of line 868 and for plant № 2 of line 1042. The heterozygous genotype (*tm-2/Tm-2²*) was observed only for plant №2 of line 981. The all remaining plants showed signals with the three AS markers.

Discussion

Identification of genes for resistance and the ability to trace them in the progenies by MAS (Marker Assistant Selection) in order to create resistant varieties is of great importance. In our study we applied SCAR, CAPS and AS markers, linked to *Tm-1* and *Tm-2* loci for *ToMV* resistance, for screening of tomato breeding lines, created at Maritsa VCRI. These markers are able to distinguish the alleles of *Tm* loci as well as their zygotic state, which is of great importance for breeding direct varieties or F_1 hybrids.

The molecular analysis for *Tm-1* gene was negative for this gene in all tested resistant lines. *Tm-1* confers resistance to pathotype 0 and 2, but not to pathotype 1. However, if the genotype possess resistant allele of *Tm-2* locus, phenotypic expression of *Tm-1* would be masked in infection tests. Genotypes possessing the resistant allele of the both loci (*Tm-1 Tm-2*, *Tm-1 Tm-2²* or *Tm-1 Tm-2/Tm-2²)* will be resistant to all three pathotypes i.e. the phenotypic expression of such genotypes will be similar to that in *Tm-2²/Tm-2²* genotypes. That's why the utilization of specific marker for *Tm-1* gene would help to recognize its presence. In our case the lack of fragment or polymorphism using dominant or co-dominant SCAR markers resp. for *Tm-1* gene is a proof for its absence in the investigated germplasm. Currently, *Tm-1* is not used as a source for resistance in modern tomato breeding programs, because of the insufficient level of resistance. However, the combination of *Tm-1* with the dominant alleles of *Tm-2* locus is recommended by some authors (Pelham, 1972; Hall, 1980).

Resistant alleles of *Tm-2* locus confer better level of resistance compared to that of *Tm-1* gene. As a result, *Tm-2* and/or *Tm-2²* are widely introduced in the modern tomato varieties by the breeders.

Comparison of the results obtained with CAPS and AS marker for *Tm-2* locus in this study showed some discrepancies. The CAPS marker revealed the presence of *Tm-2²* allele in homozygous condition in 12 plants, but the AS markers found that 10 of these 12 plants to possess genotype *Tm-2/Tm-2²* and only two plants with homozygous (*Tm-2²/Tm-2²*) genotype (Table 3). The other observed discrepancy was that the heterozygous genotype (*tm-2/Tm-2²*), found in eight plants by CAPS marker, were confirmed only in two plants by AS markers. The remaining six plants, characterized by AS markers, were found to possess all three alleles – *tm-2*, *Tm-2* and *Tm-2²*.

Based on these results we concluded that *Tm-2* AS marker was able to amplify a fragment also in individual possessing *Tm-2²* allele. Genotype of such plants would combine the two dominant alleles in the locus – *Tm-2/Tm-2²*. Close observation on the sequences of primers for *Tm-2* locus reveal that forward primer Tm2R-f1c is common, whereas the reverse primers Tm2R-r3 and Tm2aR-r3 (Table 1) differs in only three nucleotides positioned medially. The positive signal for simultaneous presence of *Tm-2* and *Tm-2²* in our case in the same genotype could be explained with possible annealing of the reverse primers to the same regions of the DNA template.

Although existence of *Tm-2/Tm-2²* genotype is possible (Pelham, 1972 and Hall, 1980) it is hard to accept presence of the recessive and the two dominant alleles of *Tm-2* locus in one genotype as indicated in seven plants (Table 3) according to AS markers. The results obtained with AS2 showed false positives for presence of *Tm-2* allele which makes the marker inapplicable for the investigated germplasm. Because of the amplification of a fragment in *Tm-2²* genotypes in majority of tested individuals AS2 marker behaves more like the AS marker (Shi et al., 2011) with primer pair Tm2R-f1c/Tm2R-r4 that amplifies DNA from tomato genotypes containing either *Tm-2* or *Tm-2²* with homozygous or heterozygous condition, indicating the primer pair can be used to select either *Tm-2* or *Tm-2²* gene. Such ambiguous behavior of AS2 marker, according to received data, would not benefit much MAS breeding. In contrast, successful application of the marker in question for identification of the dominant (*Tm2*) allele and two AS markers for *tm2* is reported by Hudcovicova et al. (2015) in experiments with 184 tomato breeding lines. Authors were able to identify 133 homozygous individuals possessing *tm-2* allele, 33 individuals possessing both alleles and only four containing *Tm-2* in homozygous condition. Provided data from Hudcovicova et al. (2015) for AS markers for *tm2* and *Tm2* did not give information about their performance in germplasm possessing *Tm2²* allele.

Conclusions

The current study provides data for successful application of CAPS marker for genotyping the *Tm-2* locus as well as revealing the confusing behavior of AS2 marker during genotyping the Maritsa VCRI tomato breeding lines. These observations should be taken with care, when the choice of the marker for characterization of a germplasm for resistance to *ToMV* and its use for MAS is made. Obviously, it is useful breeders to test as many of the available markers for identification of resistance genes in order to identify the one that works best for their specific germplasm.

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