MOLECULAR AND GENETIC STUDIES ON TOMATO MOSAIC VIRUS RESISTANCE GENES *Tm-1, Tm-2* and *Tm-2²* IN SOME LOCAL AND EXOTIC TOMATO ACCESSIONS

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The production of tomato (*Lycopersicon esculentum* Mill) plants, like many other crops, is greatly affected with virus diseases. Several viruses have been found to infect tomato such as cucumber mosaic virus (CMV), tomato yellow leaf curl virus (TyCV) and tomato mosaic virus (ToMV) (Averre and Gooding, 2000). In cultivated tomato, ToMV infections are controlled by integrations of *Tm-1, Tm-2* and *Tm-2²* resistance (R) genes **(**Pelham, 1966; Hall, 1980: Strasser and Pfitzner, 2007**)**. The *Tm-1* gene was introgressed from the wild tomato species *Lycopersicon hirsutum* and mapped to chromosome 5 **(**Meshi *et al*., 1988**)**. Both the *Tm-2* and the *Tm-2²* resistance genes were introgressed from *L. peruvianum*. They are both located close to the centromer of chromosome 9 and are considered to be allelic (Khush *et al*., 1964; Pelham, 1966; Schroeder *et al*., 1967; Hall, 1980; Tanksley *et al*., 1992; Grube *et al.,* 2000).

Polymerase chain reaction (PCR) of genomic DNA, primed with arbitrary oligomers, has been used to generate random amplified polymorphic DNA (RAPD) markers for molecular mapping (Williams *et al*., 1990). To overcome the difficulties with band reproducibility found using standard RAPD, a technique called sequence characterized amplified regions (SCARs) was devised to amplify single bands corresponding to genetically defined loci (Paran and Michelmore, 1993; Adam-Blondon *et al*., 1994; Maisonneuve *et al*., 1994). SCAR markers represent a specified genomic region that can be amplified by PCR using a pair of specific oligonucleotide primers. RAPD markers can be converted into specific, stable, and reliable sequence characterized amplified region (SCAR) markers. A SCAR marker can be obtained by cloning the amplified RAPD band, sequencing its ends, and generating extended oligomer primers specific to the determined sequences. This strategy has been used successfully to generate markers for various traits in lettuce (Xu and Bakalinsky, 1996). SCAR markers are advantageous over RAPD markers because they are identified as distinct single bands in agarose gels, and some of them show co-

dominance which differentiates heterozygotes from both types of homozygote (Adam-Blondon *et al*., 1994). Motoyoshi *et al.* (1996) reported that the gene *Tm-2* (tomato mosaic virus (ToMV) resistant), which is tightly linked to a morphological marker gene *nv* (netted virescent), resides in a heterochromatic region near the centromere of chromosome 9 in tomato. *Tm-2* and *Tm-2*^{2} are known to be allelic, and exhibit similar phenotypes to each other, but can be differentiated by their response to different ToMV strains. An inoculation experiment demonstrated that *Tm-2* helped a mutant strain of ToMV to infect a heterozygous tomato (*Tm-2/Tm-2a*). Aiming at investigating the structures of DNA around these active genes and their influence on gene activities, identify and characterize RAPD markers linked to these genes tomato. Genetic analysis using 13 RAPD markers linked to the *Tm-2* locus and cytological analysis by fluorescence *in situ* hybridization (FISH) demonstrated that the lines resistant to ToMV had a large block derived from a chromosome of *Lycopersicon peruvianum*. Among these markers, they estimated that two, OPE16 (900) and OPN31 (1000), are nearest to the *Tm-2* locus. Out of the 13 markers, six distributed within about 0.7 centi-Morgan (cM), were cloned and sequenced to be converted markers of them; four were successfully converted to SCAR markers. The six clones were also used as probes for Southern hybridization of genomic DNA from near-isogenic lines (NILs) to characterize structures around the *Tm-2* locus. One clone was estimated to be derived from a sequence that was present in one copy. The other five clones appeared to be derived from different kinds of moderately or highly repetitive sequences.

In the present research work, attempts were made to identify three genes (*Tm-1*, *Tm-2 and Tm-2*^{2}) known to influence the resistance to tomato mosaic virus in 24 local and exotic tomato genotype, using specially designed SCAR markers.

MATERIALS AND METHODS

Plant material

Twenty four tomato accessions were used in this study, as summarized in Table (1). The plants were grown in 12.5 cm pots in Levington compost/sand (3:1) mixture, and transferred to larger pots as necessary. All accessions were kept under greenhouse conditions. To test for senesitivity to ToMV infection, virus isolates were multiplied in tobacco (*Nicotiana tabacum*) and purified by the method of Gooding and Hebert (1967), and were frozen until required. To inoculate plants, infected leaf tissue was ground in 0.1 M phosphate buffer (pH 7) and rubbed onto leaves powdered with carborundum dust. Tomato plants ranged from 10-20 cm tall were selected for inoculation with ToMV virus. Three to seven plants were randomly chosen for each eccession. Twenty four accessions was obtained in (ARC) Agriculture Research Center, Horticulture Research Institute El-Dokki, Egypt, (TGRC) Tomato Genetic Resource Center, Department of Vegetable Crops, California University, Davis, California,

USA and (NGB) Nordic Gene Bank, Sweden, showed in Table (1).

DNA extraction

DNA isolation and purification was carried out on fresh leaf tissues (1gm) using CTAB (Cetyl-tetramethyl ammonium bromide) method, according to Murray and Thompson (1980).

SCAR markers

SCA15 primers were (Ohmori *et al*., 1996) used to detect the *Tm-1* locus in the present tomato accessions (Table 2). The SCG09 primers were (Sobir *et al*., 2000) used to detect the *Tm-2* locus (Table 2). While, the TomvR1 and tomvR2 primers were (Dax *et al*., 1998) used to assay the *Tm-2*^{2} locus (Table 2).

PCR amplification

The 25 µl PCR reaction mixture contained: 1.0 µl (50ng template DNA), 1.0 µl dNTPs (10 mM), 2.5 µl Mg Cl_2 (25 mM), 2.0 µl 10X buffer (10 mM Tris, pH 8.0, 50 mM KCl and 50 mM ammonium sulphate), 2.0 µl of each primer (0.5 µM), 0.25 µl Taq polymerase (5 u/µl). The volume was brought up to 25 µl by nuclease-free water. The PCR condition involved initial denaturation cycle at 95°C for 2 min. followed by 35 cycles of amplification for cycles consisted of denaturation 94°C for 1 min, annealing for 1 min at different annealing temperatures were used for each primer set and primer extension at 72°C for 2 min. A final extension at 72°C for 5 min was carried out.

RESULTS

Preliminary evaluation

Normal rather resistant reaction (symptomtess) was encountered for two accessions, rather tolerant reaction, with mild mosaic symptoms was detected for twenty one accessions, and rather susceptible reaction, with severe mosaic symptoms was detected for only one accession showed in Table (3).

SCAR marker linked to Tm-1 locus

DNA extracted from the 24 accessions was subjected for this analysis. Figure (1) illustrates the results obtained from the SCAR screening for the *T-m1* gene using the specific primer SCA15 for the present 24 accessions. Upon PCR amplification (using annealing temperature of 65°C), one band was observed for the accession LA 3276 only among all samples tested, the amplified product was approximately 1000 bp in length according to analysis by Phoretix program 1D gel analysis software version 4.01. Thus, it can be concluded that only the *L. esculentum* LA 3276 accession has the *T-m1* gene, while the rest of the accessions examined do not have this gene.

SCAR marker linked to Tm-2 locus

Electrophoretic patterns of the fragments amplified by PCR using the specific primer SCG09 are shown in Fig. (2). Upon PCR amplification (using annealing temperature of 65°C), one band was observed as shown in Fig. (2), the

amplified product was found to be approximately 700 pb in length according to analysis by Phoretix program 1D gel analysis software version 4.01. All tomato accessions examined showed one band at the right molecular weight, expect *L. pimpinellifolium* that produced a faint and non specific band (Fig. 2).

SCAR marker linked to Tm-2^2 locus

PCR assay, using the primer pair tomvR1 and tomvR2 specific to the *Tm-2^2* locus, are shown in Fig. (3). Upon PCR amplification (using an annealing temperature of 55°C) two bands were observed, but only the slower migrating band (950 bp) was found to be linked to *T-m2^2* gene (Fig. 3). This 950 bp fragment was observed in all tomato accessions tested, except the accession of *L. hirsutum* (11).

PCR amplification products of SCAR marker linked to the *Tm-2^2* gene were digestion with *Hind* III restriction enzyme as shown in Fig. (4). Accordingly, the homozygous genotype will show the intact fragment (950 bp) alone the heterozygous; will show the intact fragment in addition to several smaller fragments (150-800 bp) and the homozygous recessive will show only the smaller fragments. Absence (-) of the *Tm-2^2* allele can be detected by the presence of several *Hind* III recognition sites resulting in fragment ranging in size between 150-800 bp of the original 950 bp SCAR fragment. The present twenty four tomato accessions were genotyped for these three genes as shown in Table (4).

DISCUSSION

To gain an insight into the processes underlying virus disease resistance in tomato, the three known tomato mosaic virus (ToMV) resistance genes, *Tm-1, Tm-2* and *Tm-2^2*, were monitored in twenty four local and exotic tomato accessions. Specific SCAR markers linked to tomato mosaic virus resistance genes *Tm-1, Tm-2 and Tm-2^2* were obtained. Since SCAR markers are more easily detected than restriction fragment length polymorphism (RFLP) markers and are more distinct than RAPD markers (Paran and Michelmore 1993). The result of SCAR marker linked to *T-m1* gene is congruent with that of Ohmori *et al.* (1996), who found that the SCA15 primer expresses itself as a distant single band. The result of SCAR primer SCG09 which is linked to *T-m2* gene are in good agreement with that of Sobir *et al.* (2000), who reported that the main use of this SCAR marker is to identify the *Tm-2* locus to facilitate the breeding of ToMV resistant tomatoes.

The result of SCAR primer tomvR1 and tomvR2 linked to *Tm-2^2* durable gene congruent with that of Dax *et al*. (1998), Jing *et al.* (2004) and Ming *et al.* (2005). However, PCR amplification with these specific primers resulted in an amplified one band (SCAR) in susceptible, tolerant and resistant tomato. The amplified band from the susceptible lines could, however, be discerned from that of the resistant ones after cleavage with the restriction enzyme *Hind* III. This method enables the distinction of homo-

zygous and heterozygous individual plants, and provided a convenient and rapid genotyping assay method. These SCAR markers have clearly identified ToMV resistant, tolerant and susceptible tomatoes (Table 3). This marker turned to be a convenient tool, facilitate the selection for resistant genotypes at the first leaf stage of development. Similar studied detection of virus resistant genes in survey cultivated and wild-type tomato accessions (Grube *et al.,* 2000; Lanfermeijer *et al.,* 2005; Cillo *et al.,* 2007).

The exotic accession GCR311 (LA 3276) proved to be homozygous to *Tm-2*^{2} thus it is recommended to include such genotype in tomato breeding programs for virus resistance. Since the *Tm-2*^{2} homozygous accessions is currently the most widely utilized in greenhouse tomato cultivars breeding (Cillo *et al.,* 2007). Lanfermeijer *et al.* (2005) indicated that virulent strains may be readily established when *Tm-1* or *Tm-2* is used, but *Tm-2*^{2} confers more effective resistance. The *Tm-2* gene, like the *Tm-2*^{2} gene, encodes an 861 amino acid polypeptide, which belongs to the coiled coil/nucleotide binding site/leucine-rich repeat (CC-NBS-LRR) class of resistance proteins. The *Tm-2*^{2} and *Tm-2* open reading frames only differ in seven nucleotides, which, on a proteins (Gad and John, 2006). The *Tm-2*^{2} and *Tm-2* open reading frams only differ in seven nucleotides, which, on protein level, results in four amino acid differences, of which two are located in the nucleotide binding site and two are located in the leucine-rich repeat domain (Lanfermeijer *et al.,* 2005*).* Then the small difference between the two proteins may reflect a highly similar interaction of these proteins with the virus, which has major implications for the concept of durability.

SUMMARY

Three genes *Tm-1, Tm-2 and Tm-2*^{2} are known to confer resistance to tomato mosaic virus (ToMV) in tomato (*Lycopersicon esculentum* Mill) plants. A polymerase chain reaction (PCR) based codominant marker was developed to be linked to these genes (*Tm-1, Tm-2 and Tm-2*^{2}) using sequence characterized amplified region (SCAR) markers. The polymorphic markers co-segregated with susceptibility or resistance, as determined by biological assays for ToMV resistance. Accordingly, the homozygous genotype will show the intact fragment (950 bp) alone the heterozygous; will show the intact fragment in addition to several smaller fragments (150-800 bp) and the homozygous recessive will show only the smaller fragments. This method enables the distinction of homozygous and heterozygous individual plants in segregating populations for *Tm-2*^{2}, and provides a convenient and rapid assay for mosaic virus resistance to be used in tomato breeding programs and hybrid seed production.

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Table (1): The twenty four tomato accessions used in the present study.

No.	Accession	Source	No.	Accession	Source
1	Castle Rock	Egypt (ARC)[1]	13	Diamond	Egypt (ARC)
2	Strain B	Egypt (ARC)	14	Bonita (LA 11704)	Sweden (NGB)[2]
3	Super strain B	Egypt (ARC)	15	Tempo (LA 2050)	Sweden (NGB)
4	Edkawi	Egypt (ARC)	16	Regina (LA 11906)	Sweden (NGB)
5	Jubilee	Egypt (ARC)	17	Ailsa Craig (LA2838A)	USA (TGRC)[3]
6	Money maker	Egypt (ARC)	18	GCR626(LA 3297)	USA (TGRC)
7	Peto 86	Egypt (ARC)	19	GCR311(LA 3276)	USA (TGRC)
8	Ace	Egypt (ARC)	20	GCR267(LA 3273)	USA (TGRC)
9	Pritchard	Egypt (ARC)	21	GCR 236(LA 3268)	USA (TGRC)
10	UC 90	Egypt (ARC)	22	TY 52 (LA 3473)	USA (TGRC)
11	*L. hirsutum*	Egypt (ARC)	23	LA 1193	Egypt (ARC)
12	*L. pimpinellifolium*	Egypt(ARC)	24	Relian	Egypt (ARC)

1- (ARC) Agriculture Research Center, Horticulture Research Institute, El-Dokki, Egypt.
2- (TGRC) Tomato Genetic Resource Center, Department of Vegetable Crops, California University, Davis, California, USA.
3- (NGB) Nordic Gene Bank, Sweden.
4- (LA) line.

Table (2): The twenty four tomato accessions used in the present study and their reaction to ToMV inoculation.

No.	Accession	Reaction to ToMV inoculation
1	Castle Rock	Rather tolerant
2	Strain B	Rather tolerant
3	Super strain B	Rather tolerant
4	Edkawi	Susceptible
5	Jubilee	Rather tolerant
6	Money maker	Rather tolerant
7	Peto 86	Rather tolerant
8	Ace	Rather tolerant
9	Pritchard	Rather tolerant
10	UC 90	Rather tolerant
11	*L. hirsutum*	Rather resistant
12	*L. pimpinellifolium*	Rather tolerant
13	Diamond	Rather tolerant
14	Bonita (LA 11704)	Rather tolerant
15	Tempo (LA 2050)	Rather tolerant
16	Regina (LA 11906)	Rather tolerant
17	Ailsa Craig (LA2838A)	Rather tolerant
18	GCR626(LA 3297)	Rather tolerant
19	GCR311(LA 3276)	Rather tolerant
20	GCR267(LA 3273)	Rather resistant
21	GCR 236(LA 3268)	Rather tolerant
22	TY 52 (LA 3473)	Rather tolerant
23	LA 1193	Rather tolerant
24	Relian	Rather tolerant

Table (3): Nucleotide sequences of the used in the present study.

Primer name	Sequence	Annealing Temp.
SCA15 (F)	5'CCG AAC CCC TTA AAA ATA GTT TCA 3'	65°C
(R)	5'CCG AAC CCA ATC AGG AGG CTC ATA 3'	
SCG09 (F)	5' CTG ACG TCA CCT TGT TGG TCT TTA 3'	65°C
(R)	5' CTG ACG TCA CTT AGC TTT GTA AAC 3'	
tomvR1 (F)	5' CAC CTT TCC CTC TCC AA 3'	55°C
tomvR2 (R)	5' CAC CTT TCC CCT AAA GC 3'	

Table (4): The presence of *Tm-1*, *Tm-2*, and *Tm-2²* genes in the 24 accessions and their reaction to ToMV inoculation at greenhouse conditions.

No.	Accession	Genotypes			Reaction to ToMV inoculation
		Tm-1	*Tm-2*	*Tm-2²*	
1	Castle Rock	-/-	+/?	+/-	Tolerant
2	Strain B	-/-	+/?	+/-	Tolerant
3	Super strain B	-/-	+/?	+/-	Tolerant
4	Edkawi	-/-	+/?	-/-	Susceptible
5	Jubilee	-/-	+/?	+/-	Tolerant
6	Money maker	-/-	+/?	+/-	Tolerant
7	Peto 86	-/-	+/?	+/-	Tolerant
8	Ace	-/-	+/?	+/-	Tolerant
9	Pritchard	-/-	+/?	+/-	Tolerant
10	UC 90	-/-	+/?	+/-	Tolerant
11	*L. hirsutum*	-/-	+/?	+/+	Resistant
12	*L. pimpinellifolium*	-/-	-/-	+/-	Tolerant
13	Diamond	-/-	+/?	+/-	Tolerant
14	Bonita (LA 11704)	-/-	+/?	+/-	Tolerant
15	Tempo (LA 2050)	-/-	+/?	+/-	Tolerant
16	Regina (LA 11906)	-/-	+/?	+/-	Tolerant
17	Ailsa Craig (LA2838A)	-/-	+/?	+/-	Tolerant
18	GCR626(LA 3297)	-/-	+/?	+/-	Tolerant
19	GCR311(LA 3276)	+/?	+/?	+/+	Resistant
20	GCR267(LA 3273)	-/-	+/?	+/-	Tolerant
21	GCR 236(LA 3268)	-/-	+/?	+/-	Tolerant
22	TY 52 (LA 3473)	-/-	+/?	+/-	Tolerant
23	LA 1193	-/-	+/?	+/-	Tolerant
24	Relian	-/-	+/?	+/-	Tolerant

+ = present - = absent ? = undetected +/+ = Resistant.

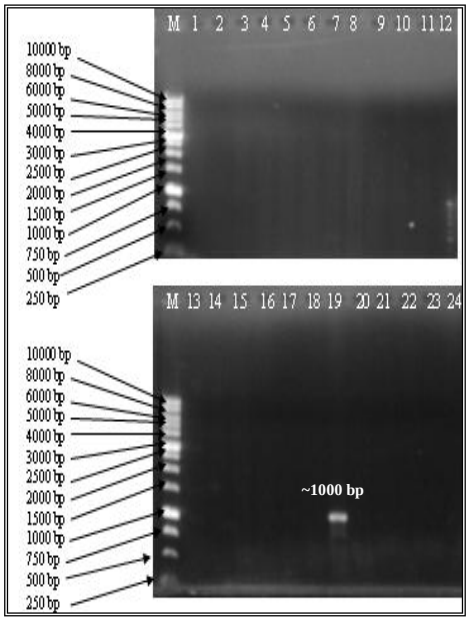

Fig. (1): SCAR screening of the genomic tomato DNA for *Tm-1* gene.

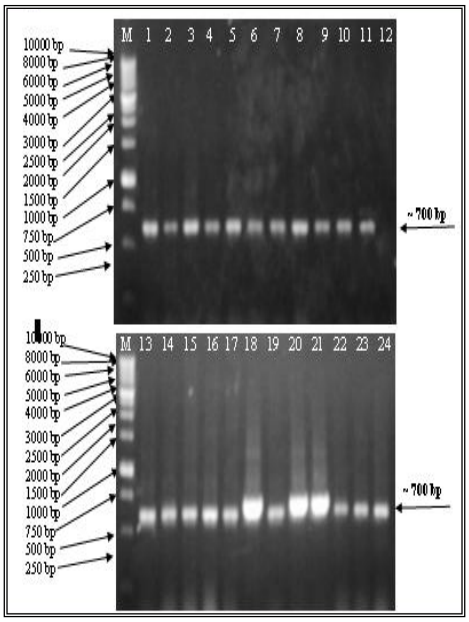

Fig. (2): SCAR screening of the genomic tomato DNA for *Tm-2* gene.

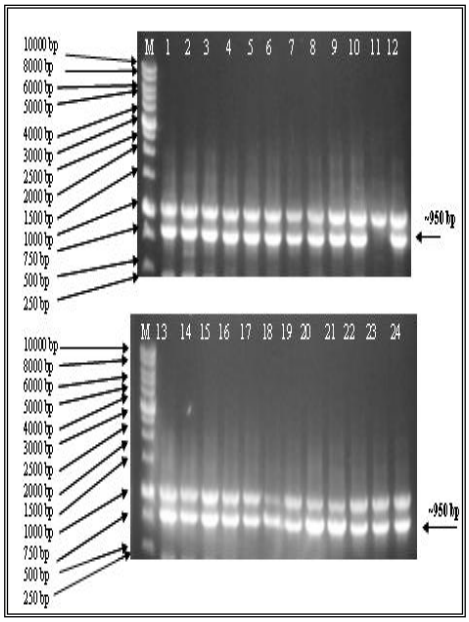

Fig. (3): SCAR screening of the genomic tomato DNA for *Tm-2*^{2} gene.

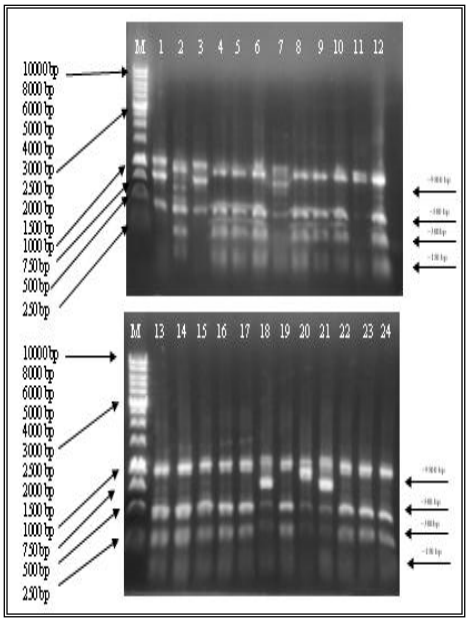

Fig. (4): SCAR markers analysis for *Tm-2*^{2} after digestion with *Hind* III.

M: 1 kb DNA marker, 1: Castle Rock, 2: Strain B, 3: Super Strain B, 4: Edkawi, 5: Jubilee 6: Money maker, 7: Peto 86, 8: Ace, 9: Pritchard, 10: UC 90, 11*: L.hirsutum*, 12: *L. pimpinellifolium,* 13: Diamond, 14: Bonita, 15: Tempo, 16: Regina, 17: LA 2838A, 18: LA 3297, 19: LA 3276, 20: LA 3273, 21: LA 3268, 22: LA 3473, 23: LA 1193, 24: Relian