

Molecular Confirmation of Intraspecific Tomato (*Solanum lycopersicum*) Hybrids and Their Evaluation Against Late Blight and Cucumber Mosaic Virus

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Abstract Tomato is one of the most consumed vegetables in the world. Diseases are the number one concern in the development of high-yield and disease-resistant tomato hybrids which is the foremost priority of breeders. Present study was conducted (1) to develop DNA-based markers for genetic confirmation of tomato F₁ hybrids, (2) to utilize sequenced characterized amplified region (SCAR) marker linked to the *Ph-3* gene for *Phytophthora infestans* resistance in tomato and (3) to evaluate male and female parental genotypes and their F₁ hybrids against late blight (LB) and cucumber mosaic virus (CMV). For molecular studies, 58 previously reported markers including RAPDs (10), SCAR (01), EST-SSR (01) and SSR (46) were applied. The SCAR marker clearly differentiated the LB3 and LB4 from Roma and T-1359 and provided evidence for *Ph-3* gene. The SCAR marker was able to confirm the *Ph-3* gene in the hybrids Roma × LB4, Roma × LB3, Riogrande × LB2, Riogrande × LB3 and Roma × LB7. Out of several tested primers, SSR-22 proved useful for genetic confirmation of F₁ hybrid TMS1 × Money Maker (MM). For LB, tested hybrids/genotypes were ranked as susceptible to highly susceptible with different infection percentage (IP). However, the pace of symptom development was slower in hybrid Rio × LB2, 45% IP after 10 days of inoculation compared with 85% disease in one of the parent genotypes (Riogrande). None of the tested

genotypes was found resistant; however, TMS1 responded as tolerant against CMV using mechanical inoculation. Under natural field conditions, TMS1 was found resistant while hybrids TMS1 × Naqeeb and TMS1 × MM were tolerant where as others were found to be susceptible. In conclusion, all tomato hybrids were genetically confirmed using DNA-based markers. SCAR marker was useful for marker-assisted confirmation of the *Ph-3* gene in parental lines and hybrids; however, this gene was unable to provide protection against the local population of *P. infestans*.

Keywords CMV · Late blight · RAPD · SSR · URPs *Ph-3* gene

Introduction

Tomato is one of the most important *Solanaceous* vegetable crops and is grown all over the world [10]. The cultivated tomato, *Solanum lycopersicum* L. (formerly *Lycopersicon esculentum* Mill.), is the second most consumed vegetable in the world after potato [6]. Across the world, different types of diseases are the major issue to fresh market and processing tomato industries. The tomato is susceptible to more than two hundred diseases caused by bacteria, nematodes, pathogenic fungi and viruses [13]. However, disease resistance has been identified and in various cases characterized for over 30 major diseases of tomato [6]. Most commercial cultivars of tomato have up to 6 (in true breeding lines) or 10 (in hybrids) disease-resistance characteristics. Except in a few cases, mostly the wild tomato species have been used as the disease resistance source [6].

Late blight (LB) is a major disease of tomato and potato. The oomycete *Phytophthora infestans* that causes

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LB in tomato can rapidly destroy the crop at any time during ontogeny [18]. Unfortunately, most of current *P. infestans* isolates are resistant to the only available fungicide metalaxyl to combat the disease [8]. Therefore, use of at least partially resistant cultivars is the most promising strategy to control LB [18]. Genes have been identified for race-specific resistance in wild tomato species, in particular in *Solanum pimpinellifolium*. *Ph-1* and *Ph-2* genes conferring resistance to *P. infestans* race 0 and 1, respectively, were identified in *S. pimpinellifolium* and subsequently introduced into cultivated varieties [6]; however, the resistance was overcome by new *P. infestans* isolates. The *Ph-3* gene was identified in *S. pimpinellifolium* accession L3708 (PI365957) (AVRDC 1994). This gene is partially dominant and confers resistance to a wide range of *P. infestans* isolates that have overcome *Ph-1* and *Ph-2* genes and was introduced into market tomato as well as tomato lines for processing industry [12]. *Ph-3* is the first cloned tomato gene for resistance to late blight and encodes a CC-NBS-LRR protein [20]. The *Ph-3* gene was mapped to the long arm of chromosome 9 in close proximity of RFLP marker TG591A [4]. One of six AFLP markers linked to the *Ph-3* gene [4] was converted successfully into a dominant SCAR marker which was reported to discriminate resistant and susceptible individuals with high stringency. The developed SCAR marker could be used for the marker-assisted selection in tomato breeding programs (P. H. [17]. Recently, *Ph-3* on-gene cleaved amplified polymorphic sequence (CAPS) marker, Ph3.gsm/HincII, able to differentiate variable susceptible alleles was developed [19].

DNA-based markers are valuable tools for plant breeding since the presence of genes can be checked at early developmental stage without phenotypic evaluations. Moreover, genetic confirmation of hybrids is also considered mandatory. Present study was conducted with the following objectives (1) to develop DNA-based markers for genetic confirmation of tomato F₁ hybrids, (2) to utilize sequenced characterized amplified region (SCAR) marker linked to the *Ph-3* gene for *Phytophthora infestans* resistance in tomato and (3) to evaluate parental genotypes and hybrids against LB and CMV.

Materials and Methods

Plant Material

Promising F₁ hybrids, their parental lines and commercial hybrids (as check) were used for molecular studies and disease response to LB and cucumber mosaic virus (CMV).

Table 1 Plant material used for molecular studies

Sr. No.	Material used for LB	Material used for CMV
1	Roma × LB3	TMS1 × Naqeeb
2	Roma × LB4	TMS1 × Money Maker
3	LB2	TMS1
4	LB3	Naqeeb
5	Roma	Money Maker
6	Riogrande × LB2	T-1359 F ₁ (check)
7	Riogrande × LB3	Salar F ₁ (check)
8	Roma × LB7	
9	Riogrande	
10	LB4	
11	LB7	
12	T-1359 F ₁ (check)	

Details of plant materials used for LB and CMV are presented in Table 1.

P. infestans Multiplication, Inoculation and Plant Material Evaluation Against LB

Fifteen tomato hybrids and genotypes were grown under low tunnel at the Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan, following the method described by Akhtar et al. [1]. A *P. infestans* isolate was obtained from naturally infected tomato plants at NIAB, Faisalabad, Pakistan, by transferring LB-infected tissues to PARP medium. The isolate of *P. infestans* was grown on V8 agar at 20 °C for 15 days, and then, the sporangia were harvested by flooding the plate with sterile distilled water. For zoospore production, sporangial suspension was kept at 4 °C for 1 h [16]. Final pathogen concentration was adjusted to 5000 zoospores per mL using a hemocytometer and sprayed on to 6–8-week-old plants of test genotypes. There were three biological replicates for each genotype and each replication comprised of three plants as technical replication per repeat. Plants were observed daily for disease development. Infection percentage (IP) of infected plants was visually observed by using a 0–5 scale described previously [3], where, 0 = no visible symptoms apparent (IP 0; immune); 1 = a few minute lesions to about 10% of the total leaf area is blighted and usually confined to the two bottom leaves (IP 0.01–10; highly resistant); 2 = leaves on about 25% of the total plant area are infected (IP 10.01–25, resistant); 3 = leaves on about 50% of the total plant area are infected (IP 25.01–40, tolerant); 4 = leaves on about 75% of the total plant area are infected (IP 40.01–60, susceptible) and 5 = leaves on whole plant are blighted and plant is dead (IP > 60.01, highly susceptible).

Evaluation of Genotypes Against CMV in Glasshouse

The inoculum of CMV for the mechanical inoculation was obtained from naturally infected tomato plants of the cultivated tomato variety Nagina and maintained in the glasshouse. Tomato leaves of susceptible check genotype Nagina with typical shoestring disease symptoms were ground in 0.02 M phosphate buffer, pH 7.4 (1 g/ml), with a pestle and mortar and squeezed through muslin cloth. Five plants of each test and check hybrids along with parents (Table 1) were grown in the glass house following CRD with three repeats. It means, there were five biological replicates for each genotype and each replication comprised of three plants as technical replication per repeat. Young leaves of four-week-old plants were dusted with 500-mesh carborandum powder and were mechanically inoculated with freshly extracted sap using cotton pads. Plants were rinsed gently with a stream of water just after inoculation to remove superfluous inoculation and were kept under insect free cages for symptoms development. The presence or absence of CMV in the test genotypes, i.e., hybrids, parent lines and check hybrid variety was assayed by double antibody sandwich procedure (DAS-ELISA) as described previously [5] with commercial polyclonal antibodies to CMV. Data were recorded 90 days after inoculation following five points on a scale from 0 to 4 of disease severity index (SI) described previously (K. P. [2]).

Evaluation of the Genotypes for CMV Response Under Field Conditions

Four- to six-inch-long nursery seedlings of seven genotypes/hybrid were transplanted to the tomato breeding field of NIAB, Faisalabad, in November, 2012, following randomized complete block design (RCBD) with three replications. The experiment was set up keeping plant to plant distance at 0.5 m and bed to bed distance at 1.5 m. Each genotype had seven plants in each replication. No plant protection measures were applied against the Aphid (*Myzus persicae*) to have the high inoculum pressure throughout the experiment. The presence or absence of CMV in the test genotypes/hybrids, parent lines and check hybrids was assayed as mentioned earlier for glasshouse experiment.

Molecular Marker Analysis

Total genomic DNA was isolated from 200 mg (sample) of young leaf tissues harvested from seedlings by using quick SDS-based method described previously [9]. Concentration and purity of extracted DNA was checked through spectrophotometer (U-2800 spectrophotometer) at the absorbance of 260 and 280 nm. Using the extracted DNA,

dilutions were prepared for subsequent PCR analysis. PCR was performed using several primers that include RAPDs, sequenced characterized amplified region (SCAR) marker, EST-SSR and SSR markers (Table S1).

RAPD Analysis

RAPD primers previously reported [21] for LB gene *Ph-3* (RAPD-CCPB272) and other primers were tested. PCR mixtures were prepared with the final volume of 20 μ l containing 2 μ l of 10x PCR buffer, 2 μ l of 2 mM dNTPs, 1.2 μ l of 25 mM $MgCl_2$, 0.20 μ l of (5 unit/ μ l) Taq polymerase, 0.4 μ l of 20 μ M primer and 4 μ l of (25 ng/ μ l) template DNA. A final volume of 20 μ l was adjusted with double distilled deionized water. PCR amplifications were performed for 40 cycles using thermal cycler (Bio-Rad T-100, USA) with initial denaturation at 94 °C for 3 min, denaturation at 94 °C for 1 min, primer annealing at 35 °C for 1 min, primer extension at 72 °C for 2 min and final extension at 72 °C for 10 min. Amplification products were separated on agarose gels and fragments were detected by ethidium bromide staining.

Sequenced Characterized Amplified Region (SCAR) Marker Analysis

The AFLP marker converted to SCAR marker linked to *Ph-3* gene for LB resistance in tomato was used [6]. PCRs were performed in 20- μ l reaction volume containing the following components: 100 ng of template DNA, 200 μ M of each of the four dNTPs, 1x Taq polymerase buffer, 1 unit Taq polymerase, 1.5 mM $MgCl_2$ and 0.4 μ M each of the two primers. Amplifications were performed in a Bio-Rad T-100 gradient thermal cycler (Bio-Rad Laboratories, USA). Initial denaturation was performed at 94 °C for 3 min followed by 35 cycles with denaturation at 94 °C for 30 s, annealing at 53 °C for 1 min and extension at 72 °C for 1 min. The final extension was done at 72 °C for 10 min. Amplification products were separated on non-denaturing 6% polyacrylamide gels and fragments were detected by ethidium bromide staining. Gels were documented using UVI pro Platinum 1.1 System and UVI-BandMap software (UVItec Ltd., Cambridge, UK).

SSR Analysis

A total of 46 SSR markers (Table S1) were screened using all genotypes included in the study. PCRs were performed in 20- μ l reaction volume containing the following components: 100 ng of template DNA, 200 μ M of each of the four dNTPs, 1x Taq polymerase buffer, 1 unit Taq polymerase, 1.5 mM $MgCl_2$ and 0.4 μ M each of the two primers. Amplifications were performed in a Bio-Rad T-100

gradient thermal cycler (Bio-Rad Laboratories, USA). Initial denaturation was performed at 94 °C for 3 min followed by 35 cycles with denaturation at 94 °C for 30 s, annealing at 50–60 °C (depending upon the primer pair) for 35 s and extension at 72 °C for 1 min. The final extension was done at 72 °C for 5 min. Amplification products were separated on non-denaturing 6% polyacrylamide gels, and fragments were detected by ethidium bromide staining. Gels were documented using UVI pro Platinum 1.1 System and UVIBandMap software (UVItec Ltd., Cambridge, UK). Molecular weight marker by BioBasic GM305 and Fermentas SM0243 were used as standards.

Results and Discussion

Evaluation Against LB

It is difficult to detect whether levels of *P. infestans* are low in the field, and by the time the disease is detected, it is often too late to save the crop through fungicide application [7]. In addition, most current *P. infestans* isolates are resistant to metalaxyl, the only available fungicide to combat the disease [8]. However, fungicides are expensive and harmful to the environment and humans. Thus, the use of partially resistant cultivars is the most promising method to control LB [18].

All the tested hybrids/genotypes were susceptible to highly susceptible with different infection percentage (IP) values ranging from 45 to 85% (Table 2). LB symptoms

started on all hybrids and genotypes in the form of small lesions on lower leaves after 3–5 days of inoculation followed by severe blight symptoms ranging from 50 to 85% IP values within 10 days and became highly susceptible within 15 days with IP values 75 to 95% with severe stem infection on all plants except for hybrid Rio × LB3 (Table 2). The pace of symptoms development was much slower in hybrid Rio × LB3 which showed 45% IP value after 10 days of inoculation, while one of parental genotype (Rio) for this hybrid had 85% IP at this time. Disease symptoms were confined to 64% of plant part of this hybrid with medium stem infection. However, other hybrids and genotypes showed 75–95% IP with severe stem infection. This level of symptoms prevails till the end of experiment (i.e., 90 days post-inoculation). The infection percentage (45%) conferred by Rio × LB3 strongly suggested the possibility of new allelic combination, due to which the susceptibility level was decreased toward lower ranks of susceptibility slab (40–60%) (Table 2). In this hybrid (Rio × LB3), male parent LB3 contained the LB-resistant gene *Ph-3* (Fig. 1) that is thought to be the strongest LB-resistant gene identified in tomato and transferred to several fresh market tomato breeding lines as well as commercial hybrid cultivars [15]. However, selected isolates of *P. infestans* have displayed virulence when conferred with *Ph-3* [14]. These studies strongly support our findings that *Ph-3* was unable to provide complete protection against the local population of the *P. infestans* (Table 2). However, depending on the genetic background this gene was able to reduce the pace of disease development with lower infection percentage during the initial period of infection.

Table 2 Response of tomato hybrids and genotypes against LB under whole plant assay

Sr. #	Hybrid/genotypes	After 10 days		After 20 days	
		Infection % age	Disease response	Infection % age	Disease response
1	Riogrande × LB2	50	S	85	HS
2	Riogrande × LB3	45	S	64	HS
3	Roma × LB3	52	S	80	HS
4	Roma × LB4	77	HS	95	HS
5	Roma × LB7	58	S	85	HS
6	LB2	63	HS	79	HS
7	LB3	65	HS	75	HS
8	LB4	61	HS	80	HS
9	LB7	62	HS	80	HS
10	Money Maker	73	HS	89	HS
11	Naqeeb	73	HS	88	HS
12	Riogrande	85	HS	95	HS
13	Roma	78	HS	95	HS
14	TMS1	78	HS	92	HS
15	T-1359 F ₁ (check)	55	S	80	HS

Fig. 1 Eletrophorogram showing SCAR marker profiles of parental genotypes and F₁ hybrids resolved on 6% PAGE followed by ethidium bromide staining

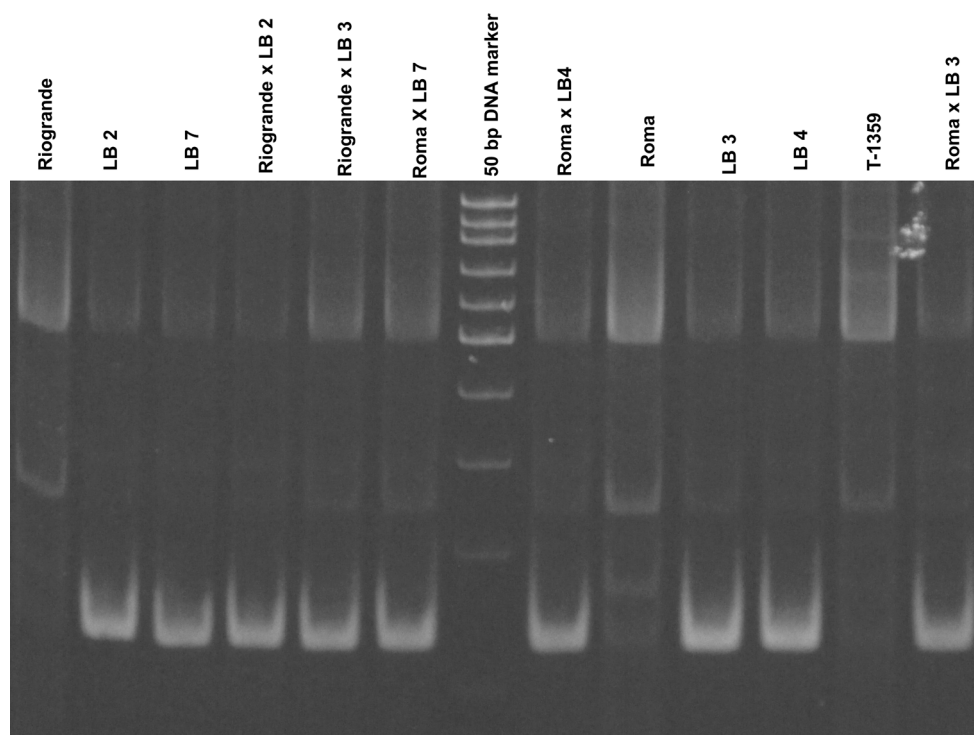


Table 3 Response of tomato genotypes against CMV using mechanical inoculation and under natural field conditions

Genotype	Mechanical inoculation		Natural field conditions	
	Severity index	Disease response	Severity index	Disease response
TMS1 × Naqeeb	3	Susceptible	2.3	Tolerant
TMS1 × Money Maker	3	Susceptible	2.3	Tolerant
TMS1	2.3	Tolerant	1.4	Resistant
Naqeeb	3.7	Highly susceptible	3	Susceptible
Money Maker	3.8	Highly susceptible	3	Susceptible
T-1359 F ₁ (check)	3.8	Highly susceptible	3	Susceptible
Salar F ₁ (check)	3.7	Highly susceptible	3	Susceptible

Evaluation Against CMV

None of tested genotypes was found resistant against CMV using mechanical inoculation. However, TMS1 responded as tolerant (Table 3). All genotypes were systemically infected with virus. Disease transmission was 100% for all tested genotypes as all inoculated plants produced diseased symptoms and reacted positively with the antiserum against the capsid protein of CMV in the TAS-ELISA. Initially, disease appeared as mild mosaic in all tested genotypes/hybrids 12 days post-inoculation which progressed into severe mosaic or mottling, leaf deformity, shoestringing. No reduction in disease severity was recorded till the end of the experiment.

A total of seven genotypes and hybrids evaluated under natural field conditions showed a wide range of disease

severity against shoestring disease caused by CMV (Table 3). Genotype TMS1 was found resistant, while hybrids TMS1 × Naqeeb and TMS1 × MM were tolerant and the remaining genotypes were susceptible.

Evaluation Through Molecular Markers

The greatest contribution of modern plant breeding to tomato improvement has been the development of cultivars with improved disease resistance [6]. Race-specific resistance genes have been identified in wild tomato species, in particular *S. pimpinellifolium*. In addition, race non-specific resistance has been reported in *S. pimpinellifolium* accession L3707 (PI365951) [11]. The *Ph-3* gene was mapped to the long arm of chromosome 9 close to RFLP marker TG591A. Park et al. [17] have successfully converted one

of six AFLP markers linked to the *Ph-3* gene [4] into a dominant SCAR marker. In the present study, this SCAR marker was used for confirmation of the LB resistance gene in the parental genotypes and then for its transfer in hybrids. The SCAR marker clearly differentiated LB3 and LB4 from Roma and T-1359 (Fig. 1). The results showed that the SCAR marker is reliable and specific to resistance gene *Ph-3*. Subsequently, this marker was used for marker-assisted confirmation of *Ph-3* gene transfer in the hybrids. The SCAR marker was able to confirm the transfer and presence of LB resistance gene *Ph-3* in the hybrids, i.e., Roma × LB4 and Roma × LB3 (Fig. 1). The results provided evidence that SCAR marker was useful for LB resistance breeding in tomato. Moreover, a RAPD marker was also polymorphic among LB-tolerant and LB-susceptible parental genotypes (Fig. 2). This primer confirmed the hybrids and resistance gene transfer from LB parents into F₁ hybrids derived from susceptible genotype Roma. This was further confirmation of results by SCAR marker.

In addition to the above-mentioned genotypes, results also confirmed the presence of *Ph-3* gene for LB resistance in male parent lines, i.e., LB2, LB3 and LB7 (Fig. 1). The SCAR marker confirmed the transfer of the *Ph-3* gene in the respective hybrids, i.e., Riogrande × LB2, Riogrande × LB3, Roma × LB4 and Roma × LB7. The SCAR marker was also applied to genotypes used for CMV resistance. The results provided marker-assisted confirmation of *Ph-3* gene for LB resistance in male sterile line

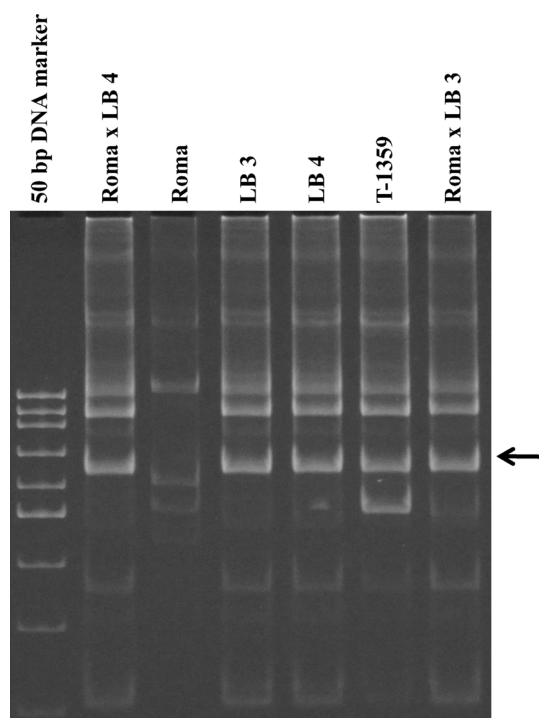


Fig. 2 Electrophorogram showing profiles of parental genotype and F₁ hybrids using RAPD primer, OPU-3 resolved on 1.5% agarose gel

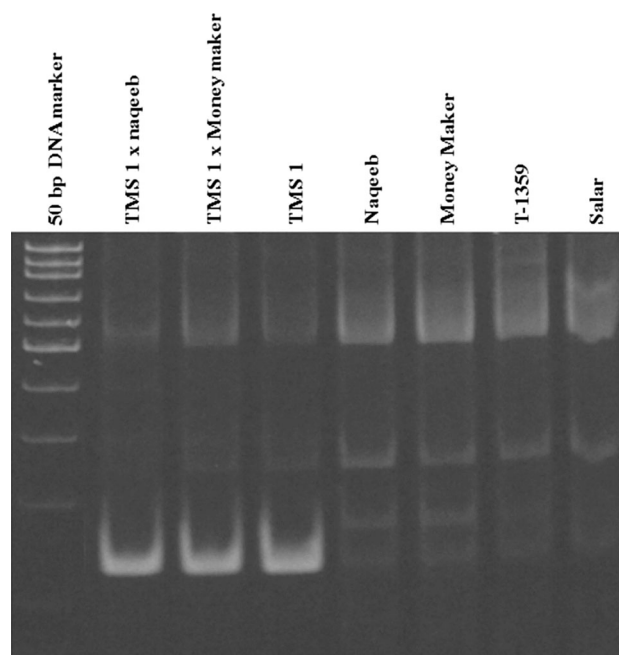


Fig. 3 Electrophorogram showing SCAR marker profiles of parental genotypes and F₁ hybrids resolved on 6% PAGE followed by ethidium bromide staining

TMS1 which was used as maternal parent with CMV resistance for F₁ hybrid development (Fig. 3). This marker also detected the *Ph-3* gene in the respective hybrids, i.e., TMS1 × Naqeeb and TMS1 × MM. Out of several tested

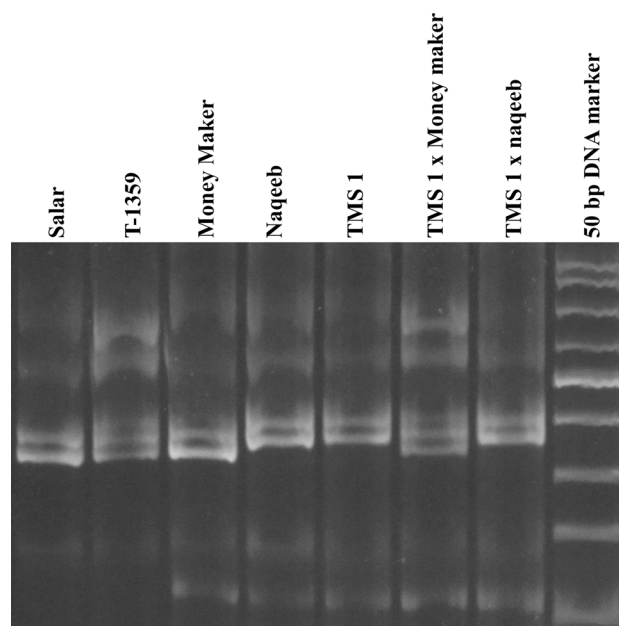


Fig. 4 Electrophorogram showing microsatellite profiles of parental genotypes and F₁ hybrids using primer, SSR-22 resolved on 6% PAGE. Three alleles, i.e., homozygous A, homozygous B and heterozygous H were detected in tested material

primers, SSR-22 was polymorphic among CMV-resistant and CMV-susceptible parental genotypes, i.e., TMS1 and Money Maker (Fig. 4). Allele A was detected in Money Maker, while allele B was detected in TMS1. A heterozygous allele H was detected in F₁ hybrid, i.e., TMS1 × Money Maker and provided evidence for its genetic confirmation. It can be concluded that genetic material having CMV tolerance also possess the gene for LB resistance and may lead to the development of F₁ hybrids with dual disease resistance. Recently, tomato inbred lines were genotyped using a new SCAR marker [18] in comparison with the previously reported SCAR marker used in present study as reference marker linked to the *Ph-3* gene (P. H. [17] and both markers gave the same results [18]. Actually, resistance to *P. infestans* can be classified into two categories: race-specific and race non-specific resistance [18]. Race-specific resistance is typically controlled by a single gene and can be quickly broken down by emergence of races of the pathogen. On the contrary, race non-specific resistance is polygenic resistance or quantitative resistance and usually governed by more than one gene and may be more durable [6].

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