



Simultaneous detection of *Mi1.2* and *Ph3* resistance alleles for root knot and late blight diseases in tomato through duplex polymerase chain reaction

BHAGYASHREE, ¹D. MAURYA AND T. CHATTOPADHYAY

Department of Plant Breeding and Genetics, ¹Department of Horticulture (Vegetables and Floriculture), Bihar Agricultural College, Bihar Agricultural University, Sabour-813210, Bhagalpur, Bihar, India

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ABSTRACT

Molecular markers have become an indispensable tool for tomato disease resistance breeding programme. For the tomato root knot and late blight diseases, molecular markers for identification of the resistance alleles of the *Mi1.2* gene and the *Ph3* gene has been reported in recent past. In this study, we report the duplex polymerase chain reaction (PCR) strategy, where both the markers for *Mi1.2* and *Ph3* resistance loci are used in a single reaction for simultaneous detection of the allelic status of the aforementioned genes present in a tomato genotype. We have authenticated the strategy using 12 tomato genotypes through separate and duplex PCR and have obtained identical results. Thus, the duplex PCR strategy will be very much useful to breeders as the strategy will reduce the amount of labour, time and reagents required for detection of these two important resistance alleles in diverse tomato genotypes.

Keywords : Duplex PCR, late blight disease, molecular markers, resistance genes, root knot disease

Tomato (*Solanum lycopersicum* L.) is a vegetable crop with global importance. Being a solanaceous crop, tomato suffers a lot from different viral, bacterial, fungal and other (like root knot nematode) diseases. With the availability of numerous basic genetics studies and whole genome sequence, a lot of resistance genes for these diseases have been identified and validated in cultivated and wild tomato accessions. Advancement in the field of biotechnology has provided the breeders with DNA-based molecular markers, which can be explored to precisely identify different disease resistance alleles in diverse tomato genotypes (Spasova *et al.*, 2001; Bendezu 2004; Devran and Elekçioğlu 2004; El Mehrach *et al.*, 2005; Garcia *et al.*, 2007; Ji *et al.*, 2007; Arens *et al.*, 2010; Shi *et al.*, 2011; Prasanna *et al.*, 2014; Jung *et al.*, 2015). As the marker assisted selection (MAS) is free from environmental factors (like presence of proper epiphytotic conditions, presence of virulent pathogen, *etc.*), this strategy is considered as a robust way for selection of parents and segregants in a tomato breeding programme for addressing disease resistance.

The resistance locus *Mi* for root knot disease has been introgressed from wild tomato *Solanum peruvianum*, and has been found to be located on chromosome 6. The locus has been documented to contain two open reading frames (*Mi1.1* and *Mi1.2*) and a pseudogene (Milligan *et al.*, 1998), where only *Mi1.2* has been proven to provide resistance against causal organisms *Meloidogyne incognita*, *M. javanica* and *M.*

arenaria. Among the different molecular markers developed for the *Mi1.2* (Bendezu 2004; Devran and Elekçioğlu 2004; El Mehrach *et al.*, 2005), the Mi23 SCAR marker (Garcia *et al.*, 2007) has been documented to be the most reliable co-dominant marker. In a similar manner, different resistance genes have been documented in wild tomato accessions for the devastating late blight disease caused by *Phytophthora infestans*. Among them, *Ph3*, a resistance gene derived from *S. pimpinellifolium* (accession L3708) has been documented to be most effective in conferring incomplete resistance against different *P. infestans* isolates (Black *et al.*, 1996; Zhang *et al.*, 2013). In a recent past, a gene-based functional co-dominant marker (Ph3-SCAR) for the *Ph3* gene has been developed (Jung *et al.*, 2015).

Identification of different resistance alleles in tomato genotypes through molecular markers involves separate polymerase chain reactions (PCRs), post-PCR agarose gel electrophoresis and documentation. Interestingly, multiplex PCR, where multiple primers of different markers are employed in a single reaction, can simultaneously identify different resistance alleles present in a particular genotype to ease the workload and cost for marker assisted breeding (Elnifro *et al.*, 2000). However, the multiplex PCR requires proper optimization, so that undesirable primer and/or amplicon interactions leading to erroneous results can be avoided. Here, we report the optimized conditions for a duplex PCR (using Mi23 SCAR and Ph3-SCAR primers) that

Table 1: Primer sequences of the used markers

Marker	Forward primer (5' – 3')	Reverse primer (5' – 3')	Reference
Mi23 SCAR	TGGAAAAATGTTGAATTTCTTTTG	GCATACTATATGGCTTGTTTACCC	Garcia <i>et al.</i> , 2007
Ph3-SCAR	CTACTCGTGCAAGAAGGTAC	TCCACATCACCTGCCAGTTG	Jung <i>et al.</i> , 2015

Table 2: Comparison of allelic status at *Mi1.2* and *Ph3* loci detected through separate PCR and duplex PCR

Genotype	<i>Mi1.2</i> allelic status with Mi23 SCAR marker	<i>Ph3</i> allelic status with Ph3-SCAR marker	<i>Mi1.2</i> and <i>Ph3</i> allelic status with Mi23 SCAR + Ph3-SCAR duplex PCR
H-86	<i>mi1.2</i>	<i>ph3</i>	<i>mi1.2/ph3</i>
BRDT-1	<i>mi1.2</i>	<i>ph3</i>	<i>mi1.2/ph3</i>
Superbug SPS	<i>mi1.2</i>	<i>Ph3</i>	<i>mi1.2/Ph3</i>
ArkaVikash	<i>mi1.2</i>	<i>Ph3</i>	<i>mi1.2/Ph3</i>
ArkaAlok	<i>mi1.2</i>	<i>ph3</i>	<i>mi1.2/ph3</i>
CLN B	<i>mi1.2</i>	<i>ph3</i>	<i>mi1.2/ph3</i>
CLN 1621 L	<i>mi1.2</i>	<i>ph3</i>	<i>mi1.2/ph3</i>
IIHR 2614	<i>Mi1.2</i>	<i>ph3</i>	<i>Mi1.2/ph3</i>
KashiChayan	<i>mi1.2</i>	<i>Ph3</i>	<i>mi1.2/Ph3</i>
VRTOLCV-16	<i>mi1.2</i>	<i>Ph3</i>	<i>mi1.2/Ph3</i>
VRTOLCV-32	<i>mi1.2</i>	<i>Ph3</i>	<i>mi1.2/Ph3</i>
H-88-78-1	<i>Mi1.2</i>	<i>Ph3</i>	<i>Mi1.2/Ph3</i>

Note : Red = susceptible allele; Green = resistant allele

allows identification of allelic status (resistant/susceptible) of *Mi1.2* and *Ph3* genes in tomato genotypes.

MATERIALS AND METHODS

Plant materials

Seeds of 12 tomato genotypes (H-86, BRDT-1, Superbug SPS, Arka Vikash, Arka Alok, CLN B, CLN 1621 L, IIHR 2614, Kashi Chayan, VRTOLCV-16, VRTOLCV-32 and H-88-78-1), were collected from Department of Horticulture (Vegetable and Floriculture) and Department of Plant Breeding and Genetics, BAU, Sabour.

Genomic DNA extraction and polymerase chain reaction (PCR)

Genomic DNA from the young leaves of the 12 tomato genotypes was isolated through a rapid protocol (Kumar *et al.*, 2017), with minor modifications. Briefly, ~50 mg of leaf tissue was crushed in 400 μ l of rapid buffer [100 mM Tris-Cl (pH 8.0), 50 mM EDTA, 500 mM NaCl, 1 % (w/v) SDS and 0.1 % (v/v) β -Mercaptoethanol] using micro-pestle. After that 128 μ l of 5M potassium acetate was added and centrifugation at 10000 RPM for 3 min was done. The supernatant was collected in a fresh tube and DNA was precipitated using equal volume of chilled isopropanol. The DNA pellet was washed in 70 % (v/v) ethanol, dried and dissolved in 150 μ l of molecular biology grade water. From the isolated genomic DNA, 2 μ l was used for a 12 μ l PCR

volume containing 1X reaction buffer [10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂ and 0.1 % (v/v) Triton X-100], 0.1 mM dNTPs, 5 pmole (or otherwise stated) of each custom synthesized (Xcelris, India) forward and reverse primers (Table 1) and 1U of *Taq* DNA polymerase (Xcelris, India). PCR was performed in an automated thermal cycler (Veriti, Applied Biosystems) as initial denaturation at 94 °C for 4 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at appropriate temperature for 30 s and extension at 72 °C for 30 s followed by final extension at 72 °C for 7 min and hold at 4 °C for 2 min. The amplicons generated through PCR were visualized and imaged in gel documentation system (Genei, Bangalore) after electrophoresis in 2.5 % (w/v) agarose gel containing ethidium bromide, using sodium borate as electrophoresis buffer. The gel was run at 150 V constant voltage for ~25 min.

RESULTS AND DISCUSSION

The suitable annealing temperatures for the Mi23 SCAR and Ph3-SCAR primers (Table 1) were first tested through gradient PCR, where 4 different annealing temperatures (*i.e.*, 53 °C, 56 °C, 59 °C and 62 °C) were used. It was found that in all the annealing temperatures, both the Mi23 SCAR and Ph3-SCAR primers produced sharp and specific amplicons (Fig. 1). Hence, the annealing temperature of 56 °C was used to separately screen 12 tomato genotypes using the Mi23 SCAR and Ph3-SCAR primers. Through the Mi23 SCAR marker,

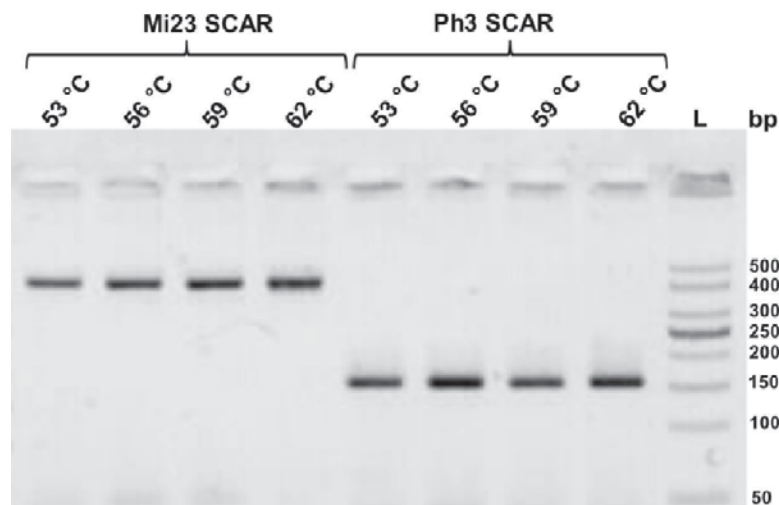


Fig. 1: Inverse image of 2.5 % agarose gel showing Mi23-SCAR and Ph3-SCAR PCR amplicons generated using different annealing temperatures. L = 50 bp DNA ladder (BioLit, SRL); bp = basepairs

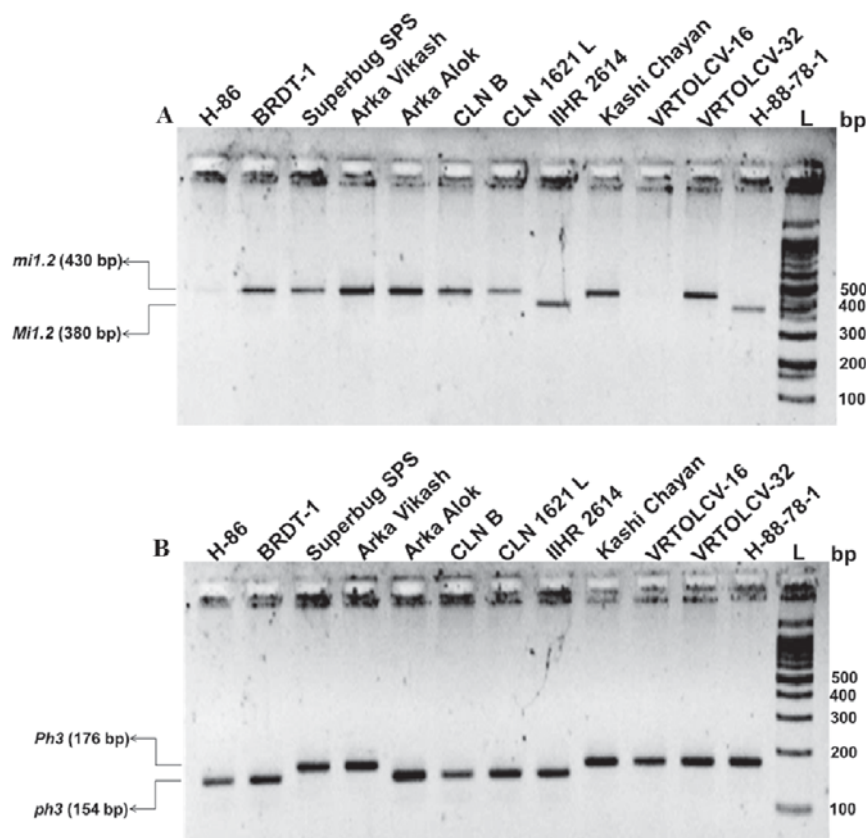


Fig. 2A: Inverse image of 2.5 % agarose gel showing of *Mi1.2* (resistant) and *mi1.2* (susceptible) allele-specific PCR amplicons of Mi23 SCAR marker in 12 tomato genotypes.

Fig. 2B: Inverse image of 2.5 % agarose gel showing *Ph3* (resistant) and *ph3* (susceptible) allele-specific PCR amplicons of Ph3-SCAR marker in 12 tomato genotypes. L = 100 bp DNA ladder (BioLit, SRL); bp = basepairs

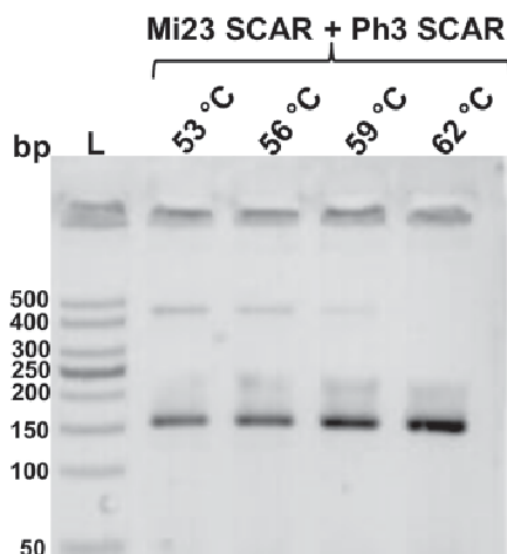


Fig. 3: Inverse image of 2.5 % agarose gel showing Mi23 SCAR + Ph3-SCAR (1:1 primer molar ratio) duplex PCR amplicons at different annealing temperature. *L* = 50 bp DNA ladder (BioLit, SRL); *bp* = basepairs

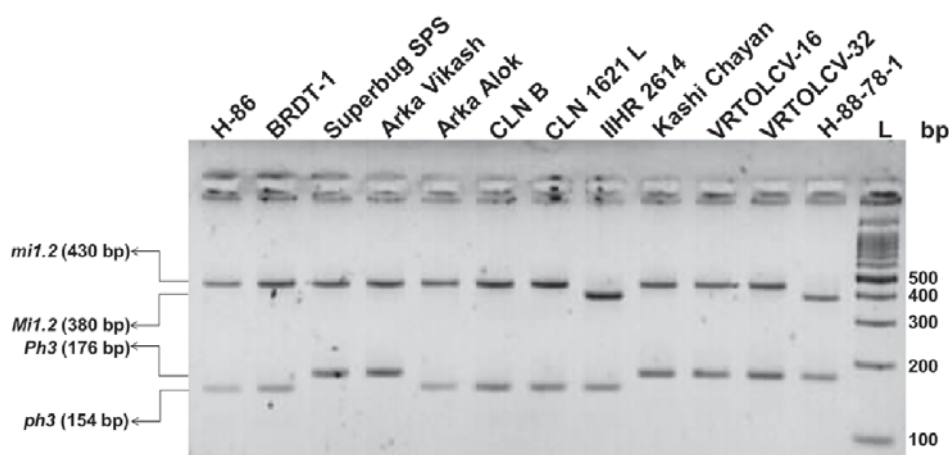


Fig.4: Inverse image of 2.5% agarose gel showing Mi23 SCAR + Ph3-SCAR (4:1 primer molar ratio) duplex PCR amplicons detected in 12 tomato genotypes. *L* = 100 bp DNA ladder (BioLit, SRL); *bp* = basepairs

two tomato genotypes (IIHR 2614 and H-88-78-1) were found to contain the *Mi1.2* resistance allele-specific ~380 basepairs (bp) amplicon, whereas the other 10 entries were observed to possess the *Mi1.2* susceptible allele-specific ~430 bp amplicon (Fig. 2A). In a similar manner, through the *Ph3*-SCAR marker, six tomato genotypes (Superbug SPS, Arka Vikash, Kashi Chayan, VRTOLCV-16, VRTOLCV-32 and H-88-78-1) were found to contain the *Ph3* resistance allele-specific ~176 bp amplicon, whereas the rest six genotypes were found to possess the *ph3* susceptibility allele-specific ~154 bp amplicon (Fig. 2B).

As the amplicon sizes of the Mi23 SCAR marker (i.e., either ~380 or ~430 bp) were sufficiently different

from the amplicon sizes of the *Ph3*-SCAR marker (i.e., either ~154 bp or ~176 bp) and both the relative differences of allele-specific amplicons could be well-resolved through 2.5 % agarose gel electrophoresis, we attempted to devise a duplex PCR (using both the Mi23 SCAR primers and the *Ph3*-SCAR primers together in a single PCR tube) for simultaneous detection of allelic status at *Mi1.2* and *Ph3* loci in tomato genotypes. For this purpose, the suitable annealing temperature of the duplex reaction was again optimized through gradient PCR. Interestingly, at 56/59/62 °C annealing temperatures, the *Ph3* allele-specific amplicon was highly amplified, whereas the amplification of the *Mi1.2* allele-specific amplicon was very poor (Fig. 3). To

overcome this problem, we increased the relative proportion of the Mi23 SCAR primers in duplex reaction, by using a 4:1 molar ratio of the Mi23 SCAR primers: Ph3-SCAR primers and carried out PCR at 56 °C annealing temperature. When duplex PCR was carried out using this altered primer molar ratio, both the *Mil.2* allele-specific and *Ph3* allele-specific amplicons were observed to be satisfactorily amplified in all the 12 tomato genotypes (Fig. 4). The allelic status at the *Mil.2* and the *Ph3* loci, as revealed by the duplex PCR was compared with the allelic status obtained through separate PCR using these 12 tomato genotypes. As per expectation, identical results were obtained (Table 2).

A simultaneous detection of allelic status at more than one locus through molecular markers saves time and labour. Moreover, this strategy saves reagents required for PCR and the valuable agarose required for post-PCR electrophoretic analysis. This strategy has been well-established in tomato genotypes for different disease resistance alleles in recent past (Masuelli *et al.*, 2000; Chen *et al.*, 2012; Fu *et al.*, 2013; Liu *et al.*, 2013; Chen *et al.*, 2015). In this article, we have presented an optimized duplex PCR condition, through which the allelic status at the *Mil.2* and *Ph3* disease resistance loci can be detected through a single PCR followed by agarose gel electrophoresis. This strategy might become well-adopted in resource-limited laboratories working in the aspect of molecular breeding for disease resistance in tomato.

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