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Development of Allele-specific PCR Markers for Tm2² Gene In Tomato

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Abstract: *Tomato Mosaic Virus* (ToMV) disease is one of the most devastating viral diseases of tomato worldwide. In order to accelerate ToMV resistance breeding, it is very important to develop a molecular marker to distinguish the resistance lines and susceptible lines. The objective of this study was to develop allele-specific PCR marker linked to Tm2² for deployment in resistant breeding. In the allele-specific PCR method, the specific primer-3 that introduced G-T mismatch could distinguish the resistant lines from susceptible lines when annealing temperature was 54°C. On the other hand, the specific primer-8 introduced C-T mismatch, which could distinguish susceptible lines from resistance lines when annealing temperature was 54±°C. In the verification experiment, the SCAR assay was consistent with the allele-specific PCR assay. The method of allele-specific PCR will provide breeders with a powerful tool in selection of Tm2² resistance genes in tomato breeding program.

Key words: Tomato, ToMV, allele-specific PCR

INTRODUCTION

Tomato Mosaic Virus (ToMV) disease is one of the most devastating viral diseases of tomato worldwide. Introgression of ToMV resistance genes into improved cultivars has been considered as a cost-effective and environmentally friendly means of minimizing the production losses due to this disease. Many reports showed that both major genes and polygenes were involved in the resistance process of the virus. Three resistance genes, Tm-1, Tm-2 and Tm2² have been identified in crossings between wild and cultivated species of tomato, all of which are used to prevent systemic mosaic symptoms and losses in fruit yield and quality caused by *Tobacco Mosaic Virus* (TMV) and *Tomato Mosaic Virus* (ToMV) (Ohmori *et al.*, 1996; Young *et al.*, 1988; Motoyoshi *et al.*, 1996; Panthee and Foolad, 2012). Among these resistant genes, the Tm2² resistant gene from tomato i.p. 128650 (*L. chilense*) has shown to be remarkably durable and effective (Hall, 1980; Lanfermeijer *et al.*, 2003; Jiang and Yang, 2003).

Molecular markers have been widely used in marker-assisted selection in tomato for disease resistance (Foolad and Sharma, 2005). For Tm2² gene, Young *et al.* (1988) isolated five different markers which tightly linked to the Tm2² locus by using near isogenic lines and Restriction Fragment Length Polymorphism (RFLP). Dax *et al.* (1994) developed a Tm2² linked RAPD assay which enabled the distinction between TMV resistant

and susceptible tomatoes. On the basis of previous studies, Sobir *et al.* (2000) and Dax *et al.* (1998) identified a co-dominant sequence Characterized Amplified Region (SCAR) marker which tightly linked to Tm-2². Shortly past, Panthee *et al.* (2013) developed a PCR-based marker based on restriction site differences from Tm2² locus-specific sequences. Each of these methods has advantages and disadvantages in a routine laboratory, but the main disadvantage is time and labor consuming. So those methods were not suitable for routine, large scale and commercial genetic analyses.

Few years ago, a method named allele-specific PCR which based on PCR technology was invented (Newton *et al.*, 1989). It only needs one PCR to distinguish the different genotypes and it has obvious advantages as to speed and cost compare with the conventional methods. At present, Allele-specific PCR has been successfully applied to the human disease screening (Huang *et al.*, 2005; Weber *et al.*, 2014), plant SNP identification (Wei *et al.*, 2006; Jin *et al.*, 2009; Sonneveld *et al.*, 2003) and assay to type *E. coli* strains (Clermont *et al.*, 2014).

The present investigation was undertaken to find out the possibility of application of allele-specific PCR technology in screening of Tm2² genes in tomato. This method could rapidly distinguish the ToMV resistant genotypes of tomato from susceptible genotypes in breeding.

MATERIALS AND METHODS

Plant materials: ToMV resistant tomato lines: CLN2264D (3165), CLN5915 (3164) (obtained from The Tomato Genetic Research Center); ToMV susceptible line viginia 73-45 (obtained from The Tomato Genetic Research Center); Hetrozygous plants 3110, F₁ generation (CLN2264D × viginia 73-45) And six commercial ToMV resistant (041, 120) and susceptible (511, 593, 620, 630) varieties were used as plant materials. In order to avoid problems in the commercial, the code name was used in this study, not mentioned the name of commercial varieties.

DNA extraction: Total DNA was extracted from leaves of tomato plant as described by Wang and Fang (2002).

Verified the enzyme site: According to Dax *et al.* (1998), a 950 bp band and a bigger band could be observed in both susceptible and resistant lines. The 950 bp-band from susceptible lines could be cleaved by Hind III into two bands of approximately 450 bp and 500 bp, while the 950 bp-band from resistant lines remained undigested.

Amplified the 950 bp-band using the primers designed by Dax *et al.* (1998).

Tm 2-2F: 5'-C A C C T T T C C C T C T C C A A-3'

Tm 2-2R: 5'-C A C C T T T C C C C T A A A G C-3'

Sequencing the 950 bp-band: The 950 bp-band was amplified with the primers Tm 2-2F/R. PCR reaction volume was 25 μL containing 0.5 μL 2.5 mM deoxynucleotide triphosphates (dNTPs), 2.5 μL 10x buffer, 2.5 μL 25 mM MgCl₂, 2.5 U Taq DNA polymerase, 2.5 μL each forward and reverse sense primer at 10 μM, 5-7 μL of DNA extract and H₂O. The PCR cycle parameters for fragment amplification were as follows: Denaturation at 94°C for 3 min, then 35 cycles 94°C for 30 sec, annealing at 54°C for 1 min and extension at 72°C for 1 min. These cycles were followed by a reaction at 72°C for 10 min and then the reaction was held at 4°C. PCR reactions were performed in the MJ DNA Engine PT200 Thermocycler™ (MJ Research Inc., Waltham, MA). PCR amplified

fragments were separated by gel electrophoresis using 1.5% agarose (Takara) in 0.5X TBE buffer, stained with ethidium bromide and visualized under Kodak Gel Logic 200 Imaging System. PCR fragments were cloned and then sequenced by Takara.

Allele-specific PCR: The 950 bp-band could be cleaved by Hind III, it was speculated that there was one or two bases mutated formed Single Nucleotide Polymorphism (SNP) in susceptible lines. The allele-specific PCR method was carried out. In this method, the primer of the reaction was moved up to the mutation site such that the 3' end of the primer sat right at the mutation. In order to improve the stability of the allele-specific PCR amplification, one or two mismatches were introduced in the specific primer. Then a common downstream primer was designed. The band would be observed that the genotype matched with the specific primer, while the band will not be observed if the genotype not matched with the specific primer.

RESULTS

Result of sequencing: The bands of 950 bp from resistant line CLN2264D and the susceptible line viginia 73-45 were sequenced. The sequencing result showed that the resistant line in the 238 and 239 loci were TA while the loci of susceptible line were GC (Fig. 1). The results showed that it was a fragment of tomato, when it compared with the sequences of Genebank(GenBank: EU139075.1).

Designed the specific primers based on the sequence: Eleven specific primers were designed (Table 1). The

Table 1: Specific primers designed

Serial number	name	sequences	the product length (bp)
1	Tmfk:	5'CCATGAGTAGTTCAACCCAATA 3'	
2	Tmfk-c:	5'CCATGAGTAGTTCAACCCA c TA 3'	
3	Tmfk-g:	5'CCATGAGTAGTTCAACCCA g TA 3'	
4	Tmfk-2c:	5'CCATGAGTAGTTCAACCCA c ATA 3'	
5	Tmfk-gc:	5'CCATGAGTAGTTCAACCCA cg TA 3'	
6	Tmfg:	5'CCATGAGTAGTTCAACCCA AGC 3'	
7	Tmfg-g:	5'CCATGAGTAGTTCAACCCA gGC 3'	
8	Tmfg-c:	5'CCATGAGTAGTTCAACCCA cGC 3'	
9	Tmfg-2c:	5'CCATGAGTAGTTCAACCCA cAGC 3'	
10	TmR801:	5' CTCTCAAGAA C ACCATAG 3' 585	
11	TmR2:	5'TTGATGTTGAAGGTGATATGATGA 3' 290	

Note: 1-5: Specific primers for resistant genotype, 6-9: Specific primers for susceptible genotype, The bases in bold were SNP, The bases in italic were introduced mismatch base

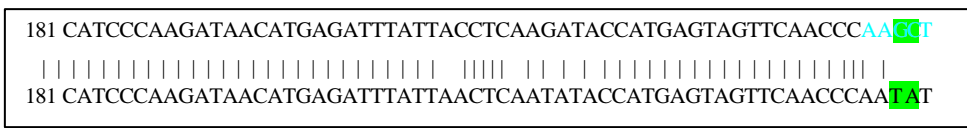


Fig. 1: Gene sequences including the SNP, the bases in blue were the sides of HindIII, the bases in green shade were SNPs. Upper line: Viginia 73-45, Lower line: CLN2264D

primer-1 to primer-5 was designed to amplify the resistant line, the primer-10 (TmR801) was the common downstream primer, the products size was about 585 bp. The primer-6 to primer-9 was designed to amplify the susceptible line, the primer-11 (TmR2) was the common downstream primer, the products size was about 290 bp. The primer-1 was a region of original sequence from resistant line and the 3' end sat right at the mutations. The sequence of primer-2 was same to the primer-1 but A at the third base from the 3' end was replaced by C, formed C-T mismatch. The sequence of primer-3 was same to the primer-1 but A at the third base from the 3' end was replaced by G, formed G-T mismatch. The sequence of primer-4 was same to the primer-1 but A at the fourth base from the 3' end was replaced by C, formed of C-T mismatch. In order to improve the stability of the allele-specific PCR amplification, two mismatches were introduced in the primer-5, AA at the third and fourth bases from the 3' end were replaced by the CG, formed CG-TT mismatch. The primer 6 was a region of original sequence from susceptible line, the 3' end sat right at the mutations. The primer 7-9 were introduced one mismatch base. In primer 7, A at the third base from the 3' end was replaced by C, formed C-T mismatch. In primer 8, A at the third base from the 3' end was replaced by G, formed G-T mismatch. In primer 9, A at the fourth base from the 3' end was replaced by C, formed of C-T mismatch.

PCR carried out using primers Tm2-2R/F and digestion with HindIII: Amplified the genomic DNA that known genotype using the primers of Tm2-2R/F, there are two bands were observed, the slower-migrating one was 950 bp (Fig. 2). The product was digested with HindIII. The 950 bp-band from susceptible line was cleaved into two bands of 500 and 450 bp, while the 950 bp-band from resistant line remained undigested. Heterozygous plant displayed all three bands (Fig. 3), thus the 950 bp band and its restricted fragments constituted a co-dominant marker. The result was consistent with the findings of Dax *et al.* (1998).

Results of PCR using different specific primers in resistant line: Amplified with the pair primers of primer-1 and TmR801. The result showed that the 585 bp-band was amplified both in the resistant line and susceptible line when annealing temperature at 50-58°C. Primer-1 could not effectively prevent base mismatch, so it could not effectively distinguish the resistant genotypes from susceptible lines (lane 1 and 2 in Fig. 4). Based on the study, mismatch introduced in further designing primers to improve allele specificity. Primer-2 was introduced C-T mismatch and primer-5 was introduced CG-TT mismatch. The results showed that the 585 bp-band was

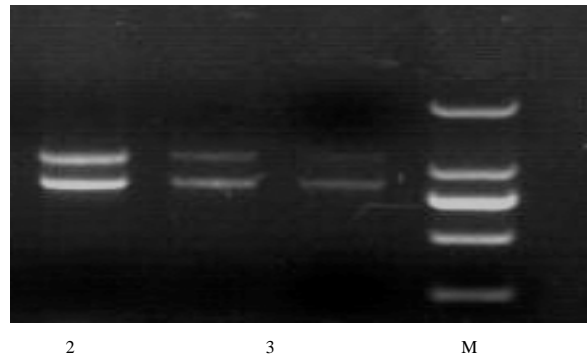


Fig. 2: PCR amplified result by primers Tm2-2R/F, (1) CLN2264D, (2) Virginia 73-45 and (3) 3110 (F₁) M. DM2000 Marker

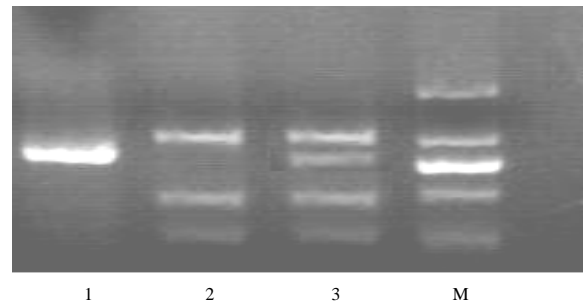


Fig. 3: Pproducts of digestion with HindIII, (1) CLN2264D, (2) Virginia 73-45 and (3) 3110 (F₁) M. DM2000Marker

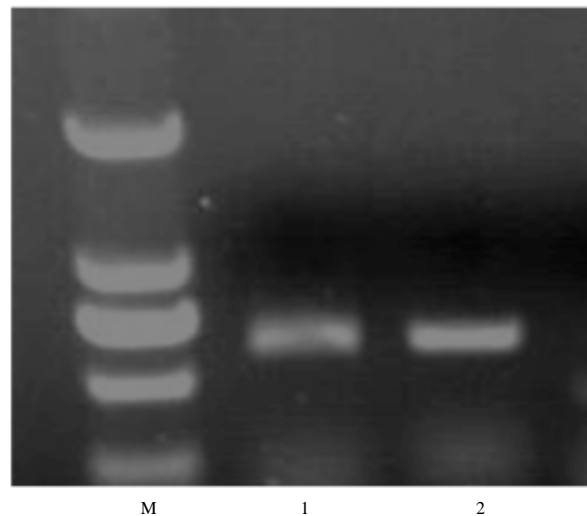


Fig. 4: PCR amplified result by primer-1, (1) CLN2264D, (2) Virginia 73-45 M. DM2000 Marker

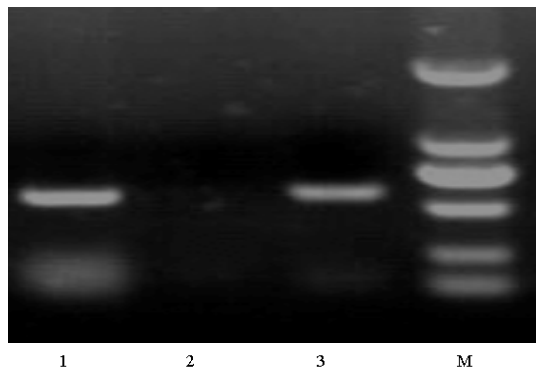


Fig. 5: PCR amplified result by primer-3, (1) CLN2264D, (2) Virginia 73-45 and (3) 3110 (F₁) M. DM2000 Marker

amplified instability with the pair of primer-2/ TmR801, while it was not observed with the pair of primer-5/TmR801 from either the susceptible line or resistant line. So they both could not effectively distinguish resistant genotype from susceptible genotype. Amplified with the primer-3 that introduced G-T mismatch, the 585 bp-band could be observed in resistant genotype and heterozygous genotype while not be observed in susceptible genotype (Fig. 5, the lane 1 was the resistance genotype; lane 2 was the susceptible genotype; lane 3 was the heterozygous genotype). So, the pair of primer-3/TmR801 could identify the genotypes of lines. The result showed that the different type of mismatch affected the results of the PCR. Amplified with primer-4 that introduced C-T mismatch, the target band could not be amplified from either the susceptible line or resistant line.

Results of amplification with different allele-specific PCR primers in susceptible line: Common downstream primer TmR2 was designed in order to make a different size from the 585 bp-band. The 290 bp-band was observed using the pair of primer-6/TmR2 both in genotype of resistant and susceptible. The designing principle of primers 7, 8, 9 was same to primers 2, 3, 4. Amplified with primer-7 that introduced G-T mismatch, no band was observed from either resistant line or susceptible line; Primer-8 and primer-9 were introduced C-T mismatch but the location was not the same. The result showed that the 290 bp-band was amplified with primer-8/TmR2 in susceptible line and hybrid genotype 3110, while not observed in resistant line (Fig. 6). Primer 9 could also distinguish the genotypes but the result was unstable.

Determination of the annealing temperature: When detecting resistant line allelic loci, only the primer-3 that

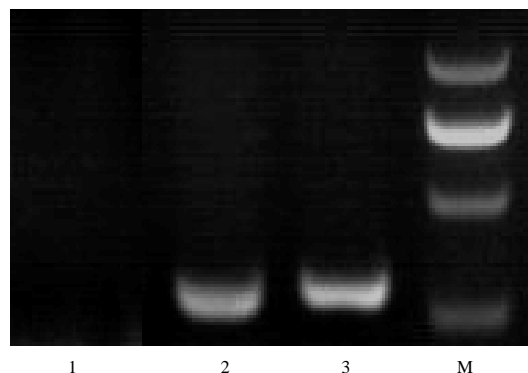


Fig. 6: PCR amplified result with primer-8/TmR2, (1) CLN2264D, (2) Virginia 73-45 and (3) 3110 (F₁) M. DM2000 Marker

introduced one mismatch in the neighbor side of SNP could identify the genotype, when the annealing temperature was 54°C. When the annealing temperature was higher 54°C, the result of amplifying was unstable, lower 54°C, the band of resistant line and susceptible line was both be amplified in resistant line and susceptible line.

When detecting the susceptible line allelic loci, the primer-8 could identify the different genotype when annealing temperature was 53-55°C. The result was same to the primer-3, when the annealing temperature was higher 55°C, the result of amplifying was unstable, when temperature was lower than 53°C, the band of resistant line and susceptible line was both be amplified in resistant line and susceptible line.

Other factors that affect the result of PCR: Except for annealing temperature, the concentrations of Mg²⁺ and dNTP were important factor on the amplification. In this trial, the range of Mg²⁺ concentration was 0.5-3.5 mM. The result showed that, 2.5 mM Mg²⁺ concentration was suitable. The range of dNTP concentration was 0.03-0.1 mM. The result showed that 0.04-0.06 mM was suitable. When less than 0.04 mM, the amplification was instability, more than 0.06 mM, there was nonspecific band.

To validate the effectiveness of specific primers: The commercial varieties 041, 120, 511, 593, 620, 630 were detected by SCAR method. The expected band was observed using Tm2-2R/F primers (Fig. 7). The PCR products were digested with HindIII. 041 and 120 were not be cleaved, 511, 593, 620, 630 were cleaved (Fig. 8). The results suggested that 041 and 120 were resistant lines, 511, 593, 620, 630 were susceptible lines.

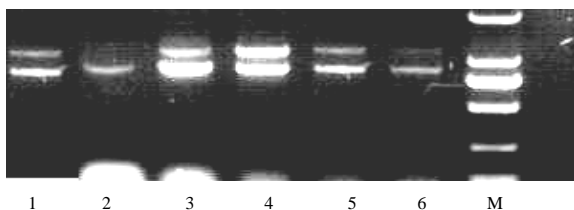


Fig. 7: PCR amplified result by primers Tm2-2R/F, 1.041, 2.120, 3.511, 4.593, 5.620 and 6.630, M. DM2000 marker

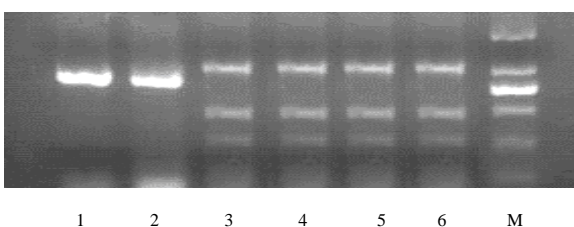


Fig. 8: Products of digestion with HindIII, 1.041, 2.120, 3.511, 4.593, 5.620 and 6.630, M. DM2000 marker

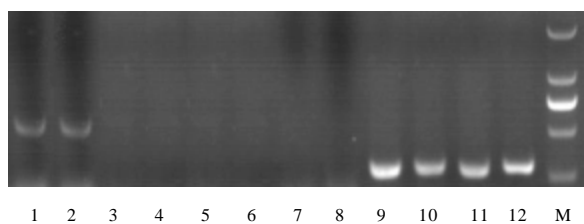


Fig. 9: PCR amplified result with primer-3/TmR801 and primer-8/TmR2

The 6 samples were amplified with primer-3/TmR801 and primer-8/TmR2, respectively. The results showed that the 585 bp-band was observed in 041 and 120 with the pair of primer-3/TmR801, while no amplification in the other 4 lines. With the pair of primer-8/TmR2, the 290 bp-band was observed in 511, 593, 620, 630 while no amplification in the other two lines. The results were consistent with the results by SCAR method to distinguish the different genotype (Fig. 9).

DISCUSSION

Screening of mismatched bases: In this study, we showed that it was feasible to detect Tm2² in tomato with allele-specific PCR method. Selecting of a specific primer was very important in this method. In order to enhance the stability of the amplification, the mismatch was

introduced when designing the specific primers. In the screening of specific primers that detecting resistant line, C-T and G-T mismatches were introduced at adjacent position of SNP. A lot of repeated experiments showed that amplified with primer-3 introduced G-T mismatch, the target band could be observed in resistant lines, amplified with primer-8 introduced C-T mismatch, the target band could be observed in susceptible line. It was reported (Ye *et al.*, 2001) that the G-T mismatch was weak, C-T mismatch was strong. In this study, TA was adjacent to G-T mismatch, CG was adjacent to C-T mismatch. In the regard of heap-based force and the three-dimensional structure, the CG was more stable than TA. Therefore, the strong mismatch which closed to CG and the weak mismatch which closed to TA required energy can be offset.

Mismatch position: When screening the specific primer to resistant line, using the primer-2 that the position of C-T mismatch at the third base from the 3' end (closely adjacent to the SNP), the amplification was instability (sometimes the band was observed, sometimes it was not observed), while using primer-4 that the position of C-T mismatch at the fourth base from the 3' end (not closely adjacent to the SNP) the band was amplified both in resistant and susceptible line.

When screening the specific primer to susceptible line, primer-8 was able to effectively prevent non-specific amplification. Primer-9 performed unexpected (sometimes the band was observed, sometimes it was not observed). In the two primers, just the position of mismatch was different. From these results, we could conclude that the position of mismatch affected the stability of amplification. The conclusion was consistent with Ye and Sun (Ye *et al.*, 2001; Sun, 2007). This result may be related to the base stacking force, hydrogen bonds, three-dimensional structure, also may be relevant to the steric hindrance caused by the interaction of the mismatch and DNA polymerase (Wei *et al.*, 2006).

Efficiency of the application of molecular markers: In this study, two SNPs were found in tomato genomic DNA by sequencing. SNPs are common and abundant variations in the plant genome. In recent years, detecting SNP methods have been improved, such as DNA microarrays (Divne and Allen, 2005) and TaqMan system (Hampe *et al.*, 2001). But these methods require precision device, so these methods were not suitable for routine, largescale and commercial genetic analyses. In this study, the resistant type and susceptible type could be distinguished only by PCR. This method was effective and rapidly and its application on plant breeding could accelerate breeding.

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