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Identification of markers tightly linked to tomato yellow leaf curl disease and root-knot nematode resistance by multiplex PCR

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ABSTRACT. Seven different commercial F_1 hybrids and two F_2 populations were evaluated by multiplex PCR to identify plants that are homozygous or heterozygous for *Ty-1* and *Mi*, which confer resistance to tomato yellow leaf curl disease and root-knot nematode, respectively. The *Ty-1* and *Mi* markers were amplified by PCR and identified by digestion of the amplicons with the *Taq*I enzyme. The hybrids E13 and 288 were found to be *Ty/ty* heterozygous plants with 398-, 303-, and 95-bp bands, and B08, 314, 198, and A10 were found to be *ty*/*ty* homozygous plants with a 398-bp band; whereas 098 did not give any PCR products. The hybrids E13 and 198 were found to be *Mi*/*Mi* homozygous plants with 570- and 180-bp bands, and 288 and A10 were found to be *Mi/mi* heterozygous plants, with 750-, 570- and 180-bp bands, and B08, 109 and 314 were found to be *mi/mi* homozygous plants with only a 750-bp band. We additionally developed a multiplex PCR technique for *JB-1* and *Mi*, which confer resistance to tomato yellow leaf curl disease and root-knot nematode. The *JB-1* marker identified the genotype of the *Ty* gene, and the plants

that produced the 400-bp band were *ty/ty* homozygous plants, whereas the plants that produced 400- and 500-bp bands were resistant to tomato yellow leaf curl disease. We conclude that multiplex PCRs can be used to reproducibly and efficiently detect these resistance genes.

Key words: Tomato yellow leaf curl disease; *Ty-1*; *Mi* gene; CAPS; Marker-assisted selection

INTRODUCTION

The tomato (*Solanum lycopersicon*) is an economically important species of the *Solanaceae* family, and it is cultivated all over the world for human consumption. Recently, tomato crops have often been infected by tomato yellow leaf curl virus (TYLCV), which causes significant yield losses in tomato (*S. lycopersicum*) crops all over the world (Picó et al., 1996; Pilowsky and Cohen, 2000; Castro et al., 2007). This has occurred since the 1990s in many regions of China including Zhejiang, Shandong, Shanghai, and Guangxi. Many defensive practices were utilized to control the disease after its rapid spread during the first outbreak, but the majority of these defensive methods are not effective. Therefore, breeding for resistance to tomato yellow leaf curl disease (TYLCD) is the most advantageous approach to controlling the damage caused by this viral disease (Castro et al., 2007; Laterrot, 1992), but no resistance has yet been found for the *S. lycopersicum* germplasm (Hassan et al., 1991; Laterrot, 1995; Ji et al., 2007). Although resistant genes have been detected in wild tomato relatives, a traditional breeding strategy requires a long time for the successful breeding of a new cultivar; therefore, it would benefit selection and gene cloning if a marker closely linked with TYLCV were found.

The resistance to TYLCD in some wild tomato relatives has been controlled by a polygenic recessive pattern (Michelson et al., 1994; Lapidot and Friedmann, 2000), in which *Ty-1* was the major gene mapped to the region around markers *TG297* and *TG97* on chromosome 6 (Zamir et al., 1994; Hanson et al., 2000). The *TG97* marker has been used in breeding practice for marker-assisted selection. In addition, another *JB-1* marker in this region that showed association with the *Ty-1* allele has revealed a polymorphism among the accessions assayed and has been found to be tightly linked to *Mi* (Castro et al., 2007).

Mi is the resistance gene to the main species of the root-knot nematode (Gilbert, 1958). The region on chromosome 6 around markers *TG297* and *TG97* harbors *Mi*, *Cf-2* and *Cf-5*, and *Ty-1* alleles, suggesting that the allele of the *JB-1* marker is also linked to the *Mi* gene. Although great strides have been made in breeding for resistance to TYLCD, most of the commercial hybrids resistant to TYLCD only contain a single resistance gene. One of the strategies for resistance is to polymerize more than one resistance gene into a single cultivar.

The purpose of this study was to develop multiplex PCRs to identify the genotype for *Ty-1* and *Mi* simultaneously. A total of 7 different commercial F_1 hybrids were evaluated. Furthermore, the F_2 individuals of E13 and A10 were screened using multiplex PCR for resistance to TYCLV and Mi. Additionally, a multiplex PCR technique was developed using *JB-1* and *Mi* conferring resistance to TYCLV and root-knot nematode separately. The results indicated that multiplex PCRs can be used to detect different genes reproducibly and efficiently.

MATERIAL AND METHODS

Plant material

The F_1 commercial hybrids used in this study were B08, 109, 314, E13, 198, 288, and A10. Of these hybrids, B08, E13, and 288 are resistant to TYLCD and 109, 314, 198, and A10 are susceptible to TYLCD in the field. Fifteen individuals of each F_1, 23 F_2 individuals of E13, and 19 F_2 individuals of A10 were planted at the vegetable research station of the College of Horticulture (P.R. China) in the spring of 2008.

Markers

Markers employed and primer sequences are listed in Table 1. The primers were synthesized by the Shanghai Shengong Science and Technology Company.

Table 1. Markers of the region used in this research.

Primer	Primer sequences	Restriction enzyme
Ty-1	CAPS1 F: 5'-TAATCCGTCGTTACCTCTCCTT-3' CAPS1 R: 5'-CGGATGACTTCAATAGCAATGA-3'	*Taq*I
Mi	CAPS2 F: 5'-TCGGAGCCTTGGTCTGAATT-3' CAPS12 R: 5'-GCCAGAGATGATTCGTGAGA-3'	*Taq*I
JB-1	JB1 F: 5'-AACCATTATCCGGTTCACTC-3' JB1 R: 5'-TTTCCATTCCTTGTTTCTCTG-3'	*Taq*I

The *Ty-1* and *Mi* primer sequences listed in Table 1 were designed by Michelson et al. (1994). The *JB-1* primer sequences listed in Table 1 were designed by Castro et al. (2007).

DNA extraction, single primer PCR, and CAPS

DNA was extracted from 30-day-old leaves as described by Chen et al. (2006). Briefly, the PCR mixture contained 0.3 U/μL *Taq* polymerase, 1.0 mM Mg^{2+}, 5 ng/μL DNA, 0.1 mM dNTP, and 0.6 μM primer. Amplification for *Ty-1* was carried out in a Mastercycler gradient by denaturation for 5 min at 95°C, followed by 1 min at 94.5°C, 1 min at 55.5°C, and 1 min at 72°C for a total of 35 cycles with a final extension step for 10 min at 72°C. For *Mi*, the optimum conditions for amplification were as follows: 94°C for 5 min, 35 cycles of 94°C for 1 min, 51.5°C for 1 min, and 72°C for 2 min, followed by an extension step of 10 min at 72°C. *JB-1* amplification was carried out at 94°C for 5 min, 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, followed by an extension step for 10 min at 72°C. After completion of all cycles, 7 μL amplified products were digested using *Taq*I. The digestion reaction consisted of 1 U *Taq*I, 1 μL buffer recommended by the supplier and double-distilled water (ddH_2O) added to a final volume of 10 μL. The PCR or digestion products (10 μL) were electrophoretically separated on a 2% (w/v) agarose gel.

Multiplex PCR and digestion

The multiple PCRs were carried out in a total volume of 20 μL containing: 1X buffer recommended by the supplier, 2.0 mM $MgCl_2$, 0.1 μM of each primer, 0.2 mM dNTPs, 0.04 U *Taq* polymerase, and 20 ng template DNA, and ddH_2O was added to bring the total volume to 20 μL.

The amplification was carried out in an Eppendorf Mastercycler. For *Ty-1* and *Mi*, the optimum conditions for amplification were: 94°C for 5 min and 35 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min, followed by an extension step for 10 min at 72°C. For *JB-1* and *Mi*, the optimum conditions for amplification were as follows: 94°C for 5 min and 35 cycles of 94°C for 1 min, 51°C for 1 min, and 72°C for 2 min, followed by an extension step for 10 min at 72°C. Restriction digestions of the amplified products (7 µL) were carried out with 10 U of the corresponding enzyme (1 µL), and 2 µL of the buffer recommended by the supplier, and ddH_2O added to bring the total volume to 20 µL. The reactions were performed at 65°C for 1.5 h. PCR and digestion products were analyzed by agarose gel electrophoresis (2%, w/v, agarose in 1X TAE buffer).

RESULTS

PCR amplification of 7 F_1 hybrids for *Ty-1* and *Mi* gene and digestion of PCR products

A 750-bp fragment was produced after amplification using *Mi* primers in 7 F_1 hybrids (Figure 1A). The PCR products were distinguishable after cleavage with the *Taq*I restriction enzyme. E13 and 198 produced 570- and 180-bp bands, indicating that they were *Mi*/*Mi* homozygous plants; 288 and A10 produced 750-, 570-, and 180-bp bands, indicating that they were *Mi/mi* heterozygous plants; and the 750-bp fragment remained in B08, 109, and 314, indicating that they were *mi/mi* homozygous plants (Figure 1B).

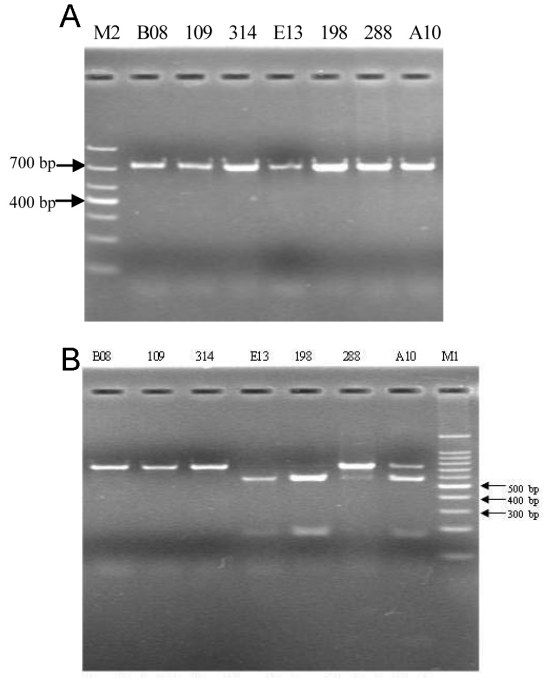

Figure 1. A. PCR profile amplified by the *Mi* primer. *Lane M2* = 50-bp DNA molecular weight marker. **B.** *Taq*I digestion of the products amplified by the *Mi* primer. *Lane M1* = 100-bp DNA molecular weight marker.

A 398-bp band was produced after amplification using *Ty-1* primers in 7 F_1 hybrids, with the exception of the hybrid 109 (Figure 2A). The PCR products were distinguishable after cleavage with *Taq*I. E13 and 288 produced 398-, 303- and 95-bp bands, indicating that they were *Ty/ty* heterozygous plants; and B08, 314, 198, and A10 produced the full-length 398-bp band without the *Taq*I digestion site, indicating that they were *ty/ty* homozygous plants (Figure 2B).

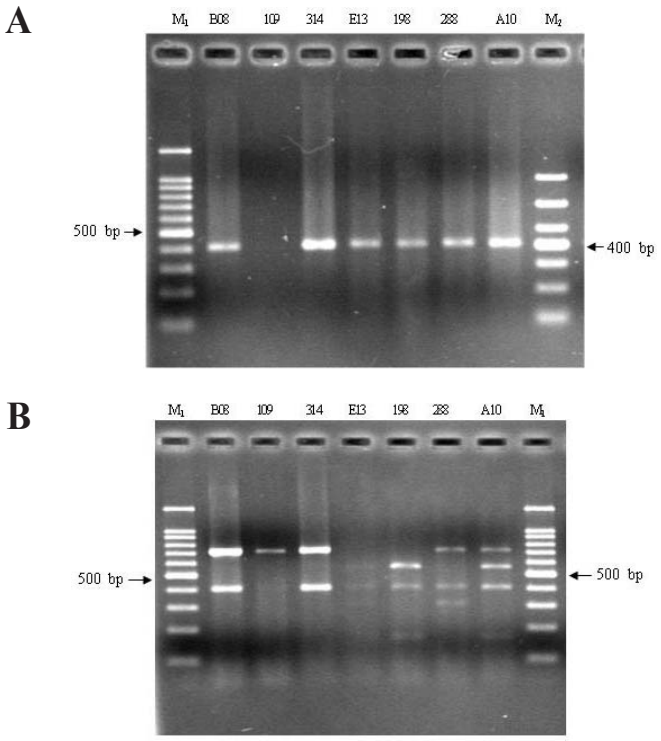

Figure 2. A. PCR profile amplified by the *Ty-1* primer. *Lane M1* = 100-bp DNA molecular weight marker; *lane M2* = 50-bp DNA molecular weight marker. **B.** *Taq*I digestion of the products amplified by the *Ty-1* primer. *Lane M1* = 100-bp DNA molecular weight marker; *lane M2* = 50-bp DNA molecular weight marker.

Multiplex PCR assays of *Mi* and *Ty-1* and digestion with *Taq*I

Multiplex PCR was further developed based on the single primer reaction, showing that multiplex PCR could be used to identify the *Mi* and *Ty-1* genes at the same time. The *Mi* primer produced 750-bp bands, whereas the *Ty-1* primer produced 398-bp bands (Figure 3A), and the products corresponded to the amplified bands produced by the single *Ty-1* and *Mi* primers. The PCR products were distinguishable after cleavage with *Taq*I. Both heterozygous plants and homozygous plants produced the original bands, which were produced using the single primer PCR. The results were reproducible after repeated experiments, showing that the multiplex PCR could be used in further experiments to distinguish the different genotypes of the plants (Figure 3B).

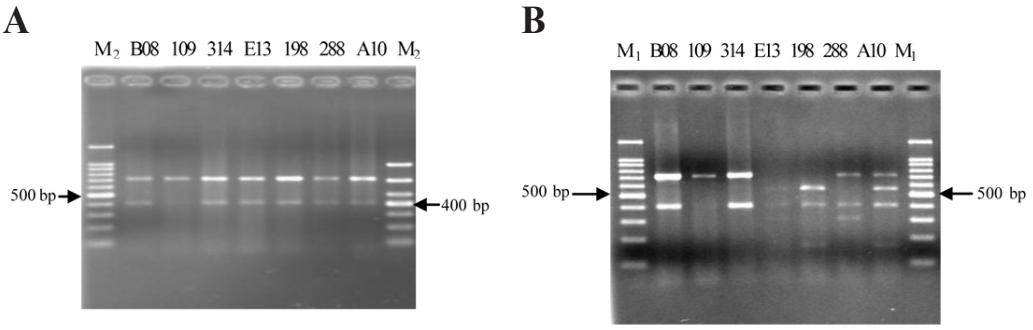

Figure 3. A. Products amplified by *Ty-1* and *Mi* primers. *Lane M1* = 100-bp DNA molecular weight marker; *lane M2* = 50-bp DNA molecular weight marker. **B.** Products amplified by the *Ty-1* and *Mi* primers digested by *Taq*I. *Lane M1* = 100-bp DNA molecular weight marker.

Analysis of F_2 materials using single PCR and multiplex PCR

The 750-bp bands were produced after amplification using *Mi* primers in 23 F_2 individuals of E13 and 19 F_2 individuals of A10. The PCR products were distinguishable after cleavage with *Taq*I, and 570- and 180-bp bands were produced after cleavage with *Taq*I in the 23 F_2 hybrids of E13. This was consistent with the conclusion that individuals of E13 were *Mi/Mi* homozygous plants (Figure 4A). Bands of 750-, 570- and 180-bp were produced in F_2 individuals 1, 4, 5, 6, 7, 8, 9, 10, 11, 13, 16, and 19 of A10, showing that these individuals were *Mi*/*mi* heterozygous plants. Bands of 570- and 180-bp were produced in F_2 individuals 3, 15, 17, and 18 of A10, showing that these individuals were *Mi/Mi* homozygous plants; however, individuals 2, 12, and 14 did not have the restriction enzyme digestion site and exhibited the 750-bp fragment, indicating that they were *mi/mi* homozygous plants (Figure 4B).

A 398-bp band was produced after amplification using *Ty-1* primers in 19 F_2 individuals of E13 and 19 F_2 individuals of A10. The PCR products were distinguishable after cleavage with *Taq*I. Bands of 398- and 303-bp were produced after cleavage with *Taq*I in F_2 individuals 1, 2, 4, 7, 9, 10, 11, 12, 15, 16, 17, 19, and 23 of E13 (Figure 5A), showing that these individuals were *Ty/ty* heterozygous plants. Bands of 303- and 95-bp were produced in F_2 individuals 3, 5, 8, and 18 of A10, showing that these individuals were *Ty/Ty* homozygous plants. Individuals 6, 14, 20, 21, and 22 did not have the restriction enzyme digestion site and exhibited the 398-bp fragment, indicating that they were *ty/ty* homozygous plants (Figure 5A). None of the individuals of A10 had the *Taq*I restriction site and therefore exhibited the 398-bp fragment, indicating that they were *ty/ty* homozygous plants (Figure 5B).

These results showed that multiplex PCR could be used to identify the genotype of *Mi* and *Ty-1* for the F_2 individuals at the same time. The products corresponded to the amplified bands produced by single *Ty-1* and *Mi* primers and separate digestion with *Taq*I. The heterozygous plants or homozygous plants could be identified using this method, and these results were reproducible and reliable. The multiplex PCR could be used to identify the different *Mi* and *Ty-1* genotype of the plants.

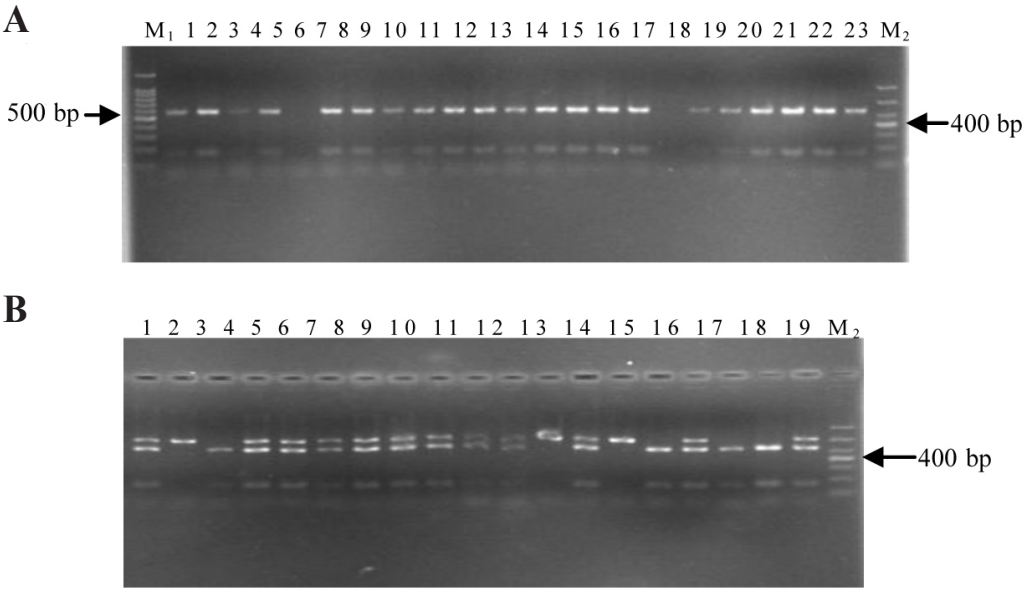

Figure 4. A. CAPS profile of E13 F_2 individuals amplified by the *Mi* primer. *Lane M1* = 100-bp DNA molecular weight marker; *lane M2* = 50-bp DNA molecular weight marker; *lanes 1-23* = F_2 individuals of E13. **B.** CAPS profile of A10 F_2 individuals amplified by the *Mi* primer. *Lane M2* = 50-bp DNA molecular weight marker; *lanes 1-19* = F_2 individuals of A10.

PCR amplification of 7 F_1 hybrids for *JB-1* and *Mi* gene and digestion of PCR products

PCR amplification of DNA from F_1 hybrids and subsequent digestions were carried out using the *Taq*I enzyme. Bands of 900 bp were obtained for the *JB-1* marker (Figure 6A). Two different alleles appeared for *JB-1* after digestion using *Taq*I, and these alleles consisted of one band slightly larger than 400 bp and a second band of approximately 400 bp. This result confirmed a report by Castro et al. (2007), in which the authors proposed that the lines showing these 2 bands had a large introgression from *Solanum peruvianum*. E13 and 288 had bands of approximately 400 bp and a second slightly larger than 400 bp (Figure 6B). The plants of B08, 109, 314, 198, and A10 had only a single band of approximately 400 bp, and they were *ty/ty* homozygous plants. E13 and 288 were resistant to TYLCD.

PCR was used to identify the *Mi* and *JB-1* markers simultaneously. The multiplex PCR products of *Mi* and *JB-1* were distinguishable after cleavage with *Taq*I. For *Mi*, E13 and 198 produced 570- and 180-bp bands, indicating that they were *Mi*/*Mi* homozygous plants; 288 and A10 produced 750-, 570- and 180-bp bands, indicating that they were *Mi*/*mi* heterozygous plants, and B08, 109, and 314 had a single band of 750-bp, indicating that they were *mi*/*mi* homozygous plants (Figure 7A and B).

The analysis of F_2 individuals using *JB-1* and *Mi* multiplex PCR

This analysis showed that 5 E13 F_2 individuals (6, 14, 20, 21, and 22) produced a 400-bp fragment after digestion, which was consistent with results of the *Ty-1* marker in that the

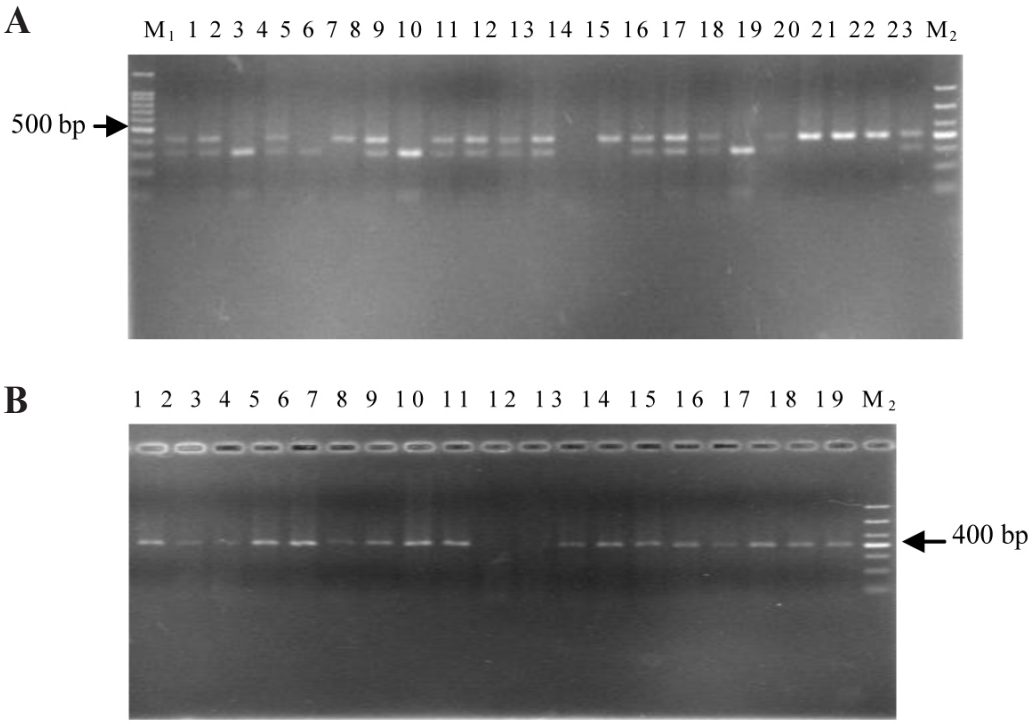

Figure 5. A. CAPS profile of E13 F_2 individuals amplified by the *Ty-1* primer. *Lane M1* = 100-bp DNA molecular weight marker; *lane M2* = 50-bp DNA molecular weight marker; *lanes 1-23* = F_2 individuals of E13. **B.** CAPS profile of A10 F_2 individuals amplified by the *Ty-1* primer. *Lane M2* = 50-bp DNA molecular weight marker; *lanes 1-19* = F_2 individuals of A10.

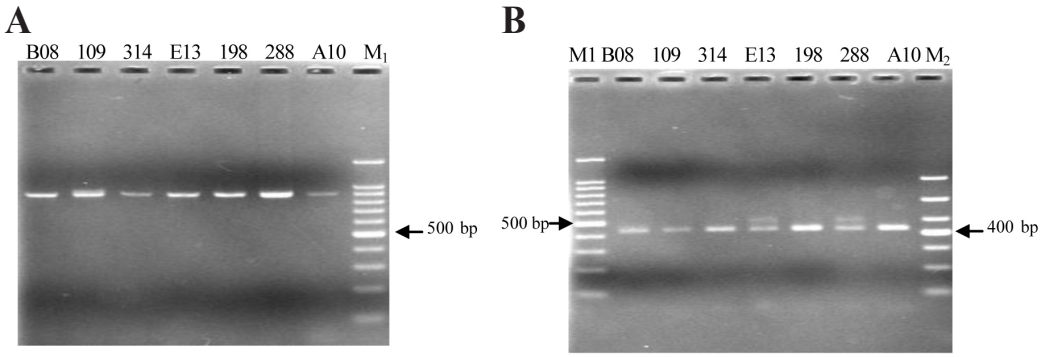

Figure 6. A. PCR profile amplified by the *JB-1* primer. *Lane M1* = 100-bp DNA molecular weight marker. **B.** *Taq*I digestion of the products amplified by the *JB-1* primer. *Lane M1* = 100-bp DNA molecular weight marker; *lane M2* = 50-bp DNA molecular weight marker.

individuals, showing that the 400-bp band produced after digestion in the *JB-1* marker were *ty/ty* homozygous plants. Individuals 2, 3, 4, 7, 8, 9, 10, 11, 12, 13, 15, 16, 18, 23, 24, and 27 showed

400- and 500-bp bands (Figure 8A and B). Comparing with the *Ty-1* results in E13 F_2 individuals, these individuals were the resistant plants that carry the *Ty* gene for TYLCD. A10 was a *ty/ty* homozygous plant, and therefore there was no separation in the F_2 individuals at this site.

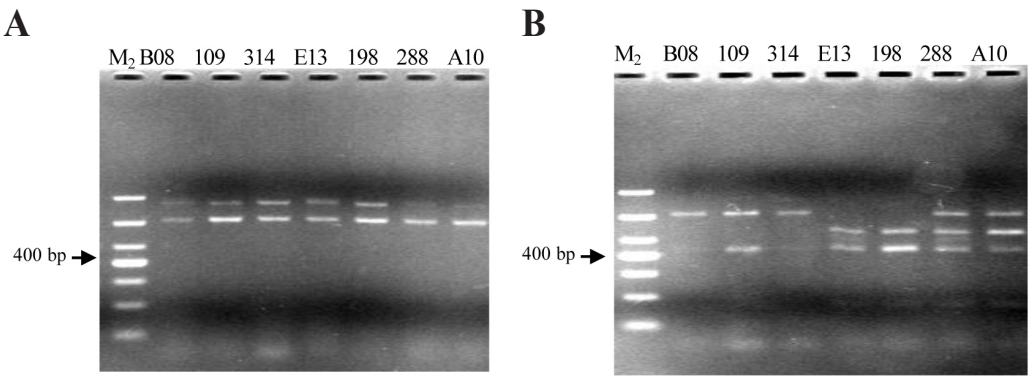

Figure 7. A. PCR profile amplified by the *JB-1* and *Mi* primers. *Lane M2* = 50-bp DNA molecular weight marker. **B.** *Taq*I digestion of the products amplified by *JB-1* and *Mi* primers. *Lane M2* = 50-bp DNA molecular weight marker.

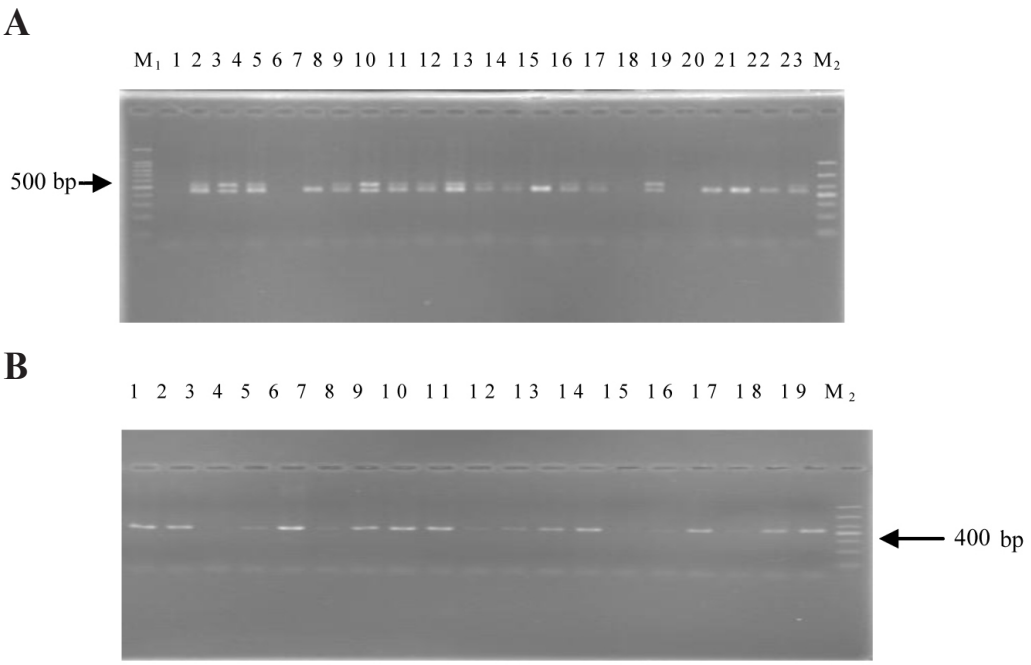

Figure 8. A. *Taq*I digestion of the products amplified by the *JB-1* primer. *Lane M1* = 100-bp DNA molecular weight marker; *Lane M2* = 50-bp DNA molecular weight marker; *lanes 1-23* = F_2 individuals of E13. **B.** *Taq*I digestion of the products amplified by the *JB-1* primer. *Lane M2* = 50-bp DNA molecular weight marker; *lanes 1-19* = F_2 individuals of E13.

Therefore, the *JB-1* marker could identify the *Ty* genotype, and the individuals that produced the 400-bp band were *ty/ty* homozygous plants, whereas the plants that produced 400- and 500-bp bands were resistant to TYLCD.

The multiple-PCR strategy was used in the F_2 individuals using the *JB-1* and *Mi* primers. The products digested by the *Taq*I enzyme corresponded to the single primer restriction fragments of *JB-1* and *Mi*, and showed that these two genes could be simultaneously identified using corresponding PCR primers at the same time (Figure 9A and B).

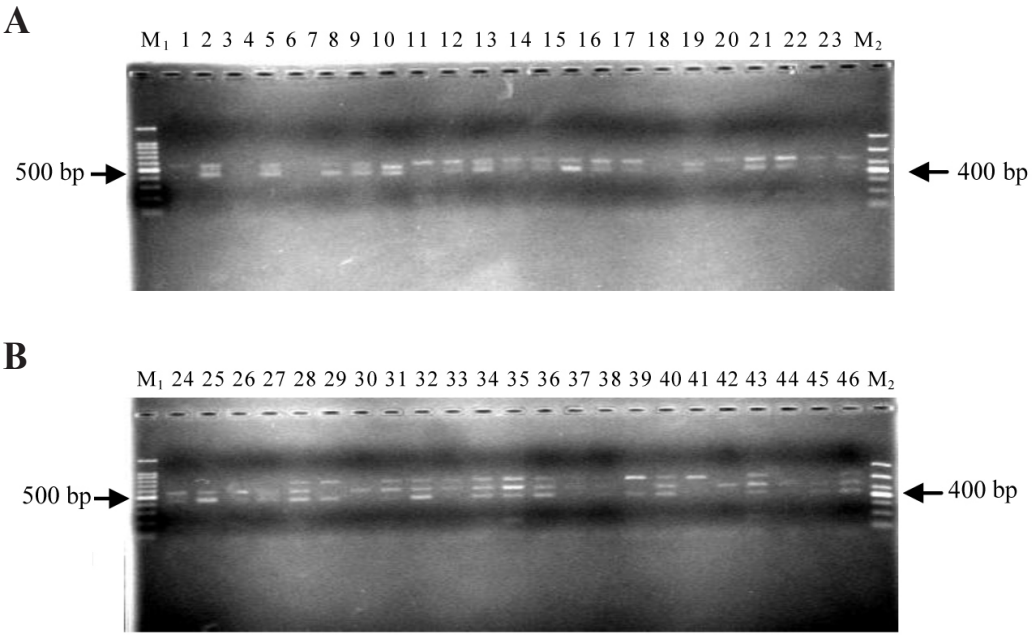

Figure 9. A. *Taq*I digestion of the products amplified by *JB-1* and *Mi* primers. *Lane M1* = 100-bp DNA molecular weight marker; *lane M2* = 50-bp DNA molecular weight marker; *lanes 1-23* = F_2 individuals of E13. **B.** *Taq*I digestion of the products amplified by *JB-1* and *Mi* primers. *Lane M1* = 100-bp DNA molecular weight marker; *lane M2* = 50-bp DNA molecular weight marker; *lanes 24-27* = F_2 individuals of E13; *lanes 28-46* = F_2 individuals of A10.

DISCUSSION

Tomato (*Lycopersicon esculentum*) is cultivated all over the world as a valuable fruit vegetable but, many crops have recently been threatened by geminiviruses. TYLCV, which belongs to the Begomovirus genus, is mainly transmitted by the whitefly *Bemisia tabaci* (Fauquet and Stanley, 2003; Fauquet et al., 2003; Kaya and Tanyolac, 2009). The tomato loss due to TYLCV has been overwhelming in most regions of China in recent years. Resistance to TYLCV has not been observed in the *S. lycopersicum* (Ji and Scott, 2006), so most breeders have to select parent plants from the wild tomato. The resistant gene can be introgressed into cultivated tomatoes from wild tomato relatives by crossbreeding.

It is well-known that resistance to TYLCD is controlled by a major incompletely

dominant gene, *Ty-1*, which is mapped to chromosome 6. The first report of a PCR-based marker for *Ty-1* gene was by Milo (2001). Castro et al. (2007) reported a *Taq*I CAPS marker using primers for *JB-1F* and *JB-1R*, and this marker is tightly linked to *Ty-1*. This suggests that this marker could be developed into a useful marker to select for TYLCD resistance.

Yu li et al. (2008) identified the *Ty-1* and *Mi* genes by multiplex PCR in the tomato. *Ty-1* and *Mi* are tightly linked in the tomato with 2 SCAR markers and have been amplified and screened using a single PCR. The PCR products corresponded to the amplified bands produced by a single SCAR primer. These reproducible results proved that these two resistance genes can be simultaneously identified using corresponding PCR primers under adaptable conditions.

In this study, we developed a multiplex PCR using *JB-1* and *Mi* primers that are tightly linked to the resistance gene *Ty-1* that can identify resistance to TLYCD and the root-knot nematode at the same time. For the *JB-1* marker, the individuals that produced 400-bp bands are the *ty/ty* homozygous plants and the individuals that produced 2 bands of 400- and 500-bp are the resistant genotypes. The multiplex PCR of *JB-1* and *Mi* will be helpful in breeding tomatoes that are resistant to *Ty* and *Mi*.

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