Production of Tomato Yellow Leaf Curl Virus-free Parthenocarpic Tomato Plants by Leaf Primordia-free Shoot Apical Meristem Culture Combined with in vitro Grafting

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Tomato yellow leaf curl virus (TYLCV) infections result in decreased tomato growth and reduced yields, and the production is almost entirely lost if plants are infected during early growth. ‘Kyo-temari’ is the commercial name for the parthenocarpic tomato ‘MPK-1’, which has been vegetatively propagated and distributed to local farmers in Kyoto City for commercial cultivation. During the winter of 2013, the typical yellow leaf curl symptoms of TYLCV were observed in 10 parthenocarpic tomato cultivars, including ‘MPK-1’, maintained as mother stock for vegetative propagation at Kyoto University. When microtissue direct polymerase chain reaction was conducted, a begomovirus-specific amplicon was detected in the plants with yellow leaf curl symptoms. Sequencing and phylogenetic analysis clarified that a TYLCV-Mild isolate was infecting the parthenocarpic tomatoes. Because signals of TYLCV were not detected in the shoot apical meristems (SAMs) of TYLCV-infected ‘MPK-1’ by in situ hybridization, elimination of TYLCV was conducted by regenerating plants from leaf primordia (LP)-free SAMs of parthenocarpic tomato cultivars. By combining the LP-free SAM culture and in vitro grafting, TYLCV-free plants were obtained in approximately three months. The technique developed in this study will contribute to the efficient elimination of TYLCV from vegetatively propagated parthenocarpic tomatoes.

Key Words: begomovirus, geminivirus, in situ hybridization, shoot apical meristem.

Introduction

Viruses in the family Geminiviridae are distinguished by their circular single-stranded DNA genomes that are packaged within geminate particles. On the basis of their genome organizations and biological properties, geminiviruses are divided into the following seven genera: Begomovirus, Becurtovirus, Curtovirus, Eragrovirus, Mastrevirus, Topocuvirus, and Turncurtovirus. According to the International Committee of Taxonomy of Viruses, 288 virus species are included in the genus Begomovirus, which makes it the largest genus of plant-infecting viruses. Moreover, begomoviruses are rapidly becoming one of the most severe plant pathogens in tropical, subtropical, and temperate regions, where they have extremely detrimental effects on the productivity of an increasing number of crops (Navas-Castillo et al., 2011).

The tomato yellow leaf curl virus (TYLCV) species in the genus Begomovirus causes the most devastating emerging disease that affects tomato (Solanum lycopersicum L.) worldwide (Czosnek, 2007). Symptoms of this disease comprise more or less prominent upward curling of leaf margins, a reduction of leaflet area, yellowing of young leaves, stunting, and flower abortion. Virus infections result in a general decrease in plant growth and reduced yields, and the production is almost entirely lost if plants are infected during early growth. Whitefly (Bemisia tabaci) is the natural vector of TYLCV (Cohen and Harpaz, 1964), and the B and Q biotypes of B. tabaci in particular have played an important role in the spread of TYLCV. In Japan, TYLCV has been reported from 37/47 prefectures, mainly in the southwest, which has a relatively warm climate (Hanada, 2012).

Parthenocarpy is a phenomenon where the fruit set
and develop without pollination and fertilization. Parthenocarpic tomatoes can produce normal fruit in conditions that are unfavorable for pollination and fertilization, such as high or low temperature. The cultivation of parthenocarpic tomatoes can reduce the fossil fuels used in horticultural production because they can be grown in unheated greenhouses in winter. ‘Kyotemari’ is the commercial name for the ‘MPK-1’ parthenocarpic tomato bred by Kyoto University possessing relatively high cold tolerance (Hosokawa et al., 2004a; Kataoka et al., 2004, 2010). ‘MPK-1’ parthenocarpic tomato produces few seeds (Takisawa et al., 2012); therefore, it is vegetatively propagated and distributed to local farmers in Kyoto City for commercial cultivation.

During the winter of 2013, the typical yellow leaf curl symptoms of TYLCV were observed in ‘MPK-1’ and other parthenocarpic tomatoes grown by farmers and in those maintained at Kyoto University. Because the maintained plants will be used as mother stock for propagation, the elimination of viruses is necessary to continue cultivation. Moreover, because ‘MPK-1’ is a genetically nonisogenic cultivar, that is, not seed-propagated but vegetatively propagated, such as by shoot-tip culture, it is required to be virus-free. Because begomoviruses including TYLCV are not seed-transmitted and tomato plants are generally seed-propagated, elimination of begomovirus or TYLCV is not conducted through tissue culture (i.e., shoot-tip culture). Shoot-tip culture is used to eliminate various viruses. The smallest explants are those that typically will be the least successful during in vitroculture but will produce the highest proportion of virus-free plants (Dale and Cheyne, 1993). Leaf primordia (LP)-free shoot apical meristem (SAM) culture, which is an improved method of conventional shoot-tip culture, can eliminate diseases that are very difficult to eliminate, such as viroids (Hosokawa et al., 2004b, 2005). In the present study, LP-free SAM culture combined with subsequent in vitrografting was conducted to eliminate TYLCV from the parthenocarpic tomatoes.

Materials and Methods

Plant materials

The parthenocarpic tomatoes ‘AQ-K’, ‘KK-oblong’, ‘MPK-1’, ‘N1’, ‘N6’, ‘N7’, ‘No.2’, ‘No.3’, ‘OK-10’, and ‘Ryukin’ were used in this study. ‘Kyo-temari’ and ‘Kyo-akane’ are the commercial names of the ‘MPK-1’ and ‘OK-10’ parthenocarpic tomatoes, respectively. During the winter of 2013, the typical yellow leaf curl symptoms of TYLCV were observed in the mother stocks of parthenocarpic tomatoes grown at the Experimental farm of Kyoto University. These plants were used in this study.

Detection of begomovirus using polymerase chain reaction

Detection of begomovirus from tomato plants grown in a greenhouse was conducted using microtissue direct polymerase chain reaction (PCR) according to Hosokawa et al. (2006). Briefly, DNA-A of begomovirus was amplified using BM-V and BM-C primers (Briddon and Markham, 1994). PCR was performed with KOD Fx Neo (Toyobo, Osaka, Japan). Wooden toothpicks were used to pierce the leaves, and the tissue templates that adhered to them were directly mixed with the PCR reaction mixture. For all PCR reactions, the reaction mixtures were initially denatured at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 3 min, with final extension for 5 min at 72°C. Electrophoresis using a 1.0% (w/v) agarose gel was performed with the amplified PCR products.

Detection of begomovirus from tomato plants obtained from tissue culture was conducted by PCR. DNA was extracted with Nucleon PhytoPure (GE Healthcare, Buckinghamshire, UK). The first PCR was conducted as described above. For the nested PCR, BM-V, BM-C primers, and 1 μL of the first PCR amplicon were used for the reaction. The reaction mixtures were denatured initially at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 3 min, with final extension for 5 min at 72°C. Electrophoresis was conducted as described above. Three technical replicates of PCR analysis were performed using independently prepared DNA from each plant.

Cloning and sequence determination of the full-length viral genome using rolling circle amplification

Full-length viral genomes of DNA-A were amplified using the rolling circle amplification (RCA)-based TempliPhi DNA amplification kit (GE Healthcare) (Inoue-Nagata et al., 2004). RCA reaction was performed according to the manufacturer’s instructions with minor modification. When adding the reaction buffer, 450 μM extra dNTPs were added to each sample (Stevens et al., 2010). For DNA extraction, a wooden toothpick was used to pierce the infected tissue, and the tissue sample obtained was mixed directly with 5 μL of the TempliPhi DNA amplification kit sample buffer. The concatamers produced in the reaction were monomerized by restriction digestion with BamHI. The digested products were resolved on a 1% agarose gel, and the bands that corresponded to 2.8 kbp were purified using the Gel DNA Recovery Kit (ZYMO Research, Irvine, CA, USA). The 2.8 kbp monomers were cloned into the BamHI site of the pBlueScript II SK (+) vector (Agilent Technologies, Santa Clara, CA, USA). Monomeric full-length clones were purified using the Zuppy Plasmid Miniprep Kit (ZYMO Research). Nucleotide sequencing was performed using an ABI PRISM 3100 genetic analyzer with an ABI PRISM BigDye

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Sequence analysis

BLASTn (National Center for Biotechnology Information) was used to search for the most similar begomovirus sequences in the GenBank database. The full-length viral sequences were aligned using ClustalW, and a phylogenetic tree was generated using Molecular Evolutionary Genetic Analysis software version 5.1 (MEGA 5.1) with 1000 bootstrap replicates (Tamura et al., 2011).

Detection of TYLCV using in situ hybridization

In situ hybridization was conducted according to Nabeshima et al. (2012). Nine SAMs of TYLCV-infected and three SAMs of TYLCV-uninfected ‘MPK-1’ were fixed with FAA solution [3.7% paraformaldehyde (w/v), 5% acetic acid (v/v), and 50% ethanol (v/v)] at 4°C overnight. The fixed tissues were dehydrated and embedded in paraffin (Paraplast Plus; Sigma-Aldrich, St. Louis, MO, USA). The tissues were cut into 12-μm sections and dried overnight. Hybridization was performed overnight at 52°C. DIG-labeled RNA probes were synthesized by T7 RNA polymerase in vitro transcription using a DIG RNA Labeling Kit (Roche Diagnostics Inc., Basel, Switzerland). Full-length TYLCV-Mild [Japan: Takatsuki] (AB921568) cloned in pBlueScript II SK (+) vector (Agilent Technologies) was used as the template for probe synthesis. Synthesized RNA probes were mixed with an equal volume of carbonic acid buffer (120 mM Na₂CO₃ and 80 mM NaHCO₃; pH 10.2) and incubated at 60°C for 27 min, in order to digest them into fragments approximately 400 bp long. After hybridization, the sections were washed twice with half-strength SSC buffer (0.3 M NaCl, 30 mM trisodium citrate) at 52°C and soaked in 0.5% blocking reagent (Roche Diagnostics Inc.) and anti-DIG alkaline phosphatase conjugate containing 0.1% BSA. After the slides were washed, TYLCV signals were detected with NBT/BCIP (Roche Diagnostics Inc.).

Plant regeneration from LP-free SAMs attached to root tips and in vitro grafting

Plant regeneration of parthenocarps from LP-free SAMs was conducted according to Hosokawa et al. (2004c) with minor modification. Lateral branches approximately 5 cm in length were collected, and the lowermost axillary bud was used for dissecting LP-free SAM. All of the leaves and LP were removed using a laser blade under a stereomicroscope (Fig. 1a). Seeds of cabbage ‘Harunami’ (Takii Seed Co., Kyoto, Japan) were sterilized with sodium hypochlorite solution containing 1% available chlorine and sown in modified Murashige and Skoog medium (Murashige and Skoog, 1962) with 1/10 strength ammonium nitrate, and 30 g L⁻¹ sucrose in a Petri dish (Fig. 1a). This medium was solidified with 3 g L⁻¹ gellan gum. Before autoclaving at 121°C for 15 min, the pH was adjusted to 5.8. An LP-free SAM dissected using a laser blade was picked up using a sharpened glass tube and transferred to the cut surface of a root tip of cabbage (Fig. 1b). Incubation conditions were 25°C and a 12 h photoperiod with light intensity of 3000 lx provided by cool white fluorescent tubes. Seeds of tomato ‘Louis 60’ (Takii Seed Co.) were sterilized with sodium hypochlorite solution and sown in modified MS medium in a Petri dish. When two LPs were detached from a Petri dish to a culture bottle approximately two weeks after in vitro grafting was conducted (f). Shoots were multiplied by nodal culture approximately a month after transplanting to a culture bottle (g). Virus-free tomatoes were transplanted to vermiculite and acclimatized for approximately three months after LP-free SAM culture was conducted (h). Bars indicate 2 cm.
BM-V and BM-C primers, begomovirus-specific ampli-
cons were observed in all parthenocarpic tomato culti-
vars showing virus symptoms (Table 1). Cuttage scions
for vegetative propagation are generally prepared from
a small number of mother stocks. Because the virus was
detected with high frequency, it was suggested that the
mother stocks had been infected by begomovirus before
cutting. In most cultivars, the virus infection rates
reached 100% by microtissue direct PCR when plants
exhibited virus symptoms (Table 1). In contrast, infec-
tion rates were 92% and 62% in ‘MPK-1’ and ‘N1’,
respectively (Table 1). Considering that the microtissue
direct PCR is a simplified technique and that nested
PCR was not conducted, those plants judged as nega-
tive may have had a low virus concentration.

To identify the infecting virus, full-length begomo-
virus DNA-A was amplified by RCA reaction. The se-
quence isolated from the ‘MPK-1’ tomato was 2787 bp
in length, and it possessed the typical sequence of
monopartite begomoviruses. A 33 bp potential stem-
loop-forming region was found in the intergenic region,
which included the conserved nonanucleotide sequence
TAATATTAC. In addition, six predicted open reading
frames were found, that is, two in the virion-sense (V1
and V2) and four in the complementary sense (C1, C2,
C3, and C4). The sequence obtained was deposited in
GenBank (AB921568).

A phylogenetic tree was constructed using the full-
length viral sequences obtained through the BLASTn
search and comparisons (Fig. 3). The isolated sequence
shared high similarity with TYLCV isolates, and it had
the highest shared nucleotide sequence identity (99%)
with the TYLCV-Mild Tokai isolate (AB439842) from
Japan. Since the first report of tomato yellow leaf curl
disease in Japan in 1996 (Kato et al., 1998), the disease
has spread and caused significant damage to tomato
production. Sequencing and phylogenetic analysis of
TYLCV isolates collected at different locations have
shown that two distinct strains of the virus are present
in Japan, the Israel strain and Mild strain (Ueda, 2008;
Ueda et al., 2004, 2005). The isolate in our study was
grouped into a cluster with TYLCV-Mild isolates, so it
was named TYLCV-Mild Takatsuki.

Furthermore, in situ hybridization was performed to
examine the TYLCV distribution on the LPs and SAM
of TYLCV-infected ‘MPK-1’ using antisense tran-
scribed TYLCV probes. When TYLCV-uninfected
‘MPK-1’ was observed, no signal was detected on

| Cultivar | Plants with virus symptom (%) | Number of TYLCV-infected plants by PCR (%) |
|----------|-------------------------------|------------------------------------------|
| AQ-K     | 39/40 (98)                    | 12/12 (100)                              |
| KK-oblong| 49/50 (98)                    | 12/12 (100)                              |
| MPK-1    | 85/88 (97)                    | 11/12 (92)                               |
| N1       | 255/267 (96)                  | 8/12 (67)                                |
| N6       | 116/143 (81)                  | 12/12 (100)                              |
| N7       | 14/15 (93)                    | 12/12 (100)                              |
| No.2     | 29/30 (97)                    | 12/12 (100)                              |
| No.3     | 22/30 (73)                    | 12/12 (100)                              |
| OK-10    | 14/14 (100)                   | 12/12 (100)                              |
| Ryukin   | 34/34 (100)                   | 12/12 (100)                              |

Fig. 2. ‘MPK-1’ tomato without TYLCV symptoms (a) and with symptoms (b). As shown in (b), leaf size decreased and no leaf lamina developed in young leaves (indicated by black triangle). Bars indicate 2 cm.

Fig. 3. Phylogenetic tree based on the alignments of full-length TYLCV DNA-A sequences. The tree was constructed using the Neighbor-Joining method with MEGA 5.1. The bootstrap values are indicated at the major nodes (based on 1000 replicates). The TYLCV-Mild Takatsuki isolate is shaded in gray.
either LPs or SAM (Fig. 4a). In contrast, strong signals were detected on the phloem tissue of LPs of TYLCV-infected ‘MPK-1’ (Fig. 4b). However, no signals were detected on the SAM of TYLCV-infected ‘MPK-1’ (Fig. 4b). TYLCV is generally regarded as limited to phloem (Cohen and Antignus, 1994; Morilla et al., 2004; Rojas et al., 2001), which is consistent with our results. InCapsicum annuum, pepper huasteco yellow vein virus and/or pepper golden mosaic virus, bipartite begomoviruses, were detected on LPs but not on SAM (Rentería-Canett et al., 2011). Elimination of TYLCV may thus be possible by regenerating plants from LP-free SAMs of parthenocarpic tomato cultivars.

Production of TYLCV-free parthenocarpic tomatoes by tissue culture

LP-free SAMs of parthenocarpic tomato cultivars were dissected and transferred to the cut surface of root tips of cabbage (Fig. 1a, b). Approximately a month after attachment to the root tip, LPs were formed on the SAMs (Fig. 1c; Table 2). Although LP formation was observed in all cultivars, some of the LP-free SAMs attached to the root tips of cabbage were unable to proceed to subsequent in vitro grafting (Table 2). Those LP-free SAMs developed no LP and gradually necrotized or developed to floral meristems. In tomato, vegetative and reproductive phases alternated regularly along the compound shoots (Samach and Lotan, 2007). Most of the LP-free SAMs developed to floral meristems in ‘No.2’ which has a vigorous reproductive phase compared with the other cultivars. This resulted in a low number of SAMs in vitro-grafted and plants obtained for ‘No.2’ (Table 2).

When eight regenerated SAMs of ‘MPK-1’, developing two to three LPs, were transferred to MS medium, further growth was not observed after three months of culturing. Novák and Mašková (1979) reported that isolated shoot tips of tomato did not grow on basal medium without plant growth regulators, and they gradually necrotized, which is consistent with our result. Although adding plant growth regulators may prompt the growth of SAMs, it is reported that adding plant growth regulators also enhances somaclonal variation in in vitro cultures (Ahmed et al., 2004; Matsuda et al., 2014). Hosokawa et al. (2004a) conducted LP-free SAM culture of tomato and the regenerated SAMs with several LPs were transferred from the root tips of cabbage to MS medium. Although they successfully regenerated tomato plants, the total culturing period was not reported. In the present study, regenerated SAMs were dissected and transferred from the root tips of cabbage to the hypocotyl of ‘Louis 60’ in order to stimulate the initial development of SAMs (Fig. 1d; Table 2). More than half of in vitro grafted SAMs started to develop vigorously. Approximately two weeks after attachment

![Fig. 4. Results of in situ hybridization of positive strands of TYLCV localizations near the SAM of ‘MPK-1’. TYLCV-uninfected (a) and TYLCV-infected ‘MPK-1’ shoot tips (b) were collected and fixed. Infected cells are stained blue. Bars indicate 100 μm.](image-url)

| Cultivar  | Number of LP-free SAMs attached | Number of SAMs in vitro-grafted (%)<sup>z</sup> | Number of plants obtained (%)<sup>y</sup> | Number of TYLCV-free plants (%)<sup>x</sup> |
|-----------|---------------------------------|-----------------------------------------------|-------------------------------------------|-------------------------------------------|
| AQ-K      | 24                              | 15 (62.5)                                    | 8 (33.3)                                  | 8 (100)                                  |
| KK-oblong | 20                              | 5 (25.0)                                     | 4 (20.0)                                  | 4 (100)                                  |
| MPK-1     | 22                              | 14 (63.6)                                    | 8 (36.4)                                  | 8 (100)                                  |
| N1        | 35                              | 12 (34.3)                                    | 4 (11.4)                                  | 4 (100)                                  |
| N6        | 37                              | 9 (24.3)                                     | 4 (10.8)                                  | 4 (100)                                  |
| N7        | 15                              | 4 (26.7)                                     | 3 (20.0)                                  | 3 (100)                                  |
| No.2      | 18                              | 1 (5.6)                                      | 1 (5.6)                                   | 1 (100)                                  |
| No.3      | 17                              | 7 (41.2)                                     | 4 (23.5)                                  | 4 (100)                                  |
| OK-10     | 35                              | 9 (25.7)                                     | 6 (17.1)                                  | 6 (100)                                  |
| Ryukin    | 22                              | 8 (36.4)                                     | 6 (27.3)                                  | 6 (100)                                  |
| Average   |                                 | (34.5)                                        | (20.5)                                    | (100)                                    |

<sup>z</sup> (Number of SAMs in vitro-grafted/number of LP-free SAMs attached) × 100.<br><sup>y</sup> (Number of plants obtained/number of LP-free SAMs attached) × 100.<br><sup>x</sup> (Number of TYLCV-free plants/number of plants obtained) × 100.
to the hypocotyl of ‘Louis 60’ in vitro, plantlets were transferred from the plastic Petri dish (Fig. 1e) to the culture bottle (Fig. 1f). Approximately a month after transfer to the culture bottle, nodal culture was conducted (Fig. 1g; Table 2). In the present study, in vitro grafting successfully stimulated the initial development of the SAMs. It is known that plant hormones, proteins, a number of small RNAs, and even mRNAs are transported between stock and scion (Aloni et al., 2010; Harada, 2010). Understanding why grafting treatments were effective for the initial development of SAM is important, so further study is needed to clarify this point.

When the DNA extracted from the leaves was analyzed by PCR, TYLCV was not detected by either first PCR or nested PCR (Fig. 5; Table 2). Approximately a month after nodal culture, the parthenocarpic tomato cultivars were acclimatized (Fig. 1h). In the present study, acclimatized tomato plants were obtained in approximately three months after conducting LP-free SAM culture. These plants were used as the mother stocks for further cutting propagation for commercial cultivation. After six months, no virus symptoms were observed in parthenocarpic tomato cultivars.

Conclusion

Strong parthenocarpy is necessary for tomato cultivation in unheated greenhouses in winter. The dilemma is that, because parthenocarpic tomatoes produce few seeds, vegetative propagation is required. Vegetatively propagated crops are under the constant threat of virus infection. The method established in this study is meaningful for two reasons. First, by combining LP-free SAM culture and in vitro grafting, acclimatized and virus-eliminated plants were obtained, including nodal culture, in approximately three months. This will enable incorporation of the tissue culture process in a fallow period of parthenocarpic tomato cultivation. Second, because LP-free SAM culture can eliminate viroids, which are very difficult to eliminate by conventional apical meristem culture methods (Hosokawa et al., 2004b, 2005), it will be possible to eliminate other pathogens that threaten tomato production, such as tomato spotted wilt virus or tomato chlorotic dwarf viroid. The technique developed in this study will contribute to the efficient elimination of TYLCV from vegetatively propagated parthenocarpic tomatoes.

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