Highly Efficient Agroinoculation Method for Tomato Plants with Tomato Yellow Leaf Curl Kanchanaburi Virus

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Tomato yellow leaf curl disease caused by begomoviruses is a serious threat to tomato (Solanum lycopersicum L.) production. If begomoviruses, transmitted by whitefly (Bemisia tabaci), infect tomato plants during early growth, production can be almost entirely lost. Tomato yellow leaf curl Kanchanaburi virus (TYLCKaV), a bipartite Begomovirus, is emerging as an important threat to solanaceous crop production in Southeast Asia. The lack of mechanical transmission of some begomoviruses is a major experimental constraint. In this study, an agroinoculation method using TYLCKaV in tomato plants was established. Partial tandem repeats of TYLCKaV DNA A and DNA B were constructed and cloned to a binary pGreenII vector, and their infectivity was tested. Co-inoculation of TYLCKaV DNA A and DNA B to Nicotiana benthamiana L. produced typical begomoviral symptoms, and both of the viral DNA components accumulated in the upper uninoculated leaves, suggesting systemic infection of TYLCKaV. Two agroinoculation methods were conducted on tomatoes. First, excised sections of tomato shoots were agroinoculated with a soaking procedure. Although two Agrobacterium tumefaciens strains were tested, approximately 40% of inoculated plants only showed viral symptoms for EHA105. Second, agrobacterium from a cultured petri dish was directly inoculated with a colony inoculation procedure. When EHA105 was used, approximately 92% of inoculated plants showed viral symptoms. Sequencing the recovered viral DNA from the upper uninoculated leaf clarified that TYLCKaV had successfully infected the tomato plants. The colony inoculation procedure is labor-saving, and viral symptoms develop in susceptible tomato plants within approximately a month from sowing the seeds. This method could contribute to simple and speedy evaluation of TYLCKaV resistance of tomato plants.

Key Words: begomovirus, geminivirus, infectious clone, Southeast and East Asia, TYLCKaV.

Introduction

The Geminiviridae family encompasses a large number of plant viruses with circular and single-strand DNA genomes that are encapsidated in twinned icosahedral particles consisting of two incomplete icosahedra (T = 1) that are approximately 22 × 38 nm (Zhou, 2013). On the basis of their genome organization 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 (ICTV), 322 virus species are included in the genus Begomovirus, making it the largest genus of plant-infecting viruses. The majority of members of Begomovirus are bipartite, with a genome composed of two similar-sized DNA components (DNA A and DNA B) (Saunders et al., 2000). DNA A encodes a replication-associated protein (Rep), coat protein, and proteins that participate in the control of replication and gene expression. DNA B encodes proteins required for nuclear trafficking and cell-to-cell movement of the viral DNA. Of note, only DNA A has been isolated for several monopartite begomoviruses, including Tomato yellow leaf curl virus (TYLCV) and...
Tomato leaf curl virus. For several monopartite begomoviruses, such as Ageratum yellow vein virus and Cotton leaf curl virus, alphasatellites and betasatellites are associated with DNA A (Hanley-Bowdoin et al., 2013). 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 begomoviruses (Cohen and Harpaz, 1964), and the B and Q biotypes of B. tabaci, in particular, have played an important role in their spread.

Over the past three decades, diseases caused by begomoviruses have contributed to production losses in solanaceous crops, particularly tomato (S. lycopersicum), pepper (Capsicum spp.), and eggplant (Solanum melongena L.), in many tropical and subtropical regions of the world (Kenyon et al., 2014). In particular, TYLCV species of the genus Begomovirus cause the most devastating emerging disease that affects tomatoes worldwide (Czosnek, 2007). Since the first report of tomato yellow leaf curl disease in Japan in 1996 (Kato et al., 1998), TYLCV has been reported in 37 out of 47 prefectures, mainly in the southwest, which has a relatively warm climate (Hanada, 2012; Koeda et al., 2015). During this period, a wide variety of distinct local begomovirus species have been identified in solanaceous crops in Southeast and East Asia; however, TYLCV has not been found there (Kenyon et al., 2014). Tomato yellow leaf curl Kanchanaburi virus (TYLCKaV) is a bipartite Begomovirus first reported to infect tomato and eggplant in Kanchanaburi Province, Thailand (Green et al., 2003). Later, TYLCKaV was reported in Laos, Vietnam, Cambodia, and Indonesia (Bagewadi and Naidu, 2016; Ha et al., 2008; Koeda et al., 2016; Tang et al., 2014), suggesting that TYLCKaV has started to emerge as an important threat to the production of solanaceous crops in Southeast Asia.

Besides control of whitefly, breeding for resistance against begomoviruses has been deployed as the main strategy in disease management. This requires appropriate and efficient screening of germplasm for begomovirus-resistant accessions. With increasing international importance of leaf curl disease in tomatoes, increased efforts have been made to identify resistance to tomato leaf curl disease and incorporate it into improved tomato cultivars (Kenyon et al., 2014). To date, five major genes for resistance or tolerance to TYLCV (Ty-1–Ty-5) have been identified from wild tomato relatives, and some major genes have been incorporated into commercial cultivars in different areas (Anbinder et al., 2009; Hutton et al., 2012; Ji et al., 2007, 2009; Lapidot et al., 2015; Verlaan et al., 2013; Zamir et al., 1994). However, whether Ty-1–Ty-5 genes also show resistance or tolerance to local begomovirus species of Southeast Asia is unknown.

The lack of mechanical transmission of some gemini-viruses is a major experimental constraint. Agroinoculation is a technique that allows the inoculation of cloned geminiviral genomes into wide variety of host plants using Agrobacterium tumefaciens (Boulton, 2008). Most begomoviruses are naturally transmitted only by whitefly, and agroinoculation has greatly simplified the study of these viruses. In many reports of plant pathology, agroinoculation of begomoviruses using infectious clones has been conducted because it is a relatively simple and inexpensive method, compared with biolistic inoculation, which needs expensive equipment such as a particle gun. Although agroinoculation is widely used for fundamental research into the study of viral functions, only a few studies have used agroinoculation for screening begomovirus resistance sources (Biswa and Varma, 2001; Tripathi and Varma, 2002). In outdoor field conditions, fluctuating environment and infection with other viruses make it difficult to evaluate the response (resistance, tolerance, and susceptibility) of each germplasm to begomoviruses. Moreover, there is a constant threat of the viruliferous-whitefly escaping in a closed laboratory space. In contrast, agroinoculation can be conducted under controlled laboratory conditions using small seedlings, which can enable accurate evaluation in a short period of time and in a small space. For the above reasons, agroinoculation seems to be a beneficial method for screening begomovirus resistance sources. However, no study to infect TYLCKaV by agroinoculation has been conducted. In the present study, a highly efficient agroinoculation method for getting TYLCKaV into tomato plants was established.

Materials and Methods

Cloning and sequence determination of the full-length DNA B using rolling circle amplification

The DNA extracted from a B6 pepper leaf sample, from which we previously recovered a full-length DNA A sequence of TYLCKaV (Koeda et al., 2016), was used to amplify a full-length DNA B sequence using a rolling circle amplification (RCA)-based TempliPhi DNA amplification kit (GE Healthcare, UK) according to Koeda et al. (2015). The concatamers produced in the reaction were monomerized by restriction digestion with BamHI. Digested products were resolved on 1.0% agarose gel and the bands corresponding to ~2.8 kbp were purified using a Gel DNA Recovery Kit (ZYMO Research, CA, USA). The 2.8 kbp monomers were cloned into the BamHI site of pBlueScript II SK(+) vector (Agilent Technology, CA, USA) and Escherichia coli (DH10B) was transformed. E. coli colonies possessing putative DNA B of TYLCKaV were selected by colony-direct PCR, T7, M13 Reverse BS-plus, and BMV (Bridgon and Markham, 1994) primers were used. PCR was performed using EmeraldAmp PCR Master Mix (Takara, Shiga, Japan). 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, 55°C for 30 s, and 72°C for 3 min, terminating with 5 min of extension at 72°C. Electrophoresis using a 1.0% (w/v) agarose gel was performed on amplified PCR products. As BM-V universal primer binds to begomovirus DNA A, when a colony possessed DNA A, two bands could be detected, one was approximately 3 kbp and the other approximately 1.2 kbp. When a colony possessed DNA B, only a single band of 3 kbp could be detected. Colonies selected for possessing putative DNA B were selected and plasmids were purified using a Zippy Plasmid Miniprep Kit (ZYMO Research). Nucleotide sequencing of four plasmids purified from different colonies was carried out in an ABI PRISM 3100 genetic analyzer with an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). Primer sequences are shown in Table 1.  

**Table 1. Primers used in this study.**

| Primer name | Primer sequence (5′–3′) |
|-------------|-------------------------|
| T7          | TAAATAGCTACCTATGATAGGG  |
| M13 Reverse BS-plus | GGGACACATGATGACAGG   |
| BM-V        | KGGGTCGACATGTGACAGG    |
| TYLCKaV fra1 F | TGGTTGAAATGGTTGAGTCACCCACATGTTCCA |
| TYLCKaV fra1 R | AACAAGTTGAGGTACCTGCTATCCTTTTCAG |
| TYLCKaV fra2 F | GAATCTCCACATGTTCCA     |
| TYLCKaV fra2 R | GTATGAGGTTGGTTGCTCCTATTCGTGTAAC |
| TYLCKaV DNA-B fra1 F | TGGTTGAAATGGTTGAGTCACCCACATGTTCCA |
| TYLCKaV DNA-B fra1 R | AACAAGTTGAGGTACCTGCTATCCTTTTCAG |
| TYLCKaV DNA-B fra2 F | GAATCTCCACATGTTCCA     |
| TYLCKaV DNA-B fra2 R | GTATGAGGTTGGTTGCTCCTATTCGTGTAAC |
| TYLCKaV F | GACCAATCAGCTTTCAGCTTGA |
| TYLCKaV R | GATAAAACCGTGGCTTCGTG |
| TYLCKaV DNA-B 2F | CTAATTATGAGAAAACACACGT |
| TYLCKaV DNA-B R | AGTCTGCTGAAATTCACGTC |

**Inoculation of TYLCKaV to Nicotiana benthamiana**

Seeds of *N. benthamiana* were sown in a horticultural soil mix. After germination, each plant was transplanted into a plastic pot containing horticultural soil mix and grown at 20–30°C of 12 h light and 12 h dark photoperiods, and a light intensity of 100 μmol·m²·s⁻¹ using day white fluorescent lights. For agroinoculation, frozen stock of pGreenII-p35S (empty), pGreenII-p35S-TYLCKaV-DNA-A, pGreenII-p35S-TYLCKaV-DNA-B, and pGreenII-p35S-TYLCKaV-DNA-A+B were thawed and plated onto a solid YEP medium (50 mg·mL⁻¹ of K and rifampicin) and cultured at 28°C for two nights. The cultured agrobacteria was inoculated into 3 mL of liquid YEP medium and grown at 28°C in a shaker overnight. These cells were separated by centrifugation (13200 rpm, 2 min, 20°C), resuspended in infiltration buffer (10 mM MES, 10 mM MgCl₂), adjusted to optical density (O.D.) 0.1–0.3, and 4 μL of 100 mM acetosyringone was added to each 1 mL of suspension. Agrobacteria possessing pGreenII-p35S, pGreenII-p35S-TYLCKaV-DNA-A, pGreenII-p35S-TYLCKaV-DNA-B, and pGreenII-p35S-TYLCKaV-DNA-A+B were cultured at room temperature for 3–5 h. The induced agrobacteria were infiltrated with a needleless 1 mL syringe into the abaxial sides of the fourth or fifth leaves of 3-week-old *N. benthamiana*.  

**Inoculation of tomatoes with TYLCKaV**

Seeds of the tomato ‘Momotaro’ (Takii seed, Kyoto, Japan), a begomovirus-susceptible cultivar, were sown in a horticultural soil mix. After germination, each plant was transplanted into a plastic pot containing horticultural soil mix and grown in the same conditions as *N. benthamiana*. pGreenII-p35S-TYLCKaV-DNA-A+B was used for the tomato agroinoculation. Agrobacte-
um mixtures were prepared as written above, except the O.D. was adjusted to 1.0. In the present study, two inoculation methods were used. First, excised sections of tomato shoots were agroinoculated with the soaking procedure (Yamaguchi et al., 2013). In brief, tomato shoots were soaked in \textit{Agrobacterium} suspension, 5 min vacuum treatment at $-0.09$ MPa was conducted, and inoculated shoots were rooted. Second, direct inoculation (colony inoculation procedure) of \textit{Agrobacterium} cells grown on the solid YEP medium of a petri plate was conducted. A small number of bacterial cells were collected by toothpick from the plate (Fig. 2a), and they were spread on the hypocotyl just below the apex of the seedlings before developing the first true leaf (Fig. 2b). Then, agrobacterium were injected into hypocotyl by stabbing the hypocotyl at about 1.0 mm with an insect pin (No. 00; Shiga, Tokyo, Japan) over bacterial cells approximately five to ten times (Fig. 2c). Inoculation of the stem, just below the apex of the seedlings developing the first true leaf, was repeated approximately 1 week after the first inoculation (Fig. 2d).

\textbf{Viral DNA detection and sequence analysis}

Newly developed upper leaves were collected from inoculated plants and DNA was extracted with Nucleon PhytoPure (GE Healthcare). DNA A of TYLCKaV was detected using TYLCKaV F and R primers by PCR. DNA B of TYLCKaV was detected using TYLCKaV DNA-B 2F and R primers. PCR was performed using EmeraldAmp PCR Master Mix (Takara). For PCR reactions, the reaction mixtures were initially denatured at 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, 67°C (DNA A) or 64°C (DNA B) for 30 s, and 72°C for 1 min, terminating with 3 min of extension at 72°C.
Electrophoresis using a 1.0% (w/v) agarose gel was performed on amplified PCR products. Primer sequences are shown in Table 1.

Amplification of full-length viral genomes of DNA A and DNA B was conducted according to Koeda et al. (2015) by a RCA method. Nucleotide sequencing was performed using an ABI PRISM 3100 genetic analyzer with an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). BLASTn (National Center for Biotechnology Information) was used to search for the most similar begomovirus sequences in the GenBank database.

**Results and Discussion**

**Viral DNA sequence analysis**

To obtain the full-length begomoviral DNA B sequence associated with yellow leaf curl symptoms, virus sequences were amplified using the RCA method. The putative begomoviral DNA B sequence was 2752 nucleotides long and contained the geminiviral conserved nonanucleotide sequence (TAATATTAC) in the intergenic-region (IR) and two predicted open reading frames, including one in the virion-sense (BV1) and one in the complementary sense (BC1) (Fig. 1a).

A phylogenetic tree was constructed using the sequences obtained through a BLASTn search (Fig. 3). Based on an ICTV threshold of 89% nucleotide sequence identity for demarcation of species (Fauquet et al., 2008), isolated in the present study was established as written below. The isolate BA_B6-1-B was grouped with the TYLCKaV isolate and had the greatest nucleotide sequence identity (98%) with the TYLCKaV pepper-isolate (KF446672) from Indonesia.

The full-length sequence of TYLCKaV-[BA_B6-1-B] was deposited in GenBank (LC177332).

**Inoculation of TYLCKaV to N. benthamiana**

*N. benthamiana*, categorized as a Solanacea species along with tomatoes and capsicums, is the most widely used experimental host in plant virology, mainly because of the large number of diverse plant viruses that can successfully infect it (Bally et al., 2015; Goodin et al., 2008). Partial tandem repeats of TYLCKaV DNA A and DNA B were constructed, and their infectivity was tested on *N. benthamiana*.

In the present study, *A. tumefaciens* strain GV2260 was used. Inoculation of either buffer, empty pGreenII-p35S or pGreenII-p35S-TYLCKaV-DNA-A alone produced no virus symptoms (Fig. 4a, b, c, d). Agroinoculation of *N. benthamiana* with pGreenII-p35S-TYLCKaV-DNA-A and pGreenII-p35S-TYLCKaV-DNA-B produced typical begomoviral symptoms (leaf curling, yellowing, mottingling, and stunting) (Fig. 4a, e). Agroinoculation of pGreenII-p35S-TYLCKaV-DNA-A+B also produced typical symptoms (Fig. 4a, f). Similar results were obtained from independently conducted experiments.

PCR was performed to examine the viral DNA A and
DNA B accumulation in plants infected with infectious clones. No accumulation of either TYLCKaV DNA A or DNA B were observed in the plants inoculated with buffer or empty pGreenII-p35S (Table 2). Accumulation of DNA A was observed in several plants inoculated with pGreenII-p35S-TYLCKaV-DNA-A alone, although none of them showed typical begomoviral symptoms (Table 2). In contrast, all of the plants inoculated with pGreenII-p35S-TYLCKaV-DNA-A and pGreenII-p35S-TYLCKaV-DNA-B or pGreenII-p35S-TYLCKaV-DNA-A+B, accumulated TYLCKaV DNA A and DNA B (Table 2). These results showed that both components are necessary for symptom expression in *N. benthamiana*. It is reported that DNA A of *Tomato leaf curl New Delhi virus*, which is a bipartite *Begomovirus*, infects *N. benthamiana* without DNA B, but the frequency of infection tends to be lower than co-infection of DNA A and DNA B (Sivalingam and Varma, 2012). Thus, some of the bipartite begomoviruses seem to be only infected by DNA A, although the pathogenicity is reduced. Our study demonstrated that constructed clones had infectivity, and agroinoculation of TYLCKaV DNA A and DNA B can successfully induce virus symptoms.

**Inoculation of TYLCKaV to tomatoes**

For tomatoes, pGreenII-p35S-TYLCKaV-DNA-A+B was used and two agroinoculation methods were conducted. First, excised sections of tomato shoots were agroinoculated using the soaking procedure (Yamaguchi et al., 2013). When *A. tumefaciens* strain GV2260 was used, 2 out of 33 plants inoculated with pGreenII-p35S-TYLCKaV-DNA-A+B showed typical begomoviral symptoms (Table 3). In contrast, when *A. tumefaciens* strain EHA105 was used, 8 out of 17 inoculated plants showed symptoms (Table 3, Exp. 1). Approximately 40% of inoculated plants showed viral symptoms after 69 days from sowing (31 days from inoculation). Agroinoculation with the soaking procedure was reported for TYLCV, a monopartite-begomovirus,

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**Table 2.** Infectivity of TYLCKaV to *N. benthamiana*.

| Construct                                      | Number of plants infected/inoculated | PCR (Exp. 3) |
|------------------------------------------------|--------------------------------------|--------------|
|                                                | Exp. 1   | Exp. 2  | Exp. 3 DNA A | DNA B |
| mock                                           | 0/18     | 0/6     | 0/6      | 0/6     |
| pGreenII-p35S                                  | 0/18     | 0/6     | 0/6      | 0/6     |
| pGreenII-p35S-DNA-A                            | 0/18     | 0/24    | 0/24     | 4/24    |
| pGreenII-p35S-DNA-A+B                         | 17/18    | 24/24   | 24/24    | 24/24   |
| N.C.                                           | 21/24    | 22/24   | 22/24    | 22/24   |

Detected with TYLCKaV F and R primer.

**Table 3.** Infectivity of TYLCKaV to tomatoes with the soaking procedure.

| Construct                                      | Number of plants infected/inoculated | PCR (Exp. 2) |
|------------------------------------------------|--------------------------------------|--------------|
|                                                | Exp. 1   | Exp. 2  | DNA A | DNA B |
| GV2260                                         | 0/2      | 0/6     | 0/6   | 0/6   |
| pGreenII-p35S                                  | 2/33     | 0/24    | 0/24  | 0/24  |
| pGreenII-p35S-DNA-A+B                          | 8/17     | 9/24    | 9/24  | 9/24  |

Detected with TYLCKaV F and R primer.

N.C. indicates not conducted.
which ensured uniform infection without escapes (Yamaguchi et al., 2013). The major difference between the two studies was that the viruses were either monopartite or bipartite, binary vectors were pBI101 or pGreenII, and \textit{A. tumefaciens} strains used were C58C1 or GV2260/EHA105. Some of the factors may have critically affected the uniformity of infectivity.

In the present study, agrobacterium from a cultured petri dish was directly inoculated with a colony inoculation procedure (Fig. 2). When the \textit{A. tumefaciens} strain GV2260 was used, 1 out of 24 plants inoculated with pGreenII-p35S-TYLCKaV-DNA-A+B showed typical begomoviral symptoms (Fig. 5a, c; Table 4). In contrast, when the \textit{A. tumefaciens} strain EHA105 was used, 23 out of 24 plants inoculated showed symptoms (Fig. 5a, d; Table 4, Exp. 3). In addition, both TYLCKaV DNA A and DNA B accumulated in all the plants with virus symptoms (Table 4). Approximately 92% of inoculated plants showed viral symptoms after 30 days from sowing (20 days from inoculation). \textit{A. tumefaciens} strain EHA105 tended to show higher infectivity compared with GV2260 using the soaking procedure or colony inoculation procedure. EHA105 and GV2260 both have a chromosomal background with C58, but the Ti plasmids are classified into different types (Hellens et al., 2000b). The transformation efficiency of tomato cells may differ between the two strains.

Viral DNAs were recovered and sequenced from the upper uninoculated leaf. The recovered DNA A and DNA B sequence had 100% identity with the infected TYLCKaV sequence (LC051116, LC177332). From these results, our study demonstrated that constructed clones had infectivity, and agroinoculation of TYLCKaV DNA A and DNA B can successfully induce virus symptoms in tomatoes.

In the present study, a highly efficient agroinoculation method for getting TYLCKaV into tomato plants was established. Because this colony inoculation procedure needs no liquid culture or preparation of agrobacterium suspension, it is a labor-saving method. Moreover, in this method, viral symptoms develop in susceptible tomato plants within approximately a month from sowing the seeds. Therefore, TYLCKaV resistance of tomato plants may be evaluated in a short period of time. To date, the resistance genes, \textit{Ty-1}–\textit{Ty-5}, have been identified from wild tomato relatives and some major genes have been incorporated into commercial cultivars. Our next goal is to evaluate the TYLCKaV resistance of commercial tomato cultivars possessing \textit{Ty} genes. Moreover, because the use of resistance to begomovirus infection is much less advanced in capsicums compared to tomatoes (Kenyon et al., 2014), efficient agroinoculation methods for capsicums also need to be established.

| Construct          | Number of plants infected/inoculated PCR (Exp. 3) |   |   |   |
|--------------------|--------------------------------------------------|---|---|---|
|                    | Exp. 1 | Exp. 2 | Exp. 3 | DNA A \(^{z}\) | DNA B \(^{y}\) |
| GV2260             |        |        |        |               |               |
| pGreenII-p35S      | 0/15   | 0/12   | 0/12   | 0/12          | 0/12          |
| pGreenII-p35S-DNA-A+B | 2/25  | 0/24   | 1/24   | 1/24          | 1/24          |
| EHA105             |        |        |        |               |               |
| pGreenII-p35S      | 0/15   | 0/12   | 0/12   | 0/12          | 0/12          |
| pGreenII-p35S-DNA-A+B | 27/28 | 20/24  | 23/24  | 23/24         | 23/24         |

\(^{z}\) Detected with TYLCKaV F and R primer.

\(^{y}\) Detected with TYLCKaV DNA-B 2F and R primer.

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