Application of Serological Techniques for the Diagnosis of Tomato Yellow Leaf Curl Disease

Bull. Natl. Agric. Res. Cent.
West. Reg. 10 : 13－27, (2011)

Application of Serological Techniques for the Diagnosis of Tomato Yellow Leaf Curl Disease

Hideki OSAKI[1,2], Koji NOMIYAMA[1] and Koichi ISHIKAWA[1]

Key words: DAS-ELISA, F(ab')$_2$-ELISA, IC-PCR, RIPA, *Tomato yellow leaf curl virus*

Contents

(Received September 17, 2010)

[1] Lettuce Big-vein Disease Research Team, National Agricultural Research Center for Western Region, NARO

[2] Vegetable Pest Management Research Team, National Institute of Vegetable and Tea Science, NARO

I Introduction

Tomato yellow leaf curl disease (TYLCD) is a severe viral disease affecting tomato crops in Japan since 1996[10]. Most commercial tomato cultivars are susceptible to the disease. It is one of the most devastating diseases of tomato crops in the world. Infected plants show severe symptoms consisting of yellowing, curling, and puckering in the top leaves; the plants become stunted, with a reduction in tomato fruits.

The causal agent, *Tomato yellow leaf curl virus* (TYLCV) is a member of the genus *Begomovirus* in the family *Geminiviridae*[7]; it is transmitted by the whitefly *Bemisia tabaci* Genn., and not transmitted by mechanical inoculation[4]. The viruliferous whitefly adults are capable of transmitting the virus throughout their lifespan[15].

A sanitation program for greenhouse tomato cultivation has been proposed as follows (http://vegetea.naro.affrc.go.jp/joho/manual/tomato_yellow_leaf_manual_h21.5.pdf). Entry of whiteflies and infected tomato plants into greenhouse, propagation of whiteflies and TYLCV in greenhouse, and leakage of whiteflies from greenhouse have to be avoided in order to control the disease. Diagnostic methods are therefore essential and important to promote the sanitation program.

Genetic diagnostic methods such as PCR[13] and LAMP[8] are now used widely for TYLCD. These diagnostic methods need several specialized expensive equipments, enzymes, and reagents. Moreover, skills and experiences are required to perform these methods. On the other hand, serological diagnostic methods do not require expensive reagents and equipments, and specialized skills to perform them. The cost of serological diagnoses is therefore generally lower than that of genetic ones.

A serological diagnostic kit for TYLCD is now sold. But it is not widely used because of the cost and the reliability of the kit. Therefore, in order to establish cheaper and more reliable diagnostic method, in this study, we purified TYLCV to obtain antiserum, and applied serological techniques, *i.e.* double antibody sandwich-enzyme-linked immunosorbent assay[3] (DAS-ELISA), $F(ab')_2$ fragment-based ELISA[2, 9] ($F(ab')_2$-ELISA), immunocapture-PCR[12] (IC-PCR) assay, and rapid immunofilter paper assay[16, 17] (RIPA), to the diagnosis of TYLCD.

II Materials and Methods

1 Whitefly and *Tomato yellow leaf curl virus*

A colony of the B biotype of *B. tabaci* was provided by Dr. T. Watanabe (Plant Protection Office of Kagawa Prefectural Agricultural Experiment Station), and reared on a cabbage (*Brassica oleracea* L. var. *capitata*) cultivar 'Harunami'.

The Tadotsu isolate in the Israeli strain[18] of TYLCV (TYLCV-IL) obtained from naturally infected tomato plants in Tadotsu, Kagawa Prefecture was propagated and purified for antiserum preparation. The Nagasaki and Tokyo isolates in TYLCV-IL were provided by Dr. S. Ueda (National Agricultural Research Center for Kyushu Okinawa Region), and by Dr. J. Ohnishi (National Institute of Vegetable and Tea Science), respectively. The Aichi isolate in the mild strain[18] of TYLCV (TYLCV-Mld) was provided by Dr. T. Tanaka, Aichi Agricultural Research Center.

Tobacco leaf curl Japan virus[7] (TbLCJV), a begomovirus indigenous to Japan, causes tomato yellow

dwarf. The symptoms are similar to those of TYLCD. TbLCJV is transmitted by *B. tabaci* JpL biotype[14, 19]. The Takamatsu isolate of TbLCJV was obtained from naturally infected tomato plants in Takamatsu, Kagawa. The Zentsuji isolate of TbLCJV was obtained by inoculating healthy tomato seedlings with *B. tabaci* JpL biotype adults, which naturally inhabit honeysuckles (*Lonicera japonica*) in Zentsuji, Kagawa. Two isolates of TbLCJV were used as an indigenous begomovirus.

The viruses were maintained on a susceptible tomato (*Solanum lycopersicum* L.) cultivar 'House Momotaro' . In experiments presented in Tables 1, 3 and 4, cv. House Momotaro was used for inoculation.

2 Purification of TYLCV

TYLCV particle was purified by an adaption of methods developed for other geminiviruses[5, 6]. Plants were homogenized in a buffer containing 0.1 M sodium phosphate, pH 7.0, 2.5 mM EDTA, 10 mM sodium sulfite, 0.1% 2-mercaptoethanol, and 1% Triton X-100. The extract was filtered through three layers of gauze. The sap was clarified with 10% chloroform and centrifuged at 8,000×g for 20 min. Polyethylene glycol (mol. wt. 7300-9300; 12 g/100 ml) and 0.2 M NaCl were added to the aqueous phase. After 30 min of stirring at 4℃, the precipitate was collected by centrifugation for 15 min at 18,000×g, and resuspended in 0.1 M phosphate buffer containing 2 mM EDTA. The suspension was loaded onto 20% sucrose cushions and centrifuged for 2 hr at 180,000×g. The pellets were resuspended in 0.1 M phosphate buffer containing 2 mM EDTA. Sucrose gradients (10-40%, 10 ml) were prepared in the resuspension buffer. Virus suspension was layered on top of each gradient and centrifuged at 240,000×g for 2.5 hr. Gradients were fractionated (2.5 ml per fraction) from the top of the gradients by using a Pasteur pipette. The presence of the virus in the sucrose gradient fractions was confirmed by electron microscopic observation and SDS-PAGE analysis. The virus-containing fractions were centrifuged for 2 hr at 190,000×g. Pellets were resuspended in 0.1 M phosphate buffer containing 2 mM EDTA. The suspension was purified by cesium sulfate density equilibrium centrifugation at 240,000×g for 20 hr. A light-scattering band was sampled, and centrifuged at 190,000×g for 2 hr. Pellets were resuspended in 0.1 M phosphate buffer containing 2 mM EDTA. Virus concentration was estimated from absorbance at 260 nm, assuming that 1 mg of the virus corresponded to 7.7 A_{260} units.

3 Antiserum and western blot analysis

Production of polyclonal antibody by a rabbit was contracted to Takara Bio Inc. Immunoglobulin G (IgG) was purified from antiserum obtained by using ImmunoPure (Protein A) IgG Purification Kit (PIERCE). Prior to purification, the antiserum was cross-absorbed with host sap.

Leaf samples were homogenized with 20 volumes (w/v) of sample buffer (50 mM Tris, pH 9.0, 2% SDS, 1% 2-mercaptoethanol, 15% sucrose, and 0.0005% bromophenol blue) for western blot. For whitefly samples, one adult or three adults were homogenized with 20 μl of sample buffer. After boiling for 3 minutes, each sample (20 μl) was separated by 12% SDS-polyacrylamide gel electrophoresis, and proteins were transferred to a PVDF membrane (BIO-RAD). Then, the membrane was treated with 0.25 μg/ml anti-TYLCV IgG in TBST (0.02 M Tris-buffered saline supplemented with 0.05% Tween 20) for 30 min at room temperature, and then alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma) at a 1:4000 dilution in TBST for 30 min. Band visualization was carried out as previously described[11]. Three 5-min washes of the membrane with TBST were performed between the steps.

4 DAS-ELISA

IgG was coupled with alkaline phosphatase using the one-step glutaraldehyde method[1]. For ELISAs, leaf extracts were prepared by grinding leaves with a mortar and pestle together with PBST (0.01 M phosphate-buffered saline supplemented with 0.05% Tween 20).

Each well of a microplate was coated with purified IgG diluted to 10 μg/ml in coating buffer (50 mM Na_2CO_3, pH 9.6), and incubated for 4 hr at 37℃. Plates were washed with PBST. Then samples were added, and incubated for 2-3 hr at 37℃. After washing, alkaline phosphatase conjugated IgG diluted to 1:500 with PBST was added and incubated for 2-3 hr at 37℃. Substrate (*p*-nitrophenylphosphate at 1 mg/ml in 10% diethanolamine buffer, pH 9.8) was added after washing and hydrolysis time was 1 hr at 37℃. The absorbance of each well was measured at A_{405} with a spectrophotometer Corona MTP-32. Absorbance values above 0.05 were considered as positive.

5 F(ab')$_2$-ELISA

F(ab')$_2$-ELISA requires F(ab')$_2$ fragments purified from IgG. The F(ab')$_2$ fragment was purified by ImmunoPure F(ab')$_2$ Preparation Kit (PIERCE). Each well of a microplate was coated with purified F(ab')$_2$ fragment diluted at 10 μg/ml in coating buffer, and incubated for 4 hr at 37℃. Plates were washed with PBST and then samples were added, and incubated overnight at 4℃. After washing, IgG (2 μg/ml) in PBST was added and microplates were incubated for 2-3 hr at 37℃. The wells were washed and re-filled with alkaline phosphatase-labelled protein A diluted at 2 μg/ml in PBST and incubated for 2-3 hr at 37℃. Adding substrate, hydrolysis, and measuring the absorbance of wells were performed as described for DAS-ELISA.

6 IC-PCR

IC-PCR assay was carried out as follows. Sterile polypropylene 0.2-ml and 96-well PCR plates (ABgene) were precoated with 20 μl of IgG (1 mg/ml diluted 1:200 in carbonate buffer) per well. The plates were incubated for 2 hr at 37℃. For IC-PCR assay, leaf extracts were prepared by grinding leaves with a mortar and pestle together with PBST. After each well of the plate was washed three times with PBST, 20-μl aliquots of extract were added and incubated again for 2 hr at 37℃. Each well was then washed twice with PBST and once with sterilized distilled water.

PCRs were carried out as follows. Amplifications were performed in 20-μl reaction mixtures, containing 0.2 μM of each primer, 0.5 unit of Ex *Taq* DNA polymerase (Takara), a dNTP mixture (containing 200 μM of each of dNTP), and Ex *Taq* reaction buffer (containing 2 mM $MgCl_2$), in a Takara PCR Thermal Cycler MP. The primers, TYmultiV and TYmultiC, were used[13]. The PCR plates were heated at 96℃ for 3 min followed by 40 reaction cycles of 30 sec at 96℃ for melting, 30 sec at 55℃ for primer annealing, and 45 sec at 72℃ for primer extension. A final step at 72℃ for 5 min was carried out prior to holding the samples at 4℃ until removal from the thermal cycler. PCR products were electrophoresed in 0.8% agarose and visualized by staining with ethidium bromide and UV illumination.

7 RIPA

The coating procedure of IgG to latex was adapted from developed RIPA procedure[16]. For coating of IgG, white latex (JSR Co., Cat. No. G24103) and red latex (JSR Co., Cat. No. G0301R) were diluted to 1% and 2%, respectively, in TBS (0.02M Tris-buffered saline). IgG was diluted to 500 μg/ml for white latex and 300

μg/ml for red latex with TBS. The latex solutions were mixed with an equal volume of IgG solution. Sensitization was performed as described by Tsuda *et al* [16].

Leaf extracts were prepared by grinding leaves with a mortar and pestle together with 0.1 M phosphate buffer, pH 7.0, containing 10 mM EDTA, 0.1% 2-mercaptoethanol, and 0.1% bovine serum albumin as extraction buffer.

The detection procedure was carried out as previously described for the two-step method of RIPA[17].

8 Time-course detection from inoculated tomato seedlings

Adult whiteflies were allowed to feed on infected tomato plants (Tadotsu isolate) for 3 days. Ten adults were transferred to each cage containing a healthy seedling at the four-leaf stage. The whiteflies were removed from the cages 3 days later. The seedling inoculated were observed and assayed.

On designated days, leaf tissue (*ca.* 20 mg) from newly-expanding top leaf of the seedlings was sampled and homogenized with 50 volumes (w/v) of PBST in experiment of Table 3, whereas, with 50 volumes (w/v) of extraction buffer for RIPA in experiment of Table 4. The leaf extract was used for $F(ab')_2$-ELISA, IC-PCR assay, and RIPA.

9 Hammer extraction for RIPA

Small amounts of leaf tissue (*ca.* 20 mg) from the newly-expanding top leaf of tomato plants were sampled, and placed into plastic bags (70×50mm). The tissues in bags were smashed with a couple of strikes by a hammer. Extraction buffer (*ca.* 500 μl) were added and mixed with the smashed tissue by fingers, and then the squeezes were used for RIPA. On experiment of Table 5, tomato leaves were sampled at greenhouses in Tadotsu, Kagawa, and extracted by a hammer for RIPA.

III Results and Discussion

1 Purification of TYLCV and antiserum

The virus was purified from infected tomato plant material. Analysis of the purified virus by SDS-PAGE showed a single band (*ca.* 30 kDa), presumed to be a coat protein of TYLCV (Fig. 1). Virus of *ca.* 2 mg was obtained from about 40 kg of infected tomato plant material. The purified virus was used for production of antiserum.

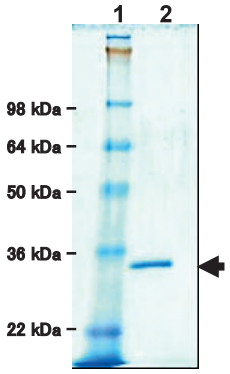

Fig. 1. Electrophoresis of purified TYLCV in SDS-polyacrylamide gel.
Lane 1: Molecular weight standards (SeeBlue Plus2 Pre-Stained Standard, Invitrogen); lane 2: Purified TYLCV. Arrow indicates coat protein of TYLCV.

In western blot analysis (Fig. 2) with IgG purified from antiserum obtained, bands were formed from samples of four TYLCV isolate-infected plants, but not from healthy and two TbLCJV isolate-infected plants. This suggested that the two strains of TYLCV, TYLCV-IL and -Mld are equally reacted with the IgG, and that the IgG is specific to TYLCV. Moreover, western blot analysis could detect the virus from the extracts of a single adult whitefly reared on infected tomato plants (Fig. 3). These results indicate that the titer of the IgG is sufficient to detect TYLCV from viruliferous whiteflies, and that the IgG will be useful in other serological detection methods for TYLCV.

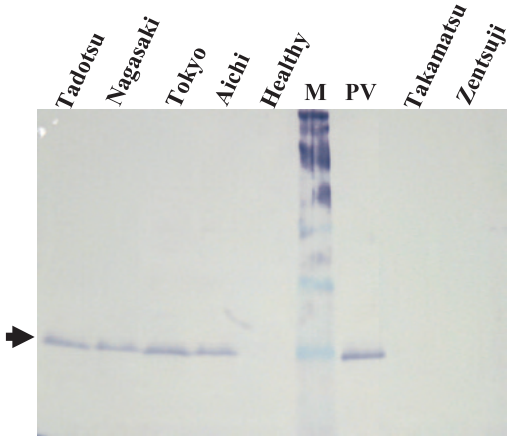

Fig. 2. Detection of TYLCV from extracts of TYLCV- and TbLCJV-infected tomato plants by western blot.
Three isolates in TYLCV-IL (Tadotsu, Nagasaki, and Tokyo), an isolate in TYLCV-Mld (Aichi), and two TbLCJV isolates (Takamatsu and Zentsuji) were used. M represents molecular weight standards (SeeBlue Plus2 Pre-Stained Standard, Invitrogen). In lane of PV, purified virus (20 ng) was electrophoresed. Arrow indicates coat protein of TYLCV.

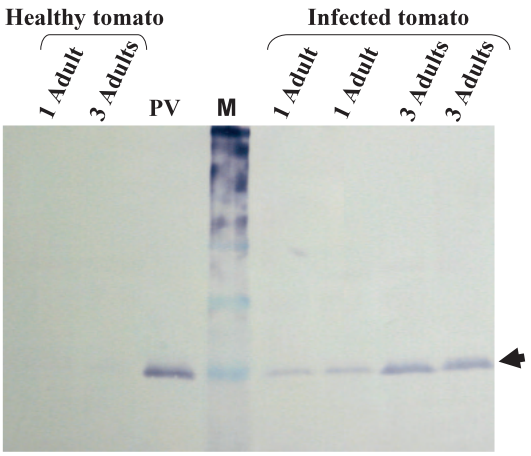

Fig. 3. Detection of TYLCV from extracts of adult whiteflies by western blot.
Adult whiteflies were reared for a week on healthy and TYLCV-infected tomato plants (Tadotsu isolate), respectively. M represents molecular weight standards (SeeBlue Plus2 Pre-Stained Standard, Invitrogen). In lane of PV, purified virus (20 ng) was electrophoresed. Arrow indicates coat protein of TYLCV.

2 ELISA, IC-PCR assay, and RIPA

The IgG was adapted to two ELISAs (DAS- and F(ab')$_2$-ELISA). DAS-ELISA detected TYLCV up to 6,400-fold diluted extract of infected leaves. Meanwhile, in F(ab')$_2$-ELISA, the detection limit of TYLCV was 12,800-fold diluted extract (Fig. 4). Since the absorbance values of F(ab')$_2$-ELISA were higher than those of DAS-ELISA, it may be possible to evaluate the results by naked eye. Thereafter, F(ab')$_2$-ELISA was particularly examined.

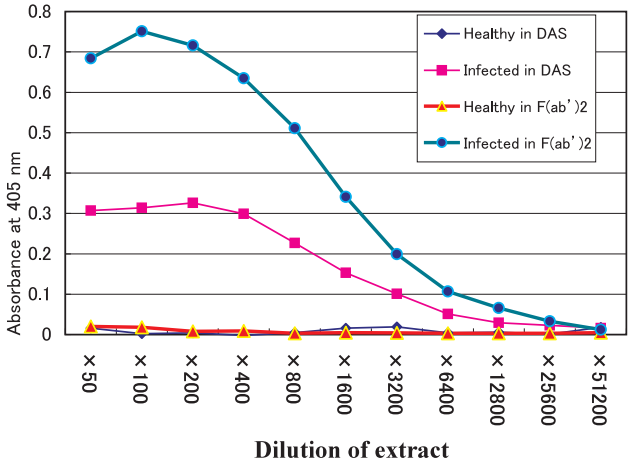

Fig. 4. Detection of TYLCV from dilutions of healthy and infected leaf extracts by DAS- and F(ab')$_2$-ELISA.
Leaf samples were homogenized with PBST. The extracts were serially diluted with PBST.
Tadotsu isolate-infected and healty tomato plants were used.

To evaluate the reliability and practical utility of F(ab')$_2$-ELISA, F(ab')$_2$-ELISA should be compared with PCR assay. Since PCR assays need laborious and time-consuming DNA extraction, to simplify preparation of DNA, the IgG was adapted to IC-PCR assay. In IC-PCR assay, the dilution end point for detection of TYLCV in leaf extract was 10^7 (Fig. 5). IC-PCR assay was 1,000-fold more sensitive than F(ab')$_2$-ELISA.

Furthermore, the IgG was also adapted to RIPA. Within several minutes, the filter paper strips showed a positive reaction to infected tomato samples, forming a red line by antigen-antibody reaction (Fig. 6). When

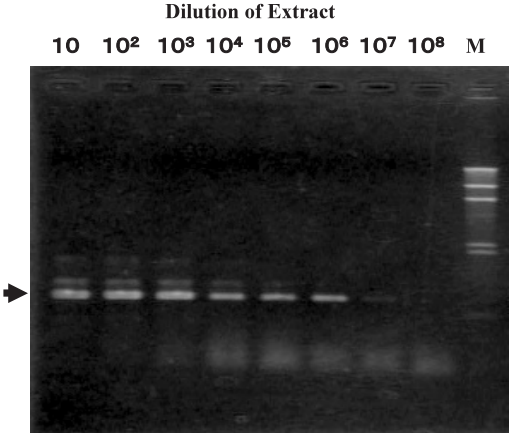

Fig. 5. Detection of TYLCV from dilutions of infected leaf extract by IC-PCR.
Leaf sample (Tadotsu isolate) was homogenized with PBST. The extract was diluted with PBST. M represents *Hin*d III-digested Lambda DNA marker.
Arrow indicates amplified specific products.

the extracts of infected tomato leaves were used, positive red line was detected up to a 3,200-time dilution (Fig. 7). This result indicated that RIPA was 4 times less sensitive than F(ab')$_2$-ELISA.

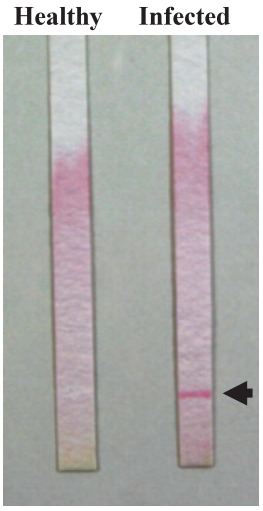

Fig. 6. RIPA of healthy and infected leaf extracts.
Leaf samples were homogenized with 50 volumes (w/v) of extraction buffer for RIPA. Red line (arrow) was formed on filter paper strip dipped into Tadotsu isolate-infected leaf extract (right strip), but not healthy (left strip).

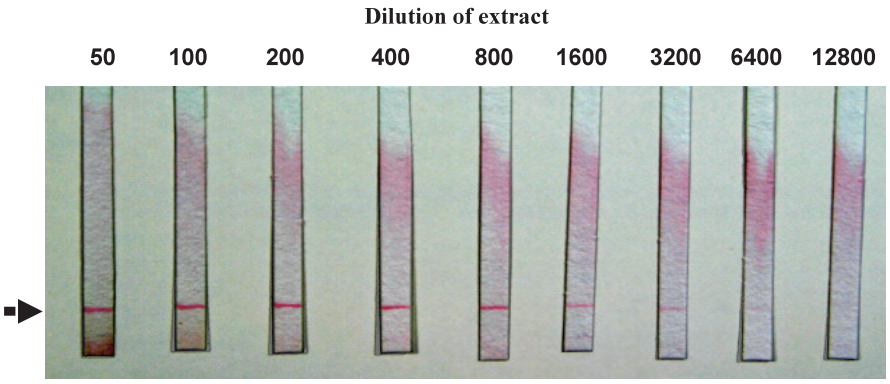

Fig. 7. Detection of TYLCV from dilutions of infected leaf extract by RIPA.
Leaf samples (Tadotsu isolate) were homogenized with extraction buffer for RIPA. The extract was diluted with the extraction buffer. Red lines (arrow) were formed on filter paper strips until 3200-fold dilution of extract by judgment with the naked eye.

3 Comparison of F(ab')$_2$-ELISA, IC-PCR assay, and RIPA for practical diagnosis

1) Assay for viruliferous whitefly

To evaluate the sensitivity of these assays and to determine whether they can identify the viruliferous whiteflies, we attempted to detect the virus in whiteflies reared on infected tomato plants.

Table 1 indicates that adult whiteflies reared on infected tomato plants for more than 90 min are considered to be potential as a vector. On samples of whiteflies reared on infected tomato plants for more than 90 min, the results of F(ab')$_2$-ELISA were consistent with those of IC-PCR assay, although F(ab')$_2$-ELISA could not detect TYLCV from whiteflies reared on infected tomato plants for 45 min (Table 2). We therefore suggest that F(ab')$_2$-ELISA is also capable in assay for viruliferous whiteflies. Meantime, DAS-ELISA and RIPA were unsuccessful for the detection of TYLCV in whiteflies reared on infected tomato plants for even 2 days (data not shown). DAS-ELISA and RIPA are not available for viruliferous whiteflies.

Monitoring for the presence of viruliferous whitefly around greenhouses is important in alerting tomato growers to deal with the threat of infection by spraying insecticides and filling gaps in greenhouses with whitefly-proof screens. F(ab')$_2$-ELISA will be a crucial tool for identifying viruliferous whitefly at low cost.

Table 1 Transmission of TYLCV by whiteflies

Period of rearing on infected tomato plants	No. of seedlings infected[1] / No. of seedlings inoculated
20 min	0/5
45 min	0/5
90 min	5/5
3 hr	5/5
6 hr	5/5
12 hr	5/5

1) Adult whiteflies were allowed to feed on infected tomato plants (Tadotsu isolate) for designated time periods. The adults (10 adults/cage) were transferred to each insect-proof cage containing a healthy tomato seedling at the four-leaf stage. After 3 days, the whiteflies were removed from the cages. The seedlings inoculated were assayed by IC-PCR on 30 days after inoculation.

Table 2 F(ab')$_2$-ELISA and IC-PCR assay of adults of B biotype of *Bemisia tabaci* reared on infected tomato plants

Period of rearing on infected tomato	No. of adults used[1]	No. of adults PCR-positve	No. of adults ELISA-positive
20 min	16	0	0
45 min	16	6	0
90 min	16	8	8
3 hr	16	10	10
6 hr	16	13	13
12 hr	16	15	15

1) In parallel with the experiment of Table 1, sixteen adult whiteflies on the leaves of infected tomato plants were sampled 20 min, 45 min, 90 min, 3 hr, 6 hr, and 12 hr later, respectively. Each adult whitefly was homogenized with 120 μl of PBST. The homogenate was used for F(ab')$_2$-ELISA (100 μl) and IC-PCR assay (20 μl).

2) Practical utilities of F(ab')$_2$-ELISA and IC-PCR assay

To compare the practical utility of F(ab')$_2$-ELISA with that of IC-PCR assay, time-course detection was done on tomato seedlings inoculated by whiteflies (Table 3). On two samples (Nos. 2 and 5), IC-PCR assay could detect the virus earlier than F(ab')$_2$-ELISA could. On the remaining samples, however, F(ab')$_2$-ELISA and IC-PCR assay detected the virus in the same way. By F(ab')$_2$-ELISA, the virus was detected 2 to 6 days before the onset of symptoms. Although IC-PCR assay is 1,000 times more sensitive than F(ab')$_2$-ELISA (Figs. 4 and 5), these results indicate that F(ab')$_2$-ELISA is sensitive enough to practically detect the virus. Additionally, F(ab')$_2$-ELISA does not require complicated sample preparation and can handle many samples at the same time. Therefore, we consider F(ab')$_2$-ELISA to be a very practical approach for diagnosis of TYLCD.

Table 3 Time-course detection by IC-PCR assay and F(ab')$_2$-ELISA of inoculated tomato seedlings

Seedling No.	Days after inoculation	5	7	9	11	13	15	17
No.1	PCR	−	+	+	+	+*	+	+
	ELISA	−	+	+	+	+*	+	+
No.2	PCR	−	−	−	+	+	+	+*
	ELISA	−	−	−	−	+	+	+*
No.3	PCR	−	−	−	+	+*	+	+
	ELISA	−	−	−	+	+*	+	+
No.4	PCR	−	−	−	+	+*	+	+
	ELISA	−	−	−	+	+*	+	+
No.5	PCR	−	−	−	+	+	+*	+
	ELISA	−	−	−	−	+	+*	+
No.6	PCR	−	−	+	+	+*	+	+
	ELISA	−	−	+	+	+*	+	+

＊：The first day of observing symptoms

On the other hand, the sensitivity of IC-PCR assay was superior to those of ELISAs. Compared with conventional PCR, IC-PCR assay does not need the use of organic solvents and centrifugation, because DNA purification, which is generally a laborious and time-consuming process, is not necessary. Therefore, it is of great advantage. Moreover, in IC-PCR assay, virion preparation and PCR amplification are both carried out completely in a single well of a 96-well PCR plate, making the assay very convenient and economical. IC-PCR assay would be a powerful tool for diagnosing a large number of samples as well as F(ab')$_2$-ELISA.

In practical terms, it is not required to apply such a sensitive but expensive and complicated method, *i.e.* PCR, for all of the samples to be assayed. It is important to apply first F(ab')$_2$-ELISA to be able to assay a large number of samples at low cost. Prior to settled planting in greenhouse, tomato seedlings grown in a nursery should be assayed by F(ab')$_2$-ELISA to prevent infected seedlings from being planted in greenhouses. Then further confirmation can be made by using more reliable method such as IC-PCR assay. F(ab')$_2$-ELISA will be very helpful for practical, inexpensive diagnosis of TYLCD in field surveys.

3) Practical utility of RIPA

Furthermore, to evaluate the practical utility and the sensitivity of RIPA, time-course detection was done on tomato seedlings inoculated by whiteflies; these results are summarized in Table 4. RIPA also showed positive before inducing symptoms. The virus was detected 2 to 6 days before the onset of symptoms.

Table 4 Time-course detection by RIPA of inoculated tomato seedlings

Seedling No.	Days after inoculation	5	7	9	11	13	15	17
No.1		−	−	+	+	+*	+	+
No.2		−	−	−	+	+*	+	+
No.3		−	+	+	+	+*	+	+
No.4		−	−	+	+	+	+*	+
No.5		−	−	−	+	+*	+	+
No.6		−	+	+	+	+*	+	+

＊：The first day of observing symptoms

These results were similar to those of F(ab')$_2$-ELISA (Table 3). Although RIPA is four times less sensitive than F(ab')$_2$-ELISA, these results indicate that RIPA is sensitive enough to practically detect the virus. RIPA is also considered to be very practical for diagnosis of TYLCD.

In our laboratory, leaf extracts were prepared for RIPA by grinding leaves with a mortar and pestle together. For field diagnosis, we developed a simple rapid extraction method using a hammer for RIPA. RIPA with the hammer extraction at tomato greenhouses in Tadotsu showed that the results were consistent with results of IC-PCR assay (Table 5). Moreover, on sample Nos. 4, 8, and 12 of Table 5, RIPA could detect TYLCV in infected plants without symptoms at incubative stage. Compared with use of a mortar and pestle, the hammer extraction could shorten the time of preparing samples. The hammer extraction permitted us to prepare a sample in about half minute, while extraction with a mortar and pestle needed a couple of minutes. Thus, we consider the hammer extraction method to be suitable for preparing samples in the field.

Table 5 Field diagnosis by RIPA with hammer extraction

Sample No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Symptoms	−	+	+	−	−	−	+	−	+	−	+	−	−	+	−
RIPA	−	+	+	+	−	−	+	+	+	−	+	+	−	+	−
PCR[1)]	−	+	+	+	−	−	+	+	+	−	+	+	−	+	−

1) After sampling, IC-PCR assay was carried out in laboratory.

Among serological diagnostic methods, ELISAs have been generally employed. The ELISAs, however, need a few equipments such as an incubator and a spectrophotometer, and take time to obtain the results, therefore those are not suitable for virus diseases diagnosis in field. RIPA method can be easily used for diagnosis of virus diseases without any equipment and in several minutes in field.

Besides, RIPA requires no skill and experience, and the results can be obtained within several minutes with the naked eyes, so that tomato growers can assay their own tomato plants by themselves. RIPA can be used as easily and simply as pH test paper.

Extremely rapid and simple diagnosis by RIPA is of great advantage to countermeasure against the spread of TYLCD. It is important to remove infected tomato plants from greenhouses as soon as possible, because infected tomato plants serve as a potential source of secondary infection to other healthy plants. Therefore, it is recommended to diagnose suspected plants using RIPA by tomato growers themselves, and then remove positive ones from greenhouse promptly. Although the sensitivity of RIPA is lower than PCR assay and ELISA, the agility of RIPA is superior to PCR assay and ELISA. The agility of RIPA is very useful for rapid removal of infected plants from the greenhouses.

Acknowledgements

We thank Mr. K. Kawanishi and Ms. A. Yonezawa (Plant Protection Office of Kagawa Prefectural Agricultural Experiment Station) for helping us to collect samples. This study was supported in part by a grant from the Research Project for Utilizing Advanced Technologies in Agriculture, Forestry, and Fisheries (No. 18002) from the Ministry of Agriculture, Forestry and Fisheries of Japan.

References

1) Avrameas, S. 1969. Coupling of enzymes to protein with glutaraldehyde. Use of the conjugate for the detection of antigen and antibodies. Immunochemistry 6：43－52.
2) Barbara, D. J. and M. F. Clark. 1982. A simple indirect ELISA using F(ab')$_2$ fragments of immunoglobulin. J. Gen. Virol. 58：315－322.
3) Clark, M. F. and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34：475－483.
4) Cohen, S. and F. E. Nitzany. 1966. Transmission and host range of the *Tomato yellow leaf curl virus*. Phytopathology 56：1127－1131.
5) Cohen, S., J. E. Duffus, R. C. Larsen, H. Y. Liu and R. A. Flock. 1983. Purification, serological, and vector relationships of squash leaf curl virus, a whitefly-transmitted geminivirus. Phytopathology 73：1669－1673.
6) Czosnek, H., R. Ber, Y. Antignus, S. Cohen, N. Navot and D. Zamir. 1988. Isolation of tomato yellow leaf curl virus, a geminivirus. Phytopathology 78：508－512.
7) Fauquet, C. M., R. W. Briddon, J. K. Brown, E. Moriones, J. Stanley, M. Zerbini and X. Zhou. 2008. Geminivirus strain demarcation and nomenclature. Arch. Virol. 153：783－821.
8) Fukuta, S., S. Kato, K. Yoshida, Y. Mizukami, A. Ishida, J. Ueda, M. Kanbe and Y. Ishimoto. 2003. Detection of tomato yellow leaf curl virus by loop-mediated isothermal amplification reaction. J. Virol. Meth. 112：35－40.
9) Ishikawa, K., S. Kusumoto and T. Kawano. 2008. Detection of Melon yellow spot virus (MYSV) from cucumber and *Thrips palmi* by TAS-ELISA. Jpn. J. Phytopathol. 74：219 (Abstr., in Japanese).
10) Kato, K., M. Onuki, S. Fuji and K. Hanada. 1998. The first occurrence of *Tomato yellow leaf curl virus* in tomato (*Lycopersicon esculentum* Mill.) in Japan. Ann. Phytopathol. Soc. Japan 64：552－559.
11) Leary, J. J., D. J. Brigati and D. C. Ward. 1983. Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose：Bio-blots. Proc. Natl. Acad. Sci. USA 80：4045－4049.
12) Nolasco, G., C. de Blas, V. Torres and F. Ponz 1993. A method combining immunocapture and PCR amplification in a microtiter plate for the detection of plant viruses and subviral pathogens. J. Virol. Meth. 45：201－218.
13) Onuki, M., S. Ueda, C. Yamamoto and S. Okazaki 2009. Discriminatory detection of *Tomato yellow leaf curl virus* and tomato-infecting indigenous begomoviruses in Japan by multiplex PCR. Kyushu Pl. Prot. Res. 55：40－44.
14) Osaki, T., H. Kobatake and T. Inouye. 1979. Yellow vein mosaic of honeysuckle (*Lonicera japonica*

Thunb.), a disease caused by Tobacco leaf curl virus in Japan. Ann. Phytopathol. Soc. Japan 45：62－69.

15) Rubinstein, G. and H. Czosnek. 1997. Long-term association of *Tomato yellow leaf curl virus* with its whitefly vector *Bemisia tabaci*：effect on the insect transmission capacity, longevity and fecundity. J. Gen. Virol. 78：2683－2689.

16) Tsuda, S., M. Kameya-Iwaki, K. Hanada, Y. Kouda, M. Hikata and K. Tomaru. 1992. A novel detection and identification technique for plant viruses：Rapid immunofilter paper assay (RIPA). Plant Dis. 76：466－469.

17) Tsuda, S., M. Kameya-Iwaki, K. Hanada, I. Fujisawa and K. Tomaru. 1993. Simultaneous diagnosis for plants infected with multiple viruses employing rapid immunofilter paper assay (RIPA) with two-step method; Multi-RIPA. Ann. Phytopathol. Soc. Jpn. 59：200－203.

18) Ueda, S., S. Takeuchi, M. Okabayashi, K. Hanada, K. Tomimura and T. Iwanami. 2005. Evidence of a new Tomato yellow leaf curl virus in Japan and its detection using PCR. J. Gen. Plant Pathol. 71：319－325.

19) Ueda, S., T. Kitamura, K. Honda and K. Kanmiya. 2006. Characterization and distribution of the biotypes of the whitefly *Bemisia tabaci* in Japan by mitochondrial cytochrome oxidase I sequence analysis. 4th International Bemisia Workshop：25 (Abstr.).

Summary

To establish cheap and reliable diagnostic method, in this study, we tried to apply three serological techniques, that is, ELISA, RIPA and IC-PCR assay, to the diagnosis of tomato yellow leaf curl disease. First of all, TYLCV was purified from infected tomato plants to obtain antiserum. Western blot analysis showed that the IgG purified from obtained antiserum was specific to TYLCV. DAS-ELISA detected TYLCV up to 6,400-fold diluted extract of infected tomato leaves. Meanwhile, in $F(ab')_2$-ELISA, the detection limit of TYLCV was 12,800-fold diluted extract. In IC-PCR assay, the dilution end point for detection of TYLCV in leaf extract was 10^7. IC-PCR assay was 1,000-fold more sensitive than $F(ab')_2$-ELISA. However, $F(ab')_2$-ELISA also could detect TYLCV from viruliferous whiteflies, and from infected plants 2 to 6 days before the onset of symptoms. By RIPA, positive red line was detected up to a 3,200-time dilution of the extracts of infected tomato leaves. RIPA also detected the virus from infected plants without symptoms at incubative stage as well as $F(ab')_2$-ELISA. $F(ab')_2$-ELISA and IC-PCR assay are considered to be very practical for diagnosing a large number of samples. On the other hand, RIPA is considered to be suitable for diagnosis by tomato growers, since it is simple and agile as a pH test paper. To hamper the spread of the disease in greenhouse, RIPA is a powerful tool for rapidly removing infected plants from greenhouses as soon as possible.

血清学的手法のトマト黄化葉巻病の診断への適用

大崎秀樹[1]・野見山孝司・石川浩一

摘　　要

安価で信頼性のある診断法を確立するため，血清学的手法，すなわちELISA，RIPA，IC-PCR検定をトマト黄化葉巻病の診断に適用した．まずTYLCVを純化し，兎に免疫し抗血清を得た．抗血清からIgGを精製しそれぞれの手法に適用した．

1．ウエスタンブロット解析の結果より，本IgGはTYLCVの系統にかかわらず反応する一方，TbLCJVには反応せず，血清学的診断法に使用可能であると考えられた．

2．本IgGをDAS-ELISAとF(ab')$_2$-ELISAに適用した．TYLCV感染トマト葉磨砕液での検出希釈限界はDAS-ELISAで6400倍，F(ab')$_2$-ELISAで12800倍であり，F(ab')$_2$-ELISAの感度が高かった．

3．F(ab')$_2$-ELISAはタバココナジラミの保毒検定が可能であった．また，トマト新葉部での発症 2 ～ 6 日前に診断が可能であった．これらの結果よりF(ab')$_2$-ELISAは，実用性があると判断された．

4．また，本IgGをIC-PCR検定に適用した．TYLCV感染トマト葉磨砕液での検出希釈限界は10^7倍であった．通常のPCR検定時に行われるDNA精製のための煩雑な作業，そして有機溶媒や遠心器等高額機材の使用が不要なため省力的であり，多試料検定にも有効であると考えられた．

5．さらに本IgGをRIPAに適用した．TYLCV感染トマト葉磨砕液での検出希釈限界は3200倍であった．検出感度はF(ab')$_2$-ELISAの1/4と劣るものの，F(ab')$_2$-ELISAと同等の発症前診断が可能であったことから実用的な診断には有効と考えられた．現場圃場での検定のため，ハンマーを用いた簡易な検体磨砕法を考案した．

以上の結果より，多試料および保毒虫の検定にはF(ab')$_2$-ELISAやIC-PCR検定が，圃場現場での迅速な診断にはRIPAがそれぞれ適していた．

近畿中国四国農業研究センター　レタスビッグベイン研究チーム
[1]野菜茶業研究所 野菜IPM研究チーム併任