Detection of Tomato Spotted Wilt Virus S RNA in Individual Thrips by Reverse Transcription and Polymerase Chain Reaction

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Key words: tomato spotted wilt virus (TSWV), RT-PCR, diagnosis, individual thrips.

Tomato spotted wilt virus (TSWV; Tospovirus, Bunyaviridae) is an only known plant virus transmitted in a persistent manner by certain species of thrips, such as Frankliniella fusca, Thrips tabaci and T. setosus. The virus is acquired only at the nymph stage, and infectious adults can transmit it throughout their lives. Detection of the virus in thrips have been generally achieved by a bioassay on host plants. Immunological or molecular biological techniques have also been developed. Paliwal demonstrated that TSWV could be serologically detected in the homogenates of 50 first-generation adults of F. fusca, reared on newly infected plants. The hemagglutination test was applied to detect the viral antigens in extracts of 20-80 insects, both Scirtothrips dorsalis and F. schultzei, exposed to infected leaves. Cho et al. has developed an enzyme-linked immunosorbent assay (ELISA) system employing the antiserum against intact TSWV particles for detection of TSWV in individual thrips. Diagnostic dot blot hybridization assay using cDNA to TSWV-RNA has been applied to individual F. occidentalis, and great variability in the quantity of TSWV was detected.

Polymerase chain reaction (PCR) is a powerful tool to amplify small amounts of DNA (or RNA by using reverse transcriptase (RT)), for various molecular analyses. It has widespread applications in diagnosis of genetic as well as infectious diseases. In this study, RT-PCR was applied to determine whether a TSWV-S RNA species was present in individual thrips throughout their lives.

A TSWV isolate from green pepper (Capsicum annuum) in Iwate Prefecture, identified as TSWV-O strain by serology to nucleocapsid protein, was used for the experiments. The virus from infected leaf was prepared with 0.1M phosphate buffer (pH 7.0) containing 10mM Na2SO3 and mechanically inoculated to Nicotiana rustica by the conventional method using carborundum. Plants were kept in a glasshouse at 23-26°C.

Non-viruliferous T. setosus were reared on Vigna sesquipedalis in laboratory, and handled as shown in Fig. 1. Insects and plants were kept in the glasshouse with a cage on a pot. Sap-inoculated Datura stramonium or green pepper plants were used as the virus source for thrips when they developed vein clearing on their top leaves. First-instar nymphs of thrips were allowed to feed on the infected leaves for 2 hr, and then reared on healthy V. sesquipedalis until adult emergence. After their emergence (about...
Infected leaf

Acquisition feeding by nymphs

Emergence

Analysis of thrips by RT-PCR

Inoculation test feeding by individual thrips

Recovery of adult thrips

Development of symptoms

Fig. 1. Overall experimental plan. The figure summarizes the successive manipulations, from acquisition and inoculation access feeding of TSWV by individual thrips to analyses by RT-PCR.

10 days passed), each viruliferous adult thrips was transferred separately to a healthy green pepper seedling and was allowed to feed for 2 days. Then thrips were removed and stored separately at −70°C. Those green pepper plants were further kept in the glasshouse for additional one month and examined for the development of symptoms.

Extraction of RNAs from nucleocapsid of purified TSWV were carried out as described by Tsuda et al.12) Isolation of total RNA from leaf tissues or insects were performed as described by Chomczynski & Sacchi3). Briefly, leaf tissues (less than 50 mg) or individual insects were ground with a mortar and pestle in 500 μl of extraction buffer containing 4 M guanidinium thiocyanate, 25 mM Na-citrate (pH 7.0), 0.5% (w/v) N-lauroylsarcosine sodium salt and 0.1 M 2-mercaptoethanol (2-ME). The extracts were mixed and shaken with an equal volume of phenol saturated with 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA (TE), 0.1 volume of 2 M sodium acetate (pH 4.0), and 0.2 volume of chloroform: isoamyl alcohol (49 : 1). Nucleic acids in the solution were precipitated by addition of 1 volume of 2-propanol, and washed with 70% ethanol. The dried pellets were dissolved in a small volume of TE.

Based on the known sequence for the TSWV-S RNA4), a set of primers (forward and reverse) was designed for the amplification of a portion of S RNA. The forward primer (5’ dGGATCCAGAGCAATTGTGTCA 3’) is complementary to the 3’ end region of the S RNA and contains BamHI site (underlined). The reverse primer (5’ dCTGCAGCTTCACCTGATCTTCATTTCAATGC 3’) containing a viral sequence from an internal region of the N protein gene and PstI site (underlined).

Nucleic acids prepared from samples were denatured with 20 mM methylmercury hydroxide for 5 min at room temperature. Reaction was stopped by adding with 20 mM 2-ME, and used as a template for cDNA synthesis. Reverse transcription was carried out at 42°C for 60 min in 50 mM Tris-HCl buffer (pH 8.4) containing 8 mM MgCl2, 30 mM KCl, 1 mM dithiothreitol, 50 pM of either of the primers, 10 mM each of four kinds of deoxyribonucleotide triphosphate, 10 units of RNase inhibitor and 10 units of avian myeloblastosis virus reverse transcriptase (Invitrogen).

The transcribed cDNA was extracted with phenol/chloroform and then amplified by PCR in a Thermal Cycler (Perkin-Elmer Cetus) for 40 cycles with a Gene Amp DNA amplification reagent kit (Perkin-Elmer Cetus) as described by Saiki et al.10) Each reaction cycle involved denaturation at 94°C for 1 min, primer annealing at 37°C for 1 min and primer extension at 72°C for 2 min. Portions of the reaction mixtures were analyzed by electrophoresis on a gel of 1.5% (w/v) agarose (NACALAI
TESQUE) with 90 mM Tris containing 90 mM borate acid and 2.5 mM Na₂EDTA (pH 8.3; TBE). DNA species on the gel were stained with ethidium bromide and examined under ultraviolet light. The size of PCR products were estimated by comparison with size markers (λ DNA digested with HindIII and φX174 DNA digested with HaeIII).

For hybridization analysis, the PCR products were electrophoresed on 1% agarose gels, transferred to Hybond-N+ membranes (Amersham International) by the alkali blotting method. The membranes were hybridized with isolated viral S RNA segments labeled with [γ-32P] ATP at the 5' end, washed twice with 2×SSC containing 0.1% (w/v) SDS at room temperature for 10 min, once with 1×SSC containing 0.1% (w/v) SDS at 65°C for 15 min and finally once with 0.1×SSC containing 0.1% (w/v) SDS at 65°C for 15 min. Membrane sheets were dried and then exposed to X-ray film (Fuji, new AIF RX) at −80°C.

The PCR products were subcloned into a Bluescript II (SK+) plasmid (Stratagene), and nested deletions of the plasmid were prepared. Sequence was determined by the dideoxynucleotide method using the enzymatic extension reactions as described in the manual provided by the manufacturer (Applied Biosystems). All sequences were determined in both directions. Analyses of the nucleotide sequences obtained were performed using DNASIS (Hitachi) on a PC-9801 computer (NEC).

In the synthesis of the first strand cDNA, the forward primer was used, and then the PCR as next step was carried out with the reverse primer. The amplified DNA was detected as band between two bands of size markers, 872 bp and 603 bp, and the band was shown to be hybridized with the isolated S RNA of TSWV (Fig. 2). Sequencing of the amplified DNA revealed a 781 bp insert which is identical to the corresponding region of the cDNA clone isolated from cDNA library of TSWV (data not shown). A band with the same length was also detected by electrophoresis, when the reverse primer was used as the first primer for RT reaction (data not shown). This fact demonstrates the presence of a complementary RNA to S RNA in the nucleocapsid, as previously found by Tsuda et al. As for the specificity to another Japanese strain (W strain) of TSWV, this system could not detect the cDNA from S RNA of W strain, as shown previously in negative result of cross-hybridization by Tsuda et al. (data not shown).

Nucleic acids extracted from each adult thrips fed on infected seedlings for 2 days were individually subjected to RT-PCR. The results were shown in Fig. 3. The thrips capable of transmitting TSWV showed bands identical in length to the positive control (P in Fig. 3). Nucleic acids isolated from male thrips which could not show the transmissibility (lane 5 in Fig. 3) also showed the band, probably because it contained a small quantity of TSWV S RNA. Therefore, TSWV was detected in all the adult...
thrips examined, even though some showed the negative results of the transmission test.

In the case of the detection of TSWV in nymphs, acquisition access feeding on infected leaf was done as described for adult thrips. Nucleic acids were separately isolated from nymphs immediately after acquisition feeding for 2 hr, and subjected to RT-PCR. Bands with correct size were detected in all the nymphs tested, except for an individual in lane 1 (Fig. 4). It is possible that the nymph (lane 1) did not acquire the virus during the access feeding on infected plant. Since nymphs generally show no or low transmitting efficiency, we did not check the presence of TSWV in viruliferous nymphs by transmission tests. This RT-PCR detection system, therefore, is a highly sensitive method for the detection of TSWV in nymphs.

These results show that RT-PCR for TSWV will extend the capability for diagnosing viral infections of vectors or plants under various circumstances. This technique will be useful in obtaining the molecular epidemiological data to predict outbreaks of this disease.

We wish to thank Dr. T. Murai, Shimane Prefectural Agricultural Experiment Station, for supplying *Thrips setosus*. 
Literature cited


和文摘要

津田新哉・藤澤一郎・花田 薫・日高 操・肥後健一・亀谷満朗・都丸敬一：RT-PCR 法によるアザミウマ1個体からのトマト黄化ウイルスの検出

Reverse transcriptase-polymerase chain reaction (RT-PCR) 法を用いてアザミウマ1頭から TSWV (O 系統) S RNA の検出を試みた。精製 TSWV RNA では予想される增幅幅に一致する約 800 塩基対のシグナルが認められた。検出されたシグナルは、S RNA をプロープとしたサザンハイブリダイゼーションとダイオキシ法による DNA シーケンシングから、S RNA を錠型にした cDNA と同定された。ダイズウスイロアザミウマ (Thrips setosus) の若齢幼虫をダチュラ (Datura stramonium) またはピーマン (Capsicum annuum) の病葉上で2時間間噴霧させた後、健全ササダに移し、約10日後羽化した成虫を1頭ずつ健全ピーマン幼苗で2日間接種試験させて伝搬の有無を調べた。次いで、接種試験した成虫1頭ずつから全 RNA を抽出して RT-PCR 法で検出した結果、TSWV を伝搬した雌雄すべての個体からシグナルが検出され、伝搬しなかった個体からもまれに検出された。以上から、本法は媒介虫 (アザミウマ) からのウイルス検出に有用と思われる。

(Received May 19, 1993)