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Detection of Tobacco mosaic virus in Petunia and Tobacco By Light Microscopy Using a Simplified Inclusion Body Staining Technique

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Abstract Tobacco mosaic virus (TMV) causes significant losses in many economically important crops. The virus caused flower break, mild mosaic and brown, brittle stems in petunia; and mosaic and mottling symptoms in tobacco leaves in northern Thailand. This study evaluated virus inclusion visualization by light microscopy (VIVLM) with virus confirmation by reverse transcription polymerase chain reaction (RT- PCR).VIVLM was accomplished by staining leaf epidermal tissue with toluidine blue O and basic fuchsin and detected tobamovirus in all symptomatic samples. TMV infection was confirmed by RT-PCR; there was a 91% agreement between VIVLM and RT-PCR in tobamovirus detection. Detection of other plant virus groups by this simplified virus inclusion visualization technique may be possible.

Keywords: petunia, tobacco, *Tobacco mosaic virus*, Inclusion bodies, Virus inclusion visualization by light microscopy, VIVLM

Introduction

Tobacco mosaic virus (TMV) is an economically important pathogen infecting tobacco, other solanaceous hosts and a large number of other crops worldwide. TMV infects 199 different species from 30 families; however, solanaceous crops incur the most dramatic losses from the disease (Christopher, 2001).

Recently, petunia (*Petunia* x *hybrid*) and tobacco (*Nicotiana tabacum*) exhibiting typical mosaic symptoms were observed in landscape and at field sites, respectively, in northern Thailand. Virus symptoms on petunia included flower break and mild leaf mosaic; stems were brittle and developed brown necrosis internally. In tobacco, mosaic and mottling symptoms were observed in leaves (Fig. 1 A, B).

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Tobamoviruses, including TMV, induce a number of inclusions that can be detected with light microscopy (Christie and Edwardson, 1994). While viruses in other groups induce similar-appearing inclusions, tobamoviruses induce several inclusion types unique to this group. These unique inclusions include aggregates of virus particles that have been described as hexagonal or rounded plates, angled-layer aggregates, X-bodies and various crystalline inclusions. These inclusions result from the way in which the rigid, elongate TMV particles orient themselves (Christie and Edwardson 1994).

Reverse transcriptase-polymerase chain reaction (RT–PCR) can be used to identify RNA plant viruses such as TMV. This procedure is rapid, specific and sensitive, and PCR-based methods are useful in high-throughput applications. Walsh *et. al.* (2001) developed an RT-PCR procedure specific for detection of TMV.

Virus inclusion visualization with light microscopy (VIVLM) of plant viruses was pioneered in the 1970s by Christie and Edwardson (1994) and others at the University of Florida and can function as a rapid first step in the identification of virus group(s). The technique can be of use to plant disease clinics lacking the infrastructure and budget to perform more specific techniques. Recently we developed a simplified VIVLM technique which was used to detect begomovirus, *Tomato leaf curl virus* (TYLCV), infection in tomato (McGovern *et al.*, 2016).

In this study, our aim was to determine the ability of the simplified VIVLM technique to detect *Tobacco mosaic virus* (TMV) in petunia and tobacco with confirmation by RT-PCR.

Material and methods

Sample collection

Symptomatic leaf samples were collected in 2014 in northern Thailand from three petunias [from gardens at Maejo University, Chiang Mai (2 samples) and in Chiang Kong, Chiang Rai (1 sample)] and eight tobacco plants [at the Tobacco Research Center, Maejo University, Chiang Mai (6 samples) and a tobacco plantation in San Kamphaeng, Chiang Mai (2 samples)]. Sampled petunia and tobacco plants were at the young stage before flowering. Representative samples obtained were freeze-dried and are maintained in the culture collection of the Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University.

Detection of tobamovirus by VIVLM

Virus infection was determined using the method developed by McGovern *et al.*, (2016): epidermal strips from expanding leaves were

cleared for 5-10 s in 25% (v/v) lactic acid (Thermo Fisher Scientific Inc.) using a microwave oven; the tissue was rinsed three times in deionized water (dH2O); stained for 1 min in a mixture containing equal volumes of 0.05% (w/v) toluidine blue O (Sigma-Aldrich Co. LLC) and 0.05% (w/v) basic fuchsin (Thermo Fisher Scientific Inc.); rinsed three times in dH2O, mounted in dH2O; and viewed with light microscopy at 1000x.

Detection of TMV by RT-PCR

PCR Primers

Primer sequences were designed based on the published sequence of TMV *CP* in Genbank. One primer pair was chosen for amplification of a specific 422 bp sequence of TMV (5′- GAC CTG ACA AAA ATG GAG AAG ATC T-3′; forward primer) and (5′- GAA AGC GGA CAG AAA CCC GCT G-3′; reverse primer) (Jacobi *et al*., 1998). The conventional PCR was performed using the above synthesized cDNA.

First-strand complementary (cDNA) synthesis

For primer annealing prior to cDNA synthesis 10 µl total RNA or 1 µl of viral RNA at 1 µl (50 µmole) reverse primer (5′- GAA AGC GGA CAG AAA CCC GCT G-3′) for a total volume of 12 µl was used. After spin down at 12,000 rcf for 10 s samples were heated for 5 min at 65 ◦C and immediately cooled on ice. For sequencing, other reaction components were added in the following order: 4 µl of reaction buffer, 2 µl of dNTP mix (10 mM), 1 µl of RiboLock Rnase Inhibitor (20 U/µl) and 1 µl of M-MLV reverse transcriptase (200U/µl), for a total volume of 20 µl. After spin down at 12,000 rcf for 10 s samples were incubated at 42 ◦C for 1 h., followed by 5 min of heating at 70 ◦C to denature the enzyme.

Amplification of cDNA by RT-PCR

The PCR reactions were performed in 25 µl volumes containing 12.5 µl. Taq DNA polymerase, 1.5 µl (50 µmole) of forward primer (5′- GAC CTG ACA AAA ATG GAG AAG ATC T-3′), 1.5 µl (50 µmole) of reverse primer (5′- GAA AGC GGA CAG AAA CCC GCT G-3′), 4.5 µl of sterile distilled water and 5 µl of cDNA product TMV reverse primer (5′- GAA AGC GGA CAG AAA CCC GCT G-3′). The reaction mixture was heated at 94°C for 3 min followed by 40 cycles at 94 °C for 30 s, 50 °C for 1 min and 72 °C for 1 min followed by a 7 min incubation at 72 ◦C. The identities of the amplicons and the specificity of the reaction were verified by agarose gel electrophoresis.

Results and Discussion

The VIVLM technique used in this study detected inclusions in the epidermis of symptomatic petunia and tobacco similar in appearance to those inclusions associated with tobamovirus infection (X-bodies and rounded plates) by Christie and Edwardson (1994) (Fig. 1 C-E).

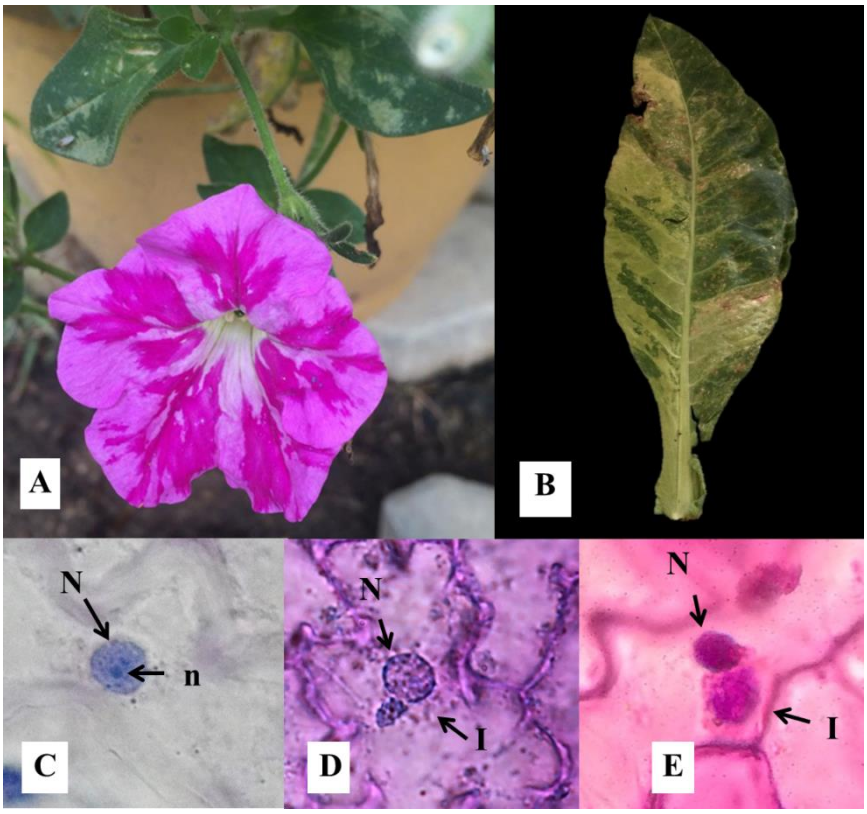

Figure 1. Symptoms of virus infection in petunia flower [A]: flower break; and tobacco leaf [B]: mosaic and mottling. Plant cells stained with toluidine blue O and basic fuchsin: virus-free cell with nucleus (N) and nucleolus (n) [C], tobamovirus-infected petunia cell showing an X-body inclusion (I) and nucleus (N) [D], and tobacco cell showing a rounded plate inclusion (I) and nucleus (N) [E]. (1000x)

Using the TMV-specific primers, the expected fragment size of 422 bp of the virus was generated from all samples except tobacco sample number 6 (Fig. 2). This detection discrepancy between VIVLM and RT-PCR could have been the result of technical or sampling error.

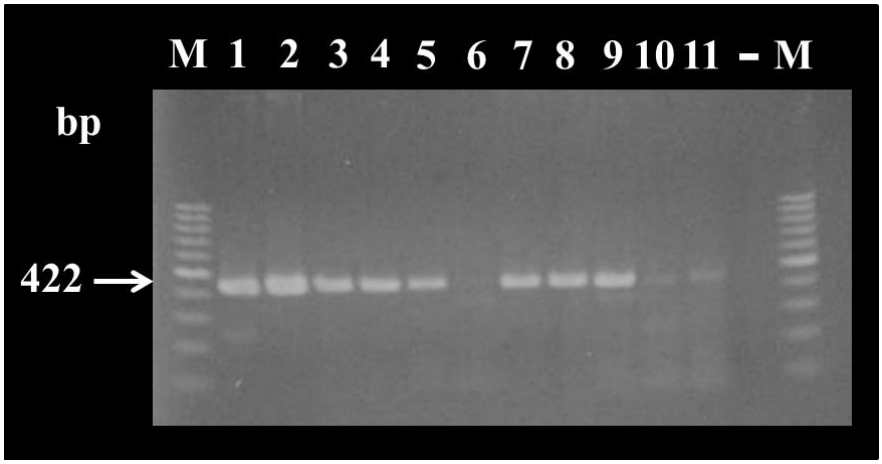

Figure 2. Agarose gel electrophoresis of RT-PCR products obtained from symptomatic samples. Lanes 1 to 3 are petunia samples from Maejo University and Chiang Kong District gardens; lanes 4 to 9 are tobacco samples from the Maejo University Tobacco Research Station; and lanes 10 and 11 are tobacco samples from the San Kamphaeng District. Lanes (M) and (-) indicate the molecular markers and negative control, respectively.

TMV appears to be endemic wherever tobacco is grown and has been reported on this host in Thailand (Sontirat *et al*., 1994). Although a mosaic disease of petunia has occurred in Thailand, the causal agent was not identified (Sontirat *et al*., 1994). To our knowledge, this is the first report of natural infection of petunia by TMV in Thailand.

TMV is very stable and can persist in soil and on inanimate surfaces, and be transmitted mechanically in plant sap and on the seed coat (Scholthof, 2000, 2005) and through vegetative propagated petunia cvs. (Lesemann, 1996). TMV is the most commonly detected virus in petunia in the USA (Nameth, 2002). Recent outbreaks of the virus have caused extensive losses to petunia production in the USA (Saalau, 2014), and have been associated with the international movement of infected, vegetatively propagated petunias (Kim *et al.,* 2014)).

The current research has demonstrated the usefulness of simplified VIVLM in detecting TMV. This result along with previous detection of TYLCV (McGovern *et al.*, 2016) suggests that this technique could serve as a preliminary diagnostic tool for other plant viruses.

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