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Tomato necrotic ring virus (TNRV), a recently described tospovirus species infecting tomato and pepper in Thailand

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Abstract Two tospovirus isolates collected from tomato and bell pepper in Thailand were studied. The isolates induced severe necrotic mottling and/or necrotic spots and rings on the leaves and fruits of the respective plants as confirmed by back-inoculation. A polyclonal antiserum raised against its nucleocapsid (N) protein reacted only with an extract from plants infected with the homologous virus. Analysis of the nucleocapsid (N) gene sequence and its deduced amino acid sequence (Mw ~31 kDa) showed 99% amino acid sequence homology with that of Tomato necrotic ring virus (TNRV). The nucleotide sequence of the 5 untranslated region and intergenic region flanking the N gene revealed typical features of the S RNA segment of tospoviruses. Mechanical inoculation of the virus on some plant species showed that most of the tested solanaceous species were susceptible to this virus. The biological, serological and molecular data presented here indicate that both isolates are identical to TNRV, a recently described tospovirus species in Thailand.

Keywords *Bunyaviridae* · Phylogeny · Serological relationship

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Diseases caused by tospoviruses have a serious impact on the production of many different crops worldwide (Mumford et al. 1996; Persley et al. 2006; Pappu et al. 2009). The genus *Tospovirus* (family *Bunyaviridae*) comprises at least 20 assigned and tentative species (Hassani-Mehraban et al. 2010) with *Tomato spotted wilt virus* (TSWV) being the type species and best studied member of this genus (Goldbach and Kuo 1996; Goldbach and Peters 1994). Tospoviruses are transmitted by a limited number of thrips species in a persistent manner. Virus particles are spherical and membrane-bound (80–120 nm in diameter). The core contains infectious ribonucleoproteins composed of the genomic RNA tightly encapsidated by the nucleoprotein (N) and small amounts of the viral RNA-dependent RNA polymerase. The RNA genome is tripartite and of negative and ambisense polarities, containing 5 open reading frames that code for 6 mature proteins (de Haan et al. 1990; de Haan et al. 1991; Kormelink et al. 1992). The small (S) RNA segment codes, in viral complementary sense, for the N protein of which the amino acid sequence is being used as major demar-

cation criterion (threshold value of 90%) for the classification of tospovirus species. Besides the N protein, biological data on host range and thrips vector species (*Thysanoptera*: *Thripidae*) (de Ávila et al. 1993a; Fauquet et al. 2005; Goldbach and Kuo 1996) are additional criteria. Tomato (*Solanum esculentum)* and pepper (*Capsicum annuum*) are highly susceptible to several tospoviruses under natural or experimental conditions. Whereas TSWV causes infections in tomato all over the world, in South America tomato is also infected by the tospoviruses Chrysanthemum stem necrosis virus (CSNV), *Groundnut ringspot virus* (GRSV) and *Tomato chlorotic spot virus* (TCSV) (Bezzera et al. 1999; de Ávila et al. 1993b). In Australia and South-East Asia, tospovirus infections of tomato other than TSWV, include Capsicum chlorosis virus (CaCV) and *Watermelon silver mottle virus* (WSMoV) (McMichael et al. 2002; Chiemsombat et al. 2008), in Iran Tomato yellow ring virus (TYRV) (Hassani-Mehraban et al. 2005) and in China Tomato zonate spot virus (TZSV) (Dong et al. 2008). Most of these tospoviruses also infect pepper. *Groundnut bud necrosis virus* (GBNV) has been reported to affect tomato in India (Umamaheswaran et al. 2003).

So far three different tospovirus species have been reported in Thailand, i.e. CaCV, WSMoV and Melon yellow spot virus (MYSV). Other than tomato, CaCV is found in pepper and groundnut, while WSMoV is additionally found in cantaloupe, *Physalis* spp., sweet pepper and watermelon. MYSV is mainly found in different cucurbitaceous plant species (Chiemsombat et al. 2008). While the thrips species *Ceratothripoides claratis* has been shown to transmit CaCV (Premachandra et al. 2005), *Scirtothrips dorsalis* and *Thrips palmi* are suspected potential vectors of the prevailing tospoviruses in Thailand (Chiemsombat et al. 2008).

Here we describe the identification of a tospovirus isolated from tomato and pepper in Thailand. The virus showed high homology to a recently characterized new tospovirus species, for which the name Tomato necrotic ringspot virus (TNRV) has been suggested (Chiemsombat et al. 2010; Seepiban et al. 2011).

In 2008, tomato plants showing distinct yellowing and necrotic rings on leaves and fruits and resembling tospovirus symptoms were observed in screenhouse crops in Chiang Mai, Thailand (Fig. 1). Samples from infected tomato (T1-isolate) and also bell pepper fruits and leaves (P1-isolate) were collected and tested for the presence of tospovirus species known to infect tomato in Asia, i.e. GBNV, TYRV, TSWV and MYSV, by ELISA using polyclonal antisera raised against their respective N proteins. Samples of infected leaf material were extracted in PBS-Tween 20 (1:30 *w*/*v*) and applied to IgG-coated plates (1:1000 dilution of a 1 mg/ml stock). Detection was performed using alkaline phosphatase (AP) conjugated IgG (1:1000 of a 1 mg/ml stock), and absorbance measured at OD_{405} after 30 min. No positive signals were obtained with any of the antisera, except for the homologous positive control samples, indicating that the T1- and P1-tospovirus isolates were likely different viruses (Fig. 2). To further substantiate this finding, RNPs were purified from the T1-isolate as described by de Ávila et al. (1990) and used for the production of a polyclonal antiserum to perform a reciprocal DAS-

Fig. 1 Symptoms on tomato and pepper plants naturally infected with T1- and P1-isolates. Necrotic rings on tomato leaf and yellow rings on tomato fruits (**a** and **b**). Necrotic rings on a pepper leaf (**c**)

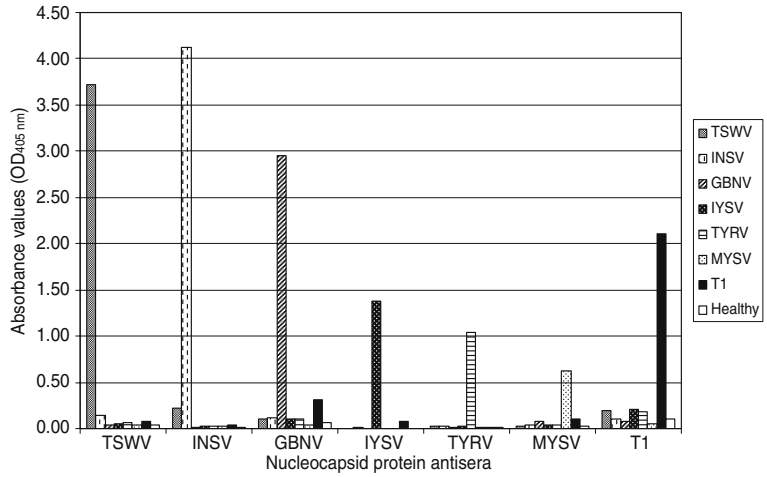

Fig. 2 Serological relationship between the T1-isolate and different tospovirus species in DAS-ELISA format using polyclonal antisera (1:1000) raised against purified nucleocapsid proteins and infected plant extract (1:30) as viral antigens. Tospovirus acronyms: TSWV=*Tomato spotted wilt virus*, INSV=*Impatiens necrotic spot virus*, GBNV=*Groundnut bud necrosis virus*, IYSV=Iris yellow spot virus, TYRV=Tomato yellow ring virus, MYSV=Melon yellow spot virus

ELISA analysis. A "Vlaamse Reus" rabbit was subcutaneously injected twice with approximately 75 μg/ml purified RNP. Serum was collected and subsequently used to purify immunoglobulin G (IgG) and produce IgG-AP conjugate. The antisera produced gave a positive reaction in DAS-ELISA with the T1- and P1-tospovirus isolates but not with any of the other tospovirus species tested (Fig. 2), not only confirming their distinct serological character but also indicating that the T1- and P1-isolates were probably the same tospovirus.

The T1-tospovirus isolate was used for biological purification from a local lesion host. *Petunia hybrida* leaves were mechanically inoculated with crude extracts from infected leaf samples in PBS buffer, pH 7.0 containing 0.1% Na_2SO_3. Necrotic local lesions were induced within 2–3 days after inoculation. After three passages of a local lesion on petunia, a single lesion was inoculated onto *Nicotiana benthamiana* plants for propagation of T1-isolate. On *N. benthamiana*, this isolate induced systemic symptoms, i.e. severe mottling, leaf deformation and finally resulting in death of the plant. The T1-isolate was used further in a host range study (Table 1). Inoculated plants were kept under greenhouse conditions for a three-week period to monitor symptom development. The T1-isolate was able to infect most of solanaceous plant species tested, including the TSWV resistant tomato cultivar, Stevens (Stevens et al. 1992) and the pepper cultivar, Ribera (Boiteux and de Ávila 1994) (Table 1).

In light of the fact that CaCV is one of the major tospoviruses threatening tomato and pepper crops in Thailand, a reverse transcription-polymerase chain reaction (RT-PCR) was performed on total RNA extracted from T1- and P1-infected leaf material to test for the possible presence of a virus with similarities to CaCV. As positive control, a sample of CaCV (kindly provided by Dr E. Maiss, University of Hannover) was included. Total RNA was extracted using Trizol according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). First strand cDNA was synthesized on 2 μg RNA using degenerate primers CaCV-R (5 - CCC**GGATCC**TTACACTTC(A/T)A(C/T)AGAAG - 3) and CaCV-F (5 - CCC**GGATCC**ATGTCTA(A/C) CGTCAGGC-3), both containing a *Bam*HI site (in boldface), and AMV reverse transcriptase (Promega Corp. Madison, WI). PCR was performed with the same (CaCV N gene) primer set during one denaturation cycle at 92°C for 2 min followed by 30 amplification cycles at 92°C for 0.5 min, 55°C for 0.5 min, 72°C for 1 min and one final extension cycle at 72°C for 7 min. For both T1- and P1-isolates three fragments differing in size were amplified (Fig. 3), whereas for CaCV only one fragment of expected size (N gene) was obtained. The large PCR fragments corresponding in size to the CaCV N gene were cloned into pGEM-T Easy vector (Promega Corp. Madison, WI) and two independent clones were sequenced. The nucleotide and deduced amino acid (aa) sequence of the large fragment of both T1- and P1-isolates turned

Table 1 Partial host range study of a tospovirus isolate (T1) collected in Thailand

Plant species	Symptoms	
	Local	Systemic
Asteraceae		
Emilia sonchifolia	…	Mo
Fabaceae		
Vicia faba	…	…
Vigna unguiculata	LGS	…
Solanaceae		
Capsicum. annuum cv. Bruinsma Wonder[a]	…	Mo, LD
C. annuum cv. Ribera[b]	…	Mo, LD
C. annuum cv. Spartacus[a]	…	Mo, LD
Datura stramonium	…	Mo, YS, LD
Nicotiana benthamiana	CS	Mo, LD, D
N. clevelandii	…	…
N. glutinosa	NL	Mo, VN, TN
N. rustica	…	Mo
N. tabacum cv. White Burley	YS, NR	VB, LD
Petunia hybrida	NLL	…
Physalis floridana	Mo	Mo, LD
Solanum esculentum cv. Moneymaker	YS	Mo, NS, TN
Solanum esculentum cv. Stevens	YS	Mo, NS, TN

CS chlorotic spots, *D* death of the plant, *LD* leaf deformation, *LGS* light green spots, *NLL* local lesions, *Mo* mottling, *NL* necrotic lesion, *NR* necrotic rings, *NS* necrotic spot, *TN* top necrosis, *VB* vein banding, *VN* vein necrosis, *YS* yellow spots, … no symptoms (no infection or systemic infection)

[a] Susceptible cultivar
[b] Resistant cultivar

out to represent the N gene but were significantly distinct from that of CaCV (57% aa sequence homology). Two other primer sets were designed based on the newly obtained sequence and used to amplify the remainder parts of the N gene: N-up (5 - GCTCTTGCAGGCTGCAAATATCTGG-3) combined with AT primer (5 - CCC**GGATCC**AGAG CAATCGAGG-3), complementary to the first 8 conserved nucleotides of the S RNA termini of Asian tospoviruses (de Haan et al. 1990; Hassani-Mehraban et al. 2005) and N-down (5 - GACTAGC TCACCTGGAACAGCTAGC-3) with UHP primer (5 CACT**GGATCC**TTTTGTTTTTGTTTTTTG 3), annealing to a part of the intergenic region (IGR) of the S RNA segment (Cortêz et al. 2001). PCR fragments were cloned, sequenced and provided the

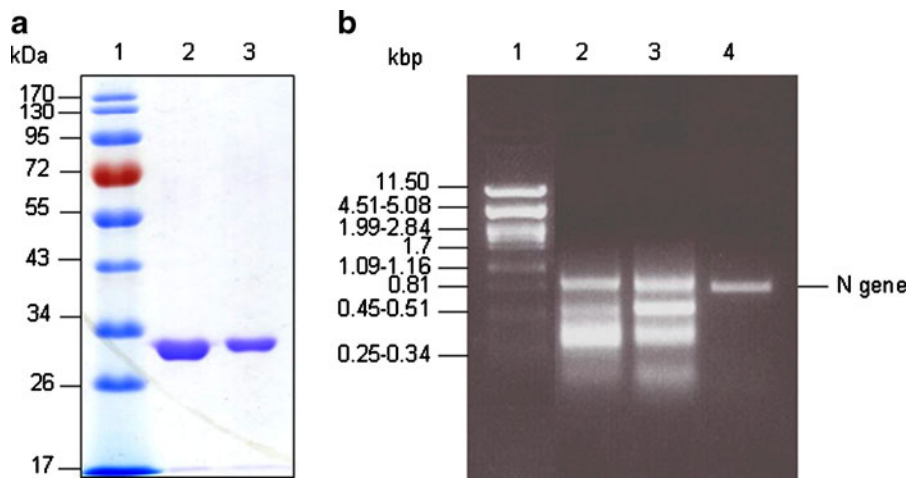

Fig. 3 Nucleocapsid (N) protein and the N gene of T1- and P1-isolates. A, nucleocapsid (N) protein of T1- and P1-isolates (lane 2 and 3) resolved in 12% acrylamide SDS-PAGE gel next to protein size ladder (lane 1) stained with Coomassie brilliant blue. B, RT-PCR product of T1- and P1-isolates (lane 2 and 3) and CaCV (lane 4) in line 4 loaded in 1% agarose gel with a DNA size marker (lane 1)

5′ and 3′ untranslated region (UTR) and the complete N gene sequence. The nucleotide sequence revealed that the CaCV primer sequences differed from the T1- and P1-N gene in only two mismatches at the CaCV-R primer site. Both T1- and P1-isolates showed an open reading frame (ORF) of 846 nucleotides, coding for an N protein of 281 aa with an approximate molecular mass of 31 kDa. Alignment of nucleotide and aa sequences of the isolates by vector NTI Advance™ 10 (Invitrogen, Landsmeer, The Netherlands) revealed that they were identical (accession no. FJ946835). T1-isolate showed 99% aa sequence identity to that of TNRV N gene sequence that was deposited in Genbank and is now published (Chiemsombat et al. 2010; Seepiban et al. 2011). Since both T1- and P1-isolates were almost completely identical, only the current T1 isolate was used for sequence alignments to other reported Asian tospoviruses and TNRV N gene sequences. Multiple sequence alignment of the N protein aa sequences from all established Asian tospovirus species showed the highest sequence identity of T1 to that of TNRV N (99%).

The comparison furthermore showed that the N proteins all shared a large conserved domain at aa positions 177–205. T The aa residues at positions 7, 47 and 48 were present in the N protein of all TNRV isolates, but were absent from the other Asian tospoviruses (Fig. 4). Among all Asian tospoviruses (CaCV, Calla lily chlorotic spot virus [CCSV], GBNV, MYSV, *Peanut chlorotic fan-spot virus* [PCFV], Peanut yellow spot virus [PYSV] Tomato zonate spot virus [TZSV], *Watermelon bud necrosis virus* [WBNV] and WSMoV), TNRV codes for the largest N protein (281 aa) (Fig. 4).

The 5 UTR of the TNRV N gene consists of 66 nts; a value that ranges between 64 and 79 nts within the analogous UTR of other Asian tospoviruses. The first 14 terminal nts [**AGAGCAAT**C(G/A)AGG(C/T)] are

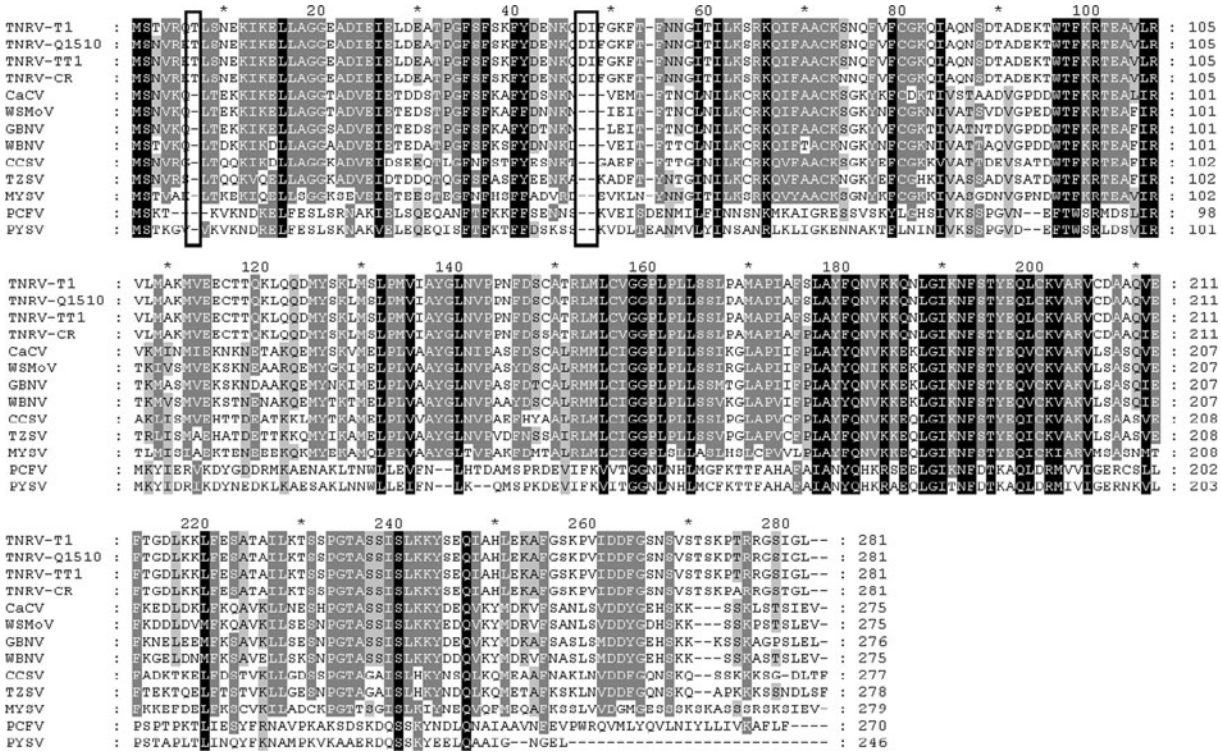

Fig. 4 Multiple sequence alignment of the deduced nucleocapsid protein sequence of Tomato necrotic ring virus (TNRV) isolates; T1(this study): FJ946835, Q1510: HM113532, TT1: FJ489600 and CR: FN677989, with those of Asian tospovirus species. The domains containing extra amino acid residues are shown in boxes. Those amino acid residues with less conservation are shaded in grays. Tomato necrotic ring virus (TNRV), Capsicum chlorosis virus (CaCV), *Groundnut bud necrosis virus* (GBNV), Calla lily chlorotic spot virus (CCSV), *Watermelon silver mottle virus* (WSMoV), *Watermelon bud necrosis virus* (WBNV), Tomato zonate spot virus (TZSV), Melon yellow spot virus (MYSV), *Peanut chlorotic fan-spot virus* (PCFV), Peanut yellow spot virus (PYSV)

conserved in all Asian tospoviral S RNA segments. Furthermore, the IGR sequence showed nucleotide stretches highly rich in A and U-residues as earlier observed within the S RNA segment of other tospoviruses.

To elucidate the taxonomic position of TNRV relative to other tospovirus species, multiple sequence alignment data (Clustal W, Thompson et al. 1994) from 21 nucleocapsid (N) proteins were used as input for the construction of a phylogenetic tree using MEGA version 4.0 (Tamura et al. 2007). In agreement with previous reports, tospoviruses are split in three clades that reflect an American, a Eurasian and Asian distribution (Pappu et al. 2009). Based on the phylogenetic tree, the TNRV isolates cluster within the Asian clade (Fig. 5.) and supports its establishment as a new tospovirus species in concurrence with another recent report (Seepiban et al. 2011).

TNRV is transmitted by the thrips species *C. claratis* and *T. palmi* (Seepiban et al. 2011). Since *Thrips tabaci* is prevalent in Thailand, (unpublished observations), transmission of this virus by this species was

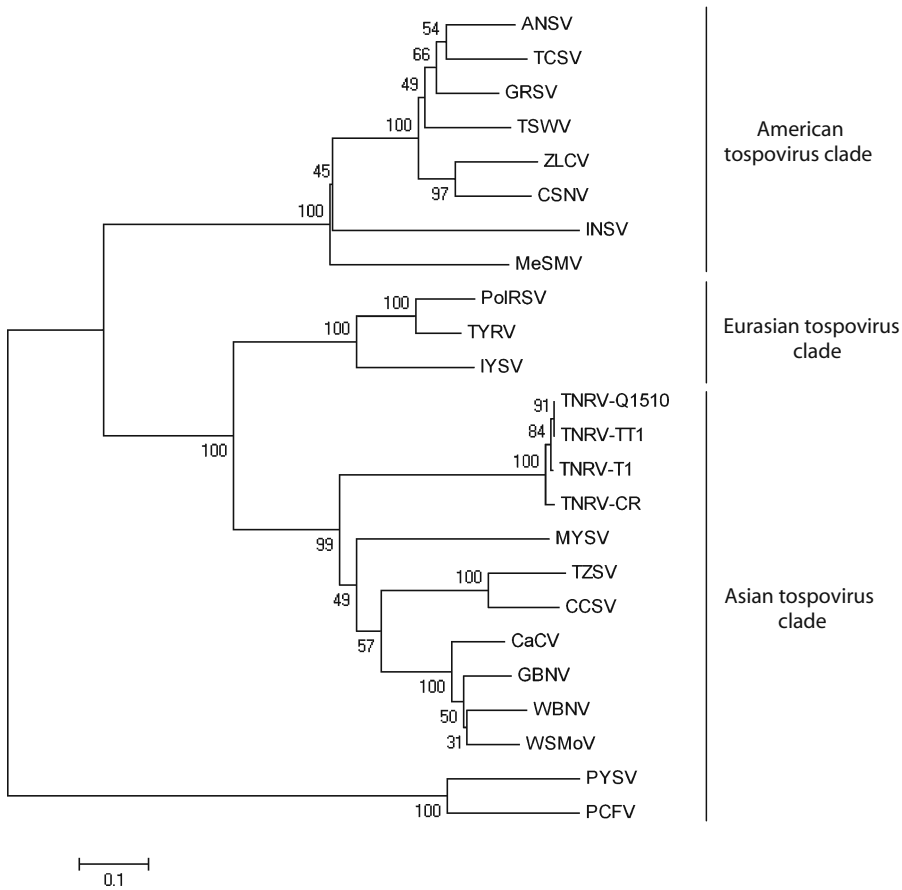

Fig. 5 Phylogenetic analysis of Tomato necrotic ring virus (TNRV) based on N protein sequence data. The phenogram was generated by the neighbour joining method with bootstrap analysis using the *MEGA* version 4.0 program. Bootstrap values are shown as percentages derived from 1,000 re-sampling. The sequences of the N proteins retrieved from the GenBank: Alstroemeria necrotic streak virus (ANSV) GQ478668, Capsicum chlorosis virus (CaCV) AY036058, Calla lily chlorotic spot virus (CCSV) AY867502, *Chrysanthemum stem necrosis virus* (CSNV) AF067068, *Groundnut bud necrosis virus* (GBNV) U27809, *Groundnut ringspot virus* (GRSV) S54327, *Impatiens necrotic spot virus* (INSV) S40057, Iris yellow spot virus (IYSV) AF001387, Melon severe mosaic virus (MeSMV) EU275149, Melon yellow spot virus (MYSV) AF067151, *Peanut chlorotic fan-spot virus* (PCFV) AF080526, Peanut yellow spot virus (PYSV) AF013994, Polygonum ringspot virus (PolRSV) EE445397, *Tomato chlorotic spot virus* (TCSV) S54325, Tomato necrotic ring virus (TNRV) T1(this study): FJ946835, Q1510: HM113532, TT1: FJ489600 and CR: FN677989, *Tomato spotted wilt virus* (TSWV) D00645, Tomato yellow ring virus (TYRV) AY686718, Tomato zonate spot virus (TZSV) EF552433, *Watermelon bud necrosis virus* (WBNV) AF04567, *Watermelon silver mottle virus* (WSMoV) Z46419, *Zucchini lethal chlorosis virus* (ZLCV) AF067069

tested. Next to this species *Frankliniella occidentalis* was also included in these tests. First instars (1–4 hour-old) of *F. occidentalis* and *T. tabaci* were produced on runner bean pods or leek leaf cuttings, and placed on TNRV-infected *Emilia sonchifolia* leaf disks. After 2 days thrips larvae were transferred to leaf disks of 2–3 weeks old *E. sonchifolia* and petunia seedlings. The disks were refreshed every 2 or 3 days until the adults died. The disks were monitored for the appearance of symptoms and necrotic lesions, respectively, and analyzed by ELISA. Neither of these two thrips species transmitted TNRV to leaf disks during three repetitions of the experiments. Due to the lack of experimental populations of *C. claratis* and *T. palmi* could not be included in these tests.

Surveys revealed the presence of TNRV in samples collected from diseased tomato and pepper, grown under protected or unprotected conditions in nine provinces in the northern part of Thailand (unpublished results). Although preliminary, these observations coupled with the reports of Chiemsombat et al. (2010); Seepiban et al. (2011) suggest that TNRV is widespread in Thailand. The availability of a TNRV-specific polyclonal antiserum now allows undertaking of more extensive surveys to obtain a better picture of its geographical distribution and impact. This information is required to indicate whether this virus poses a major threat to cultivation of tomato, pepper or other crops.

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